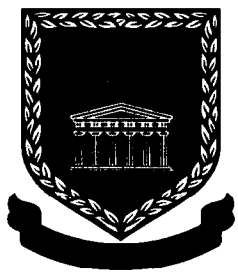
Isolation and Characterization of Natural Products from *Siphonochilus aethiopicus*



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WESTERN CAPE

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A thesis in partial fulfillment of the requirements for the degree of Magister Scientiae in the South African Herbal Science and Medicine Institute, Faculty of Natural Sciences, at the University of the Western Cape.

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DECLARATION

I declare that **"Isolation and Characterization of Natural Products from *Siphonochilus aethiopicus*"** is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the resources I used or quoted have been indicated and acknowledged by complete references.

Full Name: Dowelani Clement Ndiitwani October 2009

Signed: D.C. Ndiitwani ..... UWC, Bellville

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Isolation and Characterization of natural products from *Siphonochilus aethiopicus*

D. Ndiitwani

KEYWORDS

Siphonochilus aethiopicus

Zingiberaceae

Phytochemistry

Antimicrobial

Traditional medicine

Extraction

Chromatography

ABSTRACT

Plants have formed the basis of traditional medicinal systems that have been in existence for thousands of years. Traditional medicines play an important role in protecting, maintaining and restoring the health of people. Therefore, information on folk medicinal uses of plants has in latter times received an intense renewed interest as a source in the search for potential new therapeutic agents.

The aim of this study was to isolate and identify natural compounds from *Siphonochilus aethiopicus* which is a species from the Zingiberaceae family and is one of the most popular medicinal plants in South Africa. This species is used extensively in traditional African medicine for pain relief, asthma, coughs, colds, headaches, dysmenorrhoea and influenza.

Extraction of leaves and rhizomes were performed sequentially with hexane, dichloromethane, ethyl acetate, methanol and water. The presences of organic compounds were screened using chromatographic techniques. The screening revealed similarities between the leaves and rhizomes extracts which implies that in order to improve the sustainability of the plants only leaves need to be harvested. All HPLC chromatograms except for the methanol extract of leaves have shown prominent peak. Moreover, the HPLC results confirm that same compounds are present in both leaves and rhizomes. The antimicrobial activity of the rhizome aqueous extract was carried out against Gram positive (*Staphylococcus aureus, Mycobacterium smegmatis*) and Gram negative (*Pseudomonas aeruginosa*) bacteria as well as fungus (*Candida albicans*). GC and GC-MS were used in separating the volatile components into individual compounds. NMR and MS techniques were used for structural elucidation.

Two furanoterpenoids; 9aβ-hydroxy-4aαH-3,5α,8aβ-trimethyl-4,4a,8a,9-tetrahydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one and 4aαH-3,5α,8aβ-trimethyl-4,4a,8a,9-tetrahydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one were isolated from the hexane extract of the rhizomes using column chromatography (CC). Essential oil components as determined in the hexane extract were found to be: 1,8-Cineole, α-terpineol, terpinen-4-ol, terpinyl acetate, *cis*-4-thujanol (*cis*-sabinene hydrate), β-elemene, β-eudesmol, hedycaryol and 1,3a-ethano(*1*H)inden-4-ol, octahydro-2,2,4,7a-tetramethyl.

A polysaccharide rich fraction was extracted in order to determine its monosaccharide composition through derivatization into monosaccharide alditol acetates. The monosaccharides: rhamnose, arabinose, mannose, xylose, glucose and galactose were used as standards. GC analysis of the monosaccharide alditol demonstrated the presence of arabinose, xylose, galactose and glucose. The uronic acid content was determined using the carbazole assay.

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LIST OF ABBREVIATIONS AND SYMBOLS

CC..... Column Chromatography

TLC..... Thin layer chromatography

HPLC..... High Performance Liquid Chromatography

GC..... Gas Chromatography

GC-MS..... Gas Chromatography-Mass Spectroscopy

DCM..... Dichloromethane

EtOAc..... Ethyl acetate

$CHCl_3$..... Chloroform

MeOH..... Methanol

EtOH..... Ethanol

^1H NMR..... Proton nuclear magnetic resonance

^{13}C NMR..... Carbon nuclear magnetic resonance

NOESY..... Nuclear Overhauser effect spectroscopy

COSY..... Correlated spectroscopy

HMQC..... Heteronuclear multiple quantum correlation

HREIMS..... High resolution electron ionization-mass spectrometry

DEPT..... Distortion less enhancement by polarization transfer

J..... Coupling constant

d..... Doublet

dd..... Doublet of doublets

ddd..... Doublet of doublets of doublets

UV..... Ultraviolet

HCl..... Hydrochloric acid

H_2SO_4..... Sulphuric acid

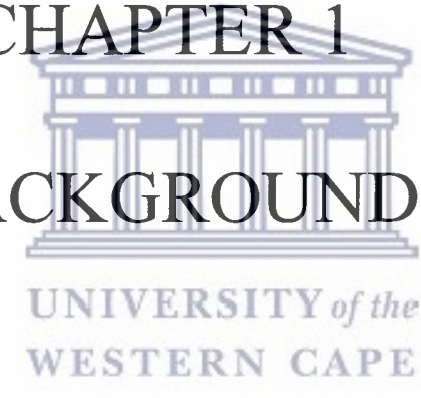
$CDCl_3$..... Deuterated chloroform

DMSO..... Dimethyl sulfoxide

m/z..... Mass-to-charge-ratio

CHAPTER 1

BACKGROUND



CHAPTER 1

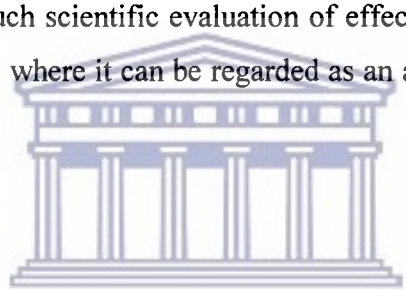
BACKGROUND

Siphonochilus aethiopicus is a highly popular plant used in traditional medicine in Southern Africa. As a herbal remedy, it is used for the treatment of a variety of ailments such as colds, coughs, pain, asthma, dysmenorrhoea and influenza. It is chewed fresh for the treatment of hysteria. This plant is commonly known as wild ginger, Natal ginger and in Zulu as 'indungulo' or 'isiphephetho,' which is frequently used to improve the health of South Africans or as a therapeutic agent. This plant grows widely in the forests of Kwazulu-Natal, Mpumalanga and Limpopo; naturally found in dams, shaded parts of the lowland or on hill slopes. The plant can be controlled and propagated for sustainable use.

Over the past years, herbal medicine has attained increasing global importance, having impacted on both world health and traditional trade. This interest is as a result of the consumer's belief that natural products are superior over conventional medicines. There have been numerous trading of medicinal plants from South Africa to other countries. Economically, *Siphonochilus aethiopicus* can be of much benefit towards uplifting the economy through trade to other countries. This again can be of benefit to the communities by providing employment. The use of herbal medicine in communities has resulted not only in economic benefits and accessibility to needed remedies, but also in building the confidence and self-image of the people. Traditional medicine has become an essential part of health systems of a large proportion of the world's population, since it has a long and uninterrupted history of use. The World Health Organization (WHO) suggests that approximately three quarters of the world's population currently use herbs (and other forms of "traditional medicine") to treat their ailments. In South Africa, 60-80% of the population still uses traditional medicine for their physical and psychological health needs. Herbal medicine plays an important role in African culture.

In countries where Western medicines are inaccessible, too expensive, or not accepted by the people, the majority of the population still relies on traditional herbal remedies as important strategies in delivering health care.

Due to the importance of this plant, interest has developed towards the screening of *S. aethiopicus* and the isolation of compounds in rhizomes which can improve treatment of several ailments. The importance of evaluating the leaves for the purpose of comparison with the rhizomes is also essential and is motivated by matters concerned with the sustainable use of this species. In South Africa, and Africa as a whole, the scientific study and evaluation of traditional medicine aims to improve efficacy, safety, availability and wider application at low cost. Such scientific evaluation of effective remedies can bring traditional medicine up to a level where it can be regarded as an acceptable alternative to the western health system.



CHAPTER 2

LITERATURE REVIEW

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

This chapter presents the history, safety and pharmacology of herbal medicines. The literature review also focuses on the discussion of medicinal plants and different genera and species of the Zingiberaceae family. *S. aethiopicus* belongs to this family and hence the family is being reviewed. Geographical distribution, traditional use, phytochemistry and phytochemical constituents of *S. aethiopicus* are discussed.

2.2 MEDICINAL PLANTS

Since time immemorial, human beings have relied on nature for the production of food stuffs, shelters, clothing, transportation, fertilizers, flavors and medicines. Gurib-Fakim, (2006) reported that plants have formed the basis of a sophisticated traditional medicine system that has been in existence for thousands of years and continues to provide mankind with new remedies. Therefore, information on folk medicinal uses of plants has recently evoked renewed interest in the search for new therapeutic agents (Parveen *et al.*, 2007).

2.3 HISTORY OF TRADITIONAL MEDICINES

Traditional medicine is more accessible to most of the population in the third world. It has been reported that 60-80% of the population in every country of the developing world tend to rely on traditional or indigenous forms of medicine for their primary health-care needs, and it can safely be assumed that a major part of traditional therapy involves the use of plant extracts or their active principles (Sofowora, 1982; Farnsworth, 1994; Akerele, 1998). With the high use of traditional medicine as an alternative form of health care, the screening of medicinal plants for active compounds is very important (Rabe and Van Staden, 1997).

The following essential drugs are derived from plants: atropine (anticholinergic), codeine (antitussic/analgesic), lapachol and vincristine (antitumor), aspirin (pain relief), quinine and artemisinin (antimalarial), penicillin (bacterial infection cure), physostigmine (cholinergic) and reserpine (antihypertensive) (Akerele, 1998). *Catharanthus roseus* is still the only source of the powerful antitumor drugs vinblastine and vincristine (Gaines, 2004; Taha *et al.*, 2008). Artemisinin was isolated from the Chinese plant *Artemisia annua* and quinine from *Cinchona ledgeriana* bark and both these drugs are natural products for fighting malaria (Gaines, 2004; Kumar *et al.*, 2009; Mishra *et al.*, 2009). Tazopsine was purified from *Strychnopsis thouarsii* stem bark and is used for the treatment of malaria (Carraz *et al.*, 2006). Traditional medicines play an important role in protecting, maintaining and restoring the health of people. Generally, a medicinal plant is defined as any plant which, in one or more of its parts, contains substances that can be used for therapeutic purposes or for the synthesis of useful drugs (Sofowora, 1982).

Traditional medicine is a comprehensive term used to refer to both traditional medicine systems such as Chinese medicine, Arabic medicine and Indian ayurveda as well as other forms of indigenous medicine. It is a system of medicine that is based on past experience, cultural beliefs and practices which are passed on from generation to generation verbally or in writing (Kabatende, 2005; Gurib-Fakim, 2006). There is usually no formal educational component to these systems and frequently the information is considered to be highly secretive and is not documented (Farnsworth, 1994). Traditional medicine has been described by WHO as one of the surest means to achieve total health coverage of the world's population (Kabatende, 2005). Most diseases can be treated with the aid of plants from the field and it was believed that the synergistic action of additives, such as animal or insect parts, yielded even stronger or more potent medicines. However, the healing action predominantly resulted from medicinal plant compounds, since the base ingredients in the majority of medicines were of plant origin (Louw *et al.*, 2002).

2.3.1 AFRICAN TRADITIONAL MEDICINE

The African continent is reported to have one of the highest rates of deforestation in the world. African traditional medicine is the oldest and perhaps the most diverse of all medicine systems. Africa is considered to be the cradle of mankind with a rich biological and cultural diversity and marked regional difference in healing practices (Gurib-Fakim, 2006). African traditional medicine in its varied forms is a holistic practice involving both the body and the mind, where the healers typically diagnose and treat the psychological basis of an illness before prescribing medicine to treat the symptoms (Gurib-Fakim, 2006). Famous African medicinal plants include *Acacia senegal* (Gum Arabic), *Agathosma betulina* (Buchu), *Aloe ferox* (Cape Aloes), *Aloe vera* (North Africa Origin), *Artemisia afra* (African wormwood), *Aspalanthus linearis* (Rooibos tea), *Harpagophytum procumbens* (Devil`s Claw), *Hypoxis hemerocallidea* (African potato) and *Prunus africana* (African Cherry) (Gurib-Fakim, 2006).

2.3.2 SAFETY OF TRADITIONAL MEDICINES

The plants commonly used in traditional medicine are assumed to be safe. Such safety is based on their history of long usage in the treatment of diseases according to knowledge that has been accumulated over centuries. Research has shown that some plants which are used as food or in traditional medicine are potentially toxic, mutagenic and carcinogenic (Fennell *et al.*, 2004). However, most medicinal plant preparations used in developing countries may be documented as having theoretical efficacy, based on the literature reports of scientific studies carried out experimentally, that is, either through *in vitro* or *in vivo* studies on extracts (Farnsworth, 1998). A large number of plants contain appreciable levels of biosynthetically produced chemical substances which have been reported to be toxic to humans. Many herbal medicines are also used as foods; nevertheless it is anticipated that some chemical constituents in herbal medicines might react adversely with normal prescription drugs. It is, however, generally accepted that herbal medicines do not present a major problem with regard to toxicity (Farnsworth, 1998).

Some herbal remedies may also contain hazardous levels of arsenic, mercury and lead as derived from surface pollutants. However, all herbal extracts are not the same, and commercially available extracts vary greatly in their quality (Pribitkin, 2005). The criteria for evaluation of safety and efficacy apply to herbal remedies as they do to other medicinal products with comparable indications. It is essential to provide the public and physicians with information on safety and standardization tests of herbs. Herbal safety must begin with botanical authentication of the source plants (Gillis, 1998).

Standardization is a method of assuring a minimum level of active ingredients in the extract and is becoming increasingly important as a means of ensuring a consistent supply of high quality phyto-pharmaceutical products. Herbal medicines contain biologically active substances that may produce non-trivial side effects when taken in excessive doses, and therefore the concentration or dosages become very important (Gurib-Fakim, 2006). Standardization also allows comparison of the clinical effectiveness, pharmacological and side effects of a series of products. Standardized products provide more security and increase the level of trust people have in herbal drugs (Gurib-Fakim, 2006). The use of herbal medicines may increase the risk of adverse effects through several mechanisms, including direct pharmacological effects, interactions with conventional prescribed drugs, and through the effects of unlisted contaminants in herbal preparations (Pribitkin, 2005).

2.3.3 PHARMACOLOGY OF HERBAL MEDICINE

Herbs have been used extensively in Asia and Europe for centuries to treat illnesses and symptoms. Herbal medicines (phytomedicines) are medicinal products that contain plant materials as their pharmacologically active components (Pribitkin, 2005). In the United States, herbal products became popular in the 1960s and there has been a remarkable increase in the number of people taking such products as well as the number of different kinds of preparations being taken (Flanagan, 2001).

In western traditions, use of medications has been based on well documented clinical trials. This justifies the use of chemotherapy, radiation modifiers, or immunomodulating therapy in the treatment of cancer. With a lack of clinical trials and evidence of efficacy and toxicity, predicting any outcome becomes very difficult.

2.3.4 IMMUNOMODULATORY ACTIVITY

During the past three decades, a number of bioactive polysaccharides and polysaccharide-protein complexes have been isolated from mushrooms, fungi, yeast, algae, lichens, and plants. These compounds have attracted significant attention because of their immunomodulatory and antitumor effects (Xie *et al.*, 2007). In recent years, polysaccharides isolated from various traditional medicinal plants have been shown to profoundly affect the immune system (Yang *et al.*, 2008). Immunomodulators are some of the most promising recent alternatives to classical antibiotic treatment for enhancing host defense responses through their ability to modulate macrophage function (Schepetkin and Quinn, 2006). Many herbal preparations alter immune function and have had an amazing array of immunomodulatory effects attributed to them (Plaeger, 2003). Plant polysaccharides have been shown to exhibit anti-inflammatory, anti-hypoglycemic, anti-bacterial, anti-tumor and anti-complementary activities (Cheng *et al.*, 2008). The traditional use and ecological roles of polysaccharides include wound healing, treatment of dermal ailments, ulcers, tumors and detoxification of foreign substances (Paulsen, 2001). Capek and Hříbalová, (2004) reported on the isolation and characterization of ion-exchange fractions of a water-extractable polysaccharide complex from sage, possessing mitogenic and co-mitogenic activities. Schepetkin and Quinn, (2006) reported that polysaccharides derived from higher plants enhance macrophage function. In particular, such compounds have been shown to increase macrophage cytotoxic activity against tumor cells and microorganisms, reactive oxygen species (ROS), nitric oxide (NO) production and enhance secretion of cytokines and chemokines (Schepetkin and Quinn, 2006). Activation of macrophages is a key event in innate immunity for the initiation and propagation of defensive reactions against pathogens. Macrophages release TNF-α, IL-6 and other inflammatory mediators when stimulated by pathological stimuli (Wu *et al.*, 2005).

Phagocytic leukocytes play multiple roles in the immune processes, serving as a link between the innate and acquired immune systems and contributing to the inflammatory response, angiogenesis, and promotion of wound healing (Xie *et al.*, 2008). Carter and Dutton, (1996) have shown that the nature of the response to a pathogen is critical in determining disease resistance and susceptibility, both in experimental animal models and in man. It was proved that Type 1 and Type 2 cytokines produce many additional secondary effects by influencing the expression of adhesion molecules, chemokine receptors and other cell-surface molecules that alter leukocyte subset trafficking and extravasation (Carter and Dutton, 1996). Breen, (2002) reported that when interleukin-10 (IL-10) was used *in vitro*, either at low concentrations that did not measurably affect the production of inflammatory cytokines or at high concentrations that clearly blocked HIV-induced TNF-α and IL-6 secretion, HIV replication within primary cells was inhibited. IL-10 may be beneficial when acting as an anti-inflammatory cytokine that suppresses the production of HIV (Breen, 2002).

2.4 THE ZINGIBERACEAE FAMILY

In view of the fact that the species which is the subject of this thesis belongs to the abovementioned family, it is important to review the family in order to highlight medicinal applications of its various species. The Zingiberaceae family is estimated as having 150 species belonging to 23 genera. Zingiberaceae is widely distributed throughout the tropics, particularly in South-East Asia (Habsah *et al.*, 2000). This study will be focused on the *Siphonochilus aethiopicus* species, since it is widespread in the Northern and Eastern part of South Africa. Makhuvha *et al.*, (1997) reported that *S. aethiopicus* is the only member of the family Zingiberaceae which is indigenous to South Africa. This is one of the most popular medicinal plants in South Africa (Viljoen *et al.*, 2002).

2.4.1 THE GENUS *Zingiber*

The genus *Zingiber* includes about 85 species of aromatic herbs from East Asia and tropical Australia (Foster, 2000). The genus *Zingiber* has been said to contain the true gingers with medicinal and culinary value in many parts of the world.

2.4.1.1 *Zingiber officinale*

Common names: Ginger, Garden ginger

Synonyms: *Amomum zingiber* L. and *Zingiber zingiber* L.

Zingiber officinale Roscoe (commercial ginger) is a monocotyledonous herbaceous perennial plant, a member of the tropical and sub-tropical Zingiberaceae, and one of the most widely used species of the ginger family (Jiang *et al.*, 2006). This type of ginger has been used traditionally from time immemorial for varied human ailments in different parts of the world. It is frequently used in Chinese traditional medicines in fresh and in dried forms (Shukla and Singh, 2007). It is used in fresh form to prepare vegetables and meat dishes and as flavoring agent in beverages and many other food preparations. Ginger rhizome is also consumed as slices preserved in syrup, candy (crystallized ginger) or for flavoring tea (Shukla and Singh, 2007). This plant originated in South-East Asia and has been introduced to many parts of the world, including subtropical parts of South Africa. It has been cultivated for thousands of years and used universally as a spice, and for medicinal and culinary purposes (Ghayur *et al.*, 2005). It has been used as a food-flavouring agent and its neutraceutical properties have been of interest in food processing and in the pharmaceutical industries (Balachandran *et al.*, 2006).

Z. officinale has been used traditionally for the treatment of poor digestion (by increasing the production of digestive fluids and saliva), heartburn, vomiting, atherosclerosis, migrane headaches, rheumatoid arthritis, high cholesterol, ulcers, depression, stroke and diabetes, as well as the prevention of motion sickness (Picaud *et al.*, 2006; Shukla and Singh, 2007; Ali *et al.*, 2008). Therapeutic properties of

ginger include the stimulation of blood circulation, removal of toxins from the body and cleansing of bowels and kidneys. Indian people use it for the treatment of cough, stomachache, worms, leprosy, skin and respiratory diseases (Charles *et al.*, 2000). In South Africa, dried rhizomes or their extracts are important ingredients of several traditional medicines, mainly used as tonics to treat indigestion and to cure stomachache, dyspepsia, flatulence and nausea (Van Wyk *et al.*, 1997; Van Wyk and Gericke, 2000; Van Wyk *et al.*, 2004). The Germans use rhizome powder for gastrointestinal distress and to prevent motion sickness (2 g/day) (Bruneton, 1999). Low doses taken regularly may be of benefit as it can reduce the risk of heart attacks and strokes (Arthur, 1996). The Japanese use ginger as a remedy for fish poisoning, especially with sushi. Ginger is thought to fight harmful intestinal bacteria (like *Escherichia coli, Staphylococcus aureus* and *Streptococcus pyogenes/pneumoniae*) without killing beneficial bacteria. Ginger aids *Lactobacillus* growth in the intestines while killing the *Schistosoma* and *Anisakis* parasites.

2.4.1.1.1 Antimicrobial and antiviral effects

Hydroethanolic ginger extract exhibit potent antibacterial activity against Gram-positive bacteria such as *Staphylococcus aureus, Streptococcus pyogenes/pneumoniae* and Gram-negative bacteria such as *Haemophilus influenzae, Pseudomonas aeruginosa, salmonella typhimurium* and *Escherichia coli* (Chrubasik *et al.*, 2005). Methanolic extract of the dried powdered ginger rhizome and isolated constituents ([6]-,[8]-,[10]-gingerol and [6]-shogoal) inhibited the growth of 19 strains of *Helicobacter pylori in vitro*.

2.4.1.1.2 Side effects and interactions

Ginger may cause heartburn, bloating, gas, belching and nausea, particularly if taken in powdered form. In doses higher than 6g, it may act as a gastric irritant and the inhalation of dust from ginger produces IgE-mediated allergy (Chrubasik *et al.*, 2005). Ginger can interfere with absorption of tetracycline, digoxin, sulfa drugs and phenothiazines as well as the digestion of iron and fat-soluble vitamins. Ginger can

also adversely affect individuals with gallstones as the herb promotes the release of bile from the gallbladder. The herb can inhibit sodium warfarin, which is a blood thinner. Consumers should check with their health-care provider for drug or other interactions. Ginger is on the U.S. Food and Drug Administration`s GRAS (generally recognized as safe), though it also interacts with several medications. The British Herbal Compendium documents no adverse effects of ginger.

2.4.1.2 *Zingiber cassumunar*

Common names: Cassumunar ginger, Bengal ginger

Synonyms: *Zingiber purpureum Roscoe*, *Zingiber montanum* (J. Koenig) Theilade

Zingiber cassumunar Roxb is a traditional medicinal plant commonly found in South-East Asia, especially in Thailand and Indonesia. It is widely used in folklore remedies as a single plant or as a component of herbal recipes (Jeenapongsa *et al.*, 2003; Lu *et al.*, 2005).

This species has been used traditionally for the treatment of inflammation and skin disease (Tewtrakul and Subhadhirasakul, 2007). Furthermore, the rhizomes of *Z. cassumunar* have been found to exhibit antioxidant and antifungal activities (Lu *et al.*, 2005). The hexane extract of *Z. cassumunar* contains an essential oil known for its effectiveness in anti-inflammatory activity (Jeenapongsa *et al.*, 2003). (E)-1-(3,4-dimethoxyphenyl) butadiene (DMPBD) is one of the compound isolated from the hexane extract of *Z. cassumunar* and reported to be an active ingredient of the essential oil.

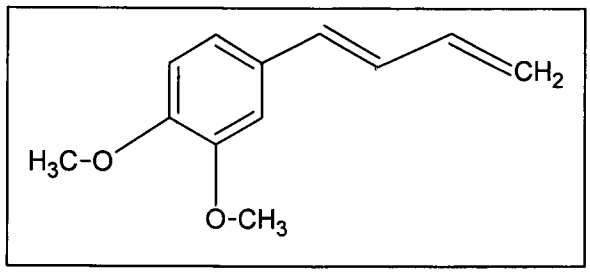


Figure 2.1: Chemical structure of (E)-1-(3,4-dimethoxyphenyl) butadiene (DMPBD) (**1**).

2.4.1.3 *Zingiber zerumbet*

Common names: Bitter ginger, Zerumbet ginger, Shampoo ginger, pine cone ginger.

Synonyms: *Amomum zerumbet* L. and *Zingiber fairchildii* (sentu Hort)

Zingiber zerumbet is a wild ginger, native to South-East Asia and it is a widely cultivated plant throughout the tropics for its medicinal properties. In South-East Asia, *Z. zerumbet* is traditionally used for the treatment of fever, constipation and to relieve pains (Somchit *et al.*, 2005). The local people in South-East Asia also use it as a cure for swelling, sores and loss of appetite. The juice of the boiled rhizomes has also been used as a medicine for worm infestation in children (Bhuiyan *et al.*, 2009). The fresh rhizomes of *Z. zerumbet* are consumed as an appetizer or to cure stomachache (Ruslay *et al.*, 2007). In Polynesia and Hawaii, the plant is used for the treatment of toothache and stomachache. The rhizomes are used as a traditional medicine for anti-inflammation (Jang *et al.*, 2004).

2.4.1.4 *Zingiber mioga*

Common names: Japanese ginger, Myoga ginger

Synonyms: *Amomum mioga*, *Zingiber echuanense*, *Zingiber oligophyllum*

Zingiber mioga Roscoe is a rhizomatous perennial plant, which is widespread in Japan and cultivated primarily for its delicious spring shoots and edible flower buds

(Gracie *et al.*, 2000; Stirling *et al.*, 2002; Gracie *et al.*, 2004). *Z. mioga* flower buds are used in soups and as a spice. Under natural conditions, *Z. mioga* undergoes a period of dormancy over winter months and regenerates in spring from the underground rhizomes (Gracie *et al.*, 2000).

2.4.2 THE GENUS *Kaempferia*

Kaempferia comprises of more than 60 species native to India, and is distributed in the tropics and subtropics of Asia and Africa. These aromatic rhizomatous herbal spices are used as ingredients of many ayurvedic drug preparations (Chithra *et al.*, 2005).

2.4.2.1 *Kaempferia galanga*

Common names: Sand ginger, Aromatic ginger, Resurrection lily.

Synonym: *Lesser galanga.*

Kaempferia galanga is a perennial plant that grows in Southern China, Indochina, Malaysia and India. It is differentiated from the others by the absence of stem and characterized by dark brown rounded rhizomes. This plant is locally known as cekur by Malays and as 'kencur' by the Indonesians (Kanjanapothi *et al.*, 2004; Othman *et al.*, 2006). It is used widely as a spice, a food flavoring, and as an ingredient in the preparation of 'Jamu', a local tonic consumed by the Malays (Othman *et al.*, 2006). *K. galanga* rhizome contains about 2.5 to 4% essential oil.

K. galanga is used traditionally for the treatment of hypertension, rheumatism and asthma (Othman *et al.*, 2006). A decoction of the rhizome is used for dyspepsia, headache, cold, toothache, abdominal pains and malaria. Rhizome extract is useful for curing skin disease, wounds, spleen disorder and coughs (Kanjanapothi *et al.*, 2004; Chithra *et al.*, 2005). Tewtrakul and Subhadhirasakul, (2007) have reported the treatment of urticaria and allergy.

2.4.2.2 *Kaempferia parviflora*

Synonyms: *Kaempferia rubromarginata, Kaempferia latiflorus and stahlianthus rubromargina.*

Kaempferia parviflora is a herb used in Thailand, and its rhizomes have been used as a traditional folk medicine for the treatment of a wide variety of illnesses (Sutthanut et *al.*, 2007). It can be used as a general health-promoting agent, an anti-inflammatory agent as well as for the treatment of gastrointestinal disorders.

Rhizomes of this plant have long been used in Thai traditional medicine (Wattanapitayakul *et al.*, 2007). It has been used to lower blood pressure and is now commonly used in nerve stimulation, antiflatulence for digestive disorders, gastric ulcer and diuresis. The alcoholic infusion (tincture) of its rhizomes has been used as a tonic drink for rectifying male impotence, body pains, relief of symptoms of stomach pain and gastrointestinal disorders (Yenjai *et al.*, 2004; Rujjanawate *et al.*, 2005; Patanasethanont *et al.*, 2007; Wattanapitayakul *et al.*, 2007).

2.5 *Siphonochilus aethiopicus*

2.5.1 BACKGROUND

Siphonochilus aethiopicus (Schweinf.) B.L. Burtt is a rhizomatous herb commonly known as wild ginger. *Siphonochilus* is an African genus of ginger that was taxonomically separated from the related Asian genus *Kaempferia*. Wild gingers are plants with large, hairless leaves developing from a small, distinctive, cone shaped rhizome. The plant can grow up to 1m in height. The flowers are broadly funnel-shaped (60-80 mm in width), pink and white in colour with a small yellow blotch in the middle (Van Wyk *et al.*, 1997; SATMERG, 1999). This species grows naturally in dams, shaded parts of the lowland or on hill slopes (Habsah *et al.*, 2000).

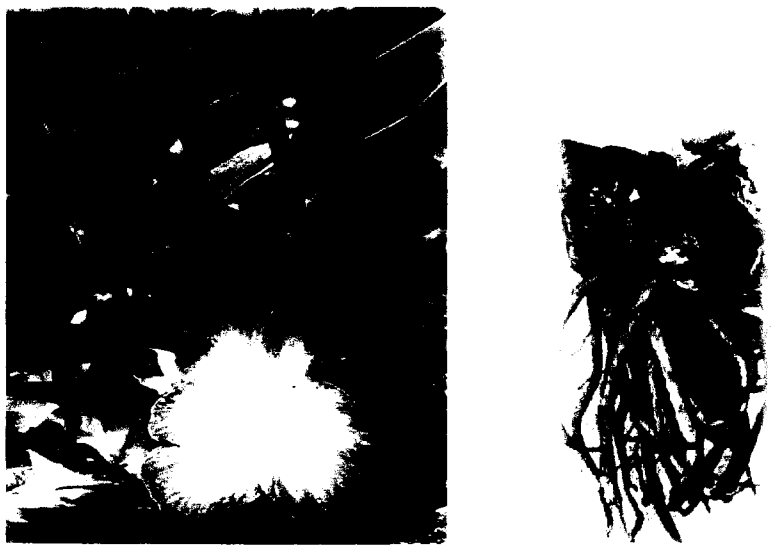


Figure 2.2: *Siphonochilus aethiopicus* plant and rhizomes as reproduced from "Medicinal plants of South Africa" (Van Wyk *et al.*, 1997).

2.5.2 SYNONYMS AND VERNACULAR NAMES

Siphonochilus aethiopicus (Schweinf.) B.L. Burtt is one of the most widely used medicinal plants in South Africa (Watt and Breyer-Brandwijk, 1962; Van Wyk *et al.*, 1997).

Different synonyms have been used previously to describe this plant species.

Synonyms: *Kaempferia aethiopica*

Kaempferia ethelae J.M. Wood

Kaempferia natalensis

Siphonochilus natalensis

Vernacular names

Zulu	: Indungulo, Isiphephetho
Swati	: Indungulu, Incathafane
English	: Wild ginger, or White's ginger, Natal ginger and African ginger
Afrikaans	: Wildegemmer
Tsonga	: Sirungulu
North Sotho	: Serokolo

2.5.3 GEOGRAPHICAL DISTRIBUTION

Siphonochilus aethiopicus is found growing in Zimbabwe, Zambia, Malawi, Mozambique, southwards Senegal and as far up as Ethiopia. It is also found in the Northern and Eastern parts of South Africa including Swaziland (Holzapfel *et al.*, 2002; Golding, 2003; Van Wyk and Wink, 2004). In South Africa, the plant used to be found growing widely in the forests of Kwazulu-Natal, Mpumalanga, and Limpopo; presently it is extinct in the wild of South Africa (SATMERG, 1999).

2.5.4 TRADITIONAL USES

Siphonochilus aethiopicus is a herb used for the treatment of several ailments (Hutchings *et al.*, 1996; Van Wyk *et al.*, 1997). Roots and rhizomes are used extensively in traditional African medicine for pain relief, inflammation, treatment of chest ailments, asthma, coughs, colds, headaches, dysmenorrhoea and influenza as well as a decongestant (Golding, 2003; Van Wyk and Wink, 2004). This herb is chewed fresh for hysteria and to clear nasal passages. The infusions of the rhizome and roots are used in the treatment of epilepsy, hysteria and malaria (Van Wyk and Gericke, 2000).

In traditional Swati medicinal use, this herb is used for the treatment of malaria and women chew it during menstruation probably to relieve pain, while the Zulus believe that the strong scental rhizome is good for catarrh, driving away snakes and warding off lightning (Watt and Breyer-Brandwijk, 1962). *S. aethiopicus* also helps in the lowering of high blood pressure and cholesterol levels, for relieving vomiting and to sooth the stomach as well as increasing sweating and to reduce body temperature in fevers. T he ethanol extract of *S. aethiopicus* has shown activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae* as well as inhibition of cyclooxygenase activity (Lindsey *et al.*, 1999; Stafford *et al.*, 2005).

2.6 PHYTOCHEMICAL SCREENING OF MEDICINAL PLANTS

Phytochemistry involves the investigation of plants, their botanical properties and their chemical components. Most drugs are derived from plants containing biologically active chemical components. It is estimated that 75% of the world's population is dependent upon plant-derived pharmaceuticals (Gaines, 2004). Drugs derived from plants have been previously illustrated in section 2.3. The following is a broad spectrum of natural products (secondary metabolites) which are generally found in plants.

2.6.1 ALKALOIDS

Alkaloids are one of several classes of secondary metabolites found in plants. They play an important role by their ability to protect the plant against predation. They may be described as basic nitrogenous compounds with definite physiological and pharmacological activities (Chhetri *et al.*, 2008). Alkaloids are biologically active substances and hence their wide use in medicine. Most are crystalline but a few (e.g. nicotine) are liquids at room temperature. Pyrrolizidine alkaloids are effective in repelling most herbivores, as they are highly toxic to vertebrates, causing damage to the liver (De Luca and Laflamme, 2001). Alkaloids have pharmaceutically significance, e.g. morphine is a narcotic analgesic, codeine used in the treatment of coughs, colchicine used in the treatment of gout and quinine used as an anti-malarial (Gurib-Fakim, 2006). Alkaloids have been found in *Aframomum danielli* of the family Zingiberaceae (Fasoyiro and Adegoke, 2007). Mayer and Dragendroff reagents have been used to test for the presence of alkaloids.

2.6.2 ANTHRAQUINONES

Anthraquinones are metabolites of commercial and pharmacological interest (Vasconsuelo *et al.*, 2004). They possess various biological activities such as anti-bacterial, anti-viral, and anti-cancer activities as well as analgesic effects (Hemwimon *et al.*, 2006). Anthraquinones have not been reported in the Zingiberaceae family.

2.6.3 ESSENTIAL OILS

Essential oils are known to possess potential as natural agents for food preservation (Ruberto and Baratta, 2000). They have been used for many years in Iran on account of the advantages of definite pharmacological activities and low toxicity (Jalali-Heravi *et al.*, 2006). Essential oils are used as functional ingredients in foods, drinks, toiletries and cosmetics (Sacchetti *et al.*, 2005). The roots and rhizomes of *S. aethiopicus* have been found to contain essential oils (Van Wyk *et al.*, 1997; Holzapfel *et al.*, 2002; Viljoen *et al.*, 2002).

Essential oils rich in phenolic compounds such as carvacrol and thymol are widely reported to possess high levels of antimicrobial activity. 1,8-Cineole has shown activity against gram positive and gram negative bacteria including *Listeria monocytogenes*. Terpinen-4-ol was active against *Pseudomonas aeruginosa*, while α-terpineol was inactive (Oke *et al.*, 2009). Carvacrol, thymol, β- caryophyllene, γ-terpinene, ρ-cymene together with linalool are the essential oils that have been reported to possess strong antioxidant activity (Ruberto and Baratta, 2000).

2.6.4 FLAVONOIDS

Flavonoids are plant secondary metabolites which are widely distributed in the plant kingdom. They have been reported to exert multiple biological effects such as anti-inflammatory, analgesic, anti-tumor, anti-HIV, anti-oxidant and immunostimulant (Gurib-Fakim, 2006; Barbosa *et al.*, 2007). They also have protective roles against heart diseases, bacterial, viral infections and allergies (Barbosa *et al.*, 2007). Flavonoids protect the plant from UV-damaging effects and play a role in pollination by attracting animals through their bright colours. Flavonoid compounds have been isolated from *Kaempferia parviflora* (Yenjai *et al.*, 2004; Sutthanut *et al.*, 2007). *Aframomum danielli* also contains flavonoids/polyphenols (Fasoyiro and Adegoke, 2007). Flavonoids were also identified in the leaves of *Etlingera elatior* the member of the Zingiberaceae family; these are kaempferol 3-glucuronide, quercetin 3-glucuronide, quercetin 3-glucoside and quercetin 3-rhamnoside (Chan *et al.*, 2007).

2.7 PHYTOCHEMICAL CONSTITUENTS OF THE ZINGIBERACEAE FAMILY

2.7.1 *Zingiber officinale*

Ginger contains up to 3% by weight of an essential oil which is responsible for the fragrance of the spice. The main constituents of ginger root include gingerols, zingibain, bisabolenel, oleoresins, starch and essential oil components which are made up of mainly sesquiterpene hydrocarbons, predominantly zingiberene, (+)-*ar*-curcumene, β-bisabolene, (-)-β-sesquiphellandrene and E,E-β-farnesene (Bruneton, 1999). Monoterpenoid hydrocarbons are present at lower percentages which include components such as 1,8-cineole, linalool (**3**), borneol, neral and geraniol as the most abundant (Shukla and Singh, 2007). Ginger's therapeutic benefits are largely due to its volatile oil and oleoresin content. Gingerol is a pungent constituent, responsible for the herbs hot taste and stimulating properties (Charles *et al.*, 2000). Essential oil and oleoresin are internationally commercialized for use in food and pharmaceutical processing (Zancan *et al.*, 2002). 6-gingerol (**4**) and 6-shogaol have been shown to have pharmacological activities such as antipyretic, analgesic, antitussive and hypotensive effects (Thomson *et al.*, 2002).

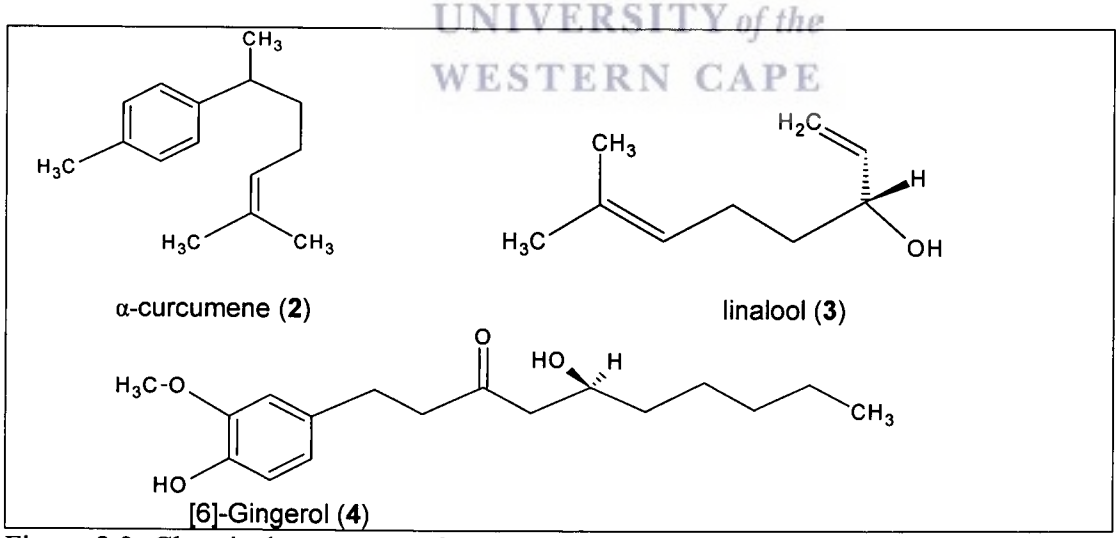
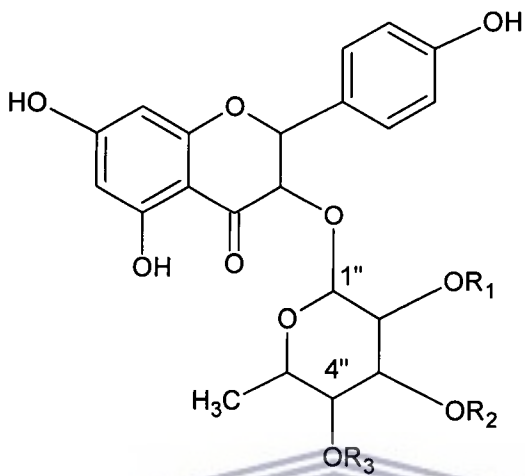


Figure 2.3: Chemical structures of selected constituents present in *Zingiber officinale*.

2.7.2 *Zingiber zerumbet*

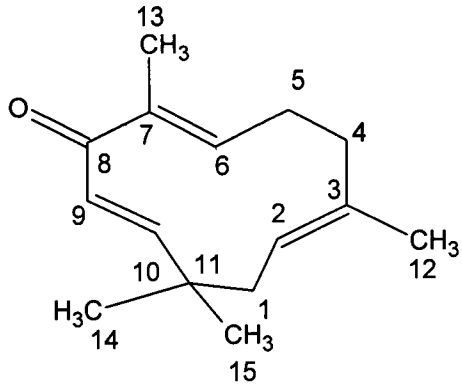
The compounds isolated from the ethyl acetate extract of the *Z. zerumbet* are shown below. No literature has been found concerning the biological activity that is associated with each of these components.



	R1	R2	R3
Kaempferol-3- *O*-rhamnoside (**5**)	H	H	H
Kaempferol-3- *O*-(2"-or 3"-acetyl) rhamnoside (**6**)	H	Ac	H
Kaempferol-3- *O*-(4"-acetyl rhamnoside) (**7**)	H	H	Ac
Kaempferol-3- *O*-(3", 4"-diacetyl rhamnoside) (**8**)	H	Ac	Ac
Kaempferol-3- *O*-(2", 4"-diacetyl rhamnoside) (**9**)	Ac	H	Ac

Figure 2.4: Chemical structures isolated from the *Zingiber zerumbet*.

Zerumbone **10** (Figure 2.5), a predominant sesquiterpene from the *Z. zerumbet*, has been studied as a food phytochemical that has distinct potential for use in anti-inflammation, chemoprevention and chemotherapy strategies (Jang *et al.*, 2004). This was found to be a potent inhibitor of 12-O-tetradecanoyl-13-acetate-induced Epstein-Barr virus activation (Tanaka *et al.*, 2001).

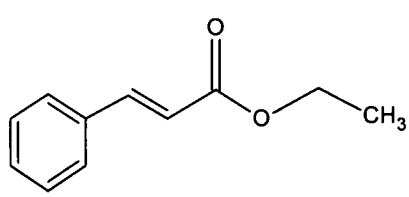


Zerumbone (**10**)

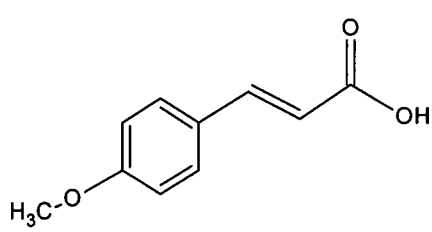
Figure 2.5: Chemical structure of zerumbone

2.7.3 *Kaempferia galanga*

The constituents of *Kaempferia galanga* rhizomes include cineol, borneol, 3-carene-5-one, camphene, kaempferol, kempferide, cinnamaldehyde ethyl cinnamate (**11**), *p*-methoxycinnamic acid (**12**) and ethyl *p*-methoxycinnamate (Kanjanapothi *et al.*, 2004; Chithra *et al.*, 2005). The rhizomes of *K. galanga* were extracted with petroleum ether and dichloromethane (CH_2Cl_2) using the Soxhlet extraction process. Othman *et al.*, (2006) characterized ethyl cinnamate (**11**) and *p*-methoxycinnamic acid (**12**) in greater percentage as well as other constituents of low percentage using GC-MS. Fractions isolated using CH_2Cl_2 as solvent exhibit vasorelaxant activity on the contracted smooth muscle of isolated rat aorta (Othman *et al.*, 2006).



Ethyl cinnamate (**11**)

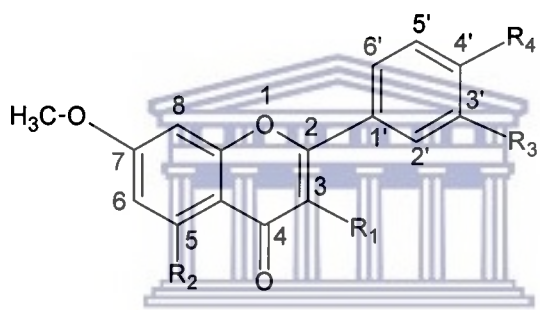


P-Methoxycinnamic acid (**12**)

Figure 2.6: Major constituents in *Kaempferia galanga*

2.7.4 *Kaempferia parviflora*

The following constituents have been isolated from *Kaempferia parviflora* (Yenjai *et al.*, 2004): 5-Hydroxy-3,7-dimethoxyflavone (**13a**, 0.2%), 5-Hydroxy-7-methoxyflavone (**13b**, 1.3%), 5-hydroxy-3,7,4'-trimethoxyflavone (**13c**, 0.08%), 5-hydroxy-7, 4'-dimethoxyflavone (**13d**, 0.3%), 5-hydroxy-3,7,3',4'-tetramethoxyflavone (**13e**, 1.5%), 3,5,7-trimethoxyflavone (**13f**, 0.14%), 3,5,7,4'-tetramethoxyflavone (**13g**, 0.6%), 5,7, 4'-trimethoxyflavone (**13h**, 1.6%) and 5,7, 3',4'-tetramethoxyflavone (**13i**, 1.01%). Compounds (**13h**) and (**13i**) exhibit antiplasmodial activity against *Plasmodium falciparum*, with IC_{50} values of 3.70 and 4.06 µg/ml, (**13g**) and (**13h**) possess antifungal activity against *Candida albicans* with respective IC_{50} values of 39.71 and 17.63 µg/ml.



Compound	R_1	R_2	R_3	R_4
13a	OCH_3	OH	H	H
13b	H	OH	H	H
13c	OCH_3	OH	H	OCH_3
13d	H	OH	H	OCH_3
13e	OCH_3	OH	OCH_3	OCH_3
13f	OCH_3	OCH_3	H	H
13g	OCH_3	OCH_3	H	OCH_3
13h	H	OCH_3	H	OCH_3
13i	H	OCH_3	OCH_3	OCH_3

Figure 2.7: Chemical structure of constituents isolated from *Kaempferia parviflora*

2.7.5 *Siphonochilus aethiopicus*

The literature gives limited information on phytochemical constituents and pharmacology aspects of *S. aethiopicus.* However, the essential oil has been found to contain compounds such as 1,8-cineole, α-terpineol, δ-3-carene (**14**), camphene, myrcene, β-elemene, γ-elemene, sabinene (**15**), β-pinene (**16**), phellandrene, (E)-β-ocimene, (Z)-β-ocimene, cis-alloocimene, trans-alloocimene (**17**) and the new sesquiterpene which is known as zingiberene (**18**) (Van Wyk *et al.*, 1997; Holzapfel *et al.*, 2002; Viljoen *et al.*, 2002). A furanoterpenoid is the major compound that has been found in both roots and rhizomes (Viljoen *et al.*, 2002); this represents 20% of the oil composition. The common name for this furanoterpenoid derivative has been suggested as siphonochilone.

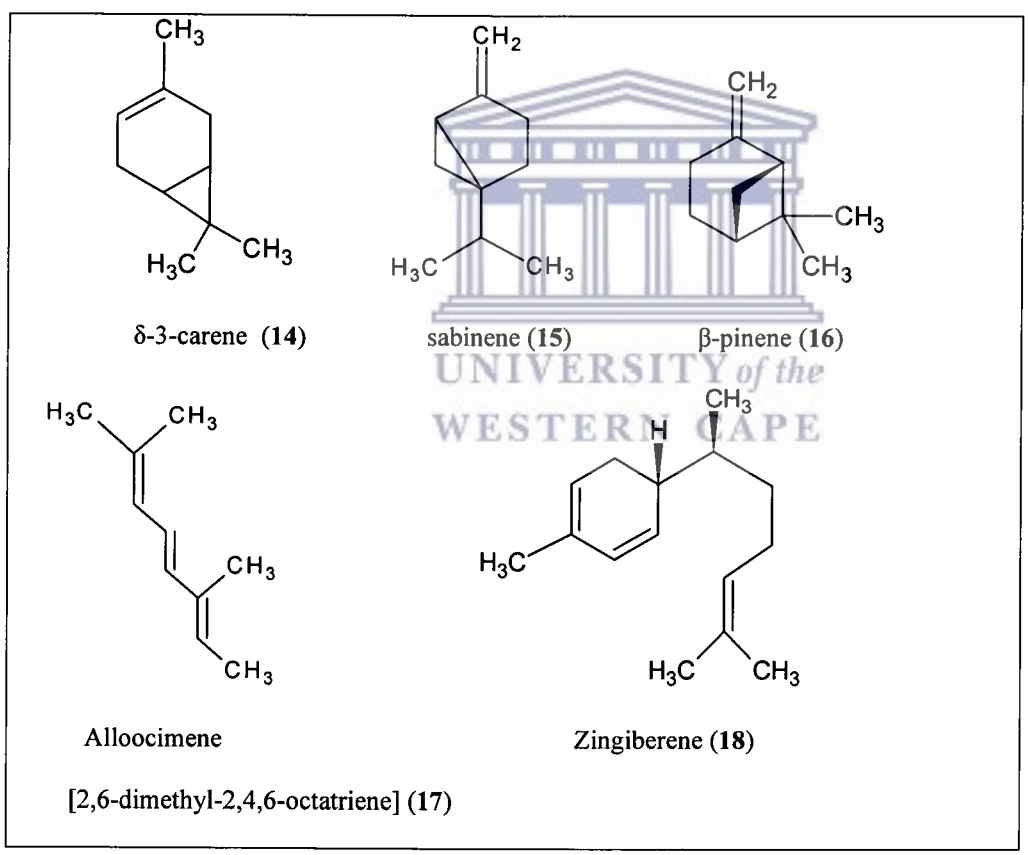
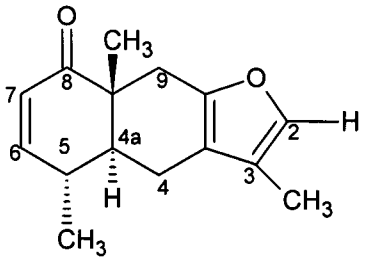
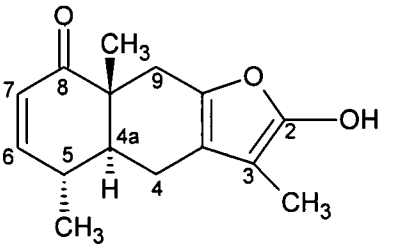


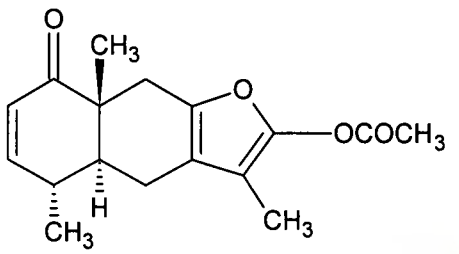
Figure 2.8: Some of the molecules found in *Siphonochilus aethiopicus.*



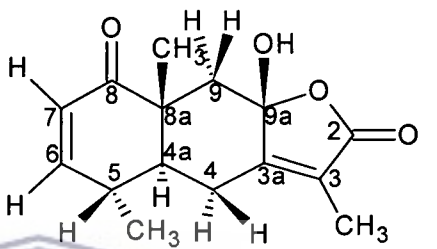
4aαH-3,5α,8aβ-trimethyl-4,4a,9-tetrahydro-naphtho[2,3-b]-furan-8-one (**19**)



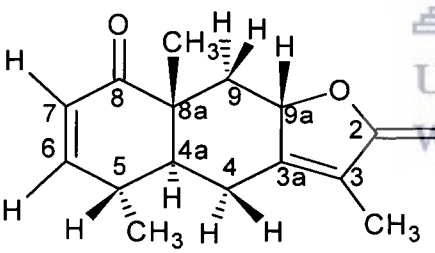
2-hydroxy-4aαH-3,5α,8aβ-trimethyl-4,4a,9-tetrahydronaphtho[2,3-b]-furan-8- one (**20**)



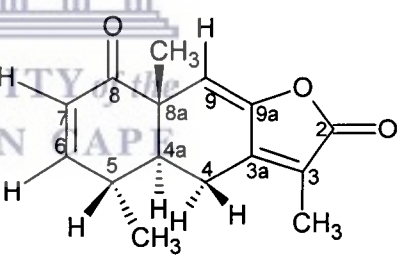
2-Acetoxy-4aαH-3,5α,8aβ-trimethyl-4,4a,9-tetrahydro-naphtho[2,3-b]-furan-8-one (**21**)



9aβ-hydroxy-4aαH-3,5α,8aβ-trimethyl-4,4a,8a,9-tetrahydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one (**22**)



4aαH-3,5α,8aβ-trimethyl-4,4a,8a,9-tetrahydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one (**23**)



4aαH-3,5α,8aβ-trimethyl-4,4a,8a-trihydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one (**24**)

Figure 2.9: Furanoterpenoid compounds found in *S. aethiopicus* reported by Holzapfel *et al.*, 2002 (19-21) and Lategan *et al.*, 2009 (22-24).

CHAPTER 3

AIM OF THIS STUDY

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AIM OF THE STUDY

3.1 HYPOTHESIS

Although both leaves and rhizomes of the *Siphonochilus aethiopicus* have been investigated before, there is a possibility of additional chemical constituents that may not have been detected, isolated and identified before. The possibility of distribution of some of the therapeutic compounds in both leaves and rhizomes cannot be overruled. Since the wellbeing of this plant is based on the availability of the rhizomes in the soil, harvesting of the rhizomes for medicinal application may lead to total extinction of this plant. It is hypothesized that the presence of such chemicals in the leaves could provide an alternative for similar therapeutic compounds and possibly additional compounds to those found in rhizomes thus leading to sustainability of the plant.

3.2 OBJECTIVES OF THE STUDY

(i) Isolation of the major compounds present in the organic extracts of *S. aethiopicus* plant

(ii) Explore any antibacterial/bioactivity exhibited by organic extracts.

(iii) Identification and characterization of the major compounds isolated using spectroscopic techniques.

(iv) Conduct a preliminary examination of the nature of the high molecular weight polysaccharides.

CHAPTER 4

METHODOLOGY

CHAPTER 4

METHODOLOGY

This chapter describes how the research was conducted. It outlines materials, chemicals and instrumentation. The uronic acid assay of the polysaccharide-rich fraction and the process of converting the polysaccharide-rich fraction into monosaccharide alditol acetates are also outlined here. The flow diagram for the extraction of rhizomes is shown in figure 4.1.

4.1 MATERIALS AND CHEMICALS

Analytical thin layer chromatography (TLC) was performed on aluminium plates coated with silica gel 60 F_{254} (Merck, *Germany*) whereas Preparative TLC was performed on 20 X 20 cm glass backed plates coated with silica gel of film thickness of 2 mm (*Sigma-Aldrich*). Column chromatography was carried out on silica gel 60 (0.063-0.200 mm). Solvents used were of analytical grade and were distilled before use.

4.2 INSTRUMENTATION

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Unity Inova 600 spectrometer (^{1}H at 600 MHz and ^{13}C at 150 MHz) and a Varian Gemini 200 spectrometer (^{1}H at 200 MHz and ^{13}C at 50 MHz). HPLC analysis was performed using an Agilent 1100 series instrument with a Diode Array Detector (DAD) and the column used was an Agilent (Eclipse XDB-C_{18}), 4.6 x 150 mm x 5µm. Gas Chromatography (GC) was performed using Agilent 7890A series. LC-MS analysis was performed using an Agilent 1200 series coupled to Agilent 6530 Accurate-Mass Q-TOF LC/MS. The column used was phenomenax (Kinetix PFP 100Å), 2.10 x 150 mm x 2.6 µm.

4.3 CHROMATOGRAPHY

4.3.1 THIN LAYER CHROMATOGRAPHY (TLC)

After elution, TLC plates were observed under UV (254 nm) and later sprayed with vanillin followed by heating on a hot plate for about 5 minutes.

4.3.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The mobile phase used was acetonitrile and 0.3% formic acid in water, mode: gradient increasing organic phase from 15% to 25% of acetonitrile over 15 minutes and running time of 33 minutes at a flow rate of 1 ml/min (Table 4.1). The UV scanning wavelength used was 254 nm. A sample of 10 µl was injected and the column temperature was 25°C (room temperature). Rutin was used for calibration purposes.

Table 4.1: HPLC gradient method

Time/ min	Acetonitrile	0.3% formic acid in H_2O
0	15	85
15	25	75 (Duration: 15 min)
33	Stop data	

4.3.2.1 *Sample preparation*

All samples were dissolved in their respective solvents to a concentration of 10 mg/ml. The samples were filtered prior to injection.

4.3.3 GAS CHROMATOGRAPHY (GC)

For GC analysis, the operational conditions were as follows: Oven temperature was at 60°C for 10 minutes and programmed to 220°C at a rate of 5°C per minutes. The temperature was then programmed to 240°C for 10 minutes at a rate of 10°C per minutes (Table 4.2). Injection temperature was 250°C and injection volume was 1 µl. Column description: Agilent HP 5: 30 m x 320 µm x 0.25 µm and the flow of carrier

gas (He) was 1.618 ml/min. The individual flow of gases used was as follows: H_2: 40 ml/min; Air: 400 ml/min; make up flow (N_2): 26 ml/min.

In: Front SS inlet and Out: Back detector FID

The Pressure of the system was 57.708 kPa and total flow was 36.952 ml/min and septum purge flow was 3 ml/min.

Table 4.2: GC method for hexane extracts

Rate °C/min	Temp (°C)	Hold time (min)	Run time (min)
	60	10	10
5	220	0	42
10	240	10	54

Table 4.3: GC method for the acetylated monosaccharide alditols derived from PFR sample

Isothermal temperature (°C)	Run time (min)
215	45

Column: DB-225 (30 m x 0.25 mm x 0.25 µm)

4.3.4 GC-MS CONDITIONS FOR HEXANE EXTRACT

GC conditions

Column: HP 5 MS (60m)

The operational conditions are indicated in Table 4.4

Table 4.4: GC-MS method for hexane extracts

Oven	Rate(°C/min)	Temp (°C)	Hold time (min)
Initial		60°C	5.00
Ramp1	5.0	220	0.00
Ramp2	10.00	280	10.00

MS conditions

Scan: 30 to 570 m/z

Solvent delay: 3min

4.3.5 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS)

The operational conditions were as follows: Column temperature was 30°C and the flow rate was 0.25 ml/min with injection volume of 1 µl. Mobile phase A (90% H_2O, 10% Acetonitrile and 0.1% formic acid) and Mobile phase B (90% Acetonitrile, 10% H_2O and 0.1% formic acid). The running time was 27.5 minutes. The UV scanning wavelength used was 254 nm. The operational method is indicated in Table 4.5.

Table 4.5: LC-MS gradient method

Time (min)	Mobile phase A (%)	Mobile phase B (%)
4	100	0
8	30	70
15	20	80
20	0	100
24	20	80
27.5	100	0

MS conditions
Ion source: APCI
Gas temperature: 250 °C
Vaporizer temperature: 350 °C
Gas flow: 6 l/min
Nebulizer: 15 psi

4.4 PLANT MATERIAL AND EXTRACTION

The plants were obtained from Kwazulu-Natal province, eThekwini municipality. One batch of rhizomes was acquired in November 2005 and a second batch in October 2007. The plants were separated into rhizomes and leaves. The plant parts (rhizomes and leaves) were washed thoroughly with water followed by milling and sequentially extracted (Figure 4.1) with hexane, dichloromethane, ethyl acetate, methanol and water for 24 hours. In a separate experiment, fresh leaves were extracted with 2 L of hexane, ethyl acetate and methanol for 24 hours in each case. Organic extracts were filtered using Whatman No 1 filter paper and the filtrate was concentrated to dryness at 40°C using a rotary evaporator (Labotech, *Germany*). The water extract was filtered using cotton wool, the filtrate was centrifuged at 3000 rpm for 10 minutes and the supernatant was freeze dried.

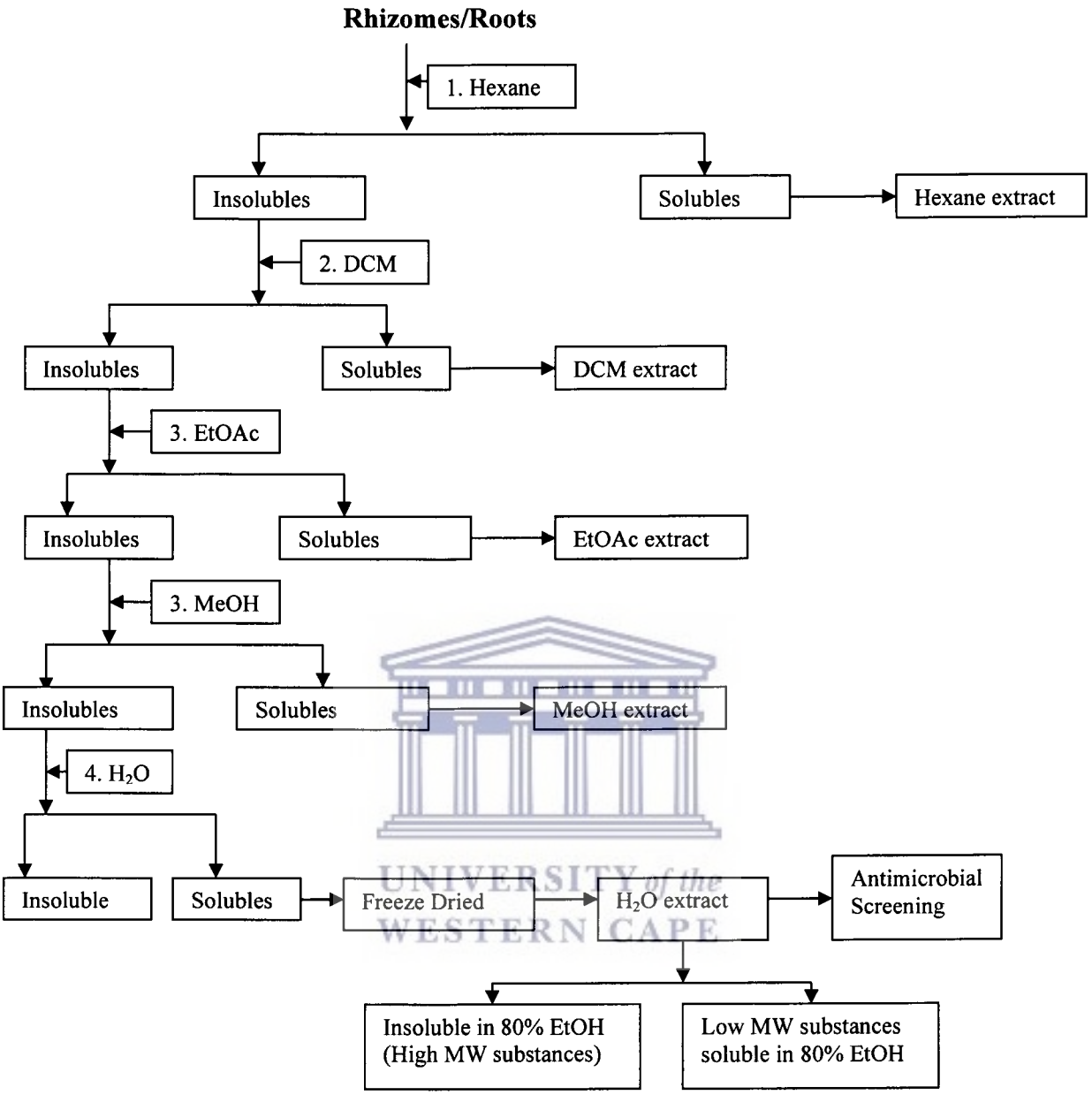

Figure 4.1: Flow diagram for the extraction of *S. aethiopicus* rhizomes

4.5 PHYTOCHEMICAL SCREENING OF *S. aethiopicus*

The plant material was screened for secondary metabolites such as alkaloids, anthraquinones and flavonoids. Phytochemical screening was performed using the method of Kabatende, 2005.

4.6 PARTIAL FRACTIONATION OF AQUEOUS EXTRACT

The freeze dried aqueous extract (14g) was re-dissolved in distilled water (20 ml) and stirred continuously, while 80 ml of absolute ethanol was added. The solution was stirred for another 15 minutes and centrifuged at 2000 rpm for 15 minutes. The supernatant was removed by decantation. The process was repeated six times until the precipitate was a lighter orange. The precipitate was washed by stirring with 60 ml of 80% ethanol, followed by centrifugation at 2000 rpm for 15 minutes. The supernatants (washings) were decanted and combined with the rest. The combined supernatants (aq)(EtOH extract) were evaporated to dryness and the yields were determined. The precipitate was reconstituted in 30 ml distilled water, frozen at -40°C and freeze-dried to obtain a brown powder.

4.7 MONOSACCHARIDE COMPOSITION OF POLYSACCHARIDE RICH FRACTION

A standard mixture of known monosaccharides: rhamnose, arabinose, xylose, mannose, galactose and glucose (5 mg each) was placed in a 50 ml conical flask and the mixture was dissolved in distilled water (3 ml). $NaBH_4$ (100 mg) was added to the solution. The contents were mixed by gentle swirling and allowed to stand overnight. The mixture was converted into alditol acetates similarly to the polysaccharide rich fraction as described below.

i) The polysaccharide rich fraction (50 mg) was hydrolysed with 2M trifluoroacetic acid (TFA) (2 ml) in a sealed Pyrex tube. The tube was heated in a water bath at 100°C for 18 hours. TFA was removed by successive addition and evaporation of methanol. The residue was reconstituted in 5 ml MeOH. 25% Aqueous ammonia solution (2 drops) was added to neutralise residual TFA and then $NaBH_4$ (100 mg) was added. The contents were mixed by gentle swirling and allowed to stand overnight. The reaction mixture was acidified to pH 6 with acetic acid followed by evaporation with MeOH. Acetic anhydride (3 ml) was added to the dried residue and the solution was heated at 100°C (steam bath) for 2 hours with occasional swirling. An ice-water mixture (30 ml) was added, followed by stirring for 30 minutes in order

to remove excess acetic anhydride. The reaction mixture was extracted with $CHCl_3$ (20 ml), and then the $CHCl_3$ layer was washed with distilled water (10 ml) three times. Anhydrous Na_2SO_4 was added to dry the $CHCl_3$ layer. Anhydrous Na_2SO_4 was filtered from the $CHCl_3$ layer.

ii) An aliquot of the reconstituted $CHCl_3$ was analysed using GC. Separation of the monosaccharide derivatives was achieved with a DB 225 capillary column (30 m x 0.25 mm internal diameter x 0.25 μm film thickness) 215°C isothermal temperature, FID detector at 300°C, injector temperature at 250°C and injector split 20:1. Flow rate was 2 ml/min. A $CHCl_3$ solution of the standard alditol acetates was injected in order to determine their retention times. The run time was 45 minutes (Table 4.3).

4.8 URONIC ACID ASSAY

The uronic acid content of the polysaccharide-rich fraction was determined by the carbazole assay. An aliquot of the polysaccharide solution (0.5 ml) at (100 μg/ml), standard solutions (5, 10, 20, 40, 50 μg/ml of uronic acid) and control (distilled water) were placed in a test tube. Reagent A (1.8 mg sodium tetraborate decahydrate; 20 ml distilled water; 180 ml of 98% concentrated H_2SO_4 that was cooled in ice) (3 ml) was added to each and mixed. The closed tubes were shaken gently at first and then vigorously with constant cooling in an ice bath. The test tubes were heated at 100°C for 10 minutes in a boiling water bath and then cooled rapidly in an ice-bath. Reagent B (100 mg carbazole; 100 ml absolute EtOH) (100 μl) was then added. The samples were re-heated at 100°C for 15 minutes, cooled rapidly to room temperature and the absorbance was measured at 525 nm.

4.9 ANTIMICROBIAL ANALYSIS

4.9.1 MICROORGANISMS

The organisms used were *Staphylococcus aureus* (ATCC 29213), *Mycobacterium smegmatis*, *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 10231). The cultures were provided by the Department of Medical Biosciences at the University of the Western Cape, South Africa. In this study, the extracts were tested against the microorganisms using the minimum inhibitory concentrations (MIC) method.

4.9.2 MINIMUM INHIBITORY CONCENTRATIONS

This is one of the measurements used to determine the minimum dose that inhibits growth of microorganisms. The MIC was determined by preparing serial dilutions of the aqueous extract. The concentrations of extracts prepared were 0.93, 1.87, 3.75, 7.5 and 15 mg/ml.

The microorganisms were inoculated into a 96 well plate and incubated at 37 °C for 24 hours except *Mycobacterium smegmatis* which was incubated for 48 hours as the organism grows more slowly. Dimethyl Thiazolyl Tetrazolim Bromide (MTT) or *p*-iodonitrotetrazolium violet (INT) was used for testing bacterial presence. Test reagent was prepared in water. Test reagent (20 µl) was then added into the microplate wells and incubated for a further 10-30 minutes. The change of suspension colour to red when using INT and change to blue when using MTT occurs as a result of no bacterial activity (Springfield, 2001). The wells which displayed no change in colour represent antibacterial activity (Light *et al.*, 2002).

CHAPTER 4

METHODOLOGY

This chapter describes how the research was conducted. It outlines materials, chemicals and instrumentation. The uronic acid assay of the polysaccharide-rich fraction and the process of converting the polysaccharide-rich fraction into monosaccharide alditol acetates are also outlined here. The flow diagram for the extraction of rhizomes is shown in figure 4.1.

4.1 MATERIALS AND CHEMICALS

Analytical thin layer chromatography (TLC) was performed on aluminium plates coated with silica gel 60 F_{254} (Merck, *Germany*) whereas Preparative TLC was performed on 20 X 20 cm glass backed plates coated with silica gel of film thickness of 2 mm (*Sigma-Aldrich*). Column chromatography was carried out on silica gel 60 (0.063-0.200 mm). Solvents used were of analytical grade and were distilled before use.

4.2 INSTRUMENTATION

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Unity Inova 600 spectrometer (^{1}H at 600 MHz and ^{13}C at 150 MHz) and a Varian Gemini 200 spectrometer (^{1}H at 200 MHz and ^{13}C at 50 MHz). HPLC analysis was performed using an Agilent 1100 series instrument with a Diode Array Detector (DAD) and the column used was an Agilent (Eclipse XDB-C_{18}), 4.6 x 150 mm x 5µm. Gas Chromatography (GC) was performed using Agilent 7890A series. LC-MS analysis was performed using an Agilent 1200 series coupled to Agilent 6530 Accurate-Mass Q-TOF LC/MS. The column used was phenomenax (Kinetix PFP 100Å), 2.10 x 150 mm x 2.6 µm.

4.3 CHROMATOGRAPHY

4.3.1 THIN LAYER CHROMATOGRAPHY (TLC)

After elution, TLC plates were observed under UV (254 nm) and later sprayed with vanillin followed by heating on a hot plate for about 5 minutes.

4.3.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The mobile phase used was acetonitrile and 0.3% formic acid in water, mode: gradient increasing organic phase from 15% to 25% of acetonitrile over 15 minutes and running time of 33 minutes at a flow rate of 1 ml/min (Table 4.1). The UV scanning wavelength used was 254 nm. A sample of 10 µl was injected and the column temperature was 25°C (room temperature). Rutin was used for calibration purposes.

Table 4.1: HPLC gradient method

Time/ min	Acetonitrile	0.3% formic acid in H_2O
0	15	85
15	25	75 (Duration: 15 min)
33	Stop data	

4.3.2.1 *Sample preparation*

All samples were dissolved in their respective solvents to a concentration of 10 mg/ml. The samples were filtered prior to injection.

4.3.3 GAS CHROMATOGRAPHY (GC)

For GC analysis, the operational conditions were as follows: Oven temperature was at 60°C for 10 minutes and programmed to 220°C at a rate of 5°C per minutes. The temperature was then programmed to 240°C for 10 minutes at a rate of 10°C per minutes (Table 4.2). Injection temperature was 250°C and injection volume was 1 µl. Column description: Agilent HP 5: 30 m x 320 µm x 0.25 µm and the flow of carrier

gas (He) was 1.618 ml/min. The individual flow of gases used was as follows: H_2: 40 ml/min; Air: 400 ml/min; make up flow (N_2): 26 ml/min.

In: Front SS inlet and Out: Back detector FID

The Pressure of the system was 57.708 kPa and total flow was 36.952 ml/min and septum purge flow was 3 ml/min.

Table 4.2: GC method for hexane extracts

Rate °C/min	Temp (°C)	Hold time (min)	Run time (min)
	60	10	10
5	220	0	42
10	240	10	54

Table 4.3: GC method for the acetylated monosaccharide alditols derived from PFR sample

Isothermal temperature (°C)	Run time (min)
215	45

Column: DB-225 (30 m x 0.25 mm x 0.25 µm)

4.3.4 GC-MS CONDITIONS FOR HEXANE EXTRACT

GC conditions

Column: HP 5 MS (60m)

The operational conditions are indicated in Table 4.4

Table 4.4: GC-MS method for hexane extracts

Oven	Rate(°C/min)	Temp (°C)	Hold time (min)
Initial		60°C	5.00
Ramp1	5.0	220	0.00
Ramp2	10.00	280	10.00

MS conditions

Scan: 30 to 570 m/z

Solvent delay: 3min

4.3.5 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS)

The operational conditions were as follows: Column temperature was 30°C and the flow rate was 0.25 ml/min with injection volume of 1 µl. Mobile phase A (90% H_2O, 10% Acetonitrile and 0.1% formic acid) and Mobile phase B (90% Acetonitrile, 10% H_2O and 0.1% formic acid). The running time was 27.5 minutes. The UV scanning wavelength used was 254 nm. The operational method is indicated in Table 4.5.

Table 4.5: LC-MS gradient method

Time (min)	Mobile phase A (%)	Mobile phase B (%)
4	100	0
8	30	70
15	20	80
20	0	100
24	20	80
27.5	100	0

MS conditions

Ion source: APCI

Gas temperature: 250 °C

Vaporizer temperature: 350 °C

Gas flow: 6 l/min

Nebulizer: 15 psi

4.4 PLANT MATERIAL AND EXTRACTION

The plants were obtained from Kwazulu-Natal province, eThekwini municipality. One batch of rhizomes was acquired in November 2005 and a second batch in October 2007. The plants were separated into rhizomes and leaves. The plant parts (rhizomes and leaves) were washed thoroughly with water followed by milling and sequentially extracted (Figure 4.1) with hexane, dichloromethane, ethyl acetate, methanol and water for 24 hours. In a separate experiment, fresh leaves were extracted with 2 L of hexane, ethyl acetate and methanol for 24 hours in each case. Organic extracts were filtered using Whatman No 1 filter paper and the filtrate was concentrated to dryness at 40°C using a rotary evaporator (Labotech, *Germany*). The water extract was filtered using cotton wool, the filtrate was centrifuged at 3000 rpm for 10 minutes and the supernatant was freeze dried.

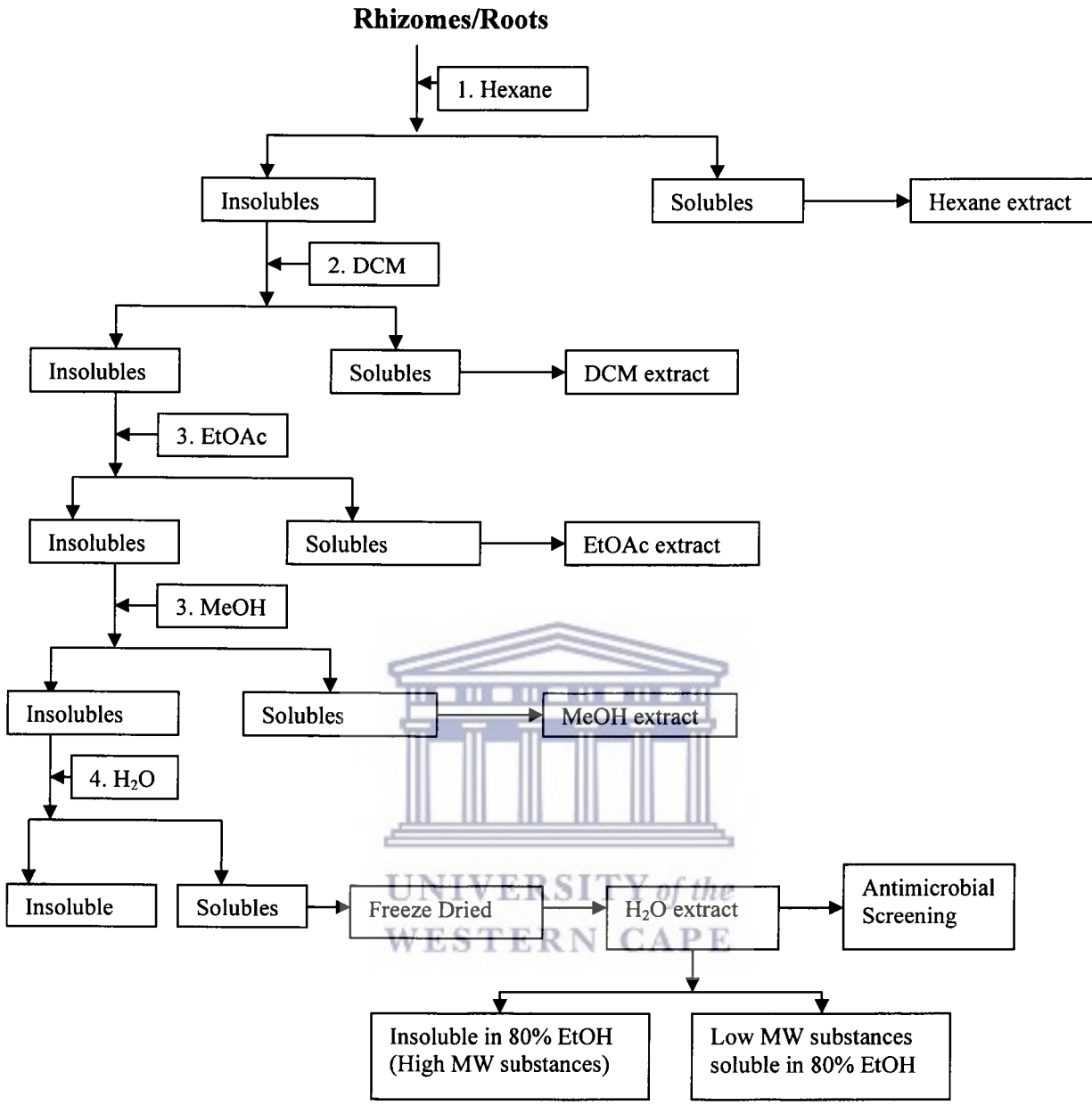

Figure 4.1: Flow diagram for the extraction of *S. aethiopicus* rhizomes

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The plant material was screened for secondary metabolites such as alkaloids, anthraquinones and flavonoids. Phytochemical screening was performed using the method of Kabatende, 2005.

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4.9 ANTIMICROBIAL ANALYSIS

4.9.1 MICROORGANISMS

The organisms used were *Staphylococcus aureus* (ATCC 29213), *Mycobacterium smegmatis*, *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 10231). The cultures were provided by the Department of Medical Biosciences at the University of the Western Cape, South Africa. In this study, the extracts were tested against the microorganisms using the minimum inhibitory concentrations (MIC) method.

4.9.2 MINIMUM INHIBITORY CONCENTRATIONS

This is one of the measurements used to determine the minimum dose that inhibits growth of microorganisms. The MIC was determined by preparing serial dilutions of the aqueous extract. The concentrations of extracts prepared were 0.93, 1.87, 3.75, 7.5 and 15 mg/ml.

The microorganisms were inoculated into a 96 well plate and incubated at 37 °C for 24 hours except *Mycobacterium smegmatis* which was incubated for 48 hours as the organism grows more slowly. Dimethyl Thiazolyl Tetrazolim Bromide (MTT) or *p*-iodonitrotetrazolium violet (INT) was used for testing bacterial presence. Test reagent was prepared in water. Test reagent (20 µl) was then added into the microplate wells and incubated for a further 10-30 minutes. The change of suspension colour to red when using INT and change to blue when using MTT occurs as a result of no bacterial activity (Springfield, 2001). The wells which displayed no change in colour represent antibacterial activity (Light *et al.*, 2002).

CHAPTER 5

RESULTS AND DISCUSSION

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5.1 EXTRACTION OF RHIZOMES

A sequential extraction of the rhizomes was followed and the yields of isolates obtained are as summarised in Table 5.1

The aqueous extract yielded higher percentages of isolates compared to the organic solvents (hexane, dichloromethane, ethyl acetate and methanol). The residues obtained from the extracts of both hexane and dichloromethane were close to each other. However, ethyl acetate yielded the lowest percentage of all the extracts.

Table 5.1: The extraction yields of rhizome

Solvents	Mass (kg)		yield (%)
	Starting material	Extracted material	
Hexane	2.8	0.024	0.86
Dichloromethane	2.8	0.020	0.71
Ethyl acetate	2.8	0.0045	0.16
Methanol	2.8	0.032	1.14
Water	2.8	0.042	1.50

The ethyl acetate extraction of the dried powdered rhizomes of *S. aethiopicus* conducted by Lategan *et al.*, 2009 yielded 3.5% of crude extract.

Partial fractionation of the aqueous extract as illustrated in section 4.6 provided results shown in Table 5.2

Table 5.2: The extraction yields of high MW and low MW fractions

Fractions	Mass (g)		yield (%)
	Starting material	Extracted material	
Low MW	14	3.76	26.86
High MW	14	8.30	59.29

MW represents molecular weight

5.2 ANTIMICROBIAL ANALYSIS

The antimicrobial analysis of the rhizome aqueous extract was carried out against Gram positive and Gram negative bacteria as well as fungi. The aqueous extract concentrations were prepared within the range 0.93 to 15 mg/ml.

Table 5.3: Antimicrobial activity of the rhizome aqueous extracts

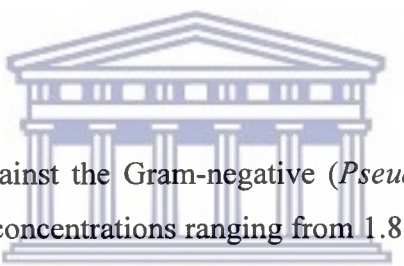
	Extracts (mg/ml)				
Microoganisms	0.93	1.87	3.75	7.5	15
Candida albicans	-	+	+	+	+
Staphylococcus aureus	-	-	-	+	+
Mycobacterium smegmatis	-	+	+	+	+
Pseudomonas aeruginosa	-	-	-	-	-

(+) Represent activity

(-) Represent no activity

In a previous study conducted by Light *et al.*, (2002), there was a slight loss of activity as a result of drying the rhizome material prior to extraction. This suggests that fresh rhizome may be more effective for medicinal use (Light *et al.*, 2002). In this study, the extraction was conducted using fresh rhizomes. The aqueous extract was tested in order to investigate whether it would show activity against bacteria. No organic extracts were tested because it has been reported that these possess a higher antibacterial activity (Light *et al.*, 2002).

The antibacterial activity results show that growth of the Gram-positive (*Staphylococcus aureus, Mycobacterium smegmatis*) bacteria were inhibited by the aqueous extract of *S. aethiopicus* at concentrations ranging from 7.5 to 15 mg/ml against *Staphylococcus aureus* and 1.87 to 15 mg/ml against *Mycobacterium smegmatis*.



There was no activity found against the Gram-negative (*Pseudomonas aeruginosa*) bacteria. Activity was shown at concentrations ranging from 1.87 to 15 mg/ml against the fungus *Candida albicans*. The aqueous extract in this study has been found to be more effective when tested against the Gram-positive bacteria than the Gram-negative bacteria we had available. In contrast, Light *et al.*, (2002) reported that in a micro dilution antibacterial assay, no activity was found in the aqueous extract of *S. aethiopicus* against the Gram-positive (*Bacillus subtilis, Staphylococcus aureus*) and Gram-negative (*Escherichia coli, Klebsiella pneumoniae*) bacteria. They did however, report that the EtOH and EtOAc extracts of *S. aethiopicus* exhibited greater antibacterial activity (ranging from 0.78 to 3.13 mg/ml) against Gram-positive bacteria than Gram-negative bacteria (Light *et al.*, 2002). In the latter study, plants were harvested in summer from the stock plants cultivated at the University of Natal Botanical Garden, Pietermaritzburg whereas in this study, the plants were harvested from the Silverglen nursery managed by the eThekwini municipality during November 2005 and October 2007. Change in climate does affect the composition of various constituents of plants, since global climate affects soil moisture, soil temperature and litter quality in ways that affect decomposition processes.

The difference in location could additionally result in varying the properties of plants since Pietermaritzburg is inland and Silverglen nursery is in the coastal region. Nitrogen fertilization typically leads to plant tissue having higher concentrations of nitrogen and lower concentrations of secondary metabolites. Alternatively, reduced radiation tends to depress plant carbon and to lower secondary metabolism (Ayres, 1993). Therefore, the difference in plant collection periods could result in a varying activity profile of the constituents. Most importantly, this could have resulted by performing the extraction of the rhizomes while still fresh.

5.3 PHYTOCHEMICAL ANALYSIS

Phytochemical screening of rhizomes was performed and the results indicated the presence of alkaloids, anthraquinones and flavonoids. Test controls (Table 5.4) were used in demonstrating positive results.

Table 5.4: Phytochemical composition of *S. aethiopicus* rhizome

Test	Result	Positive control
Alkaloids	+	*Catharanthus roseus*
Anthraquinones	+	*Cassia angustifolia* (Senna pods)
Flavonoids	+	Morin hydrate/ *Artimisia afra*

Key: + (Positive) means present, - (Negative) means absent

The results for the screening of selected secondary metabolites are shown in Table 5.4. Based on the literature, alkaloids are one of several classes of secondary metabolites found in plants. Alkaloids are pharmaceutically significant as exemplified by active compounds such as codeine for the treatment of coughs and quinine as an anti-malarial (Gurib-Fakim, 2006). Alkaloids have been found in rhizomes of *Zingiber officinale*, seeds of *Kaempferia galanga* and rhizomes of *Curcuma longa*, which all belong to the Zingiberaceae family (Chowdhury *et al.*, 2008). Flavonoid compounds have been isolated from *Kaempferia parviflora* and the leaves of

Etlingera elatior of the family Zingiberaceae. Flavonoids have been reported to possess multiple biological effects such as anti-inflammatory, analgesic, anti-tumour, anti-oxidant and immunostimulant (Gurib-Fakim, 2006; Barbosa *et al.*, 2007).

5.4 HPLC ANALYSIS (AGILENT 1100 SERIES)

HPLC screening of the extracts was performed in order to compare the compounds profiles of both rhizomes and leaves in the hexane, ethyl acetate and methanol extracts. Should the profiles show similarities between rhizomes and leaves, it will improve the sustainability of the plants since only leaves required to be harvested.

The results from HPLC screening of the crude extracts obtained from hexane, ethyl acetate and methanol of both rhizomes and leaves are shown below. Rutin was used for the purpose of calibration of the instrument and other peaks are due to impurities.

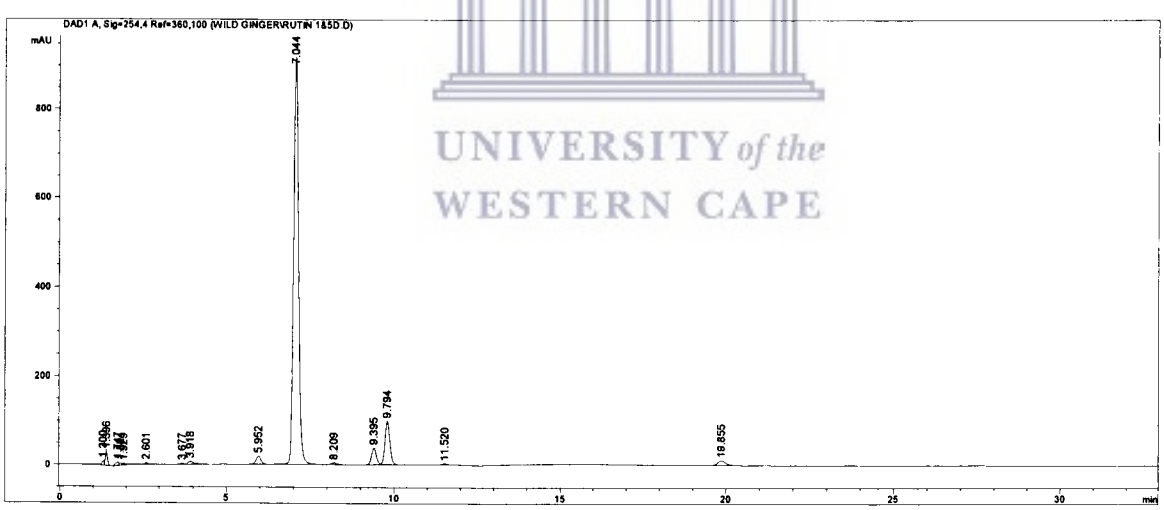


Figure 5.1: HPLC chromatogram of Rutin (Reference standard)

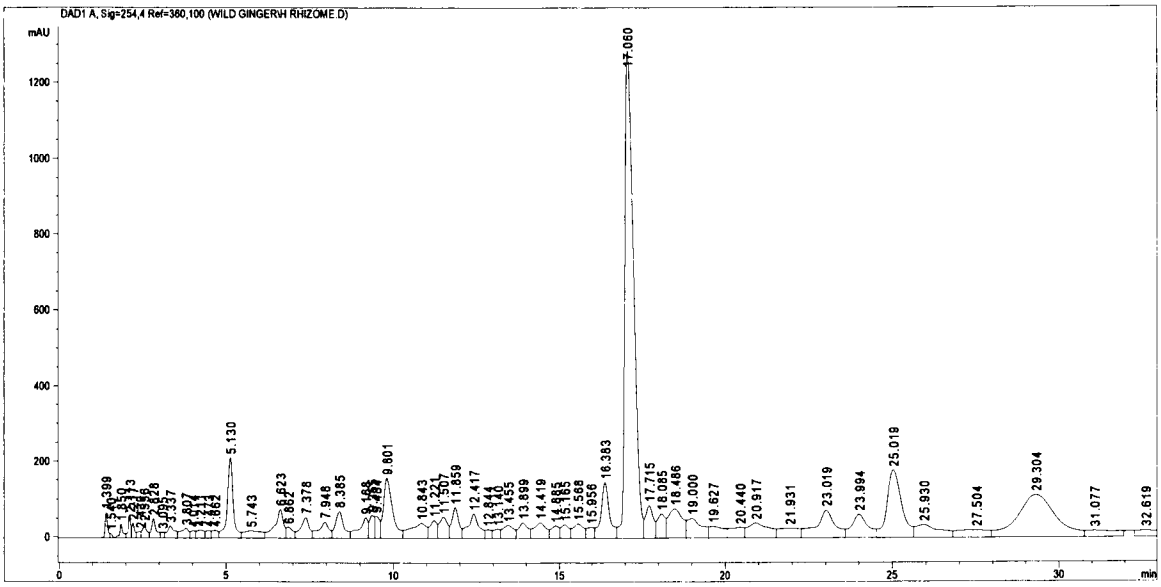


Figure 5.2: HPLC chromatogram of the hexane rhizome extracts

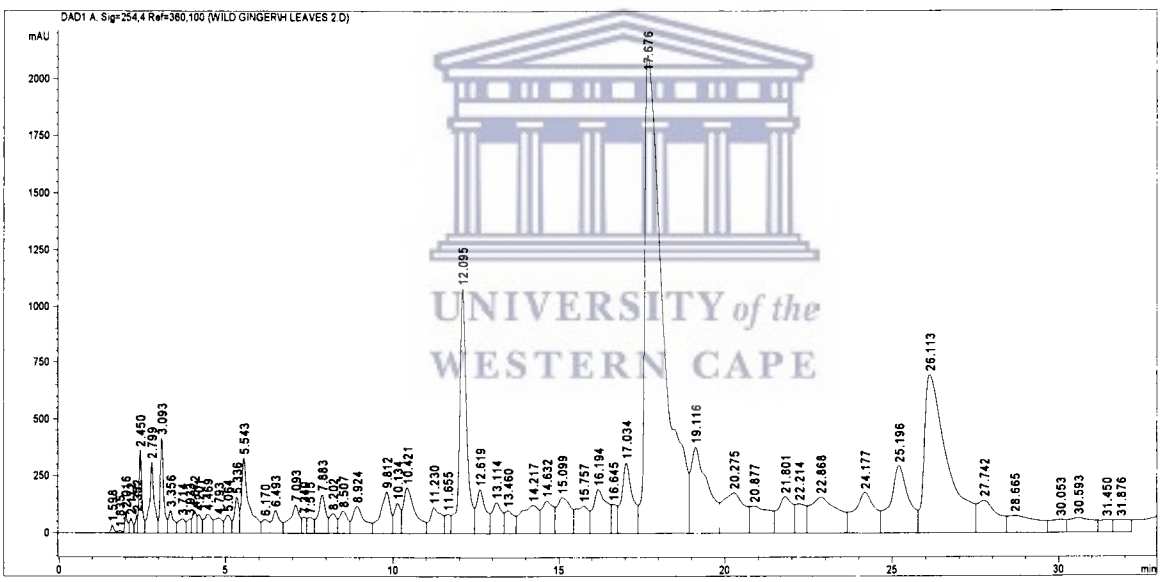


Figure 5.3: HPLC chromatogram of the hexane leaf extract

Figure 5.2 represents the chromatogram of the hexane rhizome extract analysed at wavelength 254 nm. The chromatogram shows one prominent peak at 17 minutes. Figure 5.3 represents the chromatogram of the hexane extracts of the leaves and clearly has a similar prominent peak at 17 minutes and additional peaks that are absent in Figure 5.2. In the interest of sustainability of the plant, leaves could be considered to be a suitable alternative for obtaining similar therapeutic compounds and possibly additional compounds which are absent from the rhizomes.

The chromatograms of the ethyl acetate extracts of the rhizomes and leaves are shown below.

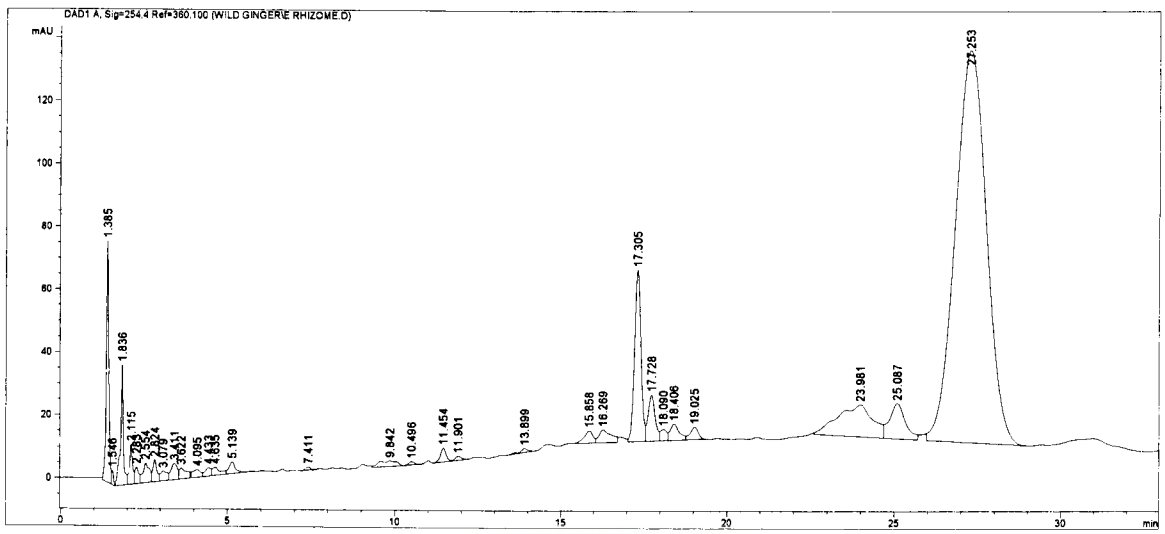


Figure 5.4: HPLC chromatogram of the ethyl acetate rhizome extracts

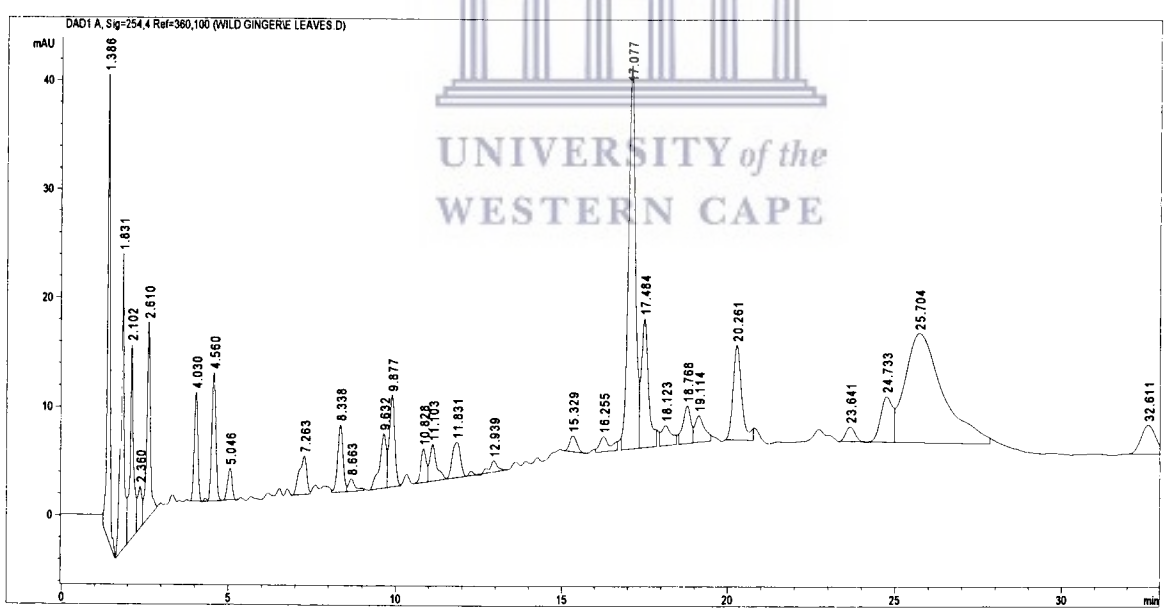


Figure 5.5: HPLC chromatogram of the ethyl acetate leaf extract

Figure 5.4 shows two prominent peaks at 17.30 and 27.25 minutes. Figure 5.5 shows a prominent peak at 17.07 and additional peaks at 20.26 and 25.70 minutes that are absent in Figure 5.4. The Leaf extract also had a peak at 17.48 which appears to be similar to the rhizome extract at 17.3 minutes.

The chromatograms of the methanol extracts of the rhizomes and leaves are shown below.

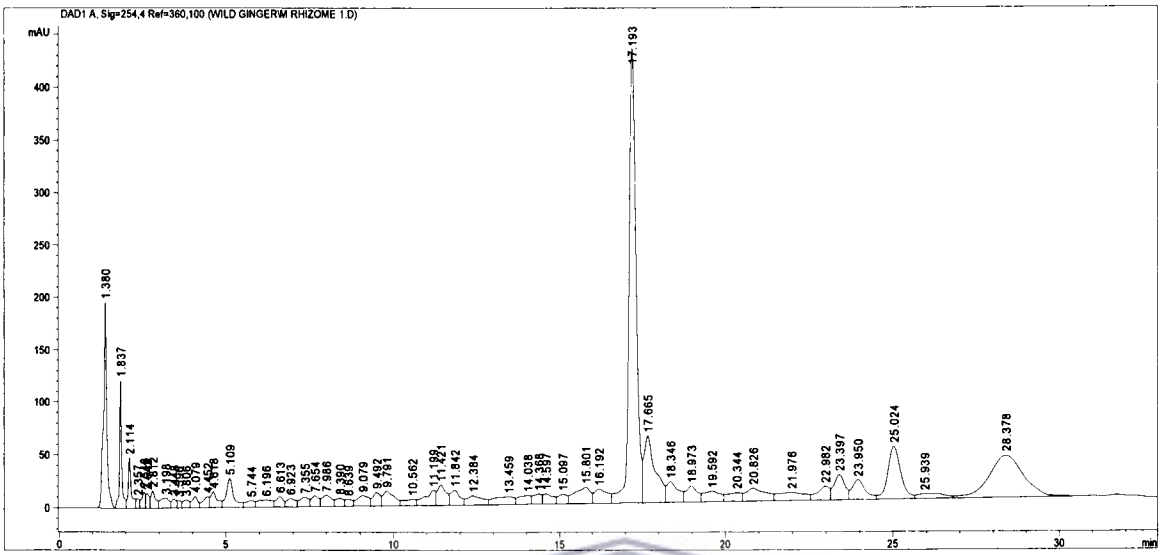


Figure 5.6: HPLC chromatogram of the methanol rhizome extracts

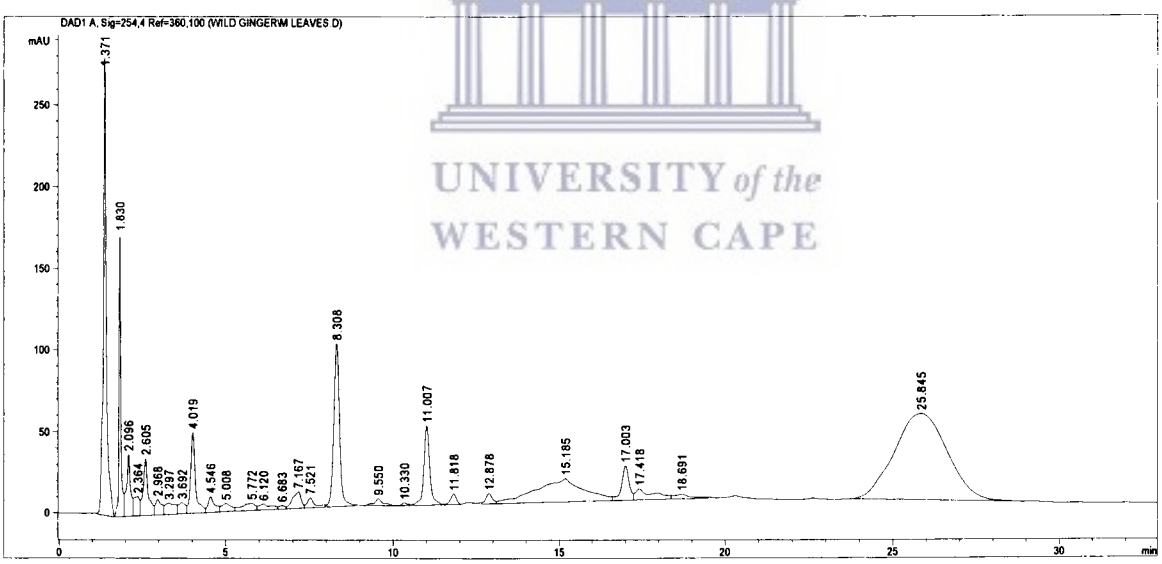


Figure 5.7: HPLC chromatogram of the methanol leaf extract

Figure 5.6 shows the prominent peak at 17.19 minutes which is found to be the minor peak at 17.00 minutes in Figure 5.7. Figure 5.7 show peaks at 4.01, 8.30, 11.00 and 25.84 that are absent in Figure 5.6. The minor peak shown at 17.00 minutes in Figure 5.7 could be that furanoterpenoids are deposited on the gland at the edges of the leaves.

Hexane and ethyl acetate were found to be the more suitable solvents for extraction of furanoterpenoids than methanol. Moreover, rhizomes seem to have a uniform distribution of the same compounds throughout.

5.5 GC ANALYSIS (7890A SYSTEM)

The hexane extract was analysed by GC since it was expected to contain essential oils. The dried sample was dissolved in DCM (5 ml) prior to analysis, and the resulting chromatogram is shown below (Figure 5.8).

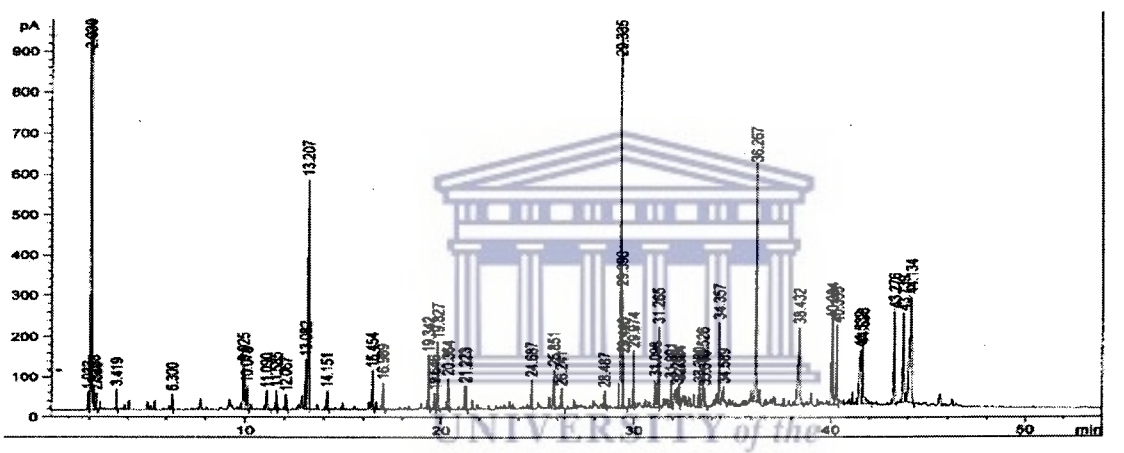


Figure 5.8: GC chromatogram of the hexane extract of *S. aethiopicus*

The identification of the essential oil constituents was achieved through further GC-MS analyses. Figure 5.9 shows the essential oils profile and the constituents found are listed in Table 5.5 together with their molecular weights.

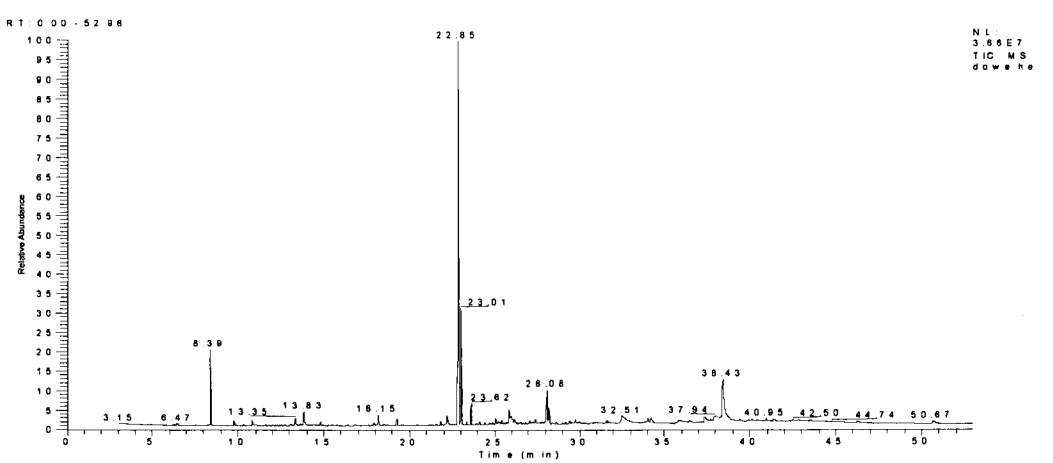


Figure 5.9: GC-MS chromatogram of the hexane extract of *S. aethiopicus*

1,8-cineole, α-terpineol, δ-3-carene, camphene, myrcene, β-elemene, γ-elemene, sabinene, β-pinene, phellandrene, (E)-β-ocimene, (Z)-β-ocimene, cis-alloocimene and trans-alloocimene were found in *S. aethiopicus* (Van Wyk *et al.*, 1997; Viljoen *et al.*, 2002) and such data has been compared with the data found in this study and also data found in other medicinal plants.

Table 5.5: GC-MS analysis of the essential oils from rhizome hexane extracts

Compound	t_R(min)	MW
1,8-Cineole	8.39	154
cis-4-thujanol (*cis*-sabinene hydrate)	9.77	154
Trans-4-thujanol	10.81	154
Terpinen-4-ol	13.35	154
α-terpineol	13.83	154
α-terpinyl acetate	18.15	196
β-elemene	19.26	204.35
γ- elemene	22.19	204.35
*1,3a-ethano(*1*H)inden-4-ol	23.01	222.34
Hedycaryol	23.33	222.36
β-eudesmol	25.84	222.36

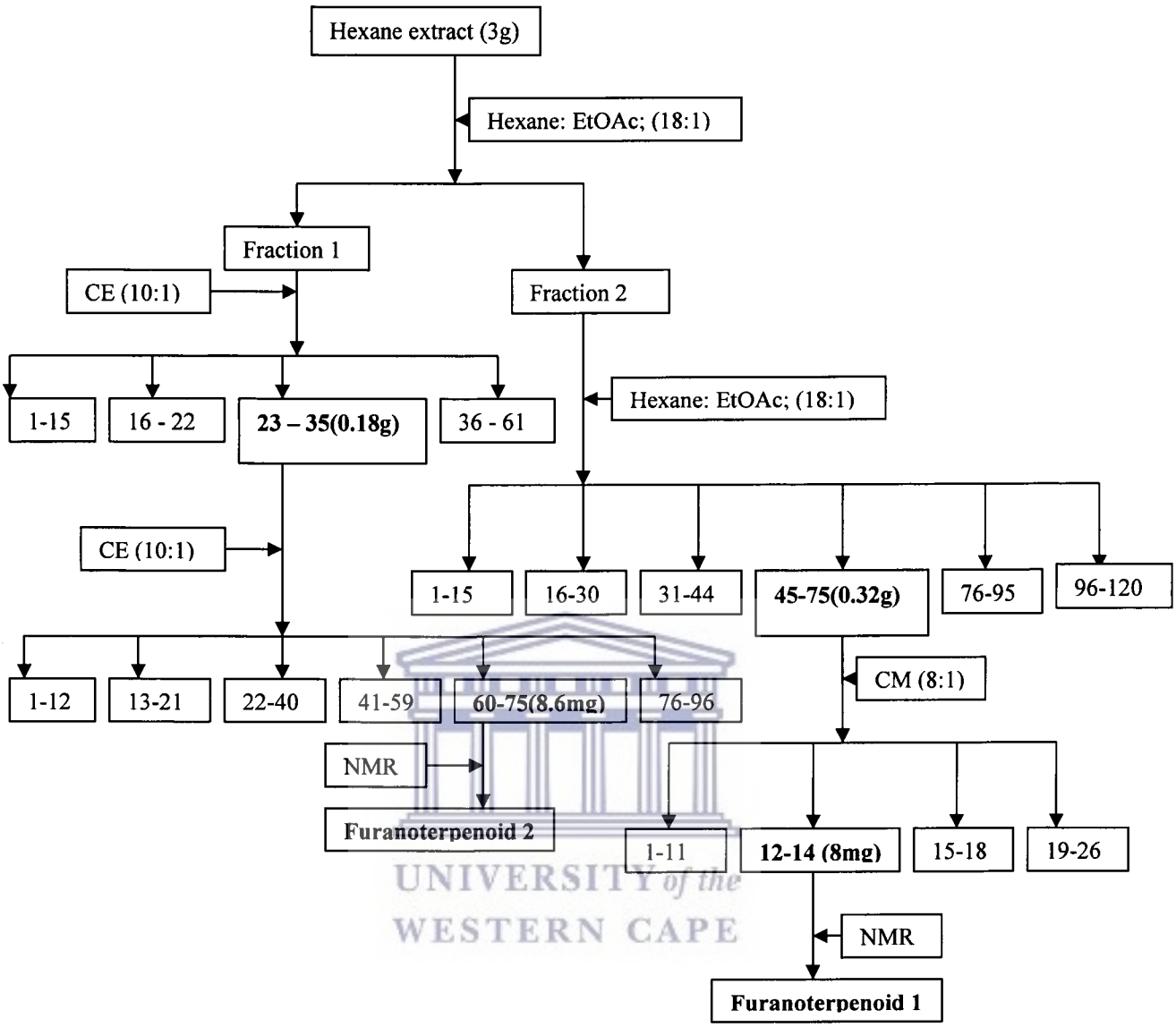
*1,3a-ethano(*1*H)inden-4-ol, octahydro-2,2,4,7a-tetramethyl-

All the essential oils given in the table above have previously been found to be contained in *S. aethiopicus* (Viljoen *et al.*, 2002). It is the first time in our knowledge that hedycaryol and 1,3a-ethano(*1*H)inden-4-ol, octahydro-2,2,4,7a-tetramethyl are being reported in this plant. 1,8-Cineole, α-terpineol and β-elemene have also been found in *Zingiber officinale* (commercial ginger) (Nigam *et al.*, 1964; Zancan *et al.*, 2002; Ma and Gang, 2006). The leaves and rhizomes of *Alpinia conchigera* Griff and *Aframomum corrorima* contain essential oils such as 1,8-Cineole, terpinen-4-ol, α-terpineol and β-elemene (Eyob *et al.*, 2008; Ibrahim *et al.*, 2009).

Based on the literature, 1,8-Cineole demonstrated activity against Gram positive and Gram negative bacteria (Oke *et al.*, 2009). Terpinen-4-ol was active against *Pseudomonas aeruginosa*, while α-terpineol was inactive against *P. aeruginosa* (Oke *et al.*, 2009). The minor essential oil constituents were not observed due to their extremely low concentration. Among the many constituents contained in the rhizomes and roots, a furanoterpenoid was the major compound representing as much as 20% of the oil composition.

5.6 ISOLATION AND STRUCTURAL ELUCIDATION OF FURANOTERPENOID 1

The flow diagram leading to the isolation of furanoterpenoids **1** and **2** is shown in Figure 5.10. Furanoterpenoid **1** has been characterised by NMR data (Table 5.6 and 5.7) and MS analysis which confirmed the chemical structure shown in (Figure 5.11).



CE represents $CHCl_3$ and EtOAc

CM represents $CHCl_3$ and MeOH

Figure 5.10: Flow diagram for the isolation of furanoterpenoid **1** and **2** using column chromatography

The hexane extract (3g) was fractionated using column chromatography on silica gel (75-230 mesh) and eluted with a mixture of hexane and ethyl acetate (18:1) to yield fractions 1 and 2. TLC of fraction 2 reflected a spot under UV (254 nm), with an R_f value of 0.25 and the spot gave a purple colour against vanillin spray. Fraction 2 was then rechromatographed to yield a fairly concentrated fraction of a residue (0.32g).

This was finally purified using an eluent comprising a mixture of $CHCl_3$ and MeOH (8:1) which led to the isolation of a pure compound (8 mg) which was dissolved in $CDCl_3$ for NMR analysis. This isolated pure molecule was assigned the structure of the known furanoterpenoid **1** and named 9aβ-hydroxy-4aαH-3,5α,8aβ-trimethyl-4,4a,8a,9-tetrahydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one (Table 5.6).

The crude fraction 1 was in turn rechromatographed and eluted with $CHCl_3$ and EtOAc (10:1) yielding a semi-pure fraction (0.18g) which was rechromatographed and purified by eluting with a mixture of $CHCl_3$ and EtOAc (10:1) which lead to the isolation of furanoterpenoid **2** (8.6 mg). This compound was UV (254 nm) active and gave a yellow colour against the vanillin spray. The above isolated compounds have been previously isolated (Lategan *et al.*, 2009). Furanoterpenoid **2** was dissolved in $CDCl_3$ for NMR analysis which established it to have the structure of the known furanoterpenoid **2** and named 4aαH-3,5α,8aβ-trimethyl-4,4a,8a,9-tetrahydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one (Table 5.8).

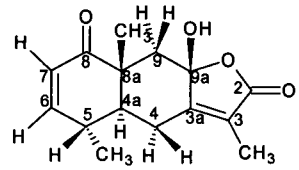


Table 5.6: NMR data of furanoterpenoid 1 in $CDCl_3$

Position	δ^1H, 200 MHz (ppm)	δ^{13}C, 50 MHz (ppm)
2	-	171.5
3	-	122.6
3a		158.0
4α	2.84 dd (13.6; 3.6 Hz)	24.1
4β	2.38 dd (13.8; 13.0 Hz)	
4a	1.60 ddd (9.0; 8.9; 3.6 Hz)	49.9
5β	2.50 tq (7.2; 2.5 Hz)	33.8
6	6.65 dd (10.0; 2.0 Hz)	153.3
7	5.87 dd (10.2; 2.8 Hz)	126.2
8	-	202.4
8a	-	44.8
9α	2.66 d (14.4 Hz)	43.9
9β	1.72 d (14.4 Hz)	
9a	-	103.1
3-Me	1.83 d (1.2 Hz)	8.3
5-Me	1.29 d (7.2 Hz)	18.2
8a-Me	1.35 s	16.7
9a-OH	3.23 br s	

The ^{1}H and ^{13}C NMR data obtained from the 200 MHz NMR spectrometer agreed well with previous data measured on a 600 MHz NMR spectrometer with a very close correlation (Lategan *et al.*, 2009). The very minor discrepancies may be due to the fact that no solvent information is given in the literature.

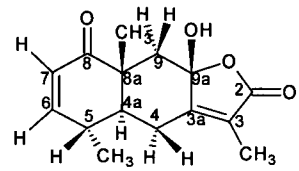


Table 5.7: NMR data of furanoterpenoid 1 in DMSO

Position	δ^1H, 600 MHz (ppm)	δ^{13}C, 150 MHz (ppm)
2	-	171.4
3	-	120.4
3a		159.9
4α	2.94 dd (13.2; 3.0 Hz)	20.7
4β	2.78 dd (13.8; 3.0 Hz)	
4a	1.60 ddd (9.0; 8.9; 3.6)	49.9
5β	2.50 tq (7.2; 2.5 Hz)	33.8
6	6.71 dd (10.2; 9.6 Hz)	155.4
7	5.74 dd (10.2; 9.6 Hz)	123.3
8	-	202.1
8a	-	44.6
9α	2.66 d (14.4 Hz)	43.9
9β	1.51 d (13.8 Hz)	
9a	-	103.3
3-Me	1.72 d (1.2 Hz)	7.9
5-Me	1.23 d (4.2 Hz)	22.0
8a-Me	1.29 s	19.2
9a-OH	3.32 br s	

Table 5.6 and 5.7 displayed NMR data of furanoterpenoid **1** as measured in $CDCl_3$ and DMSO-*d*6 respectively.

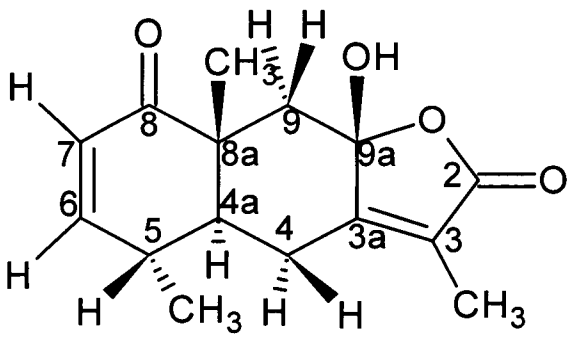


Figure 5.11: Chemical structure of 9aβ-hydroxy-4aαH-3,5α,8aβ-trimethyl-4,4a,8a,9-tetrahydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one.

In the HREIMS, it gave a $[M+1]^+$ ion at 263.1287 allowed one to deduce that the compound had a molecular formula as $C_{15}H_{19}O_4$ (calculated: 263.1283). An analysis of the ^1H, ^{13}C and 2D NMR spectra allowed the following deductions to be made. The molecule possesses an α,β-unsaturated ketone moiety, a furan unit, 3-methyl groups, 2 methylene groups and 2 methine carbons. A downfield pair of 1-proton double doublets at δ 6.65 and δ 5.87 are assigned to H-6 and H-7 of the α,β-unsaturated C-8 ketone in the ^1H NMR spectrum. The HMQC spectrum demonstrated coupling between H-6 and the C-5 CH_3 in addition to C-4a. H-5 was identified as a multiplet at δ 2.50 and was supported by coupling to C-4a and C-8a in the HMQC spectrum. A 3-proton doublet at δ 1.83 (J=1.2 Hz) was assigned to the C-3-Me and also shows coupling to C-2, C-3 and C-3a in the HMQC spectrum which supports the furanone ring structure. A ddd at δ 1.60 (J=9.0, 8.9 and 3.6 Hz) is assigned to H-4a which allowed the identification of the adjacent methylene hydrogens at C-4. The HMQC spectrum has demonstrated coupling between H-4a and C-8, C-8a and the C-8a-CH_3. A 3-proton doublet at δ 1.29 (J=7.2 Hz) was assigned to the C-5-Me. Support for this was evident in the HMQC spectrum which revealed coupling to C-4a, C-5 and C-6. Finally, NOESY correlations were observed between the C-8a-Me and H-5; between H-4a and H-9α; between C-5-CH_3 and H-4α; between C-8a-CH_3 and H-4β and between H-5 and H-4β. A comparison between the δ values of C-9a in the ^{13}C NMR spectra clearly establishes the position of the OH group at C-9a in furanoterpenoid **1** viz., δ 103.1 compared to δ 77.9 in furanoterpenoid **2**.

5.7 STRUCTURAL ELUCIDATION OF FURANOTERPENOID 2

The data of furanoterpenoid 2 is illustrated below.

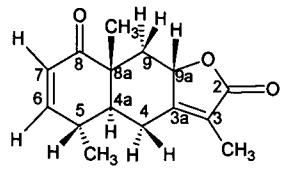


Table 5.8: NMR data of furanoterpernoid 2 in $CDCl_3$

Position	δ^1H, 200 MHz (ppm)	δ^{13}C, 50 MHz (ppm)
2	-	174.0
3	-	120.8
3a	-	159.6
4α	2.97 dd (14.1; 4.7 Hz)	25.7
4β	2.27 dd (14.1; 11.8 Hz)	
4a	1.62 dd (13.9; 4.3 Hz)	48.4
5β	2.30 (m)	34.2
6	6.64 dd (10.2; 2.1 Hz)	153.3
7	5.89 dd (10.2; 3.0 Hz)	126.3
8	-	201.7
8a	-	44.9
9α	2.78 dd (13.2; 6.2 Hz)	39.7
9β	1.63 dd (13.2; 4.0 Hz)	
9a	4.80 dd (11.6; 5.8 Hz)	77.9
3-Me	1.81 d (1.8 Hz)	8.3
5-Me	1.20 d (8.0 Hz)	18.2
8a-Me	1.24 s	15.8

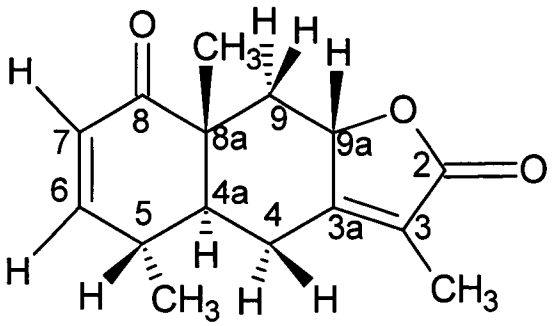


Figure 5.12: Chemical structure of 4aαH-3,5α,8aβ-trimethyl-4,4a,8a,9-tetrahydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one.

For furanoterpenoid **2**, the HREIMS gave a M^+ ion at 246.1256 which established a molecular formula of $C_{15}H_{18}O_3$ (calculated: 246.1260). This loss difference of 16 amu indicates that **1** and **2** differ in their structures at C-9a (shown in the ^{13}C spectrum), in which **1** has an OH while **2** has a H at that carbon attached. By and large the NMR spectra of furanoterpenoid **1** and **2** are quite similar as may be observed by comparing table 5.6 and 5.8. Discussion is therefore limited to the changes at C-9a. The first noticeable difference is in the ^{13}C NMR spectra in which C-9a resonates as a singlet at δ 77.9 in furanoterpenoid **2** compared to a singlet at δ 103.1 in furanoterpenoid **1**. A 1-proton dd at δ 4.80 (J =11.6 and 5.8 Hz) in the ^1H NMR spectrum is assigned to H-9a and is coupled, as shown in the COSY spectrum, to H-9α and H-9β, both resonating as dd's at δ 2.78 and δ 1.63 respectively. In the HMQC spectrum coupling was observed between H-9a and C-3a lending further credence to the structure. Finally a NOESY spectrum demonstrated correlation between H-9a and the clearly identifiable C-8a-CH_3 which confirmed its orientation to be β-in the furanone ring.

A third very minor fraction was obtained but the material was insufficient to purify for any spectral analyses.

5.8 LC-MS ANALYSIS (AGILENT 1200 SERIES/ 6530 Q-TOF LC-MS)

Based on the HPLC results obtained previously (5.4), LC-MS was performed in order to find the retention time of the corresponding mass of the isolated compounds as well as comparing the profiles of both rhizomes and leaves as their hexane, ethyl acetate and methanol extracts.

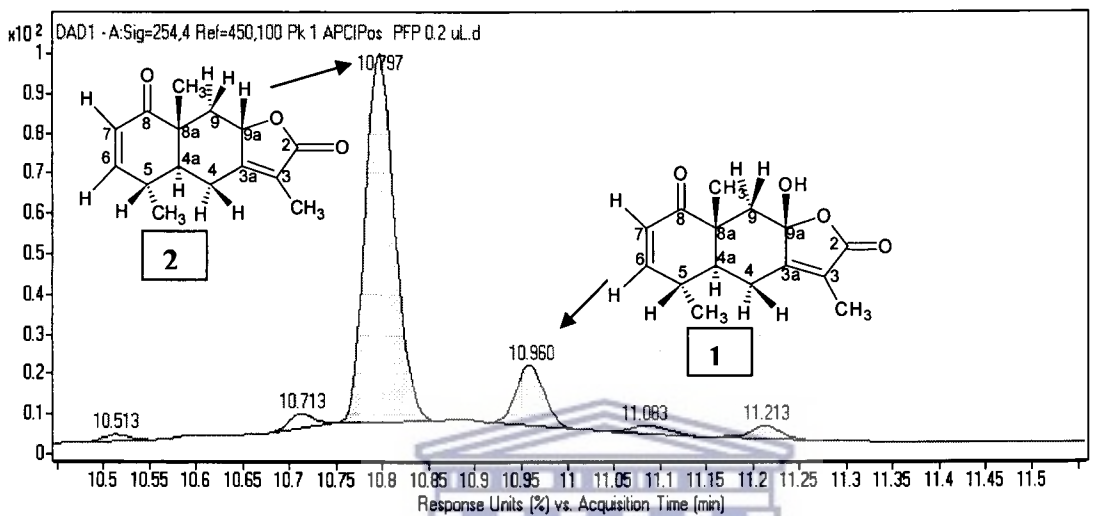


Figure 5.13: LC-UV chromatogram of furanoterpenoid 1 and 2 standard measured at 254 nm

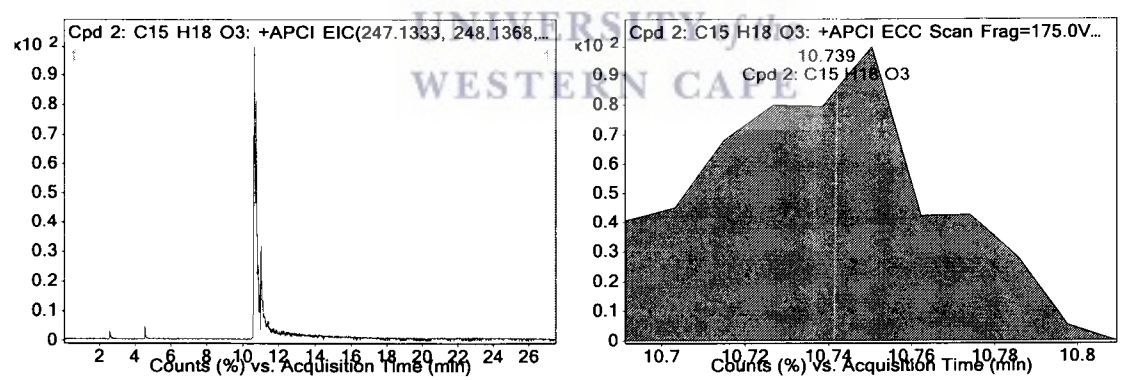


Figure 5.14: APCI chromatograms of empirical formula $C_{15}H_{18}O_3$ [**2**]

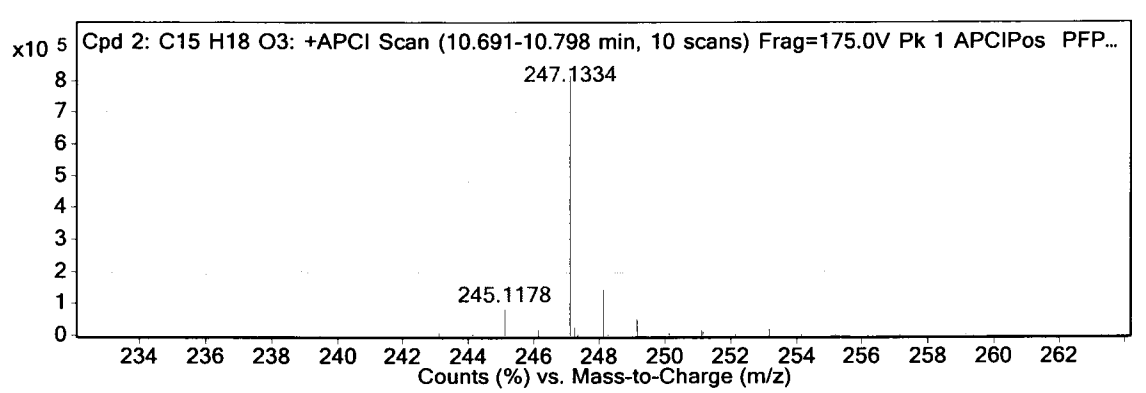


Figure 5.15: Mass spectrum of furanoterpenoid **2** showing $[M+1]^+$ at 247.1334

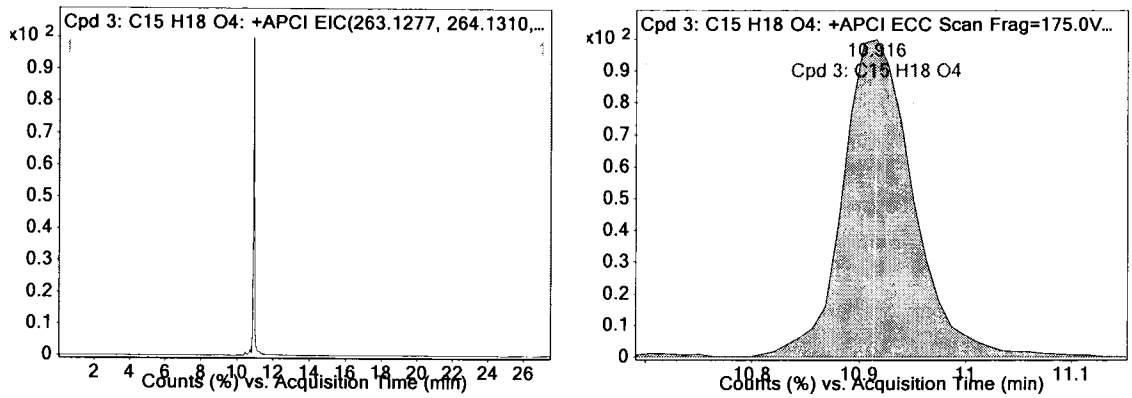


Figure 5.16: APCI chromatograms of empirical formula $C_{15}H_{18}O_4$ [1]

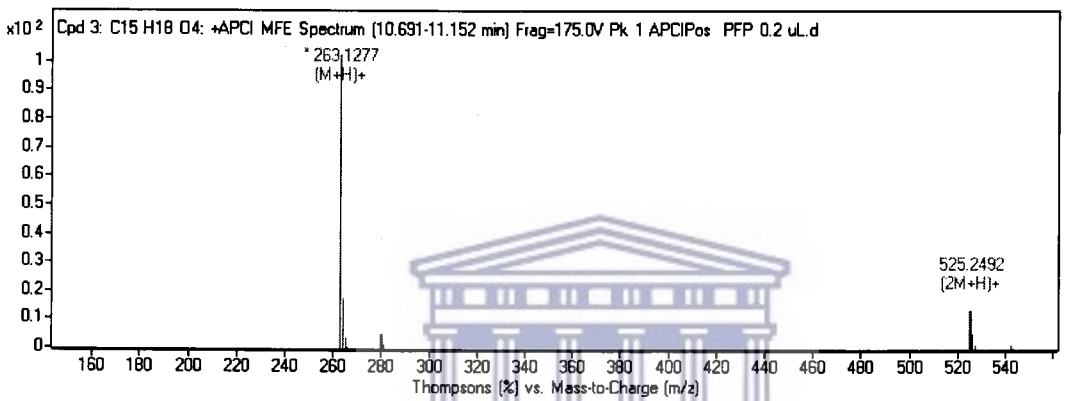


Figure 5.17: Mass spectrum of furanoterpenoid **1** showing $[M+1]^+$ at 263.1277

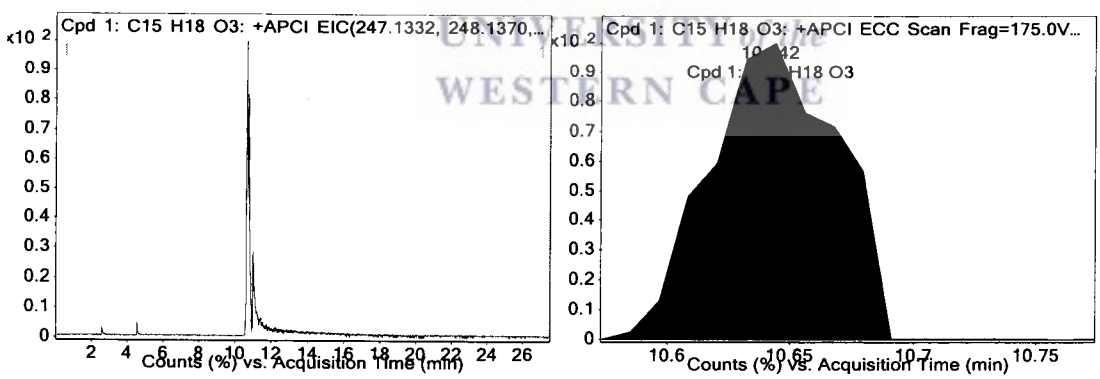


Figure 5.18: APCI chromatograms of empirical formula $C_{15}H_{18}O_3$

The total ion chromatograms of the standard mixture (Figure 5.14 and 5.16) shows the presence of the two compounds also isolated in this study (furanoterpenoid **1** and **2**) at retention times 10.797 and 10.960 respectively (Figure 5.13). The mass spectra of furanoterpenoids **1** and **2** are shown in figure 5.15 and 5.17. Figure 5.18 show the total ion chromatograms of empirical formula $C_{15}H_{18}O_3$ which occurs at retention time different from furanoterpenoid **2**.

This compound was found in figure 5.13 at retention time 10.713. The mass spectrum of this compound also show an $[M+1]^+$ ion at 247.1333. The compounds were established by the use of mass spectra and the APCI scans which gave the empirical formula relative to the retention time. Figure 5.15 show $[M+1]^+$ ion at 247.1334 which lead to the establishment of molecular formula as $C_{15}H_{18}O_3$. Figure 5.17 gave a $[M+1]^+$ ion at 263.1277 which established a molecular formula of $C_{15}H_{18}O_4$. The molecular weight of both compounds is in agreement with the previous data analysed on HREIMS.

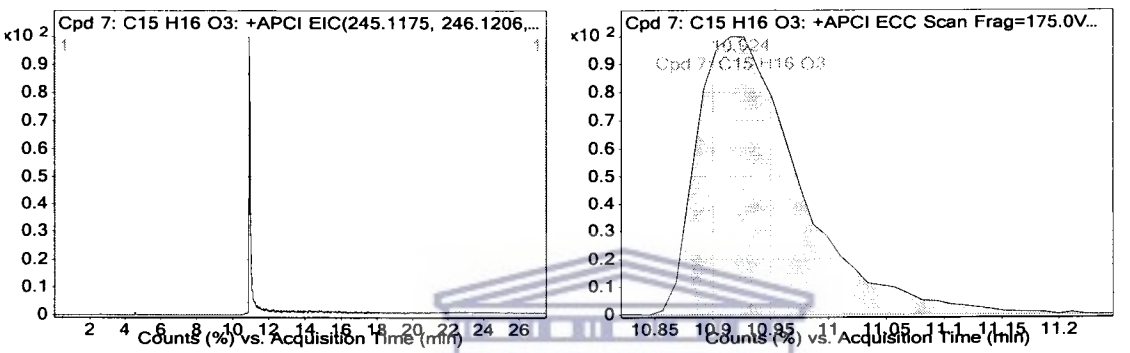
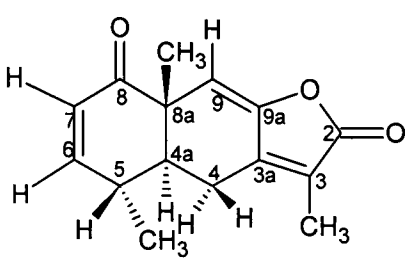


Figure 5.19: APCI chromatograms of empirical formula $C_{15}H_{16}O_3$ [**3**]

The total ion chromatogram (Figure 5.19) represent a compound that has not been isolated in this study, but has been reported previously (Lategan *et al.*, 2009). This compound conforms to the empirical formula of $C_{15}H_{16}O_3$ and the molecular weight calculated as 244.1102.



3

Figure 5.20: 4aαH-3,5α,8aβ-trimethyl-4,4a,8a-trihydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one

Figure 5.20 show the structure of compound whose data appear in figure 5.19 and its mass spectrum is given below (Figure 5.21).

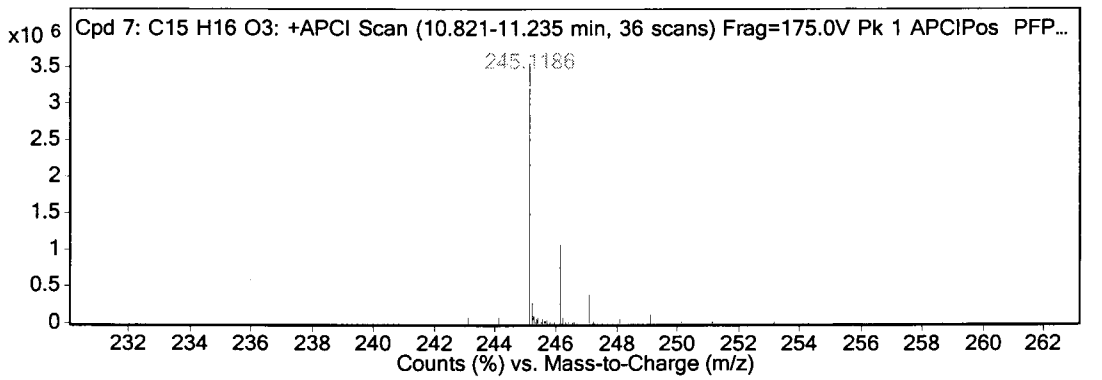


Figure 5.21: Mass spectrum of furanoterpenoid **3**

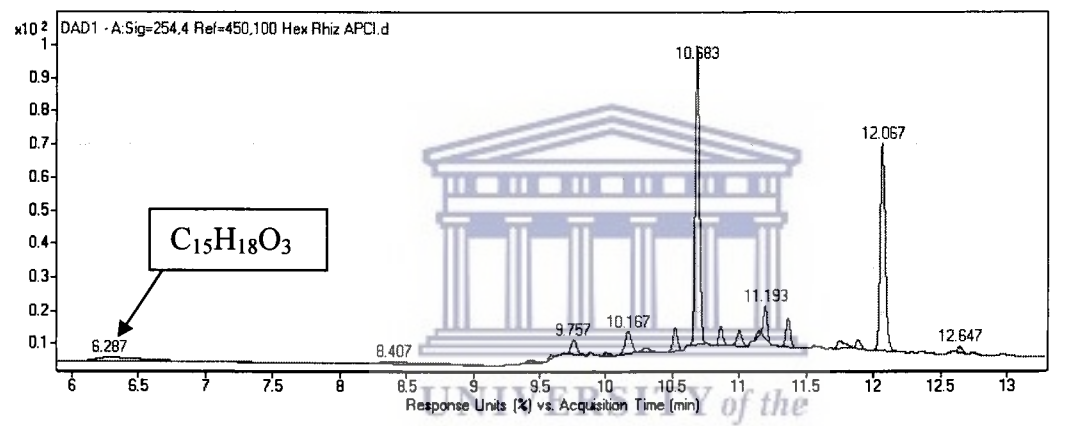


Figure 5.22: LC-UV chromatogram of hexane rhizome extracts

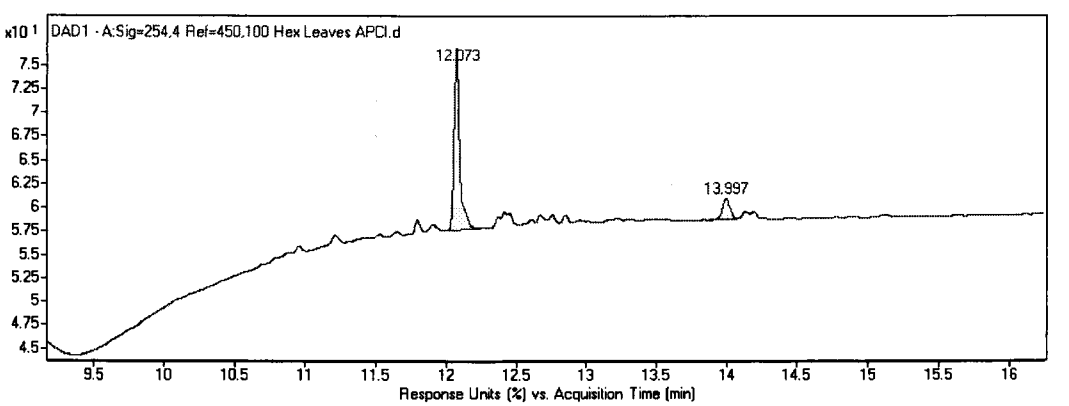


Figure 5.23: LC-UV chromatogram of hexane leaf extracts

The LC-UV chromatogram of hexane rhizome extract shows the presence of the two compounds isolated in this study (furanoterpenoid **1** and **2**) at the retention time of 10.683 and 11.193 respectively (Figure 5.22).

The very minor compound was found at the retention time of 6.28 min corresponding to an empirical formula of $C_{15}H_{18}O_3$. This compound has an empirical formula similar to furanoterpenoid **2**. One of the isomeric compounds isolated by Holzapfel *et al.*, (2002) had the empirical formula $C_{15}H_{18}O_3$ and which had the structure illustrated in figure 2.9 (ii). Therefore, the hexane rhizome extracts have two compounds with the same empirical formula, viz; $C_{15}H_{18}O_3$ but with different retention times. Furanoterpenoid **3** was found at a retention time of 12.067 min and which had the empirical formula $C_{15}H_{16}O_3$. Figures 5.22 and 5.23 shows the presence of furanoterpenoid **3** at the retention time of 12.07 min. Furanoterpenoid **3** is noted as the only prominent peak found in hexane leaf extracts. Rhizome extracts contain a prominent peak and other additional peaks that were not found in leaves. The structures of furanoterpenoid **1**, **2** and **3** were established by the use of mass spectra and the APCI scans which gave the empirical formulae relative to the retention time. The mass spectra and the total ion chromatograms confirm the above compounds (Appendix E).

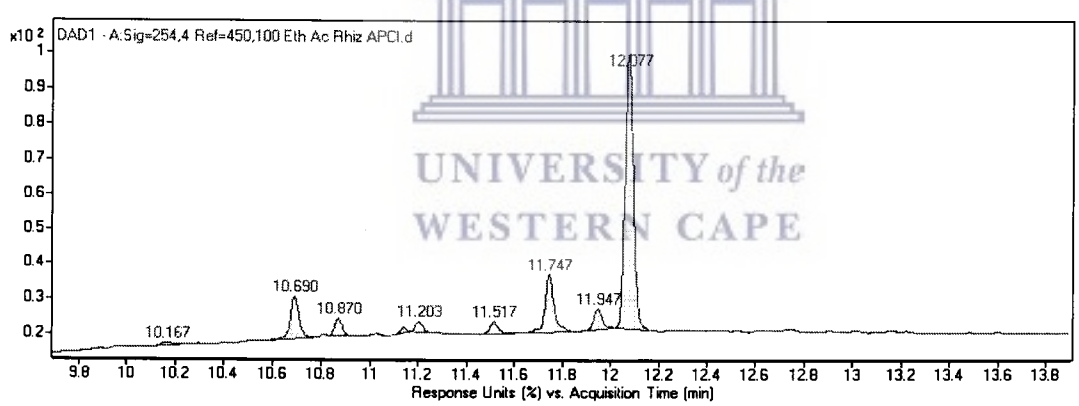


Figure 5.24: LC-UV chromatogram of ethyl acetate rhizome extracts

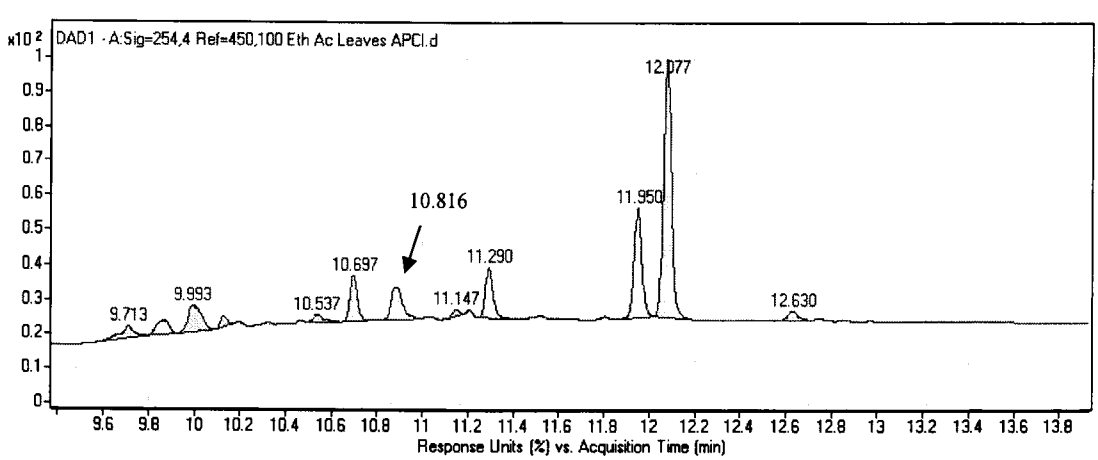


Figure 5.25: LC-UV chromatogram of ethyl acetate leaf extracts

Figure 5.24 and 5.25 shows the presence of furanoterpenoid **2** at the retention time of 11.517 and 11.29 min. The retention time signals at 12.07 min indicate the presence of furanoterpenoid **3** with isomeric compound at the retention time range of 10.82-10.87 min. However, furanoterpenoid **1** was not found in any of the ethyl acetate extract. Both rhizome and leaves chromatograms are similar to each other, but the rhizome extract contain an additional peak at the retention time of 11.747 min. The compounds were established by the use of mass spectra and the APCI scans which gave the empirical formula relative to the retention time. The APCI analysis gave the confirmation of the presence of furanoterpenoid **2** in ethyl acetate extract. The total ion chromatogram and the mass spectrum of furanoterpenoid **2** are given in appendix E.

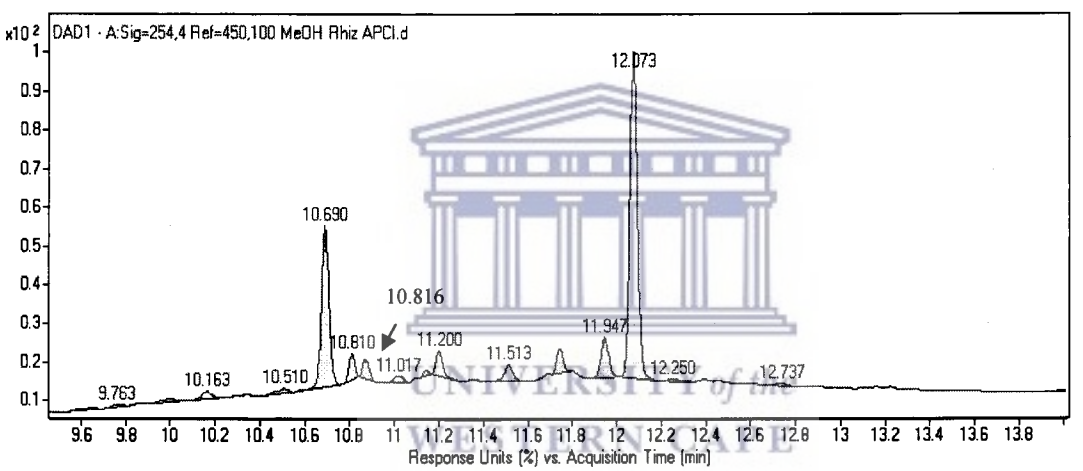


Figure 5.26: LC-UV chromatogram of methanol rhizome extracts

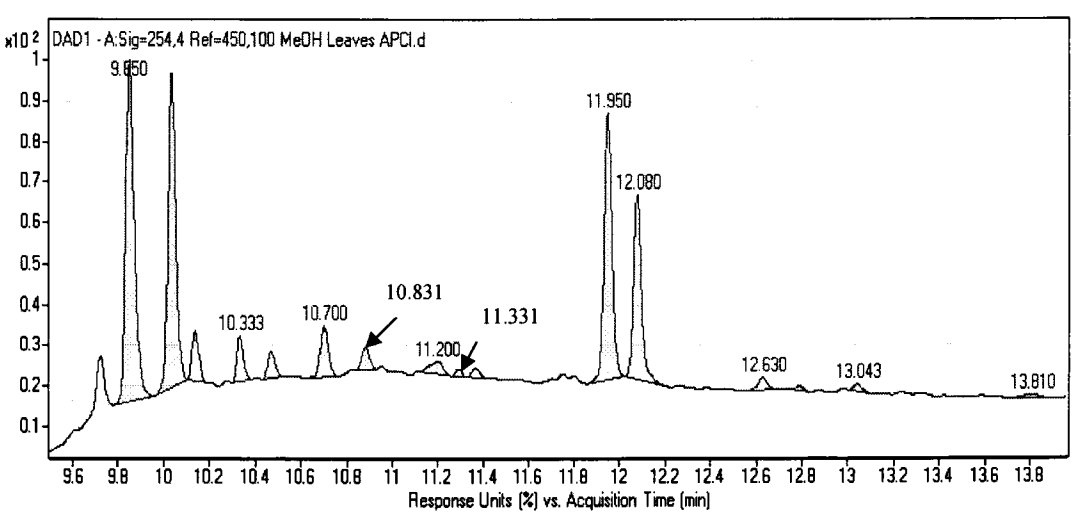
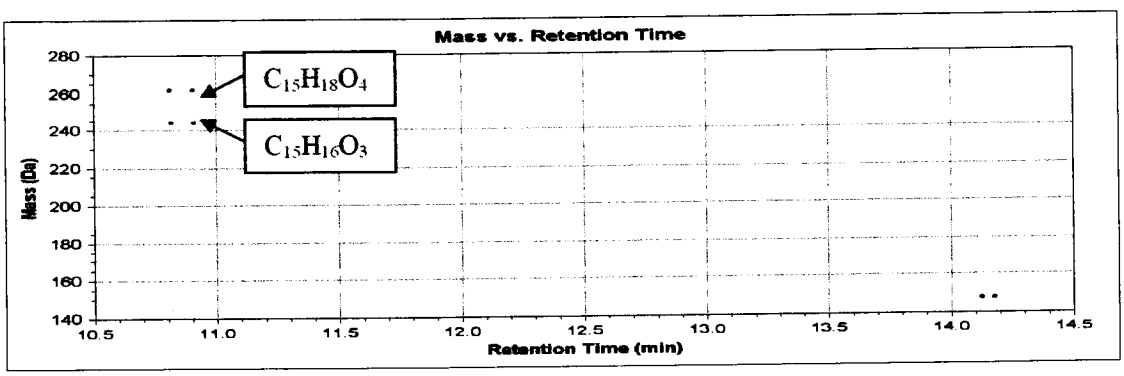


Figure 5.27: LC-UV chromatogram of methanol leaf extracts

The LC-UV chromatogram of the methanol rhizome extract shows the presence of two compounds (furanoterpenoid **1** and **3**) at retention times 10.810 and 12.07 respectively. Methanol rhizome and leaf extracts have two compounds with the same empirical formula, viz; $C_{15}H_{16}O_3$ but with different retention times. Therefore, the compounds at retention times of 10.83 and 12.07 are isomeric which represent furanoterpenoid **3**. Figure 5.26 and 5.27 shows the presence of furanoterpenoid **2** at the retention time of 11.20. Furanoterpernoid **1** was not found in methanol leaves extract and this could indicate that such compound is mainly found in the rhizome extracts. The compounds were established by the use of mass spectra and the APCI scans which gave the empirical formula relative to the retention time (Appendix E). However, rhizome hexane extracts contain all compounds (furanoterpenoids **1**, **2** and **3**).

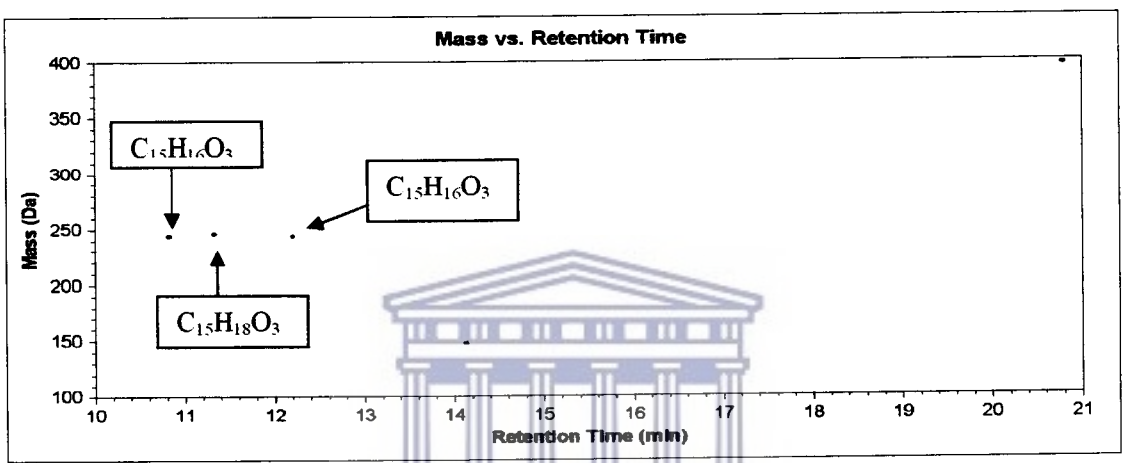
The prominent peak shown in all LC-UV chromatograms at retention time 12.07 appears to be furanoterpenoid **3**. This relates well with HPLC chromatograms, where one prominent peak was shown at retention time of 17 min. All HPLC chromatograms except for the methanol extract of leaves have shown this prominent peak. Considering the fact that different column, percentage of formic acid and ratio of the mobile phase were used in HPLC, the prominent peaks appear to be found in both HPLC and LC-UV chromatograms. Therefore, HPLC can be used as a base for the quality control purposes.



Rhizome = red and standard = blue

Figure 5.28: The molecular mass of furanoterpenoid **1** and **3** against retention time of methanol rhizome extract and standard

The Agilent mass hunter profiling software based on accurate Q-TOF MS data gave the molecular weight against the retention times of the methanol rhizome extract and standard. Figure 5.28 shows two compounds with molecular weights of 244.1102 and 262.1204 associated with retention times of 10.80 and 10.90 min respectively. The mass of 244.1102 and 262.1204 corresponded to the empirical formulae of $C_{15}H_{16}O_3$ and $C_{15}H_{18}O_4$ respectively. The presence of the compounds was compared between rhizome extract and standard despite the fact that there was a shift in retention time by 0.1 min. The molecular mass confirmed the similarity of the compounds between rhizome extract and standard.



Leaves = red and rhizome = blue

Figure 5.29: The molecular mass of furanoterpenoid **2** and **3** against retention time of methanol leaves and rhizome extracts

Leaves and rhizomes contain both compounds as indicated by both dots overlaying each other at retention time 10.82 and 11.32 min. Leaves contain two compounds with empirical formula $C_{15}H_{16}O_3$ at retention times of 10.82 and 12.19 min as well as $C_{15}H_{18}O_3$ at 11.32 min. The structures of the molecules having the same empirical formula $C_{15}H_{16}O_3$ could be due to two isomeric molecules. The mass hunter profiling can be used as a qualitative comparison between rhizome and leaf extracts.

5.9 MONOSACCHARIDE COMPOSITION OF WATER SOLUBLE POLYSACCHARIDE-RICH FRACTION

As mentioned before, polysaccharides isolated from various traditional medicinal plants have shown effects on the immune system, viz., immunomodulatory and antitumor effects. It was deemed necessary to determine the monosaccharide composition of the water soluble fraction to identify a possible contribution from this source.

5.9.1 URONIC ACID ANALYSIS

The uronic acid content was determined using the carbazole assay.

Table 5.9: Glucuronic acid standard curve values

Concentration (µg/ml)	Absorbance (525 nm)
0	0
5	0.026
10	0.067
20	0.117
40	0.245
50	0.307
Sample	0.075

The sample analysis demonstrated absorbance of 0.075 measured at wavelength of 525 nm. The uronic acid concentration of the sample was determined from the glucuronic acid standard curve.

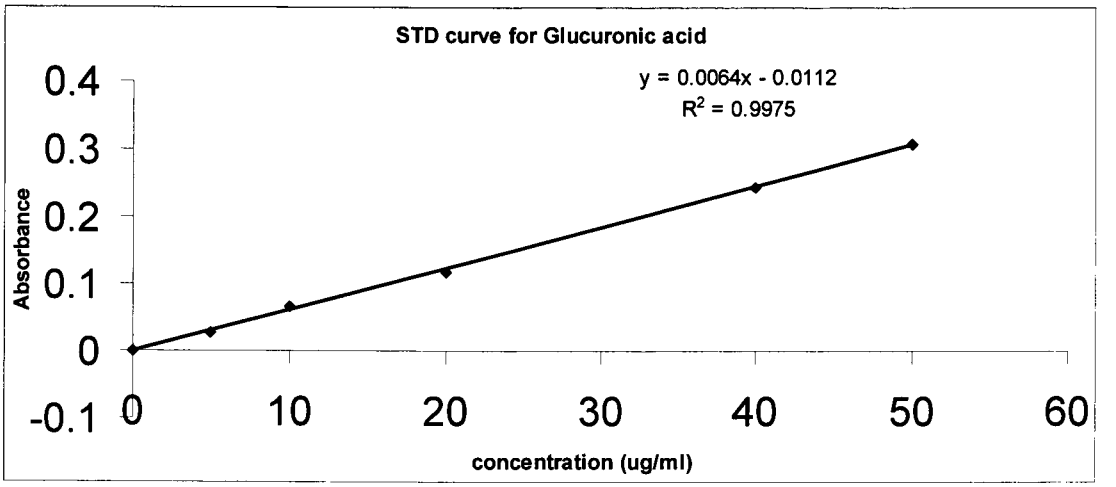


Figure 5.30: Glucuronic acid standard curve.

Using the formula: y = mx + c, where **m** is a slope, **c** is intercept and **y** is absorbance of a sample. The **x** value is the concentration of a sample.

y = 0.075, m = 0.0064, c = -0.0112

Therefore: 0.075 = 0.0064x – 0.0112

0.0862 = 0.0064x

13.468 = x

The sample concentration obtained is 13.468 µg/ml.

Uronic acid content = (13.468 µg/ml uronic acid/100 µg/ml PRF) X 100%

= 13.5%

The uronic acid content of the polysaccharide-rich fraction (PRF) of *S. aethiopicus* was calculated to be 13.5%.

5.9.2 GC ANALYSIS OF MONOSACCHARIDE SUGARS

The confirmation of monosaccharide sugars present and quantification was done by the use of GC. The sugars were identified as acetylated monosaccharide alditols and were characterised using relative retention times of the standard sugar derivatives.

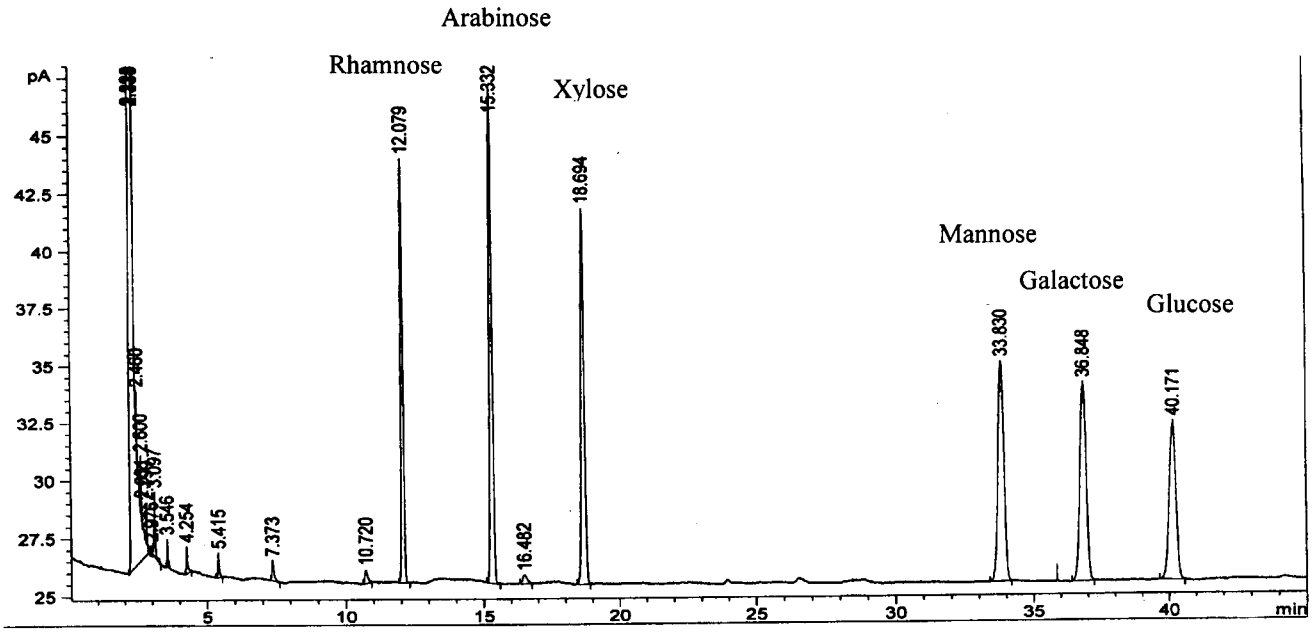


Figure 5.31: GC analysis of monosaccharide alditol standards

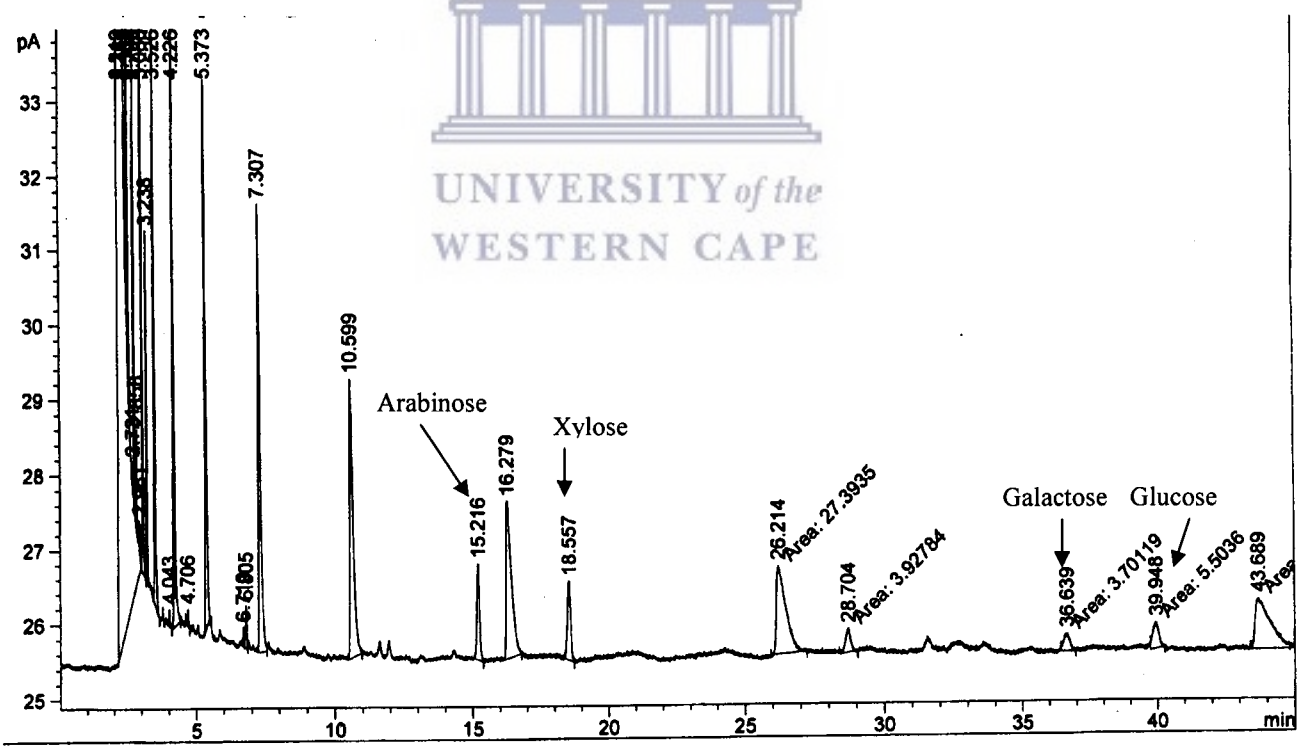


Figure 5.32: GC analysis of acetylated monosaccharide alditols derived from PFR sample

No GC-MS confirmation of the sugars was conducted. Being aware of the unavailability of the MS data, therefore the detected sugars were only assigned on the basis of comparing their retention times with that of the standards.

Table 5.10: Monosaccharide composition of the polysaccharide-rich fraction from *S. aethiopicus*

Monosaccharides	Yield (%) Relative
Arabinose	28.46
Xylose	28.37
Galactose	11.94
Glucose	17.73

Yields of neutral sugars are expressed as the percentage of the area of each sugar over the sum of the total areas of the four sugars.

Figure 5.32 indicated that arabinose and xylose are the more prominent monosaccharides of the four that were identified. The total monosaccharide content is 86.5 % and the uronic acid content is 13.5 %. Paulsen, (2001) reported that most polysaccharides possessing biological activity contain uronic acids e.g. D-galacturonic acid. Different types of polysaccharides isolated from plants used in traditional medicine are identified for their activities on the organism e.g. arabinans, arabinogalactans and rhamnogalactans (Paulsen, 2001). The GC trace has shown arabinose (28%) and xylose (28%) to be the prominent types of monosaccharide, whereas glucose and galactose were the minor sugars accounting for 17% and 11% respectively of the total carbohydrate. No information has been reported on the determination of the carbohydrate composition for any member of the Zingiberaceae family.

Based on the literature, Yang *et al.*, (2008) reported that polysaccharides isolated from various traditional medicinal plants have been shown to profoundly affect the immune system. In our study, the *S. aethiopicus* monosaccharide composition of the polysaccharide-rich fraction could exhibit potential effects towards immune-modulating. Parker, (2004) reported that the aqueous extract of a 3-year old *S. aethiopicus* rhizome has been found to possess good immune-modulating effects on Interleukin-6(IL-6). The plant extract increases the levels of IL-6, thus having a pro-inflammatory action. The extract (78µg/ml) increases IL-6 levels to 288pg/ml and 2500 µg/ml of the extract increases IL-6 levels to 409pg/ml. *S. aethiopicus* aqueous extract has been reported to be more effective on IL-6 than the commercial ginger (Parker, 2004).

5.10 SUMMARY OF MAIN RESULTS

The screening of aqueous extract of *S. aethiopicus* against microorganisms was performed by the MIC method with MTT being used as an indicator. The aqueous extract shows activity against the Gram-positive bacteria (*Staphylococcus aureus, Mycobacterium smegmatis*) and the fungus *Candida albicans*. No activity was found against the Gram-negative bacteria (*Pseudomonas aeruginosa*). However, EtOH and EtOAc extracts of *S. aethiopicus* have demonstrated greater antibacterial activity against Gram-positive bacteria than Gram-negative bacteria (Light *et al.*, 2002).

Screening for selected secondary metabolites such as alkaloids, anthraquinones, and flavonoids was performed. Alkaloids, anthraquinones and flavonoids were detected in *S. aethiopicus*. Such secondary metabolites have also been detected in other members of the Zingiberaceae family. The presence of essential oils in *S. aethiopicus* was also investigated.

GC-MS analyses revealed the essential oils as well as their molecular weights (Table 5.5). 1,8-Cineole and 1,3a-ethano(*1*H)inden-4-ol, octahydro-2,2,4,7a-tetramethyl were found in major quantity. 9aβ-hydroxy-4aαH-3,5α,8aβ-trimethyl-4,4a,8a,9-tetrahydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one and 4aαH-3,5α,8aβ-trimethyl-4,4a,8a,9-tetrahydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one which were

previously isolated from *S. aethiopicus* by Lategan *et al.*, 2009. The LC-MS analysis of the *S. aethiopicus* extracts also show the presence of an additional compound as 4aαH-3,5α,8aβ-trimethyl-4,4a,8a-trihydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one which was also isolated by Lategan *et al.*, 2009.

The flow diagram for the isolation of furanoterpenoid compounds has been illustrated in figure 5.10. Furthermore, the sugar content was investigated from the aqueous extract of the rhizome through derivatization of the polysaccharide-rich fraction into acetylated monosaccharide alditols. GC analysis of the derived sample indicate the presence of arabinose, xylose, galactose and glucose. The total uronic acid content was determined from the glucuronic acid standard curve (Figure 5.30).

5.11 CONCLUSION

Isolation of the furanoterpenoids 9aβ-hydroxy-4aαH-3,5α,8aβ-trimethyl-4,4a,8a,9-tetrahydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one **1** and 4aαH-3,5α,8aβ-trimethyl-4,4a,8a,9-tetrahydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one **2** has been successfully accomplished by CC. These compounds have been previously isolated from the rhizomes of *S. aethiopicus* and tested for antiplasmodial activity (Lategan *et al.*, 2009). A third furanoterpenoid was isolated in very low yield and its structure was deduced to be furanoterpenoid **3** by Mass Spectroscopy and also based on the literature. (4aα,5β,8α)-3,5,8a-trimethyl-4,4a,9-tetrahydro-naphtho[2,3-b]-furan-8(5H)-one is the furanoterpenoid which is the major compound that has been found in both the roots and rhizomes (Viljoen *et al.*, 2002). It also represents 20% of the oil composition. 4aαH-3,5α,8aβ-trimethyl-4,4a,9-tetrahydro-naphtho[2,3-b]-furan-8-one (**19**), 2-hydroxy-4aαH-3,5α,8aβ-trimethyl- 4,4a,9-tetrahydronaphtho[2,3-b]-furan-8-one (**20**) and 2-Acetoxy-4aαH-3,5α,8aβ-trimethyl-4,4a,9- tetrahydro-naphtho[2,3-b]-furan-8-one (**21**) were other furanoterpenoid derivatives isolated from the roots of *S. aethiopicus* (Holzapfel *et al.*, 2002).

HPLC screening of extracts in the leaves and rhizomes were performed, which show similarities and as a result that will improve the sustainability of the plants since only leaves need to be harvested. All HPLC chromatograms except for the methanol

extract of leaves have shown prominent peak. Based on the HPLC results, LC-MS was performed in order to find the retention time of the corresponding mass of the isolated compounds as well as comparing the profiles of both rhizomes and leaves as their hexane, ethyl acetate and methanol extracts. The prominent peaks appear to be found in both HPLC and LC-UV chromatograms. Therefore, HPLC can be used as a base for the quality control purposes.

The antimicrobial analysis of the rhizome aqueous extract was carried out against Gram positive (*Staphylococcus aureus, Mycobacterium smegmatis*) and Gram negative (*Pseudomonas aeruginosa*) bacteria as well as fungus *Candida albicans*. The results has shown that there was no activity found against the Gram-negative (*Pseudomonas aeruginosa*) bacteria. Activity was shown at concentrations ranging from 1.87 to 15 mg/ml against the fungus *Candida albicans*. The activity was also found at concentrations ranging from 7.5 to 15 mg/ml against *Staphylococcus aureus* and 1.87 to 15 mg/ml against *Mycobacterium smegmatis*. From these results, the future work should determine the lowest concentration of the extracts which inhibit the bacteria and the toxicity when using higher concentrations.

It has been shown from GC and GC-MS analysis that *S. aethiopicus* contains essential oils such as 1,8-cineole; α-terpineol; *cis*-4-thujanol; terpinen-4-ol; α-terpinyl acetate; β-elemene; β-eudesmol and 1,3a-ethano(*1*H)inden-4-ol,octahydro-2,2,4,7a-tetramethyl. The sugar composition of the polysaccharide rich fraction was determined to be arabinose, xylose, galactose and glucose in relative quantities of 28%, 28%, 11% and 17% respectively. No MS confirmation of the sugars was conducted and being aware of the unavailability of the MS data, therefore the detected sugars were only assigned on the basis of comparing their retention times with that of the standards. To have a complete data, MS should be used in future for confirmation of the sugars presence.

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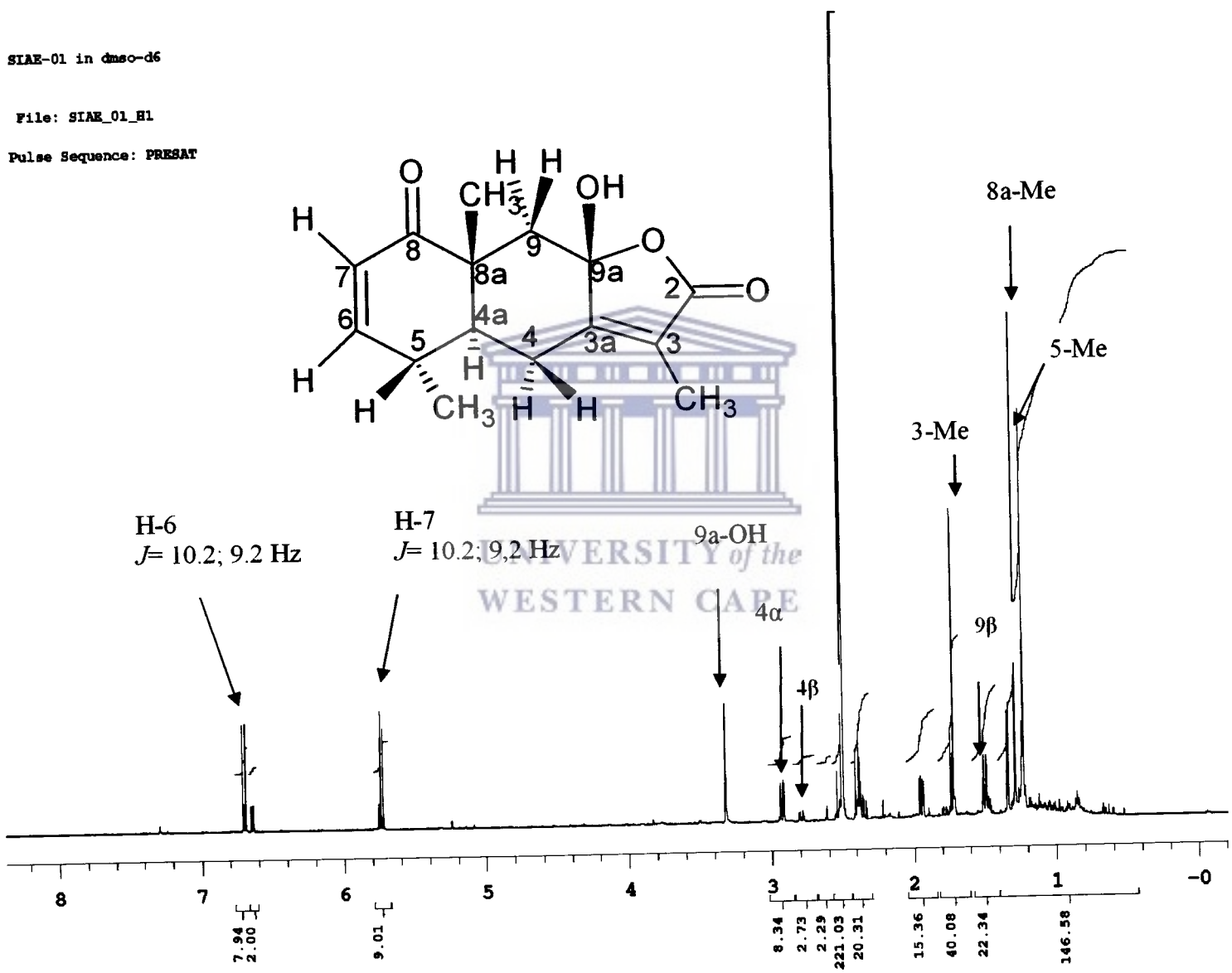
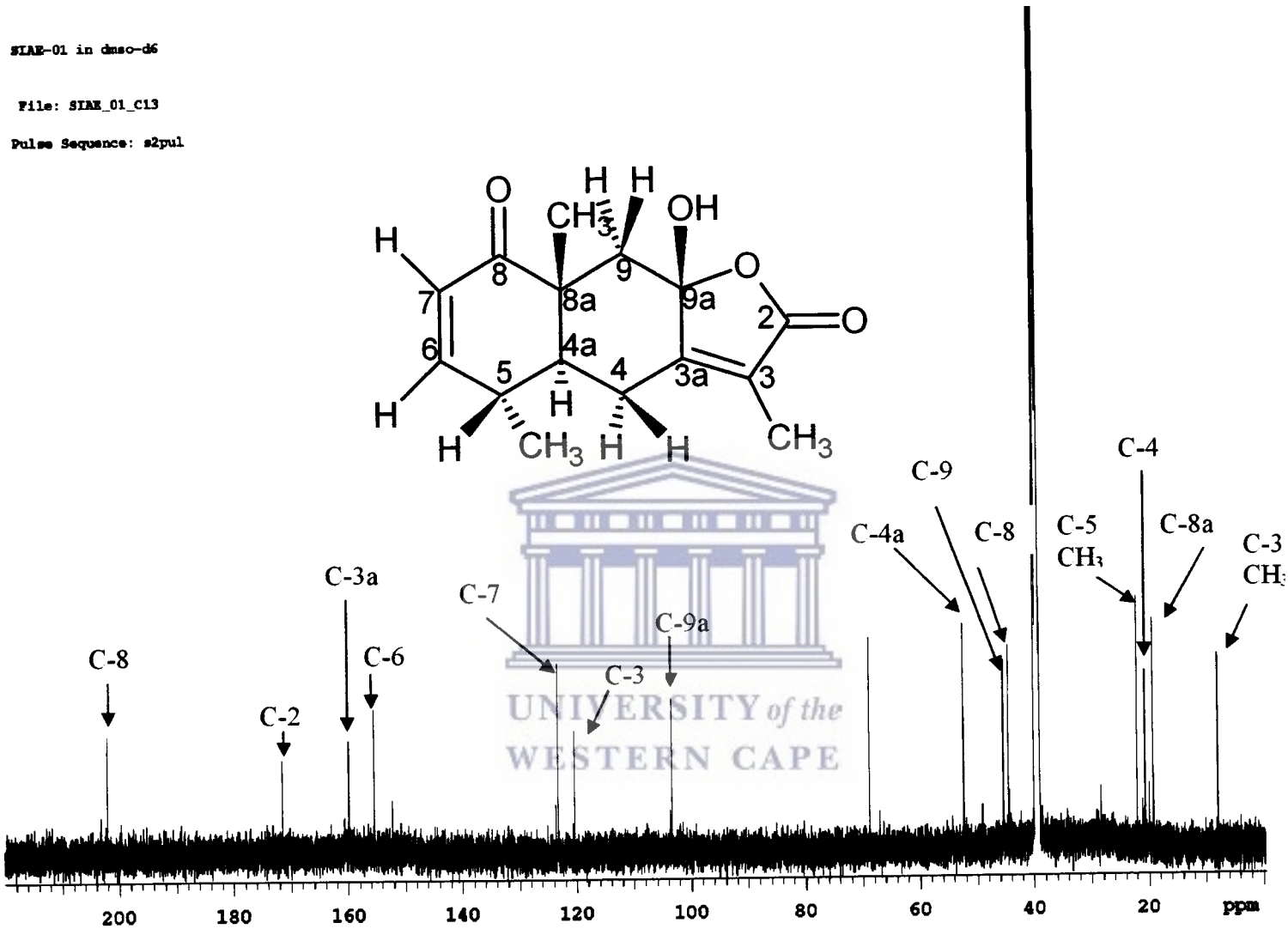
APPENDIX A

^{1}H NMR of Compound 1

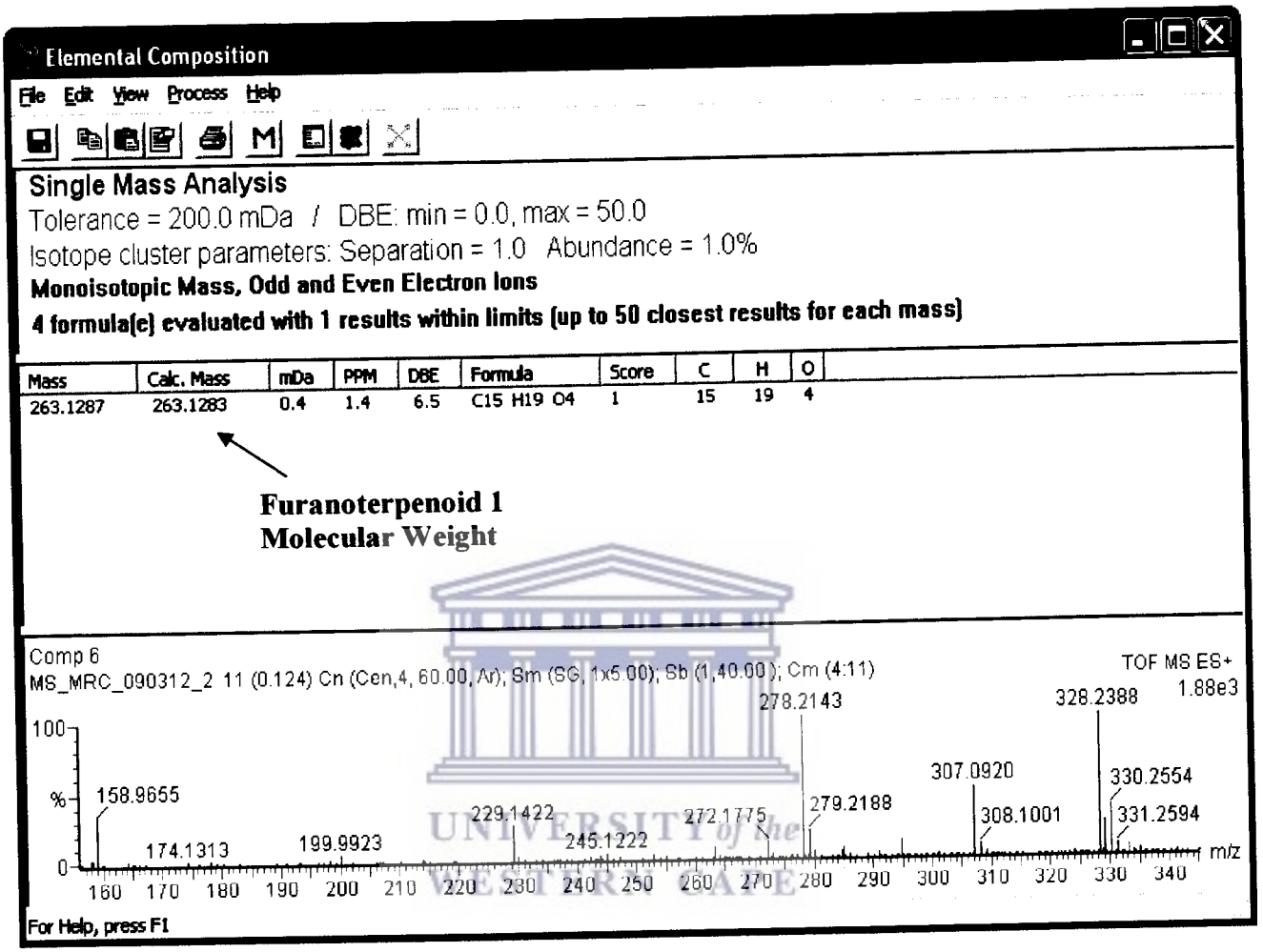


^{13}C NMR of Compound 1



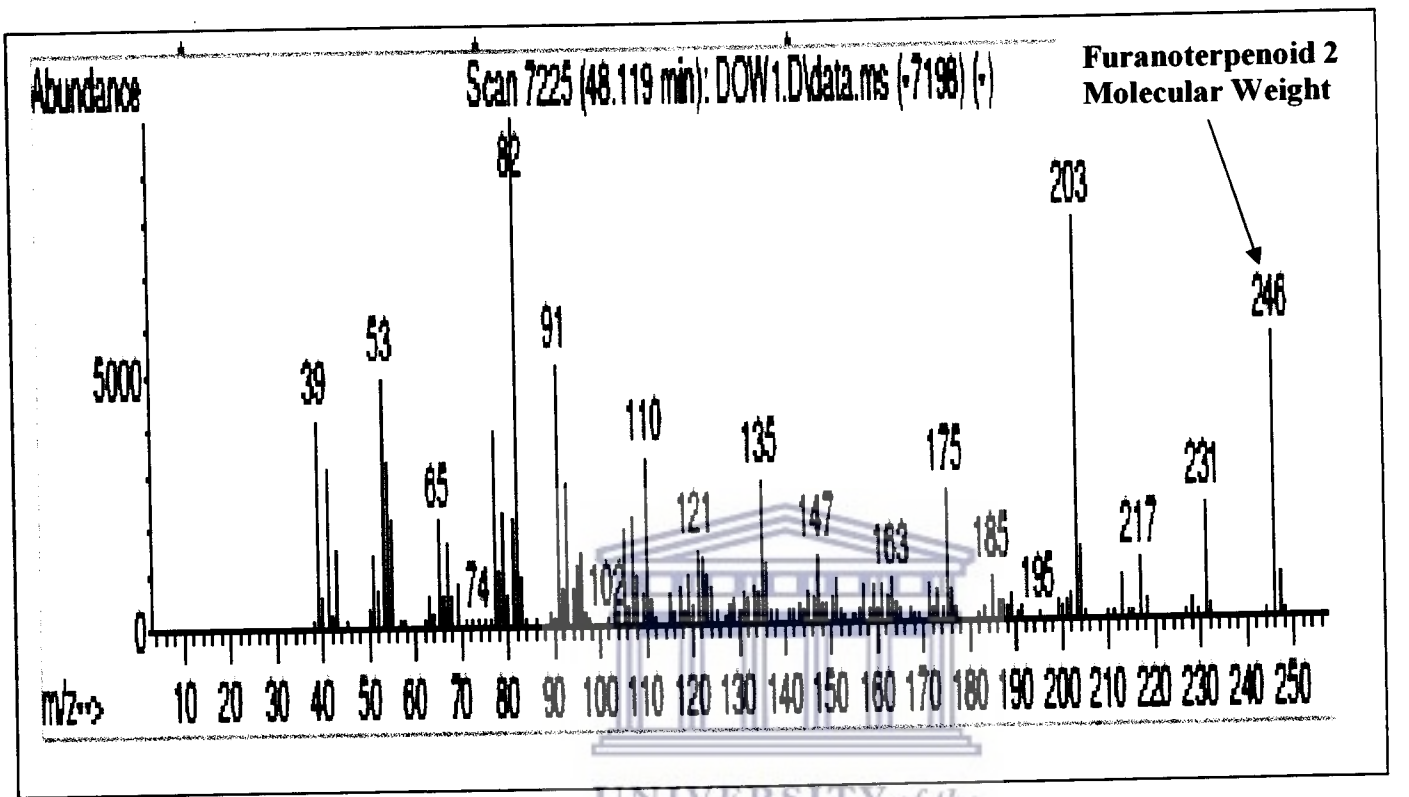
APPENDIX B

Calculated mass of furanoterpenoid 1



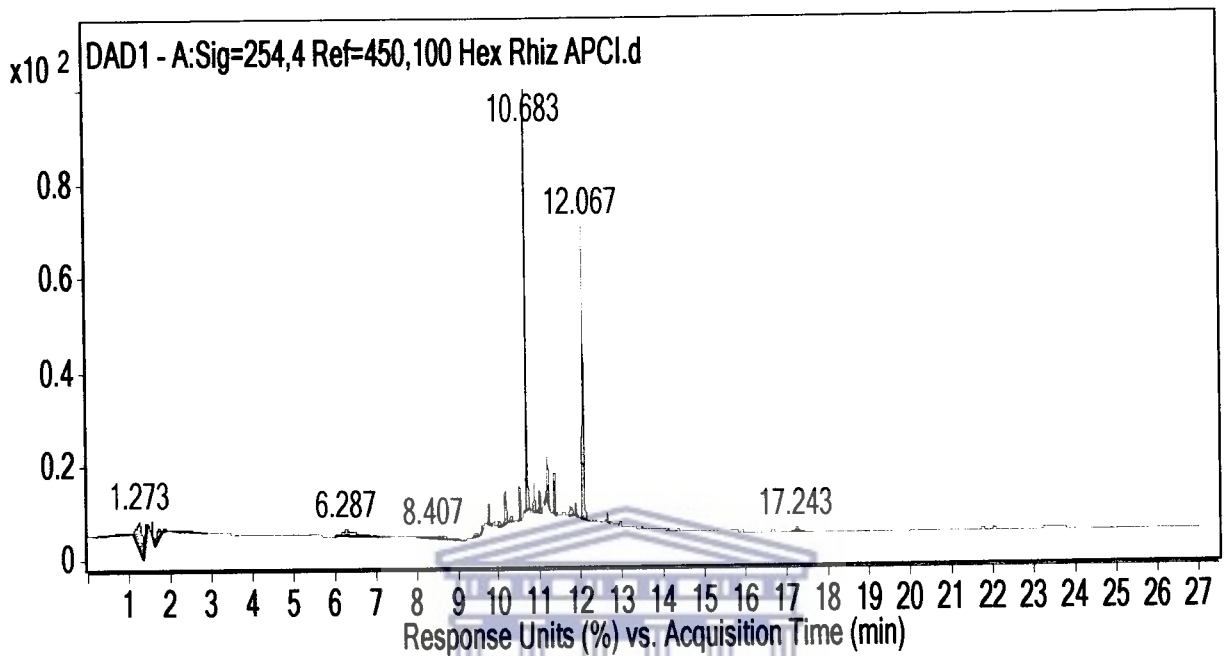
APPENDIX C

The MS of Furanoterpenoid 2

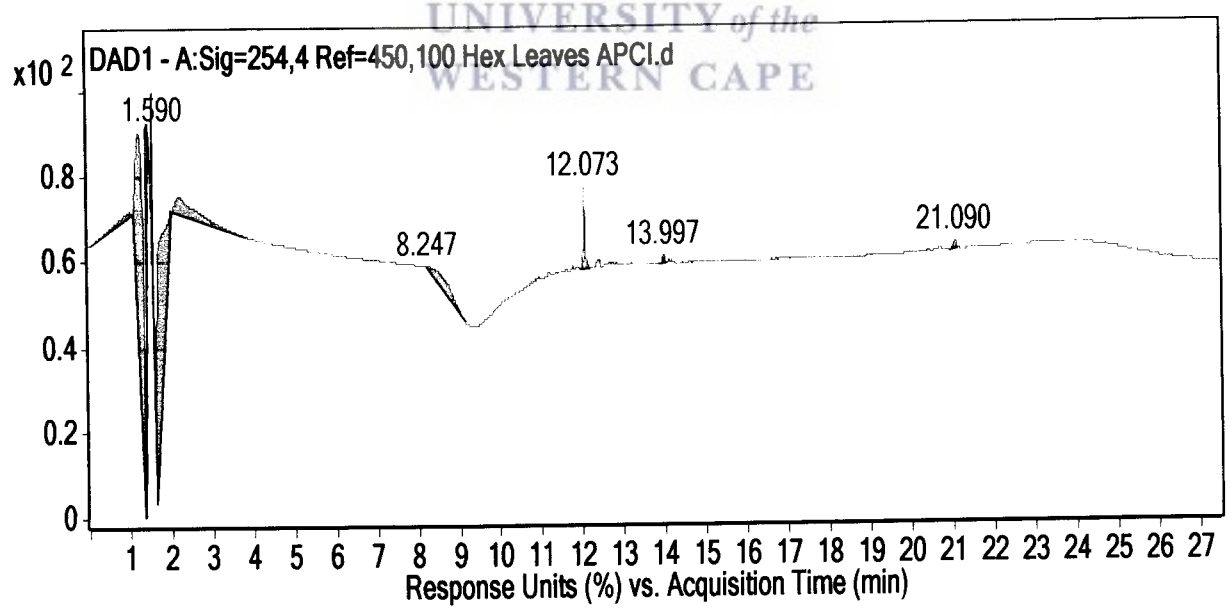


APPENDIX D

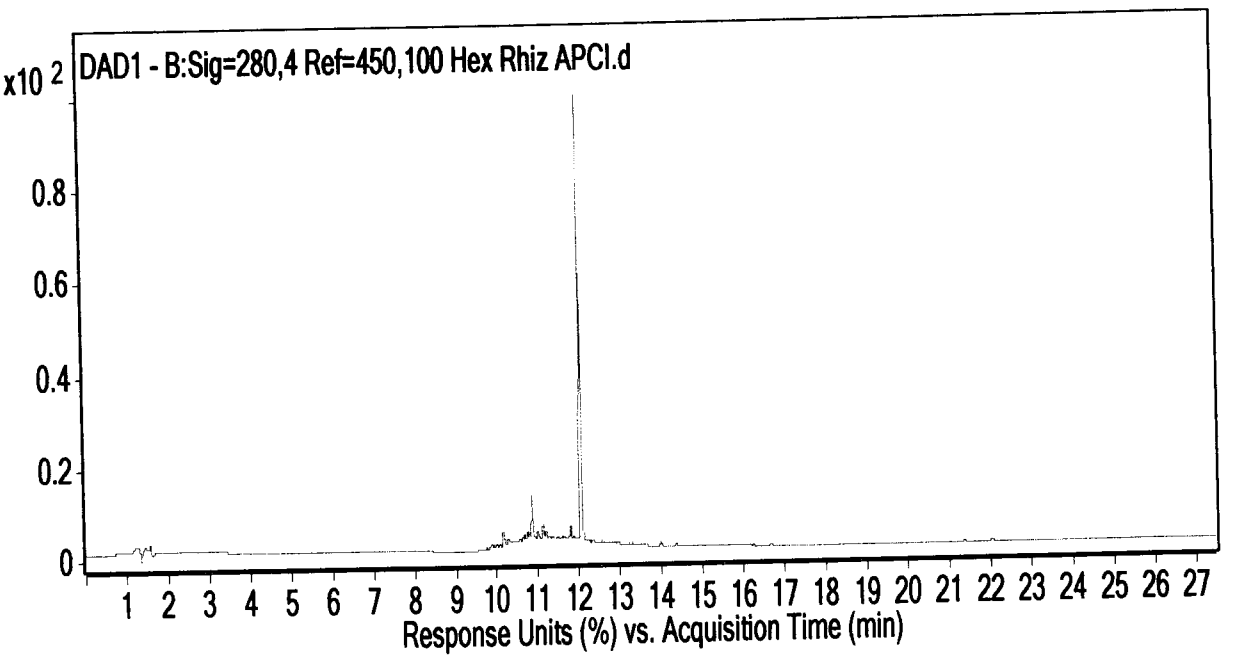
LC-UV chromatograms



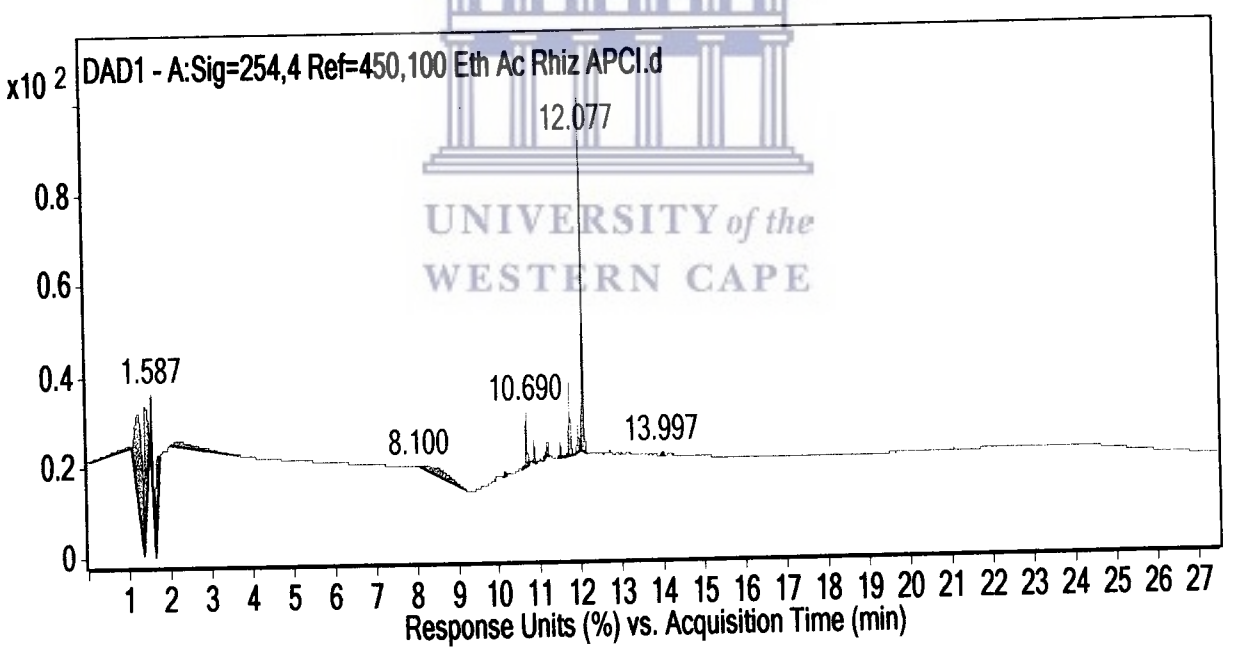
LC-UV chromatogram of hexane rhizome extract analyzed at 254 nm



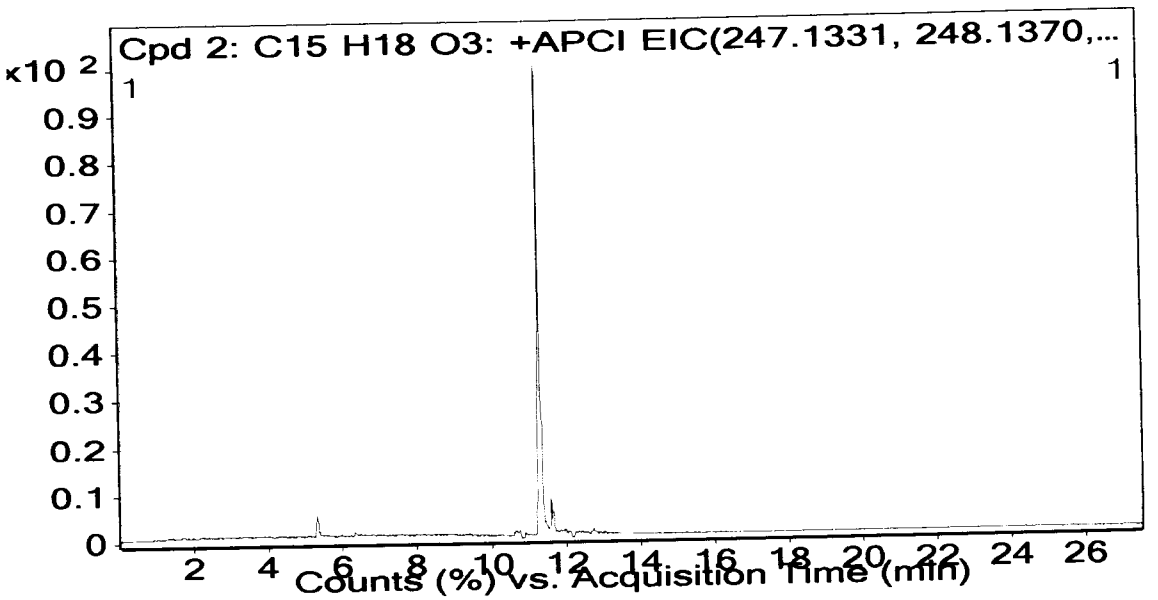
LC-UV chromatogram of hexane leaves extract analyzed at 254 nm



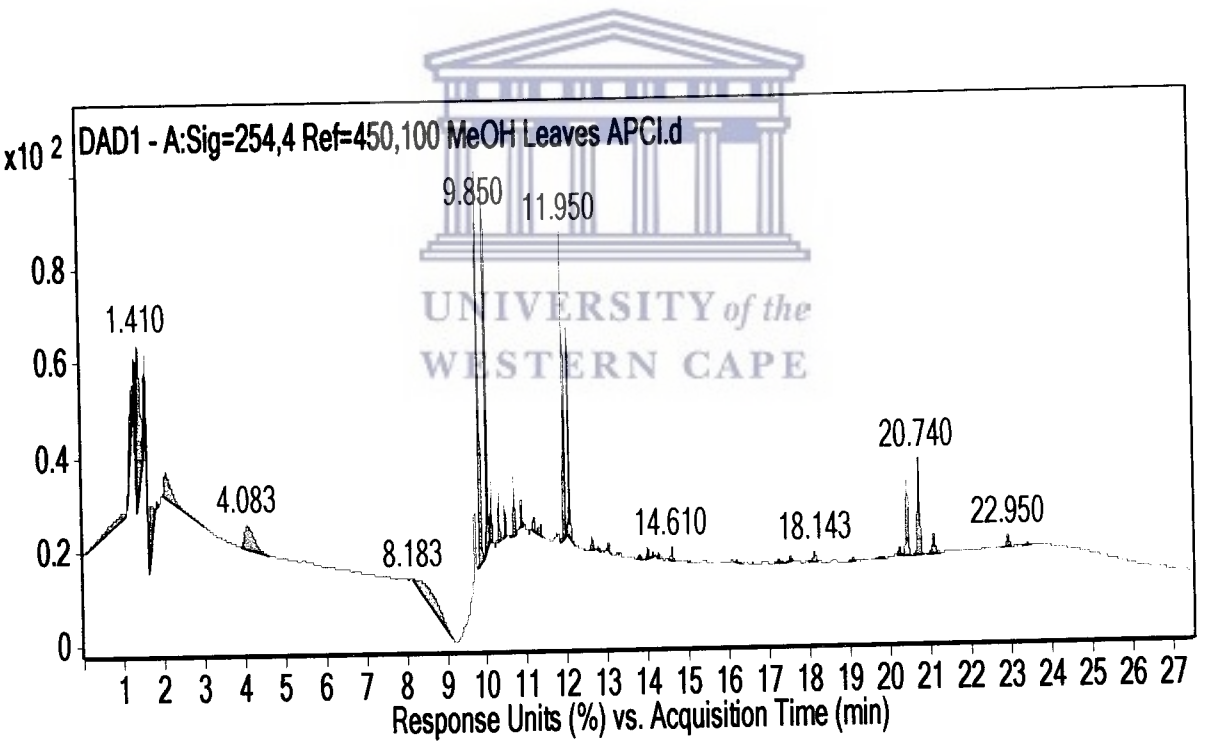
LC-UV chromatogram of hexane rhizome extract analyzed at 280 nm



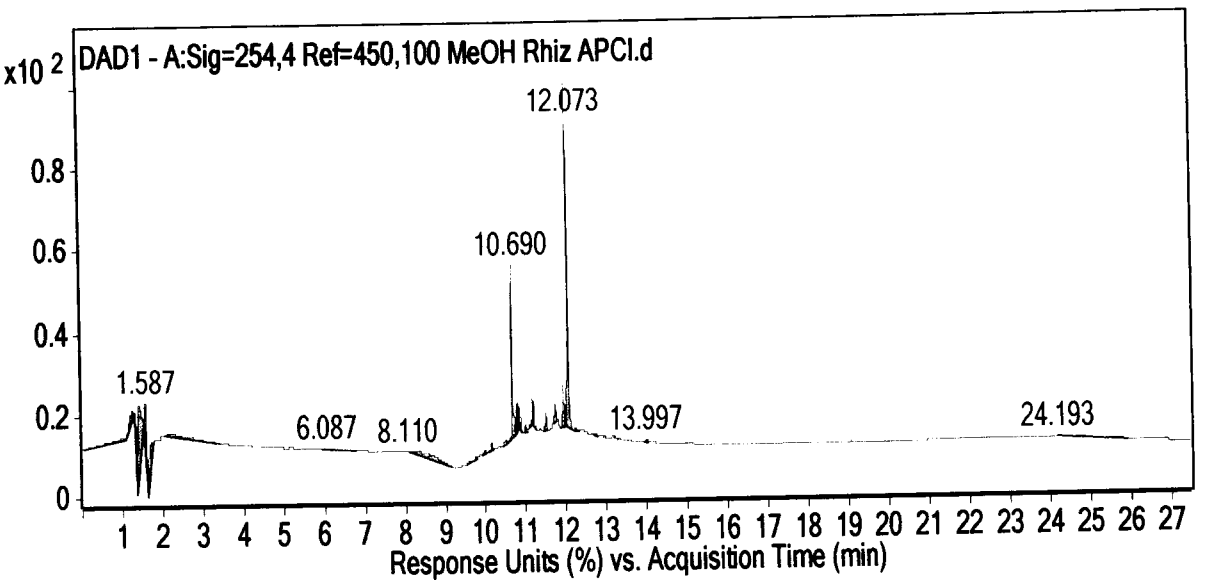
LC-UV chromatogram of ethyl acetate rhizome extract analyzed at 254 nm



LC-UV chromatogram of ethyl acetate leaves extract analyzed at 254 nm



LC-UV chromatogram of methanol leaves extract analyzed at 254 nm

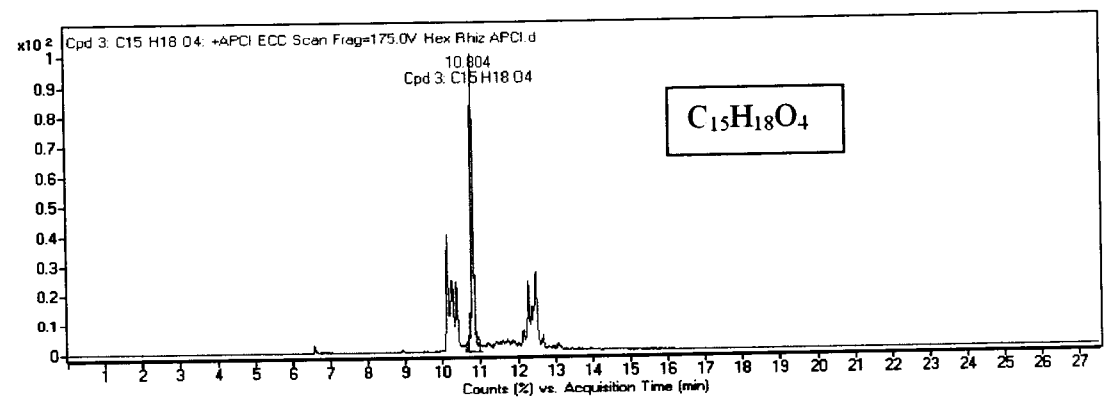


LC-UV chromatogram of methanol rhizome extract analyzed at 254 nm

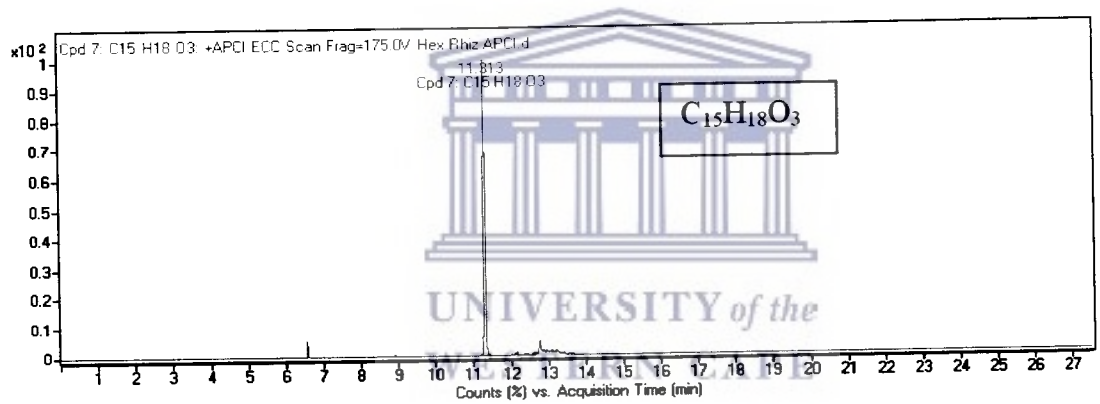
APPENDIX E

APCI chromatograms and mass spectra

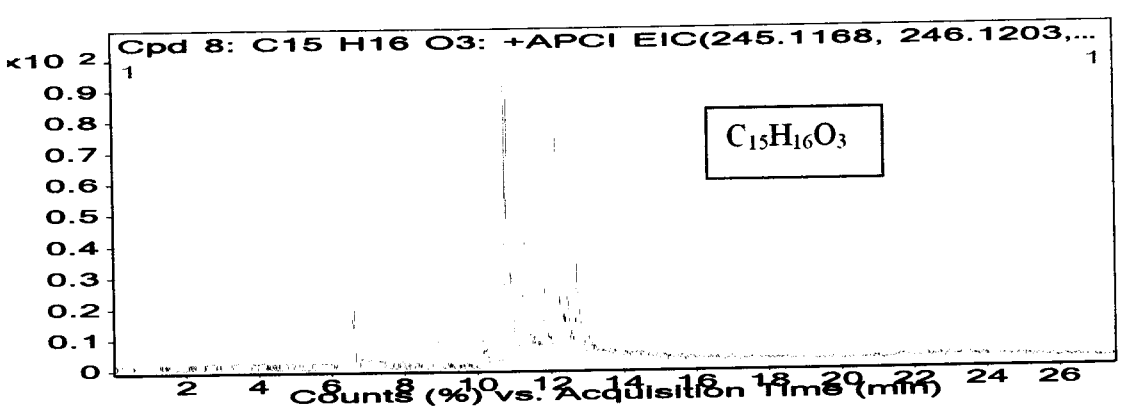
Hexane



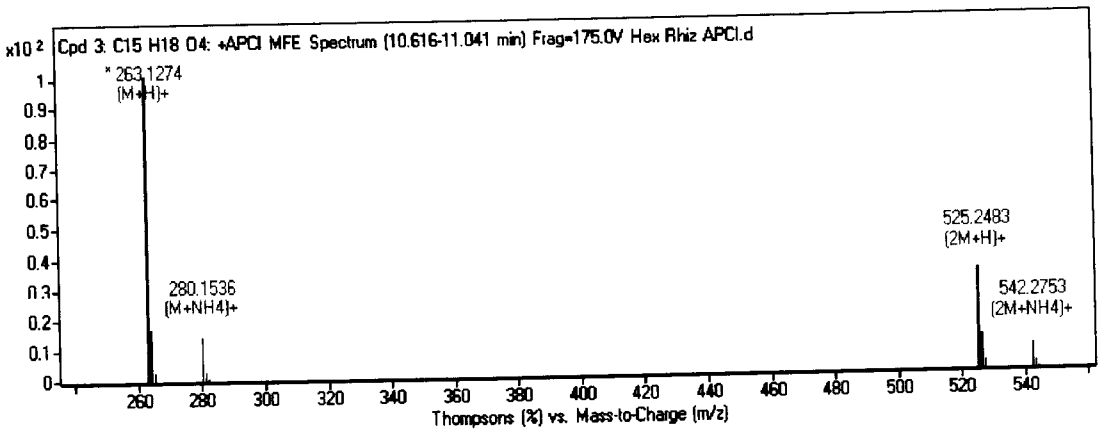
APCI chromatogram of furanoterpenoid **1**



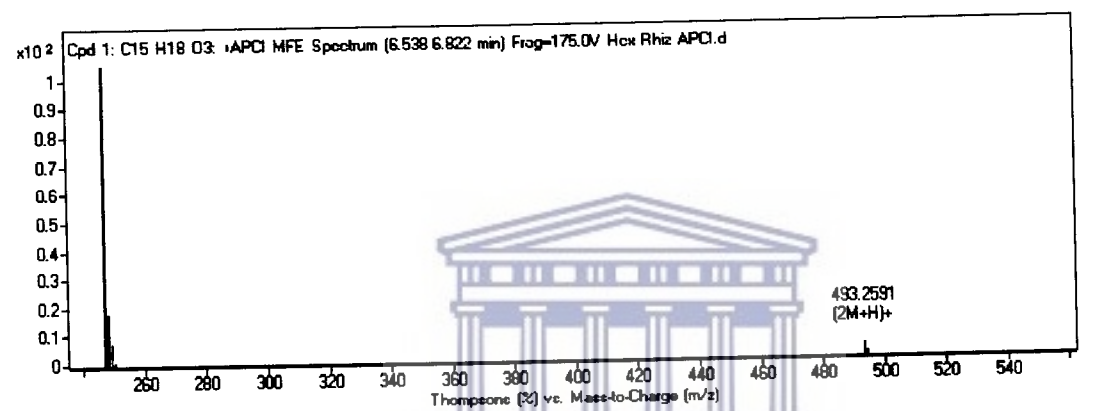
APCI chromatogram of furanoterpenoid **2**



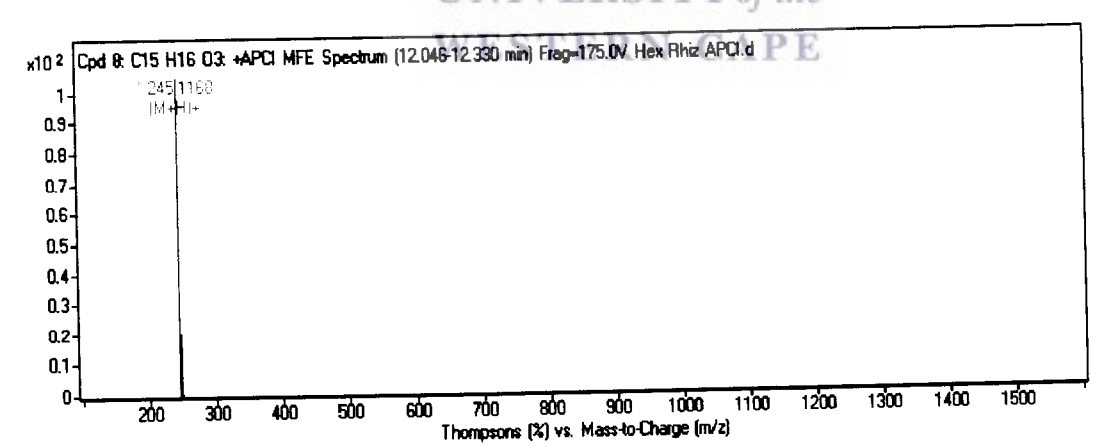
APCI chromatogram of furanoterpenoid **3**



Mass spectrum of furanoterpenoid **1**

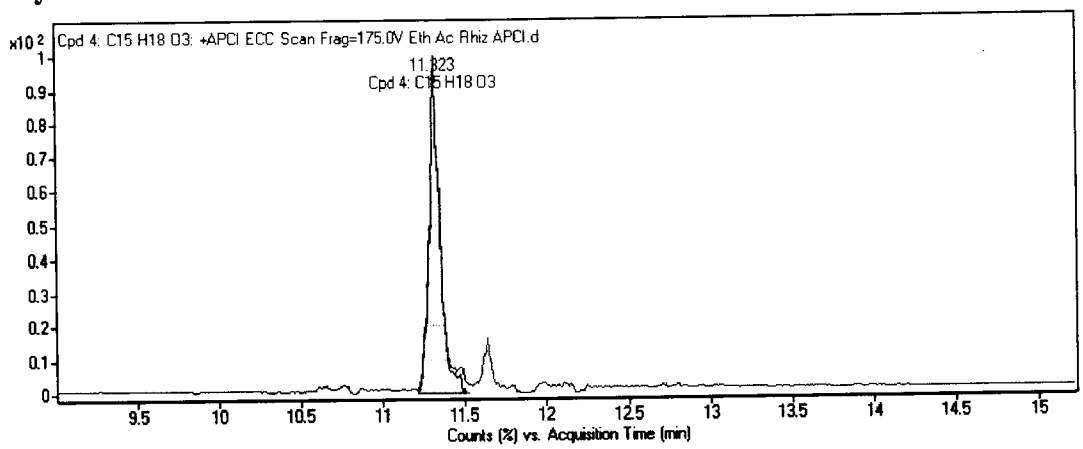


Mass spectrum of furanoterpenoid **2**

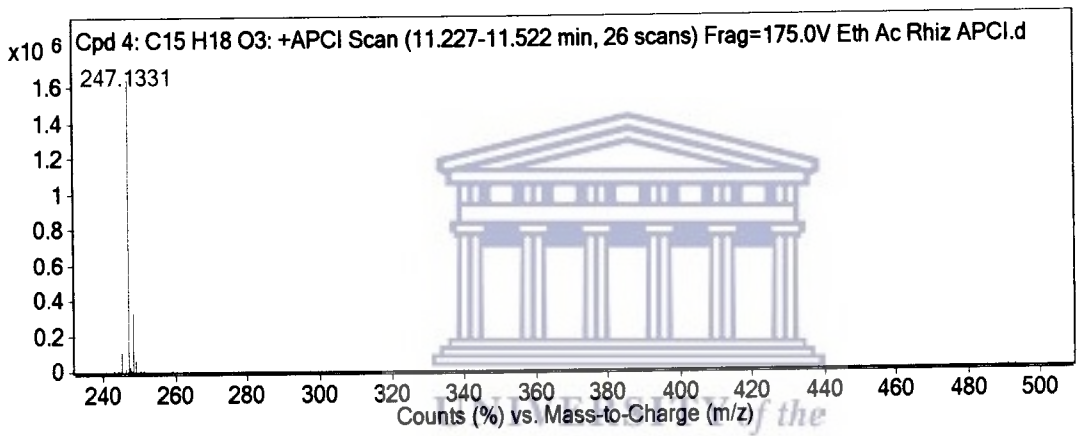


Mass spectrum of furanoterpenoid **3**

Ethyl acetate

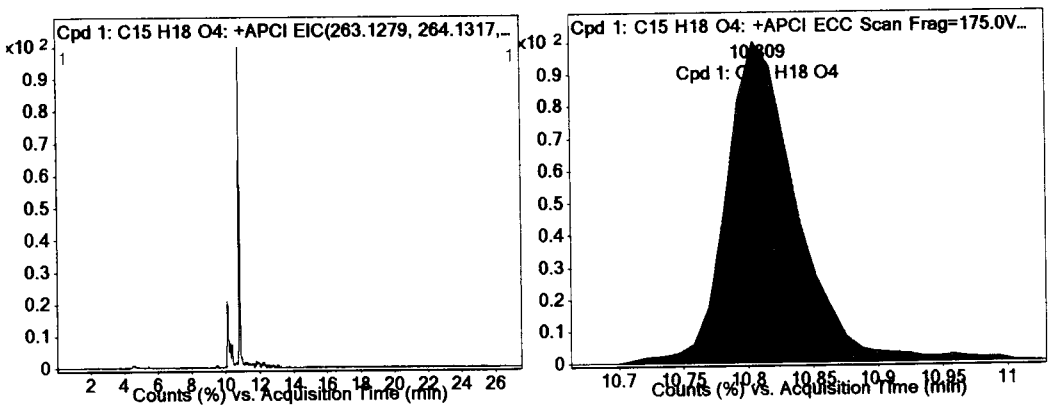


APCI chromatogram of furanoterpenoid **2**

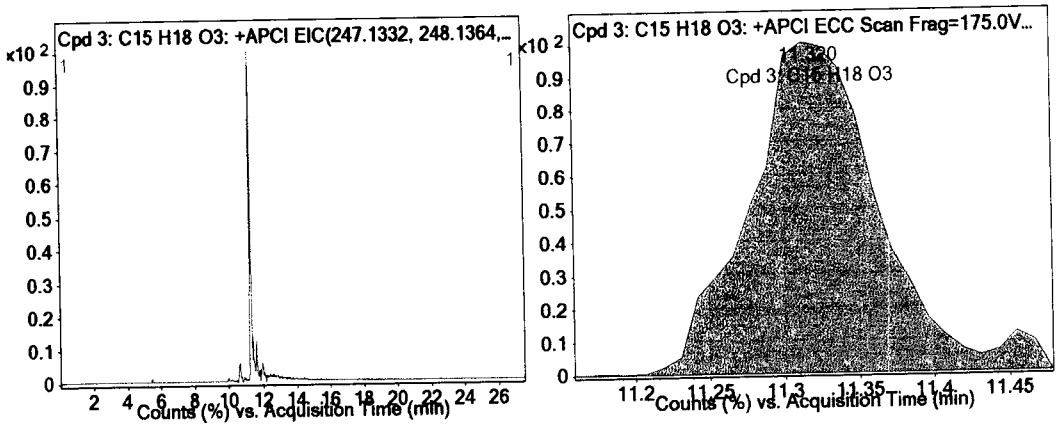


Mass spectrum of furanoterpenoid **2**

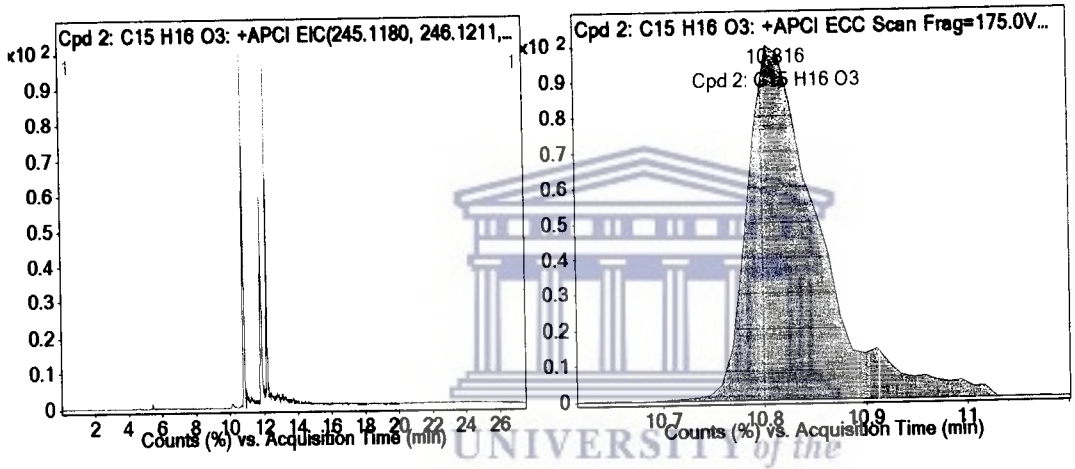
Methanol



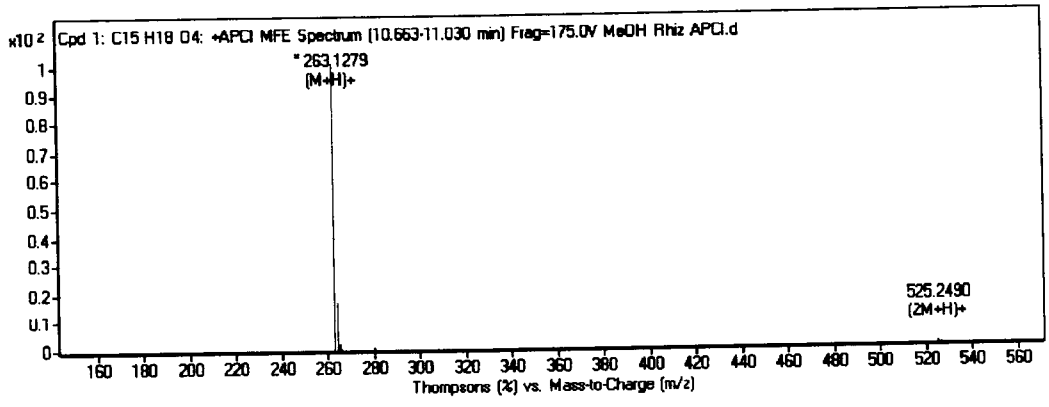
APCI chromatogram of furanoterpenoid **1**



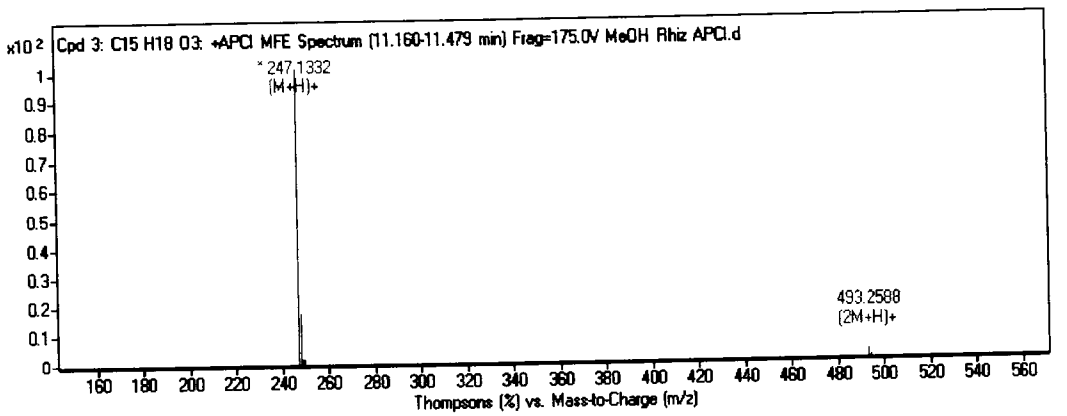
APCI chromatogram of furanoterpenoid **2**



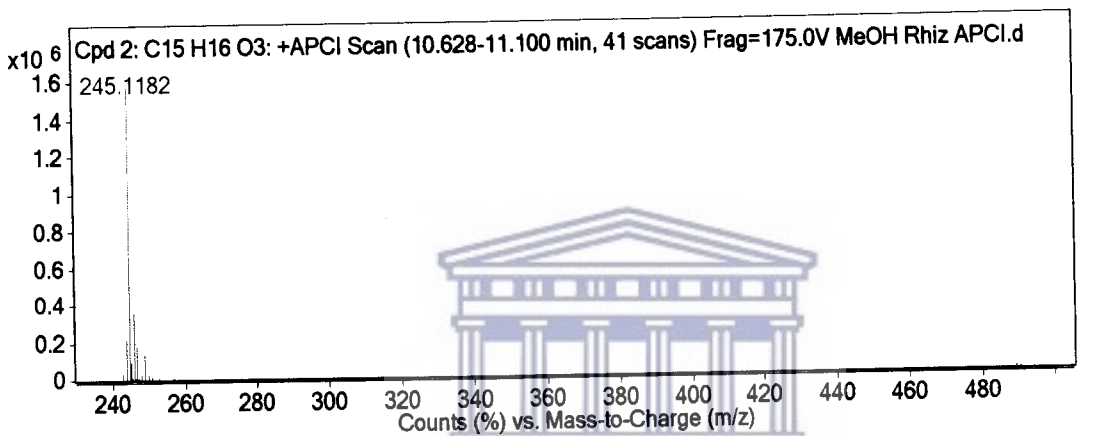
APCI chromatogram of furanoterpenoid **3**



Mass spectrum of furanoterpenoid **1**



Mass spectrum of furanoterpenoid **2**



Mass spectrum of furanoterpenoid **3**