

## CHAPTER 1

### 1. Background

The indiscriminate use of antibiotics has led to drug resistance of many bacterial strains. The emergency of antibiotic resistance by bacteria has become a medical catastrophe and we may be entering a 'post-antibiotic' era where antibiotics are no longer effective. Development of new microbial compounds for resistant organisms is becoming critically important (Martini and Eloff, 1998). Plant-derived medicines have been part of traditional health care in most parts of the world for thousands of years and there is increasing interest in plants as a source of agents to fight microbial diseases (Palombo et al, 2001).

More than 80% of the population in developing countries depend on plants for their medicinal needs. Medicinal and poisonous plants have always played an important role in African society. Traditions of collecting, processing and applying plants and plant-based medication have been handed down from generation to generation (Fyhrquist et al, 2001). It appears that science is becoming a full circle. In the beginning, all drugs were natural, since everything we used to treat our illness, cure our discomforts, and protect us came from the world around us, which is from plants, herbs, and in some cases, the animal world (Katz, 2002).

Use of medicinal plants as a source of relief and cure from various illness is as old as humankind itself. Even today, medicinal plants provide a cheap source of drugs for majority of world's population. Plants have provided and will continue to provide not only directly usable drugs, but also a great variety of chemical compounds that can be used as a starting points for the synthesis of new drug with improved pharmacological properties (Ballabh,

2008). A large proportion of the South African population use traditional medicine to serve human and animal health needs. Medicinal plants have become the focus of intense study recently in terms of conservation and whether their traditional uses are supported by actual pharmacological effects or merely on folklore (Masoko et al, 2005). Medicinal herbs and their preparations (hot and cold infusions, decoction, and tinctures) are widely used by human beings all over the world (Arpadjan, 2008).

It is estimated that around 27 million South Africans depend on traditional medicine for their primary health care needs. The reliance of such a large portion of the population on traditional medicine can be attributed to a number of factors, relatively good accessibility to the plants, affordability and extensive local knowledge and expertise amongst the local communities (Street et al, 2008). Because of this strong dependence on plants as medicine, it is important to study their safety and efficacy (Masoko et al, 2005). Herbal medicines have been used for centuries in rural areas by local healers and have been improved in industrialised countries. A number of substances used in modern medicine for treatment of serious diseases have originated from research on medicinal plants (Lall and Meyer, 1999).

Herbal medicine is the oldest and most tried and tested form of medicine. In a sense it forms the basis of all medicine. It is the original medicine, the mother of all remedies used today. It has been used by all cultures for centuries and is still the main form of medical treatment among 80% of the world population. Herbal medicine is the most important medicine for the majority of people on the planet, especially those who cannot afford expensive drugs (McKenna, 1996). Many traditional healers still use plants in their crude form (herbal remedies), although western technologies have transformed some plant products into more palatable forms like tablets, capsules and syrups. Extracts from some of the medicinal plants

being used by traditional healers have properties that inhibit the growth of bacteria, viruses and other microbes (Mativandlela, 2005). Traditional knowledge of medicinal plants has always guided the search for new cures. In spite of the advent of the modern high throughput drug discovery and screening techniques, traditional knowledge systems have given clues to the discovery of valuable drugs (Surveswaran et al, 2006). South Africa is a country rich in plant and cultural diversity. The region has some 24,000 species of flowering plants, accounting for almost 10% of the world's higher plants (Steenkamp, 2006).

During the last decade, the use of traditional medicine has expanded globally and is gaining popularity. It has continued to be used not only for primary health care of the poor in developing countries, but also in countries where conventional medicine is predominant in the national health care system (Tadeg et al, 2005). The reliance on plants as source of medicines warrants scientific validation of their safety, efficacy, quality and the appropriate dosage of the plant material. With the increasing acceptance of herbal medicine as an alternative form of health care, the screening of the medicinal plants for active compounds is becoming increasingly important (Shai et al, 2008). Plants commonly used in traditional medicine are assumed to be safe. The safety is based on their long usage in the treatment of diseases according to the knowledge accumulated over centuries (Fennel et al, 2004).

However, recent scientific research has shown that many plants used as food or in traditional medicine are potentially toxic, mutagenic and carcinogenic (Fennel et al, 2004). Drawbacks of traditional medicinal plant remedies include seasonal unavailability of plants, the possibility of infective or harmful treatments, uncertain dosages and lack of standardisation (McGaw et al, 2005). The prescription and use of traditional medicine in South Africa is currently not regulated, with the result that there is always the danger of misadministration, especially of

toxic plants. The potential genotoxic effects that follow prolonged use of some of the more popular herbal remedies, are also for cause alarm (Fennel et al, 2004). During the early stages of antibiotic development it was clear that some bacteria could survive and multiply in the presence of antibiotics. In the early 1980s, a number of hospitals in Melbourne were plagued with infections which were resistant to almost all antibiotics. Only one antibiotic remained effective, vancomycin, a drug which is expensive and toxic (Mckenna, 1996). Reserpine can be produced synthetically at a cost in the USA of \$1.25 per gram, the large scale production of the alkaloids from the roots of *Rauwolfia* species is as much cheaper process producing reserpine at a cost of only \$0, 75 per gram (Safowora, 1982). Traditional medicinal plants are often cheaper, locally available and easily consumable, raw or as simple medicinal preparation (Surveswaran et al, 2006).

Approximately 25% of the drugs worldwide come from plants and of the drugs considered as basic and essential by WHO, 11% are either exclusively of plant origin or synthetic drugs obtained from natural precursors (Martini and Eloff, 1998). The thalidomide disaster in the 1950s was a major warning sign of the dangers of using synthetically-produced medicines. In the 1980s, Opren an anti-inflammatory drug used to treat arthritis killed a number of patients suffering from that condition. In June 1986, all children's medicine containing aspirin had to be taken off the market because a number of children had died from brain and liver damage (McKenna, 1996).

Scientists have also analysed an herbal medicine called Meadowsweet and found that it contains a natural aspirin which can be used as a pain killer. The beauty of this product is that Meadowsweet contains a tannin and mucilage, both of which act to protect the lining of the stomach. Hence it does not produce the side effects seen when only synthetic aspirin is used

(McKenna, 1996). Herbal medicine has been improved in developing countries not only as a way to rescue ancient traditions but also as an alternative solution to health problem (Martinez et al, 1996).

Isolation and characterization of the bioactive agent in a plant leads to the possible synthesis of a more potent drug with reduced toxicity. The pure compound is required to assess the possible toxicity or side effects (Safowora, 1982). The chemicals from plants may possess complex chemical structures that are not available in synthetic compound libraries. There are estimated to be 250 000 plant species in the world, and only 5-15% of these species have been tested for potentially useful biologically active compounds (McGaw and Eloff, 2008). The use of plant-derived drugs for the treatment malaria has a long and successful tradition. Isolation of quinine *Cinchona* bark and *Artemisia annua*, which illustrate the potential value of investigating traditionally, used antimalaria plants for the development of pharmaceutical antimalaria drugs (Muthaura et al, 2007).

There are hundreds of chemical substances that have been derived from plants for use as drugs and medicines. Many more await discovery (Taylor, 2000). Medicinal plants are well-known natural sources for the treatment of various diseases since antiquity. About 20,000 plant species used for medicinal purposes are reported by WHO. Furthermore, natural products, either pure compounds, or as standardized plants extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity (Maregisi, 2008).

**Table 1:** Selected drugs originally derived from plants (Taylor, 2000 and Safowora, 1982).

Plant	Drug	Use
<i>Aloe vera</i>	Resin	Purgative
<i>Cinchona succirubra</i>	Quinine	Bitter tonic
<i>Claviceps purpurea</i>	Ergometrine	Uterine stimulant
<i>Digitalis purpurea</i>	Digoxin	Myocardial stimulant
<i>Hammamelis virginiana</i>	Gallic acid	Astringent
<i>Papaver somniferum</i>	Morphine	Analgesic
<i>Penicillium</i>	Penicillin	Antibiotic
<i>Rauwolfia serpentina</i>	Reserpine	Antihypertensive
<i>Slix alba</i>	Salicin	Analgesic
<i>Cassia species</i>	Sennoside A, B	Laxative
<i>Berberis vulgaris</i>	Berbine	Baccillary dysentery
<i>Ephedra sinica</i>	Ephedrine	Sympatho mimetic
<i>Vasicinece cerebral</i>	Vinca minor	Cerebral stimulant
<i>Chondodendron tomentosum</i>	Tubocuranine	Skeletal muscle relaxant

### 1.1 Secondary metabolites

Plants produce a diverse array of secondary metabolites with many functions, such as defense against herbivores, diseases and parasites (McGaw and Eloff, 2008). Secondary metabolites are molecules that are not necessary for the growth and reproduction of a plant, but may serve some role in herbivore deterrence due to astringency or they may act as phytoalexins, killing bacteria that the plant recognizes as a threat. Secondary compounds are often involved in key interactions between plants and their abiotic and biotic environments that influence them (Gibson, 2002). Medicinal plant parts (roots, leaves, branches/stems, barks, flowers, and fruits) are commonly rich in phenolic compounds such as flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans and lignins (Surveswaran et al, 2006).

### **1.1.1 Phenolic compounds**

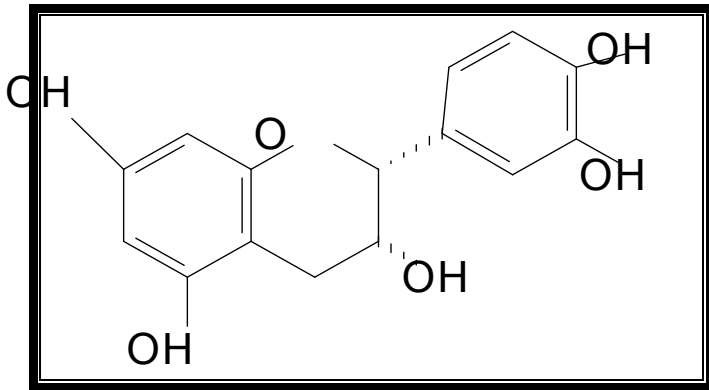
Phenolics are widespread in nature and are found in many natural compounds with aromatic rings and hydroxyl groups. This group of secondary metabolites includes anthocyanidins, anthocyanins, anthraquinones, coumarins, flavonoids, naphthaquinones, simple phenolic compounds and tannins (Evans, 1989). Phenolic compounds are secondary metabolites in the plant materials that are known to possess antioxidant effects. The consumption of certain plant materials may reduce the risk of chronic diseases related to oxidative stress on account of their antioxidant activity and promote general health benefits (Kosar et al, 2006). Phenolic compounds can play an important role in preventing body cells and the organs of man from damage of unsaturated fatty acids by lipid peroxides and absorbing and neutralizing free radicals (Ao, 2007).

### **1.1.2 Flavonoids**

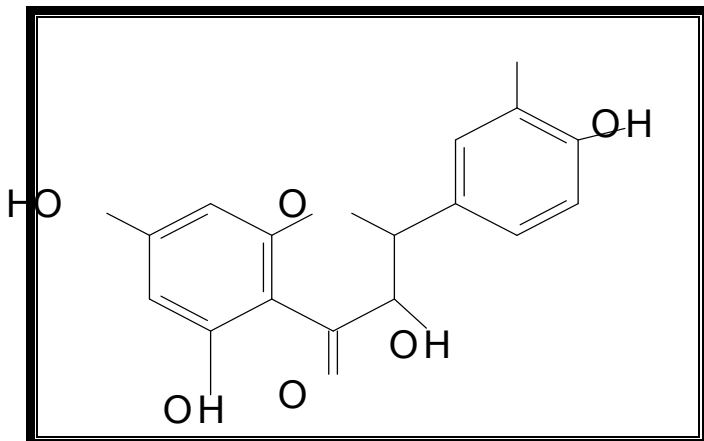
Flavonoids are 3-ringed phenolic compounds consisting of a double ring attached by a single bond to a third ring (Figure 1.1.2a-c). Flavonoids can be described as polyphenolic compounds possessing 15 carbon atoms; two benzene rings joined by a linear three carbon chain. The six major subgroups are: chalcones, flavone, flavono, flavanone, anthocyanins and isoflavonoids (Corbett, 1974). Flavonoids exhibit a wide range of biological effects both in vitro and in vivo (Ren et al, 2007).

Flavonoids are commonly found in most plants, and are the pharmacologically active constituents in many herbal plant medicines. Many flavonoids exhibit several pharmacological properties, acting as vasodilatory, anticarcinogenic, anti-inflammatory,

antibacterial, immunestimulating, anti-allergic, and antiviral agents (Lee et al, 2002). Flavonoids, including flavones, flavanone, flavanols, and isoflavones, are polyphenolic compounds which are widespread in foods and beverages and possess a wide range of biological activities, of which antioxidation has been extensively explored (Dai et al, 2005).

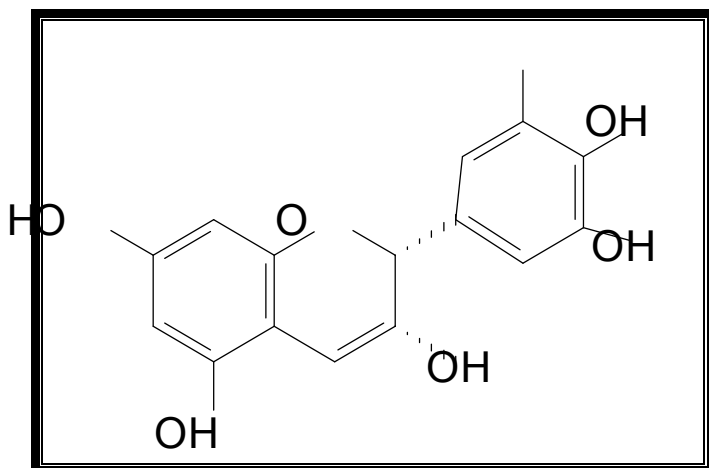


**Figure 1.1.2a** Structure of flavonoid (Quercetin).



**Figure 1.1.2b** Structure of flavonoid (Epicatechin).

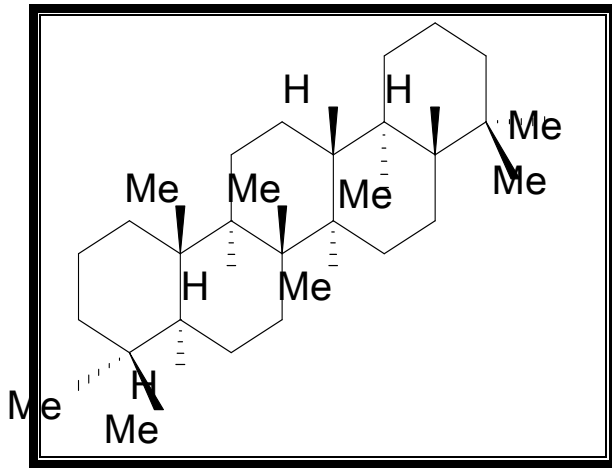




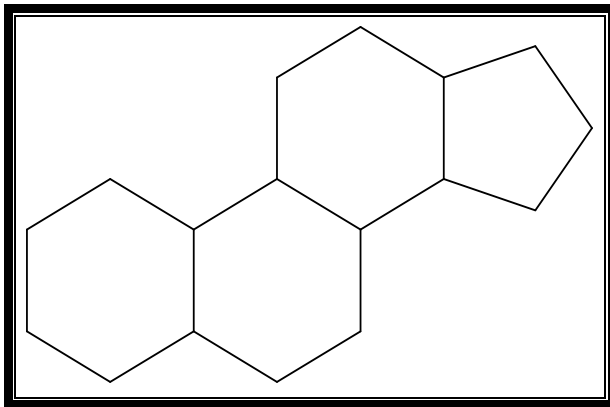
**Figure 1.1.2c** Structure of flavonoid (Epigallocatechin).

### 1.1.3 Terpenoids

Terpenoids can be divided into groups according to their structures. Terpenoids are formed when isoprene units are linked together. They occur as sesquiterpenoids ( $C_{15}$ ), diterpenoids ( $C_{20}$ ), triterpenoids ( $C_{30}$ ) and tetraterpenoids ( $C_{40}$ ). Triterpenoids are present in plants in the form of saponins. Monoterpenoids and sesquiterpenoids are common constituents of volatile oils (Van Wyk et al, 1997). Triterpenes have a wide spectrum of biological activities such as antitumour, antiviral, bactericidal, fungicidal, analgesic, anti-inflammatory spermicidal and cytotoxic activities (Mahato et al, 1996 and Patocka, 2003). In plants triterpenoids function as hormones (gibberellins, abscisic acid), photosynthetic pigments (phytol, carotenoids) and electron carriers (ubiquinone, plastoquinone) (McGarvey and Croreau, 1995). Carotenoids may protect human against certain types of cancer, cardiovascular and other diseases associated with ageing. The dihydro caretenids lutein and zeaxanthin have the macular pigment of the human retina (Aman et al, 2005).



**Figure 1.1.3a** Structure of Terpenoids (Hopane).



**Figure 1.1.3b** Basic Structure of triterpenoids.

## CHAPTER 2

### 2. Literature Review on *Rhus* species

#### 2.1 Botanical information about some species of *Rhus*

*Rhus* spp were selected for investigation based on its wide traditional use, widespread occurrence all over Southern Africa and its antibacterial activity in preliminary assays.

*Rhus* is a *genus* of approximately 250 species of *flowering plants* in the family *Anacardiaceae* or mango family. They are commonly called sumac or sumach. Some species (including *Poison ivy*, *poison-oak*, and *poison sumac*) are often placed in this genus. The leaves of the most of *Rhus* species are divided into three leaflets and they have thinly textured, bright green, narrow leaves with toothed margins (Coates, 2002).

The *Anacardiaceae* includes 76 genera with over 600 species in which 25 of those genera contain poisonous species. The principle function of the secondary chemicals in this family is presumably as defence against herbivores. *Anacardiaceae* compounds are of chemical interest and they hold great promise in the search of new medicinal and commercial agents (Mitchell, 1990). A number of South African plants are traditionally used to treat epilepsy and convulsions and previous studies have shown that the extracts of *Rhus* species have good affinity to the GABA receptor. GABA is the most important inhibitory neurotransmitter in the human central nervous system (Svenningsen, 2005).

*Rhus* is a valuable agent in treating pneumonia, bronchitis, flue, and phthisis, when the patient is extremely irritable and suffers from gastric irritation. *Rhus* is an extremely useful remedy in

the various disorders of the skin. Redness, intumescence, and burning are the indications in cutaneous diseases (Felter, 1989). The common names for *Rhus leptodictya* are mountain karee, rock karee (English); bergkaree, klipkaree (Afrikaans); inHlangushane (siSwati) and Mohlwehlwe (Northern Sotho). A useful way to distinguish the mountain karee from other, similar *Rhus* species is that the two lateral leaflets are at right angles to the terminal one. Its natural distribution stretches across the four Northern provinces in South Africa and includes the Northern Free State. It is also found in Swaziland, Botswana, Zimbabwe and Mozambique.

Domestic animals feed on the tree during times of drought and this apparently does not cause as much tainting of milk, as when stock feed on *Rhus lancea*. Beer is made from fermenting the fruit, which are edible but small and sour. Small household articles can be made from the wood. It is commonly used as a street tree as it does not have an aggressive root system (Coates, 2002). Native Americans used the leaves and berries of smooth and staghorn sumacs with tobacco in a traditional smoking mixture. Beer is brewed from the fruits of *R. leptodictya* and various parts are used in traditional medicine (Schmidt et al, 2002).

The Manyika people use powdered roots of *R. leptodictya* for acute pain in the chest and abdominal areas. The Xhosa people use roots for gall sickness in cattle. Branch lotion and smoke from burning is used for eye complaints by Swati people (Hatchings et al, 1996). *R. leptodictya* has been screened for in vitro anticancer activity against breast, renal and melanoma human cell lines which showed moderate anticancer activity (Fouche et al, 2008).



Tree



Fruits



Leaves

**Figure 2.1** *Rhus leptodictya* showing a tree, fruits and leaves.

## 2.2 Chemical compounds isolated from *Rhus* by other scientists

Plants contain numerous biologically active compounds, many of which have been shown to have antimicrobial properties (Palambo, 2001). The search for biologically active extracts based on traditionally used plants is still relevant due to the appearance of microbial resistance of many antibiotics and the occurrence of fatal opportunistic infections (Tshikalange et al, 2005). Four phenolic acids are present in the sumac extracts as determined by HPLC analysis, gallic acid was the main component, protocatechuic acid, p-OH-benzoic acid and vanillic acid in the extracts (Kosar et al, 2006).

A benzofuran lactone, rhuscholid, 5-hydroxy-7-(3,7,11,15-tetramethylhexadeca-2,6,10,11-tetraenyl)-2(3H)-benzofuranone, betulin, betulonic acid, moronic acid, 3-oxo-6 beta-hydroxyolean-12-en-28-oic acid and 3-oxo-6 beta-hydroxyolean-18-en-28-oic acid were isolated from the stems of *R. chinensis* (Gu et al, 2007). The methanol extract from *R. glabra* was subsequently fractionated and monitored by bioassays leading to the isolation of three antibacterial compounds, the methyl ester of 3,4,5-trihydroxybenzoic acid (methyl gallate), 4-methoxy-3,5-dihydroxybenzoic acid and gallic acid (Saxen, 1994). Bioassay-directed fractionation of the n-hexane extract of the stem of *R. semialata* (Anacardiaceae) has led to the isolation of 6-pentadecylsalicylic acid (Kuo et al, 1999).

Two biflavonoids with activity in the H-Ro 15-1788 (flumazenil) binding assay were isolated by high pressure liquid chromatography (HPLC) fractionation of the ethanol extract of the leaves from *R. pyroides* (Svenningsen et al, 2006). Extracts from *Rhus verniciflua* had the following flavonoids, protocatechuic acid, fustin, fisetin, sulfuretin and butein (Lee et al, 2004). The extracts of *R. pyroides* contain agathisflavone and amentoflavone (Svenningsen et

al, 2006). The biflavanone(2S,2''S)-7,7''-di-*O*-methyltetrahydroamentoflavone and five flavonoids, 7-*O*-methylnaringenin, 7, 3'-*O*-dimethylquercetin, 7-*O*-methyleluteonlin and eriodictyol are present in the leaves of *R. retinorrhoea* (Ahmed et al, 2001).

### **2.3 Ethnomedicinal use of *Rhus***

*Rhus* is an extremely useful remedy in the various disorders of the skin (Mitchell, 1990). The native Indians use *R. glara* in the treatment of bacterial disease, such as syphilis, gonorrhoea, dysentery and gangrene. The species was included in antibiotic screening of British Colombian medicinal plants (Hartley, 2007). Other reports indicated that sumac has antimicrobial activity with limited information on its antioxidant activity and potential as a source of antioxidative substances. Sumac extracts had remarkable antioxidant activity against inhibition of lipid peroxidation and scavenging activity on DPPH radical (Kosar et al, 2007).

Rhuscholidide isolated from the stems of *R. chinensis* has significant anti-HIV-1 activity (Gu, 2007). Gallnuts from oak and sumac contains the highest naturally occurring level of tannin (gallotannin): 50-75%. They also contain 2-4% each of the smaller molecules gallic acid and ellagic acid that are polymerized to make tannins. The tannin of gallnuts has been used for centuries for tanning of leather (a process involving coagulating proteins). Now, gallnut extracts are widely used in pharmaceuticals, food and feed additives, dyes, inks, and metallurgy (Dharmananda, 2003). The extract from *R. glabra* was subsequently fractionated and monitored by bioassays leading to the isolation of three antibacterial compounds, the methyl ester of 3, 4, 5-trihydroxybenzoic acid (methyl gallate), 4-methoxy-3, 5-dihydroxybenzoic acid and gallic acid (Saxen, 1994). As preventive and therapeutic measures

to deal with diseases caused by oxidative stress become more common, the opportunities for the biochemical and clinical application of natural antioxidants increase. Consequently, numerous compounds have been used to treat diseases by reducing oxidative stress in particular, plant-derived antioxidants, such as  $\alpha$ -tocopherol, ascorbic acid and flavonoids (Lee et al, 2002).

**Table 2.3:** Medicinal uses of the *Rhus* species.

<i>Rhus</i> species	Medicinal indication
<i>R. retinorrhoea</i>	For malaria
<i>R. pyroides</i>	For epilepsy
<i>R. verniciflua</i>	For gastritis, stomach cancer and arteriosclerosis
<i>R. typhina</i>	For sore throats and intestinal parasites
<i>R. taitensis</i>	For boils
<i>R. semialata</i>	For pain, inflammation and swelling
<i>R. chinensis</i>	For HIV
<i>R. glabra</i>	For diarrhoea and ulceration of throat
<i>R. diversiloba</i>	For dysmenorrhoea
<i>R. aromatica</i>	For the relief of urinary, bowel and hemorrhagic disorders

#### 2.4 Phytochemical information of *Rhus*

Some species of the genus *Rhus* are used in traditional medicine either as antimicrobial concoctions or for their cytotoxic properties while others display insecticidal properties.



Biflavonoids have been isolated from a number of *Rhus* species (Ahmed et al, 2001). Sumac is one example, which is widely used in Turkey and Middle East. It's dried and ground leaves have been used as a tanning agent due to their high tannin content. Previous phytochemical studies of this plant reported that its leaves contained flavones, tannins, anthocyanins, and organic acids (Kosar et al, 2006).

Leaves of staghorn sumac (*Rhus typhina*) contain several galloyltransferases that catalyze the beta-glucogallin dependent transformation of 1, 2, 3, 4, 6-pentagalloylglucose to gallotannins. Among these, an enzyme has been isolated that preferentially acylates the 3-position of the hexagalloylglucose, 3-O-digalloyl-1, 2, 3, 4, 6-tetra-O-galloylglucose, to afford the corresponding heptagalloylglucose being characterized by a 3-O-meta-trigalloyl side-chain (Niemetz and Gross, 2001). Dried leaves of *R. taitensis* were extracted with petrol by percolation at room temperature to give, after evaporation of solvent, a crude extract. Fractionation of petrol extract led to isolation of triterpenes, 3B, 20, 25-trihydroxy lupine, 3, 20-dihydroxylupane, 20-hydroxylupane-3-one, 20, 28-dihydroxylupane-3-one, 3, 16-dihydroxy lup-20(29)-ene and 28-hydroxy-B-amyrone (Yuruker et al, 1997).

## **2.5 Pharmacological use of *Rhus***

Sumac extracts are most notable for their antimicrobial activities, although some limited information is available on their antifungal and antiviral activities. Crude methanolic extracts of *R. glabra* branches exhibited both the widest zones of inhibition in a disc assay, and the broadest spectrum of inhibition (active against all of the following 11 species of bacteria tested: *Bacillus subtilis*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Mycobacter phlei*, *Pseudomonas aeruginosa*, *Serratia marcescens* *Staphylococcus aureus*,

*Staphylococcus aureus*, and *Salmonella typhimurium* (Rayne and Mazza, 2007). *Rhus leptodictya* has been screened for in vitro anticancer activity against breast, renal and melanoma human cell lines which showed moderate anticancer activity (Fouche et al, 2008).

## 2.6 Problem Statement and Hypothesis

Throughout the years, the indiscriminate use of synthetic drugs, particularly antibiotics, has led to bacterial resistance. Hence, there is a great need to develop new forms of drugs that are effective against bacteria.

Traditional medical practitioners in developing countries are known to use plants as forms of drugs to heal all kinds of diseases. However, most plants used in this way by traditional healers have not been scientifically tested. Because of this strong dependence on plants as medicine, it is important to study their safety and efficacy. In this study the antibacterial activities of *Rhus leptodictya* will be examined. To the best of my knowledge, no reports have been published in this regard.

## 2.7 Aim and Objectives of the study

Medicinal plants have become the focus of intense study recently in terms of conservation and as to whether their traditional uses are supported by actual pharmacological effects or merely based on folklore. Various parts of *R. leptodictya* are used in traditional medicine. The aim of this study is to isolate, characterize the chemical components of *R. leptodictya* and evaluate the antibacterial activities of extracts obtained from the leaves and stems of the plant to justify its continued usage in traditional medicine. The study focuses mainly on the following objectives:

2.7.1 Extraction of dried leaves and stems of *R. leptodictya* with different extracting solvents of varying degrees of polarity to determine best extractant.

2.7.2 Evaluation of the antibacterial activity of the extracts against four bacterial pathogens namely, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Escherichia coli* of the plant extracts using bioautography and microdilution essay method.

2.7.3 Isolation of chemical compounds from the fractions by applying chromatographic techniques.

2.7.4 Characterization of the isolated compounds using NMR spectroscopy and MS techniques.

2.7.5 Evaluation of the antibacterial activity of the isolated compounds against four bacterial pathogens namely, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Escherichia coli*.

## CHAPTER 3

### 3. Materials and methods

#### 3.1. Plant collection

The leaves and twigs of *Rhus leptodictya* were collected from the botanical garden at the University of Pretoria in May 2008. The plant was identified by the Botanist, Ms Laurens Middleton of the Department of Botany. A voucher specimen was deposited at the herbarium of Phytomedicine Programme, Department of Paraclinical Sciences, University of Pretoria Onderstepoort.

#### 3.2 Plant preparation

The leaves and twigs of *R. leptodictya* were dried in dark room in the Phytomedicine laboratory at University of Pretoria for two weeks. The dried leaves and twigs were ground to fine powder using a Macasalab mill (Model 200 Lab). The fine powder materials were stored in closed bottles at room temperature in the laboratory until needed.

#### 3.3 Preliminary extraction procedure

Separate aliquots of 4g of the fine powdered material of *R. leptodictya* were extracted with 40 ml of seven different solvents of increasing polarity (hexane, chloroform, dichloromethane, ethyl acetate, acetone, methanol and water) in 50 ml centrifuge tubes. The tubes were shaken on a Labotec shaking machine for four hours. The extracts were centrifuged using an EBA 30

Hettich centrifuge at 6000 x g for twenty minutes and the supernatant was filtered through Whatman No.1 filter paper into pre-weighed glass vials and placed under a stream of cold air to dryness and the mass of the extract was determined. Concentrations of 10 mg/ml were prepared in acetone for biological assays.

### **3.4 Chromatographic analysis**

Chemical constituents of the extracts were analysed by thin layer chromatography (TLC) using aluminium-backed TLC plates (Merck, 60 F<sub>254</sub>). The TLC plates were developed with one of the three eluent systems developed in Phytomedicine laboratory for the separation of components of *R. leptodictya* extracts, i.e., ethyl acetate/methanol/water(10:1.35:1) (EMW, polar); chloroform/ethyl acetate/formic acid (10:8:2) (CEF, intermediate polarity) and benzene/ethanol/ammonia hydroxide (18:2:0.2) (BEA, non-polar). Aliquots of 10 µl (100µg) of the extracts were loaded with a micropipette on each of three TLC plates. The developed chromatograms were air dried in the fume cupboard and thereafter visualized under UV light (254 and 360 nm Camac Universal UV Lamp). For further detection of chemical compounds, the plates were sprayed with a mixture of vanillin-sulphuric acid (0.1g vanillin: 28ml methanol: 1ml sulphuric acid). The plates were heated at 110<sup>0</sup>C to optimal colour development.

### **3.5 Biological activity methods**

#### **3.5.1 Bacterial cultures**

Bacterial test organisms used in screening tests were *Staphylococcus aureus* (Gram-positive) (American Type Culture collection [ATCC 29212], *Enterococcus faecalis* (Gram positive ATCC 29212), *Pseudomonas aeruginosa* (Gram-negative ATCC 27853) and *Escherichia coli* (Gram-negative ATCC 25922). Bacterial cultures cells were maintained at 37<sup>0</sup>C on Muller-Hilton (MH) agar on slants until needed. The MH broth was prepared and 100ml of the media was poured into each of four Erlenmeyer flasks. The flasks were autoclaved, and then the bacteria species were inoculated in Erlenmeyer flasks for each bacterium. The flasks were incubated at 37<sup>0</sup>C for 16 hours.

#### **3.5.2 Bioautographic assay of the plant extracts**

Thin layer chromatography (TLC) plates were loaded with 100µg of each extract, and dried in a stream of air before developing in mobile phases of varying polarities (BEA, CEF and EMW). Ten ml of a dense fresh bacterial suspension was added into two centrifuge tubes and centrifuged at 3500 x g for 20 minutes to concentrate the bacteria. The supernatant was discarded and the pellet was re-suspended in 2-4 ml of fresh MH broth with a vortex shaker. The chromatograms were sprayed with concentrated cultures of *S. aureus*, *E. faecalis*, *P. aeruginosa* and *E. coli* until completely moist with aid of spraying gun. The chromatograms were incubated under 100% relative humidity 16 hours to allow the microorganism to grow on the plates. The plates were then sprayed with 2 mg/ml of p-iodonitrotetrazolium violet (INT) (Sigma) and incubated for 30 minutes. The emergence of purple-red colour resulting from the

reduction of INT into its respective formazan was a positive indicator of cell viability. Clear zones indicated growth inhibition of the compounds present in the extract with the specific  $R_f$  value.

### **3.5.3 Minimal inhibitory concentration assay of the plant extracts**

Minimal inhibitory concentrations (MIC) are regarded as the lowest concentration of extract that inhibits growth of test organisms. The method of Eloff (1998b) was used. The assay was initiated by pouring sterile water aliquots (100  $\mu$ l) into wells of microtitre plates. Exactly 100  $\mu$ l of 10 mg/ml extract prepared in acetone was added in row A and mixed using a micropipette. From row A 100  $\mu$ l was aspirated and added into row B and mixed. The procedure was repeated until all the wells filled. An additional 100 $\mu$ l in row H was discarded. Two columns were used as sterility control (no cultures were added) and negative growth control (the extracts were replaced with 100  $\mu$ l of acetone). Concentrated suspensions of microorganisms (100  $\mu$ l) were added to each well except the sterility controls. The microtitre plates were sealed in a plastic bag with a plastic film sealer (Brother) before incubating at 37<sup>0</sup>C in a 100 humidified incubator for 18 hours. After incubation 40  $\mu$ l of 0.2 mg/ml INT was added to each well and plated were incubated for a further 2 hours before observation in antibacterial activity assays. The development of red colour, resulting from the formation of the red/purple formazan, was indicative of growth (positive indicator of cell viability). MIC values were regarded as the lowest concentrations of the extracts that inhibit the growth of the test organisms (decrease in the intensity of the red formazan colour). Gentamicin was used as a positive control in the antibacterial tests. The experiments were performed in triplicate.



### **3.5.4 Antioxidant assay of the plant extracts**

The 2, 2-diphenyl-picrylhydrazyl (DPPH) assay used by Braca (2002) was employed in this study. This is an indicator of free radical scavenging capacity. DPPH radical is reduced from a stable free radical, which is purple in colour to diphenylpicryl hydrazine, which is yellow. Extracts were separated by TLC, air dried and then sprayed with 0.2% DPPH in methanol. Chromatograms were examined for colour change over 30 minutes. Antioxidant compounds would change the purple DPPH colour to yellow.

### **3.5.5 Cytotoxicity assay of isolated compounds**

The cytotoxicity of the isolated compounds was determined using the MTT assay. The MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) reduction assay is widely used for measuring cell proliferation and cytotoxicity. MTT (yellow) is reduced into a formazan (purple) by viable cells. The colour intensity of the formazan produced, which is directly proportional to the number of viable cells, is measured using a spectrophotometer (Mosmann, 1983).

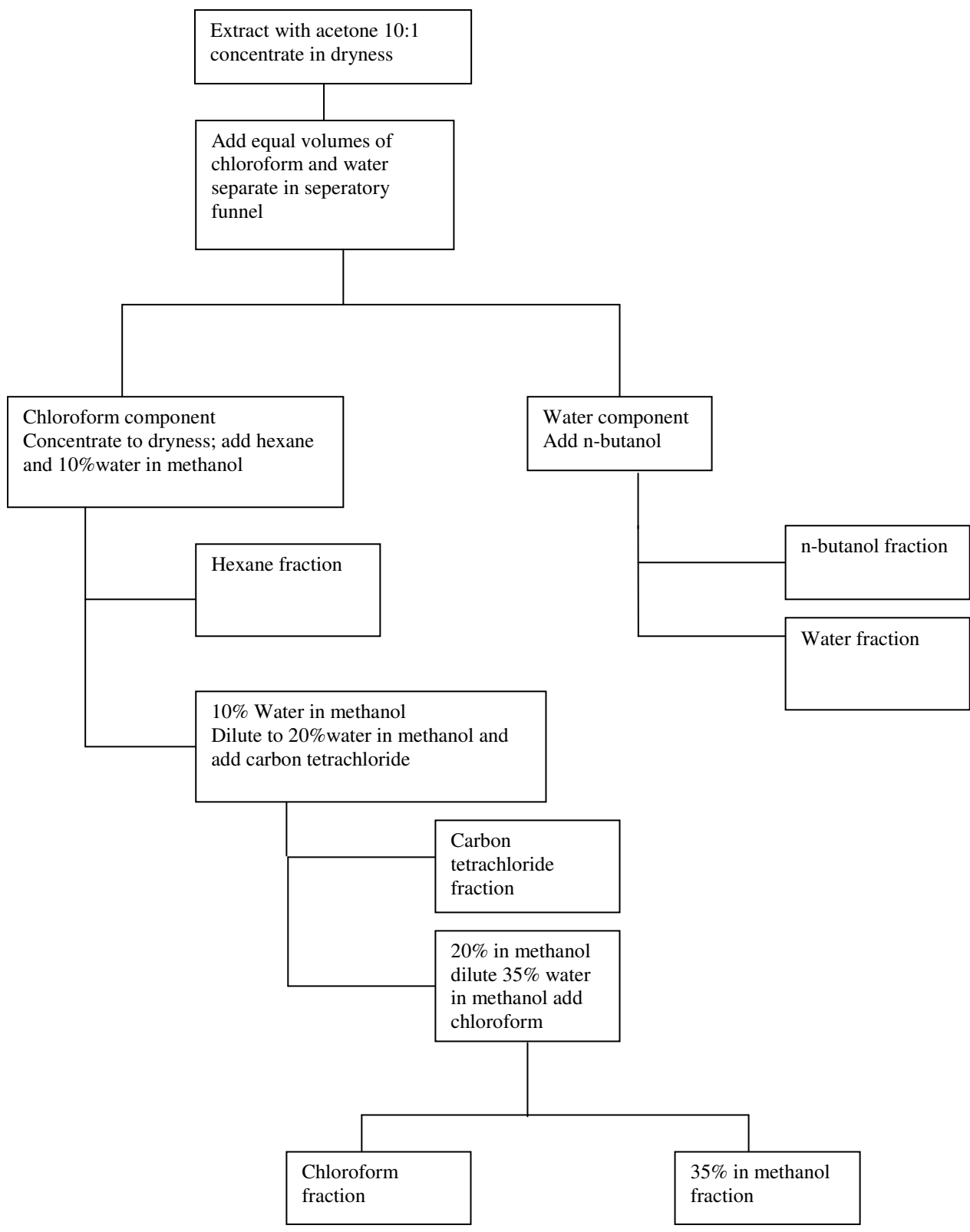
Vero African monkey kidney cells (Vero cells) of a subconfluent culture were harvested and centrifuged at 200 x g for 5 min, and the cell pellet resuspended in growth medium to a density of  $2.4 \times 10^3$  cells/ml. Minimal Essential Medium (MEM) (Highveld Biological, South Africa) supplemented with 0.1% gentamicin (Sigma) and 10% foetal calf serum (Highveld Biological, South Africa) was used. Cell suspension (200  $\mu$ l) was added into each well of columns 2 to 11 of a sterile 96-well microtitre plate. Growth medium (200  $\mu$ l) was added into wells of columns 1 and 12 to minimize the “edge effect” and maintain humidity. The plates

were incubated for 24 h at 37°C in a 5% CO<sub>2</sub> incubator, until the cells were in the exponential phase growth. The medium was then removed from wells using a thin tube attached to a hypodermic needle and immediately replaced with 200 µl of test compound or berberine chloride (Sigma) (positive control) at various known concentrations (quadruplicate dilutions prepared in growth medium). The microtitre plates containing treated and untreated cells were incubated at 37°C in a 5% CO<sub>2</sub> incubator for a defined contact period. MTT (30 µl) (Sigma) (stock solution of 5 mg/ml in phosphate-buffered saline [PBS]) was added to each well and the plates incubated for a further 4 h at 37°C. The medium in each well was carefully removed without disturbing the MTT crystals in the wells. The MTT formazan crystals were dissolved by adding 50 µl of DMSO to each well, followed by gentle shaking of the MTT solution. The amount of MTT reduction was measured immediately by detecting absorbance at 570 nm using a microplate reader (Versamax). The wells in column 1, containing only medium and MTT but no cells, were used to blank the reader. The LC<sub>50</sub> values were calculated as the concentration of test compound or plant extract resulting in a 50% reduction of absorbance compared to untreated cells. Selective activity of the most active extracts was calculated.

### **3.6 Bulk extraction procedure with acetone**

Acetone extracts with the most antibacterial compounds was further extracted in bulk. The powdered leaves and stems (500g) of *R. leptodictya* was extracted with five litres of acetone three times with fresh five litres solvent each time and shaken vigorously for five hours on a Labotec shaking machine to facilitate the extraction process. The supernatant was filtered through Whatman No. 1 filter paper using a Buchner funnel and acetone was evaporated at 40°C using a Buchi rotavapor R-114 Labotec. The reduced extract was poured into pre-weighed glass bottle and left under a stream of cold air to dryness and then yielded 26g. This

was further fractionated using solvent-solvent extraction procedure recommended by USA National Cancer Institute as described by Suffness and Douros (1979) adapted by Eloff (1998) as the first step towards attempted isolation of the bioactive compounds. This process led to six fractions separated based on solubility characteristics of the constituents. Figure 3.6 below represents the scheme procedures of solvent-solvent extraction.



**Figure 3.6** Solvent-solvent fractionation of plant extracts developed by National Cancer Institute.

### **3.7 Chromatographic analysis and bioautography of fractions from solvent-solvent extraction**

The dried seven fractions were dissolved to a concentration of 10 mg/ml in acetone and TLC was performed as described in Section 3.4. The three mobile systems were again used, namely CEF (intermediate polarity), BEA, (non-polar) and EMW (polar). A duplicate set of TLC plates was sprayed with vanillin and placed in an oven at 110<sup>0</sup>C for few minute until optimal colour development. The TLC plates not sprayed with vanillin were left for three days under stream of air to allow the mobile phase to evaporate completely from the plates to avoid lethal effects of solvents during bioautography.

The TLC plates were sprayed with concentrated suspensions of an actively growing culture of *S. aureus* and incubated overnight in a humid environment. After incubation, the TLC plates were sprayed with 2 mg/ml of INT solution and incubated for 30 minutes until optimum colour change, where the colourless INT solution turns red indicating growth of organisms on the plates. Clear zones of inhibition can be observed against a red background indicating bacterial growth inhibition of separated plant constituents on the TLC plates.

TLC analysis of the chosen fractions with different solvent system was used to select the best solvent system that could be used as mobile phase for subsequent column chromatography. Various chromatographic techniques were employed in an attempt to isolate bioactive constituent.

### **3.8 Isolation procedure**

#### **3.8.1 Isolation of compounds from carbon tetrachloride fraction**

About 6 g of carbon tetrachloride fraction which has the most antibacterial compounds was applied to column chromatography. Silica gel 60 (Merck) was used as stationary phase. Dry silica gel powder was suspended in hexane and stirred gently until an even suspension was formed. The suspension was slowly poured into a column that was 70% full of eluent. Carbon tetrachloride fraction (6g) was dissolved in a small volume of ethyl acetate, mixed with 10g of silica gel 60 (Merck), allowed to dry under stream of cold air and loaded at the top of the column until it was eventually distributed over the whole surface. Initially the column was eluted with 100% hexane and subsequently with gradient elution using solvent systems of gradually increasing polarity. Essentially, a volume of 500ml of 100% was initially used, followed by the same volume of each of the following solvent mixtures: 5% EtOAc, 10% EtOAc, 20% EtOAc, 25% EtOAc, 30% EtOAc, 40%EtOAc, 50% EtOAc, 60% EtOAc, 70% EtOAc, 80% EtOAc, 90% EtOAc, all in Hexane, 100% EtOAc and finally the column was washed with Acetone. The fractions 1-57 were collected and placed under a stream of air to concentrate.

#### **3.8.2 Isolation from D2 fraction**

Silica gel 60 (Merck) was mixed with hexane and packed in a column. D2 fraction (669 mg) was dissolved in chloroform and mixed with small amount of silica gel 60 (Merck), dried and loaded on top of the packed column and the column was eluted with 2% Methanol,

4% Methanol, 10% Methanol and all in chloroform. Fractions 1-20 were collected and placed under a stream of air to concentrate.

### **3.8.3 Chromatographic analysis of isolated fractions**

After every subsequent isolation of the plant constituents the collected fractions were loaded onto 10 cm × 20 cm TLC plates and developed in CEF mobile phase which gave a good separation and sprayed with vanillin spray reagent heated in an oven at 100 °C until colour development. Similar fractions were combined together and kept for further isolation until pure compounds were obtained.

## **3.9 Spectroscopic analysis**

### **3.9.1 Sample preparation for NMR spectroscopy analysis**

The Varian Unit Innova 300 MHz NMR system (Oxford instruments) and Brüker DRX – 400 instrument were used for  $^{13}\text{C}$  and  $^1\text{H}$  NMR. The compounds isolated from *R. leptodictya* were dried, weighed (10-20 mg) and dissolved in a maximum of 2 ml of deuterated acetone (Merck) because the isolated compounds were soluble in acetone. The solutions were then pipetted into NMR spectroscopy tubes (commonly of 5 mm diameter to a depth of 2-3 cm) using clean Pasteur pipettes and taken to the Department of Chemistry and Biochemistry, University of Limpopo (Medunsa Campus) and University of Botswana for proton ( $^1\text{H}$ ), carbon 13 ( $^{13}\text{C}$ ) and distortionless enhancement through polarization transfer (DEPT).

### **3.9.2 Sample preparation for MS analysis**

The analytical VG 7070E mass spectrometer (Quattro (VG Biotech, England) using electron impact at 70 eV as ionization technique was used for mass spectroscopy. Approximately 20 mg of each isolated compound was dried, placed into a 2 ml glass vial and sent to University of Pretoria.



## CHAPTER 4

### 4. Extraction and preliminary fractionation of plant extracts

#### 4.1 Introduction to extraction of plant material

The plant material used for extraction as a source of extract for the secondary plant metabolites could be fresh or dried. Dried plant material is preferred because traditional healers use many, if not most plants in dried form, there are fewer problems associated with large scale extraction of dried plant material than with fresh material because water presence in fresh leaves can complicate extraction with non-polar solvents, and the time delayed between collecting plant material and processing it, make it difficult to work with fresh material because physiological change can take place before extraction (Eloff, 1998).

There are several methods of extraction of chemical soxhlet extraction of dried plant material with various solvents is frequently used since it is performed at high temperature it is not suitable for thermolabile compounds. In order to circumvent the effect of high temperature extraction can be done under reduced pressure, but this is complicated and expensive. The choice of solvent depends also on what is intended with the extract. If extraction is to screen plants for microbial components, the effect of the extractant on subsequent separation procedures is not important, but the extractant should not inhibit the bioassay procedure. If the plant material is extracted to isolate chemical components without using bioassay, toxicity of the solvent is not important because the solvent can be removed before subsequent isolation procedures (Eloff, 1998). Eloff (1998a) concluded that acetone was the best solvent for extraction of screening of plants for activity. Acetone is volatile, miscible with polar and non

polar solvents and is also less toxic to plant materials. The non-polar solvents such as chloroform are toxic to the test organisms and the extracts would not mix with water in MIC assay, the extracts of the non-polar solvents were dried and dissolved with acetone. The main reason for using solvents of different polarities is to determine if any of these solvents could selectively extract compounds with antibacterial activities. To isolate antibacterial compounds a different extraction was used in section 3.3 and section 3.6.

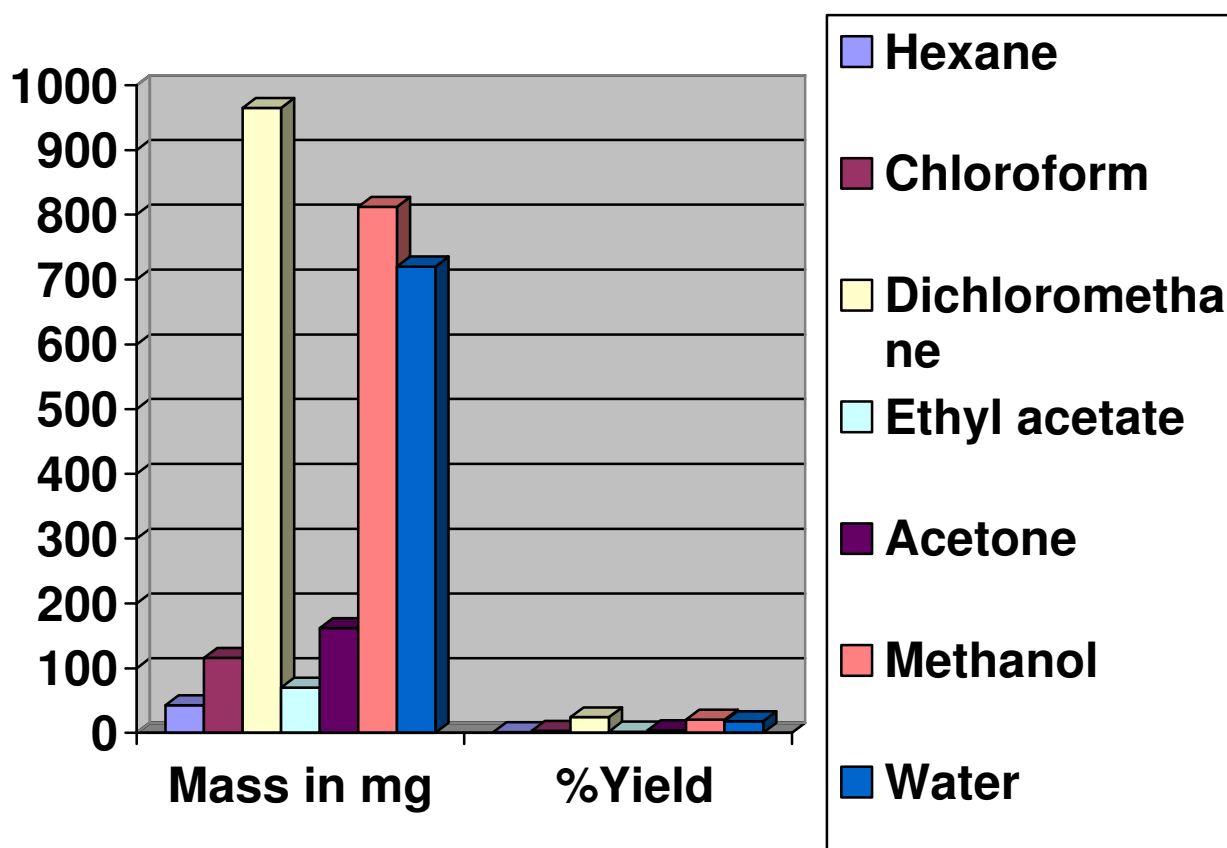
Chromatography is an analytical method that is used for the separation, identification and determination of the chemical components in complex mixtures. No other separation method is powerful and generally applicable as is chromatography. Thin Layer Chromatography has become the workhorse of the drug discovery for the all-important determination of product purity. It was also found widespread use in clinical laboratories and is the backbone of many biochemical and biological studies (Skoog et al, 1991).

#### **4.2 Extraction yield of plant extracts**

Separate aliquots of 4 g of the leaves and twigs of *R. leptodictya* powdered were extracted with 40 ml of seven different solvents of increasing polarity in 50 ml centrifuge tubes described in section 3.3. Dichloromethane (DCM) with 24.1% was the best extracting solvent amongst the seven solvents because it generally extracted the highest quantity of plant material compared to the other solvents, followed by methanol (MeOH) with 20.3 %, acetone (Act) with 4.1%, chloroform (Chl) with 2.9%, water (Wat) with 18.0%, ethyl acetate (Eth) with 1.8%, and hexane (Hex) with 1.1% extracting the lowest quantity. It shows that the leaves and twigs of *R. leptodictya* contain more polar solvents than non-polar solvents since methanol extracted the highest quantity of compounds and hexane with the lowest quantity.

**Table 4.2:** Quantity extracted by different solvents of *R. leptodictya* leaves and twigs.

Extractants	Mass in mg	%Yield
Hexane	43	1.1
Chloroform	116	2.9
Dichloromethane	965	24.1
Ethyl acetate	70	1.8
Acetone	162	4.1
Methanol	812	20.3
Water	720	18.0



**Figure 4.2:** Quantity extracted by different solvents of *R. leptodictya* from 4 g leaves and twigs.

#### 4.3 Chromatographic analysis of plant extracts

Leaves and twigs extracts were subjected to qualitative analysis using a thin layer chromatography (TLC) technique as described in section 3.4. This was done to investigate the chemical composition of the extracts. The larger the variety of the compounds that can be extracted by the extractants, the better the chance that biologically active compounds will also be extracted. There were several compounds circled with a pencil on the chromatograms which are visible under UV light and others quenched fluorescence indicator added to the plates. There was a difference in the composition of the extracts sprayed with vanillin reagents.

CEF was the best mobile phase system for separation of almost all the extracts, followed by BEA and EMW with the least, because many compounds were separated, efficiently using CEF (Fig 4.3). The separation in CEF indicates a number of separated intermediate polar compounds and BEA with non-polar compounds. It is expected that the more polar extractants would extract few of the very non-polar compounds. The separations of the extracted compounds in EMW system were not as efficient because the majority of the extracted compounds were too non-polar and intermediate polar for these solvent system and moved to the top of the TLC plate making it difficult to count the number of extracted compounds visible after spraying with vanillin sulphuric acid spray reagent (Fig 4.3).

This can probably be explained by the presence of saponin-like components present in the plant material. Similar to soap with a polar and non-polar ends, these compounds could make non-polar compounds soluble in polar extractants (Kotzé and Eloff, 2002). In CEF it appeared that the methanol extract contained the lowest number of compounds compared to hexane, chloroform, dichloromethane, ethyl acetate and acetone extracts, with ethyl acetate and

acetone extracting the greatest number of compounds followed by hexane, chloroform and then DCM. CEF solvent system separated intermediate polar compounds better with BEA solvent system separated non-polar compounds better but EMW was not good in separating polar compounds. This indicates that the separated compounds are more of intermediate and non-polar compounds. To find out which extracts contained the antibacterial compounds, extracts were subjected to bioautography.

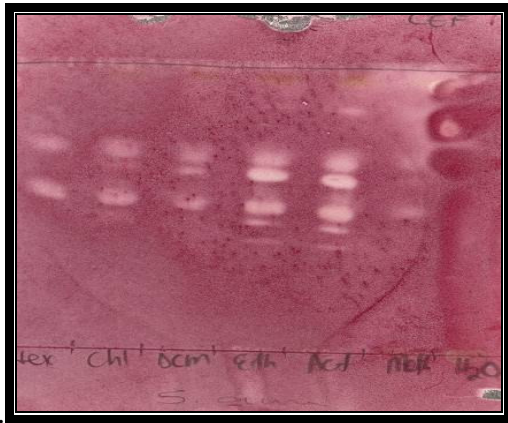


**Figure 4.3** TLC chromatograms of *R. leptodictya* leaves and twigs extracted from left to right with hexane (Hex), chloroform (Chl), dichloromethane (DCM), ethyl acetate (Eth), acetone

(Act), Methanol (Met) and water (Wat), developed in BEA (top), CEF (middle) and EMW (bottom) and sprayed with vanillin reagent.

#### **4.4. Bioautography of plant extracts**

The TLC bioautogram of *R. leptodictya* of seven solvent extracts are shown in Fig 4.4. The TLC plates were developed in CEF mobile system. The highest number of compounds inhibiting the growth of *S. aureus* (6) was observed for the acetone extract followed by the ethyl acetate (5). Acetone and ethyl acetate extracts inhibited the growth of *S. aureus* of most compounds it shows that most antibacterial compounds had an intermediate polarity. Acetone extracted the least with (612mg) of plant constituents compared to dichloromethane (965mg) and methanol (812mg) extracts; but acetone extracted most antibacterial compounds compared to dichloromethane and methanol. This implies that nature of the active constituents tends more towards intermediate polarity and that acetone extraction already led to an extract with fewer inactive compounds. In EMW solvent little separation took place and system there was inhibition of growth of *E. coli* suspension in almost all extracts at the top of TLC plates.

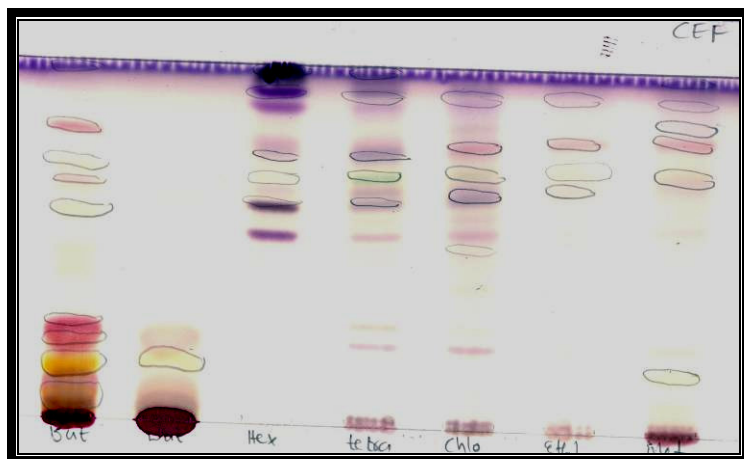


**Fig 4.4** Bioautogram of *R. leptodictya* leaves and twigs extracted with hexane (Hex), chloroform (Chl), dichloromethane (DCM), ethyl acetate (Eth), acetone (Act), Methanol (Met), water (Wat); developed in CEF (top) and EMW (bottom) and sprayed with an actively growing *S. aureus* and *E. coli* suspension and INT solution.

#### 4.5 Chromatographic analysis of solvent-solvent fractions

The powdered leaves and stems (500g) of *R. leptodictya* was extracted with five litres of acetone three times with fresh five litres solvent each time and shaken vigorously for five hours on a Labotec shaking machine to facilitate the extraction process as described in section 3.6. The extract was separated into seven fractions by solvent-solvent fractionation. The chromatogram of the seven fractions obtained by solvent-solvent extraction is shown in Fig

4.5. Carbon tetrachloride and chloroform fractions had greatest number of compounds followed by hexane and water fraction with the least number of compounds. Hexane, carbon tetrachloride and chloroform fractions separate almost similar compounds.

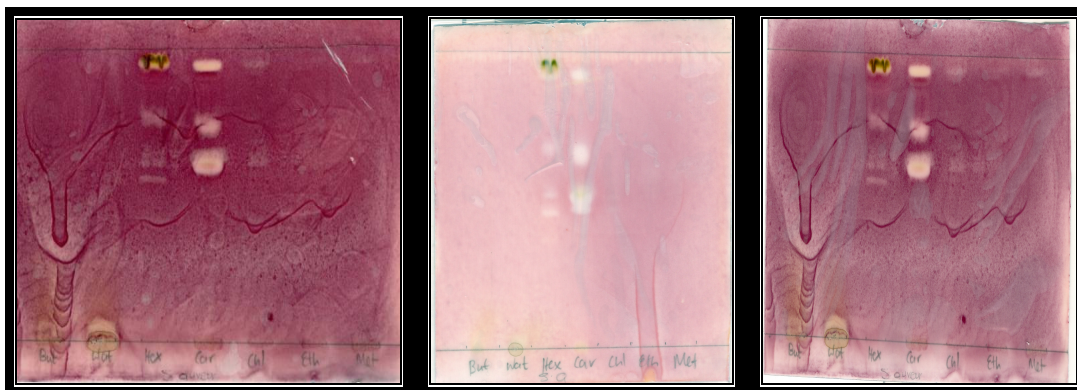


**Figure 4.5** TLC chromatogram of different fractions of *R. leptodictya* leaves and stems extract obtained by solvent-solvent fractionation. Lanes from left to right with butanol (But), water (Wat); hexane (Hex), carbon tetrachloride (Car), chloroform (Chl), ethyl acetate (Eth) and methanol (Met) fractions developed in CEF and sprayed with vanillin reagent.

#### 4.6. Bioautography of solvent-solvent extraction

TLC chromatogram of different fractions of *R. leptodictya* leaves and twigs extract obtained by acetone solvent-solvent extraction Fig 4.6. The TLC plates were developed in CEF mobile system. Carbon tetrachloride and hexane fractions inhibited the growth of *S. aureus* compared to other fractions. The highest inhibition of growth of *S. aureus* is observed for carbon tetrachloride followed by hexane, which is characterized by six and five respectively major clear spots, while other fractions does not show inhibition. Therefore carbon tetrachloride and hexane fractions were taken for isolation of antibacterial compounds.

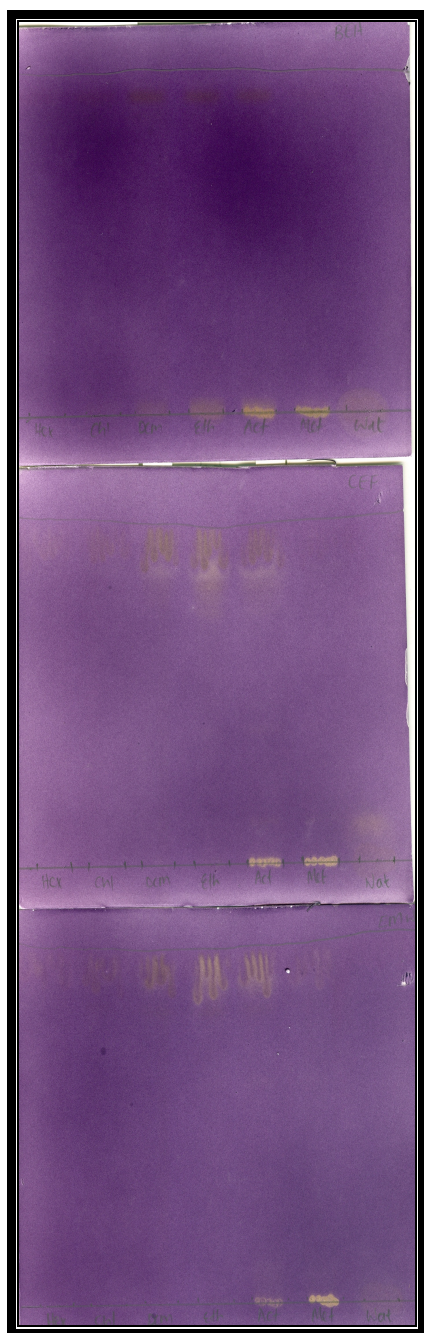




**Figure 4.6** Bioautogram of different fractions of *R. leptodictya* extract obtained by solvent-solvent extraction. TLC chromatograms developed in CEF mobile phase and sprayed with actively growing *S. aureus* suspension and INT solution.

Colourless areas denote inhibition of bacterial growth. Lanes from right to left with butanol (But), water (Wat), hexane (Hex), carbon tetrachloride (Car), chloroforms (Chl), ethyl acetate (Eth) and methanol (Met) fractions.

#### 4.7. Antioxidant activity of plant extracts



**Fig 4.7** TLC chromatograms of *R. leptodictya* leaves and twigs extracted from left to right with hexane (Hex), chloroform (Chl), dichloromethane (DCM), ethyl acetate (Eth), acetone (Act), Methanol (Met), water (Wat); developed in BEA (top), CEF (Middle) and EMW (bottom) and sprayed with DPPH.

The antioxidant activity was determined by using DPPH assay as described in section 3.5.4. The leaf and twig extracts in BEA, CEF and EMW solvent systems showed the activity at the origin of the TLC plates with the exception of acetone and methanol extracts and other extracts with no activity. In the chromatograms eluted with CEF and EMW there are some activity at the top of the plate of the polar fractions. The polar extractants, methanol and acetone show some activity in all solvents systems compared to other extracts. This is due to the polarity of the components of the compounds present in the extractants.

#### **4.8. Quantification of antibacterial activity of plant extracts.**

The quantity of the antibacterial compounds present depends on the MIC values of the extract. To determine the total activity in ml the quantity in mg extracted has to be divided by the MIC in mg/ml (Eloff, 2000). MIC is the lowest concentration of test sample expressed in mg/ml that leads to an inhibition of the growth (i.e. the lowest concentration that gave a decrease in colour). To determine the activity in ml the quantity in mg extracted has to be divided by MIC in mg/ml. The average MIC value and the total activity of *R. leptodictya* extracted with seven different solvent systems against *S. aureus*, *E. faecalis*, *P. aeruginosa* and *E. coli* are presented in Table 4.8. This table shows that the water extract had a very low MIC average value of 0.16mg/ml against *S. aureus* and *E. faecalis* and with no activity against *E. coli* and *P.aeruginosa* followed by acetone extract with the average MIC value of 0.36mg/ml against all four bacteria. This shows that acetone extract was more active than the other extracts because the lower the MIC values the more active the extracts. Hexane extract had the highest MIC average value with 2.2mg/ml and this shows that hexane extract was less active against four bacterial. The MIC value of the extracts did not match the results from Bioautography Fig 4.5 well. This may be due to the fact that some of the compounds that

have antibacterial activity in MIC evaporated when air-drying the plates overnight. The highest total activity was found with DCM and Met extracts with values 12063ml and 10150ml respectively. This means that the quantity present in DCM extract can be diluted to 12063ml but would still inhibit the growth of the bacteria. The total activity indicates the volume to which the biologically active compounds present in gram of dried plant material can be diluted and still kill the bacteria. Acetone extract has the lowest MIC values of all four test organisms, therefore acetone solvent was selected for bulk extraction and this facilitates the isolation and characterization of active constituents.

**Table 4.8:** Quantification of antibacterial activity of plant extracts from 4g of plant material, average MIC values and total activity of seven extracts against *S. aureus*, *E. faecalis*, *P. aeruginosa* and *E. coli*.

Plant extracts	Quantity extracted (mg)	MIC values (ml/g)				MIC average	Total activity (ml/g)			
		<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>E. faecalis</i>		<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>E. faecalis</i>
Hexane	43	0.63	0.63	0.31	0.63	2.20	68	68	139	68
Chloroform	116	0.31	0.63	0.08	0.16	1.18	725	184	1450	725
Dichloromethane	965	0.31	0.63	0.08	0.16	1.05	6031	1532	12063	6031
Ethyl acetate	70	0.16	0.16	0.04	0.16	0.44	875	438	1750	438
Acetone	162	0.08	0.16	0.04	0.08	0.36	2025	1013	4050	2025
Methanol	812	0.16	0.16	*	0.08	0.40	5075	5075	*	10150
Water	720	0.08	*	*	0.08	0.08	9000	*	*	9000

\* No activity

## CHAPTER 5

### 5. Isolation of antibacterial compounds

#### 5.1 Introduction to isolation of compounds

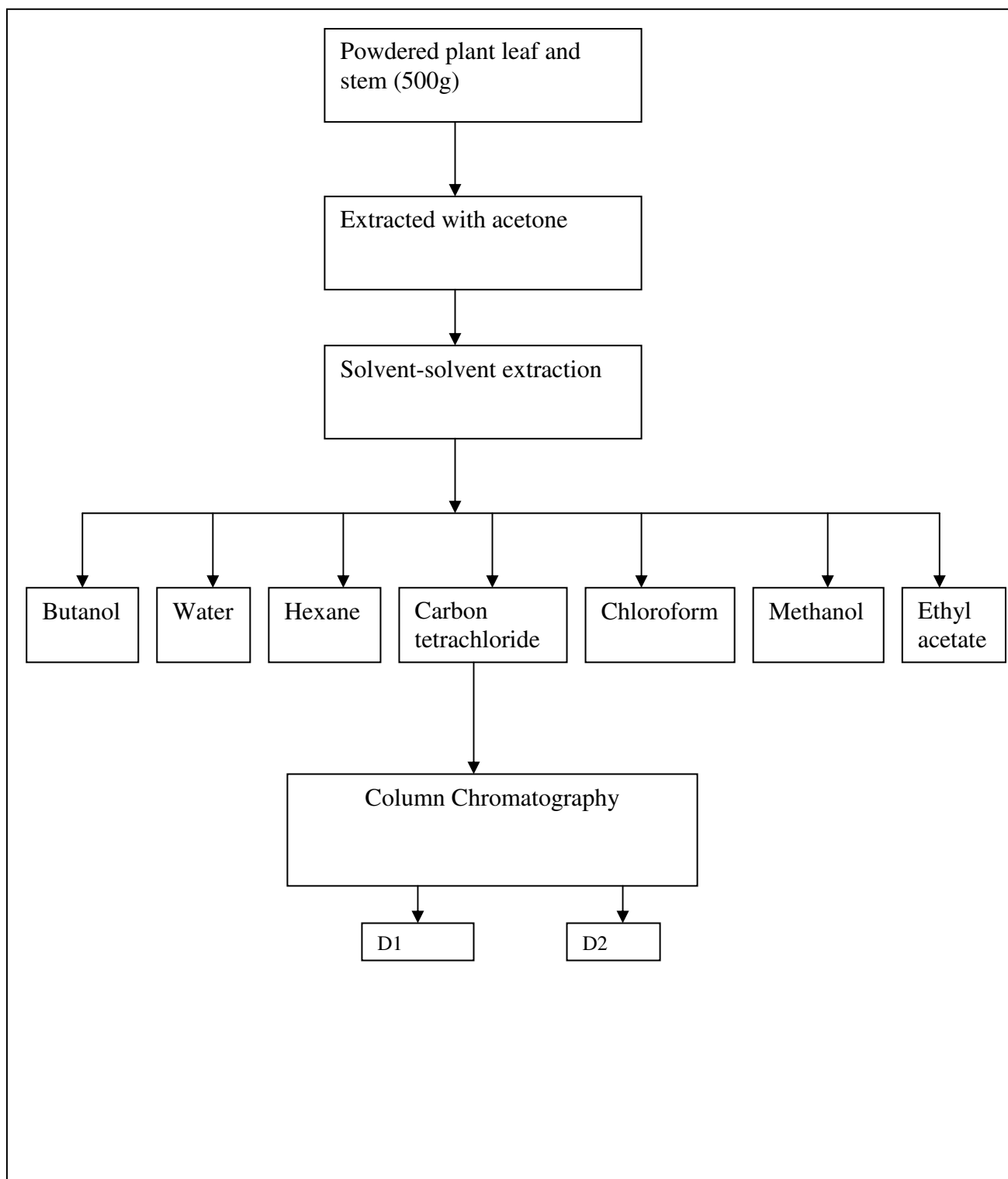
Chromatographic techniques such as thin layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC) are frequently used for the analysis of plant medicines. TLC is an important method for the isolation, purification and confirmation of natural products. This method is considered to be reproducible and accurate (Wen *at al.*, 2004).

Column chromatography is one of the most frequently used techniques in the isolation of natural plant constituents. In principle, plant constituents are distributed between the solid phase (for example silica gel or Sephadex) and the mobile phase, which comprises an eluting solvent. In silica gel the separation of compounds from each other in an extract is based on a number of factors including the polarity of compounds, hence compounds are eluted from the column with solvent systems of differing polarity. Silica gel has polar ends which interact strongly with polar compounds and they are eluted later from the column. In Sephadex gel filtration the separation of constituents in an extract depends on the size of the molecules. Constituents with a small molecular weight interact strongly with the matrix of the gel and tend to move slowly through the gel and they are eluted later while the large molecular weight constituents are eluted early because they move fast through the column.

The successful isolation of bioactive compounds from indigenous medicinal plants will validate indigenous knowledge adding value to plants and support plant conservation and knowledge preservation. It may also contribute to research and development in the production of new pharmaceutical drugs for the treatment of various infectious diseases caused by micro-organisms such as fungi, bacteria and viruses.

## **5.2 Chromatographic techniques used to obtain pure compounds**

In a preliminary screening with a small quantity of dried finely ground leaves and stems of *R. leptodictya*, acetone extracts were chosen for isolation of compounds because they contained more antibacterial compounds than other extracts based on the bioautography results. Acetone extracts were taken further for solvent-solvent extraction from which seven fractions were obtained. Column chromatography was employed to fractionate carbon tetrachloride fraction because it containing more antibacterial compounds than other fractions. Initially a large column was packed with a silica gel suspension in hexane under vacuum. The procedures used are described in section 3.8. The flow diagram shown in figure 5.2 shows the steps taken to get pure compounds.

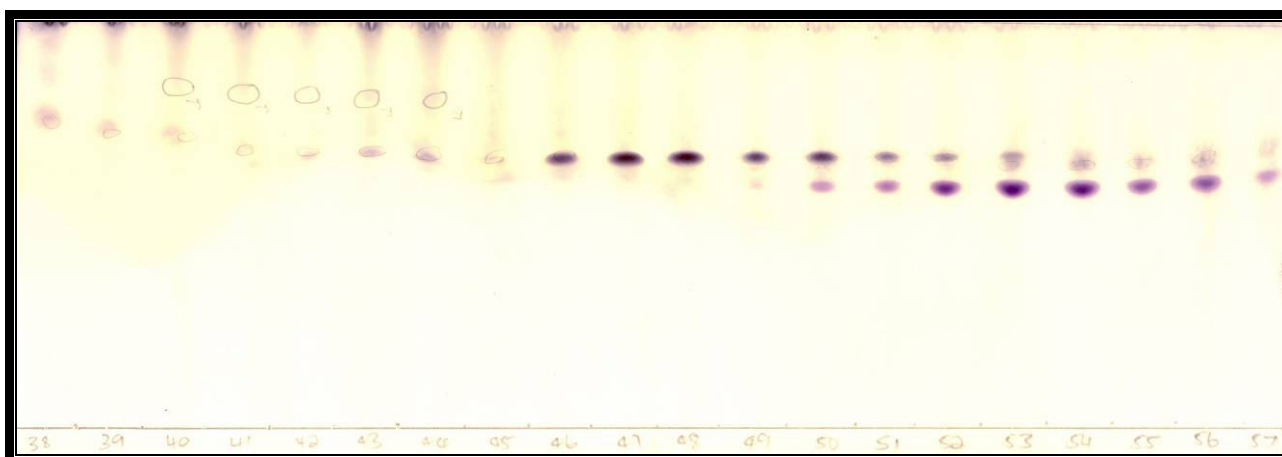


**Fig 5.2** Schematic representation, indicating the isolation pathway of the compounds from bulk acetone extraction.

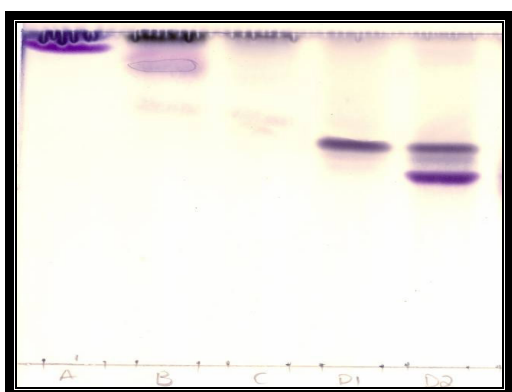
### **5.3 Isolation of compounds from carbon tetrachloride fraction**

The isolation of compounds from the carbon tetrachloride fraction using the silica gel column chromatography described in section 3.8 which in fractions 1-57 shown in figure 5.3a. The fractions with similar constituents were combined and developed on a TLC plate using CEF and sprayed with vanillin spray reagent and heated at 110<sup>0</sup>C in an oven until optimal colour development as shown in Figure 5.5b. Fractions A (14-19), B (33-37), C(38-39), D1 (46-48) and D2 (49-57), from the carbon tetrachloride fraction were combined, developed in CEF and sprayed with vanillin spray reagent and heated at 110 in an oven until optimal colour development shown in figure 5.5b. Fraction D1 yielded (100mg) of pure compound which was analysed with to NMR spectroscopy and MS techniques. Fraction D2 was further isolated using silica gel column chromatography described in section 3.8.2 and yielded (41mg) of a pure compound which was analysed with NMR spectroscopy and MS techniques.

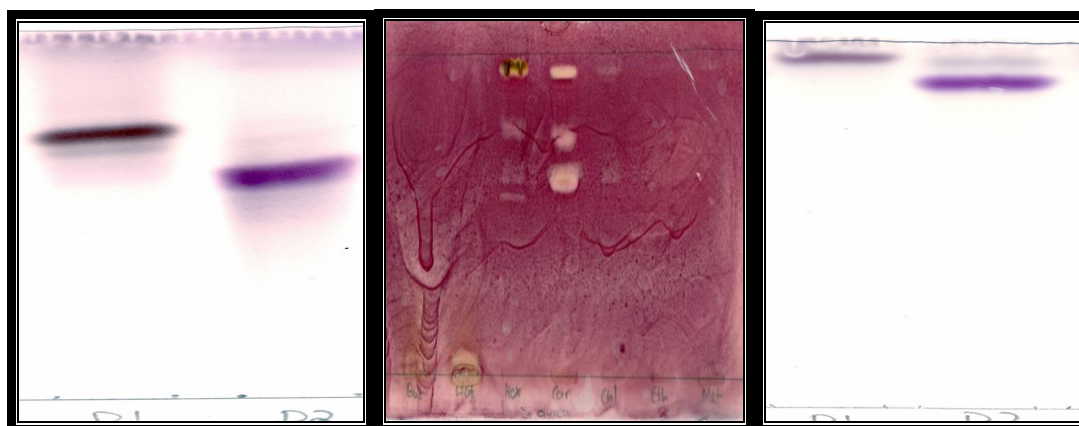




**Figure 5.3a** TLC chromatogram of the carbon tetrachloride fraction showing the number of compounds present in different fractions developed in CEF and sprayed with vanillin.



**Figure 5.3b** TLC chromatogram of the combined similar fractions from carbon tetrachloride fraction showing the number of compounds present in different fractions developed in CEF and sprayed with vanillin.



**Figure 5.3c** TLC chromatogram of isolated compounds from carbon tetrachloride fraction developed in CEF from the left, developed in EMW from the right sprayed with vanillin and at the middle solvent-solvent extraction developed in CEF mobile phase and sprayed with *S. aureus* suspension and INT solution.

As an aid to replicating isolation of antibacterial compounds from carbon tetrachloride fraction the  $R_f$  values in our commonly used TLC solvent systems were determined. The  $R_f$  values of the isolated compounds were 0.7 for D1, 0.6 for D2, 0.5, 0.6, 0.8 and 0.95 from bioautography of solvent-solvent extraction in CEF. These  $R_f$  values were calculated from figure 5.3c. Compound D1 and D2 has the same  $R_f$  value as one of the  $R_f$  values of carbon tetrachloride fraction in figure 4.6, this show that compounds D1 and D2 are active against *S. aureus* from the calculated  $R_f$  value.

## CHAPTER 6

### 6. Structural elucidation and characterization of isolated compounds

#### 6.1 Spectroscopic analysis of isolated compounds

The different interactions of electromagnetic radiation with organic compounds based on their structural features forms the basis of the application of spectroscopy in the structural elucidation of organic compounds. The identification of compounds involves a diverse range of analytical techniques and methods such as nuclear magnetic resonance (NMR), ultraviolet (UV) and infrared (IR) spectroscopy, and mass spectroscopy (MS). In this study NMR and MS techniques were used as tools for the analysis and identification of the compounds isolated from *R. leptodictya*. Structure elucidation of isolated compounds was achieved by a combination of nuclear magnetic resonance (NMR) and mass spectrometric (MS) analysis. One of the most important techniques for the characterization, or structure determination of carotenoids is H-NMR spectroscopy (Moss, 1976 and Englert, 1985).

##### 6.1.1 $^1\text{H}$ and $^{13}\text{C}$ - NMR (One dimensional spectrum)

One dimensional NMR spectra were recorded for the isolated compounds on Varian 300 Spectrophotometer using deuterated methanol as solvent. 2D NMR (COSY, HMBC and HMQC) data were also obtained using the same instrument and solvent system.

### **6.1.2 HMQC $^{13}\text{C}$ - $^1\text{H}$ correlation**

Heteronuclear multiple quantum correlation (HMQC) was used for the determination of substitution patterns at the different carbon atoms, correlation of carbon shifts, multiplicity and proton shifts were compiled from a two dimensional heteronuclear correlation spectrum. The combined spectral information allowed a view of carbon atoms showing different functionalities and substitutions.

### **6.1.3 COSY - $^1\text{H}$ - $^1\text{H}$ 2D**

Two dimensional spectra were obtained by recording a series of conventional one dimensional NMR spectra in which two parameters are changed incrementally. The most readily established connections between the individual carbon atoms were derived from connectivities through couplings between the protons as compiled from a COSY spectrum. From these correlations, the main part of the proton spin systems was outlined and several structural fragments were identified.

### **6.1.4 HMBC long range $^1\text{H}$ - $^{13}\text{C}$ correlation**

Heteronuclear bond correlation (HMBC) experiment provided information on the direct  $^{13}\text{C}$  and  $^1\text{H}$  heteronuclear connectivity. The method relies on the indirect detection of the  $^{13}\text{C}$  by observing their effects on the more sensitive proton nuclei to which they are coupled. It not only shows the connection of unprotonated carbon atom to the proposed elements, but also indicated vicinal proton relationships not resolved in the COSY spectrum. Analysis and

interpretation of the spectroscopic data obtained led to the structures proposed for the active compounds D1 and D2 in chapter 3.

## **6.2 Identification of compounds**

Inspection of the Bioautogram in figure 4.6 showed major active spots. Column chromatographic separation of the bioactive fraction led to the isolation of two bioactive compounds. Compound D1 and D2 were sufficiently pure for subsequent structurally related elucidation. The structures of the two isolated compounds are discussed below.

### **6.2.1 Compound D1**

Compound D1 was isolated as yellowish-reddish amorphous solid. One of the most important techniques for the characterization of carotenoids is H-NMR spectroscopy (Moss, 1976). The NMR spectra and spectral data analysis of this compound is presented in Figure 6.2.1a-f. Inspection of the carbon spectra showed the presence of 40 carbon chemical shifts, with signals in the up field and downfield region. The spectra of the isolated compound showed a characteristic pattern for carotenoids [C40 derivatives]. There were 20 signals in the vinylic region. There were 20 signals downfield (120-180 ppm) and 20 carbons upfield (10-48). This indicated a long conjugated system as seen in carotenoids. The interconnectivity between the various carbons in the compound are worked out as described in various 2D NMR data stated below. The carbon spectra of carotenoids suggested the basic skeleton of the isolated compound. The multiplicity of absorption in the proton spectrum is because the isolated compound (a carotenoid) has two parts in its structure which are mirror image of each other and most of the proton have similar chemical environment, hence similar chemical shift of

their signals. The  $^{13}\text{C}$  chemical shift was compiled and the data was compared with previously data on  $^{13}\text{C}$  shifts for previously isolated carotenoids. The  $^{13}\text{C}$  for isolated compound compare perfectly with data from a previously isolated carotenoid lutein (Arigon et al, 1999). The carbon data for the isolated compound and lutein are presented in table 6.2.1.

**Table 6.2.1:** The carbon data of the isolated compound BSRL-1 and lutein (Arigon et al, 1999).

Carbon	Lutein	BSRL-1
1	37.09	37.11
1'	34.01	34.16
2	48.30	48.35
2'	44.60	44.64
3	65.04	65.09
3'	65.89	65.94
4	42.46	42.49
4'	124.39	124.45
5	126.13	126.17
5'	137.98	137.98
6	137.68	137.56
6'	54.87	54.95
7	125.53	125.59
7'	128.79	128.71
8	138.47	138.49
8'	137.68	137.56
9	135.68	135.68
9'	135.06	135.06
10	131.28	131.31
10'	130.77	130.81
11	124.90	124.93
11'	124.77	124.81
12,12'	137.53	137.56
13	136.40	136.40
13'	136.48	136.46
14,14'	132.56	132.58
15	130.09	130.09
15'	130.04	130.04
16	30.26	30.26
16	24.19	24.19
17	28.72	28.68
17	29.50	29.46
18	21.62	21.63
18	22.86	22.88
19	12.75	12.76
19	13.10	13.10
20,20	12.81	12.81

### 6.2.1.1 BSRL-1 HMQCGP

From the HMQC spectra in Figure 6.2.1d, which showed the substitution pattern directly on the carbon atom, the following prominent linkages were clearly observed.

C-14 (132.56) ----- H-14 (6.21)

C-7' (128.79) ----- H-7' (5.43)

C-10' (130.77) ----- H-10' (6.67)

C-12 (137.53) ----- H-12 (6.37)

C-5 (126.13) ----- H-12(6.15)

C-10 (131.28) ----- H-10 (6.20)

C-8 (138.47) ----- H-8 (6.13)

C-19 (13.11) ----- H-19 (1.89)

### 6.2.1.2 COSY (Correlation Spectroscopy)

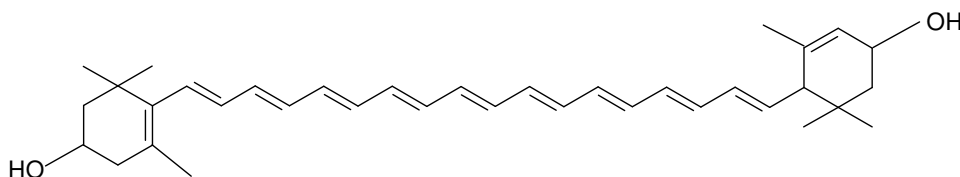
COSY spectra (Figures 6.2.1e) which indicate  $^1\text{H} - ^1\text{H}$  direct correlation clearly showed the proton at H-10 with a chemical shift of 6.11 ppm is coupled to the proton at H-11 with a chemical shift of 6.61. Acetylenic proton at H-7' (5.4ppm) is coupled to a doublet at H-6' (2.38ppm). The proton at H-3 (4.2 ppm) position which exists as a multiplet is coupled to H-2 with a chemical shift of 1.9. The various linkages are in agreement with the proposed structure.

### 6.2.1.3 HMBC

Long range  $^{13}\text{C}-^1\text{H}$  in Figure 6.2.1f showed a strong correlation from the olefinic carbon (C-4') at 128 to the proton at H-6'. Further correlations clearly observed are from C-2 at 48.30



ppm to H- 3. The carbon at C-6 position with a chemical shift of 137.68 ppm is linked to H-7. The carbon at C-14 showed correlation to the proton at H-15. The  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR and 2DNMR (HMQC, HMBC, COSY and DEPT) of compound D1 support the structure proposed as shown below.



**Figure 6.2.1** Proposed structure Lutein.

### 6.2.2 Compound 2

This compound showed a molecular ion at  $m/z$  539[M - 1] which is consistent with molecular formulae of  $\text{C}_{30}\text{H}_{20}\text{O}_{10}$ . The fragmentation pattern is consistent with the characteristic pattern of a biflavonoid which consist of a flavonone and a flavone as shown in the fragmentation pattern in figure 6.2.2a. The biflavonoid with molecular ion 539 fragmented to its monomer form which consists of a flavonone and a flavone with mass units of 269 and 267 respectively. The loss of  $\text{CH}_2$  from the flavonone and the flavone substituents gave rise to the peaks at 255 and 253 respectively. The resulting substituents further fragment with the loss of carbonyl group [-CO] which resulted in the peaks observed at 227 and 225. The consistent difference of 2 in the mass units of the monomers and their substituents further confirm the nature of the isolated biflavonoid – a flavonone and a flavone subunits; The absence of a

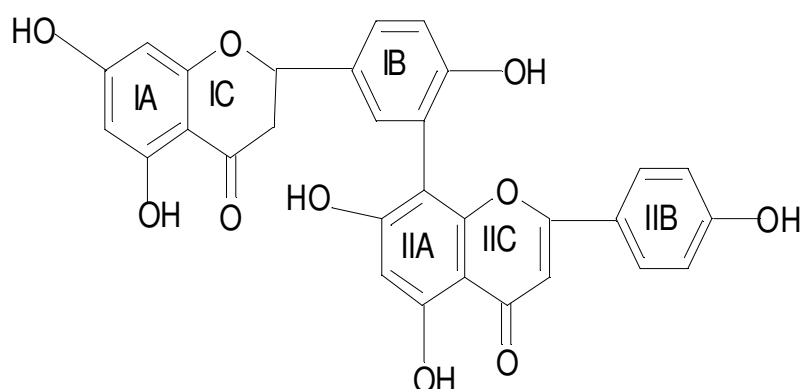
double bond between the C-2 and C-3 positions of a flavonone [present in flavone] explains the consistent difference of 2 mass units in their resultant fragments.

<sup>1</sup>H NMR spectra of BSRL-2 shown in figure 6.2.2b indicated a biflavonoid which consist of a flavonone and a flavone unit linked through the C-3 of a flavonone ring to the C-8 of the flavone ring. This class of compound consists of three ring systems A, B, and C. Ring B which has a hydroxyl substitution at C-4 often gives a typical 4 peak pattern of two doublets (AA'BB' system) with a characteristic coupling constant; this pattern was clearly shown in C-3, C-5, C-2 and C-6 with resonance at 7.99 ppm and 7.07 ppm respectively and a coupling constant of 9 Hz at ring IIB. The protons at H-6 and H-8 in ring A consist of 2 doublets with signal at 5.99 ppm and 5.96 ppm respectively with a coupling constant of 2.1 Hz; this is due to the meta-coupling of these two related protons. This is often characteristic of flavonone which contain the 5, 7 dihydroxy substitution as observed in compound A. This is differentiated from the substitution pattern in flavone because the metacoupled proton at 5, 7 dihydroxy substitution occurs further downfield when compared with flavonone in the isolated compound. The flavonone component is further confirmed by the two doublets at 5.54 and 5.50 ppm, this is due to the proton at C-2 positioned which is coupled to the two protons at the C-3 positions. The two proton at the C-3 also showed characteristic signal at 3.31 and 2.82 ppm which exist as doublet of doublet because the two protons are coupled to each other and the proton at the C-2 position. Two 1H signals at 13.45 and 12.21 ppm are consistent with two chelating protons in the biflavonoid structure in the isolated compound. Two distinct singlets were observed at 6.72 and 6.83 ppm due to the singlets at II-H-3 and II-H-6 positions respectively.

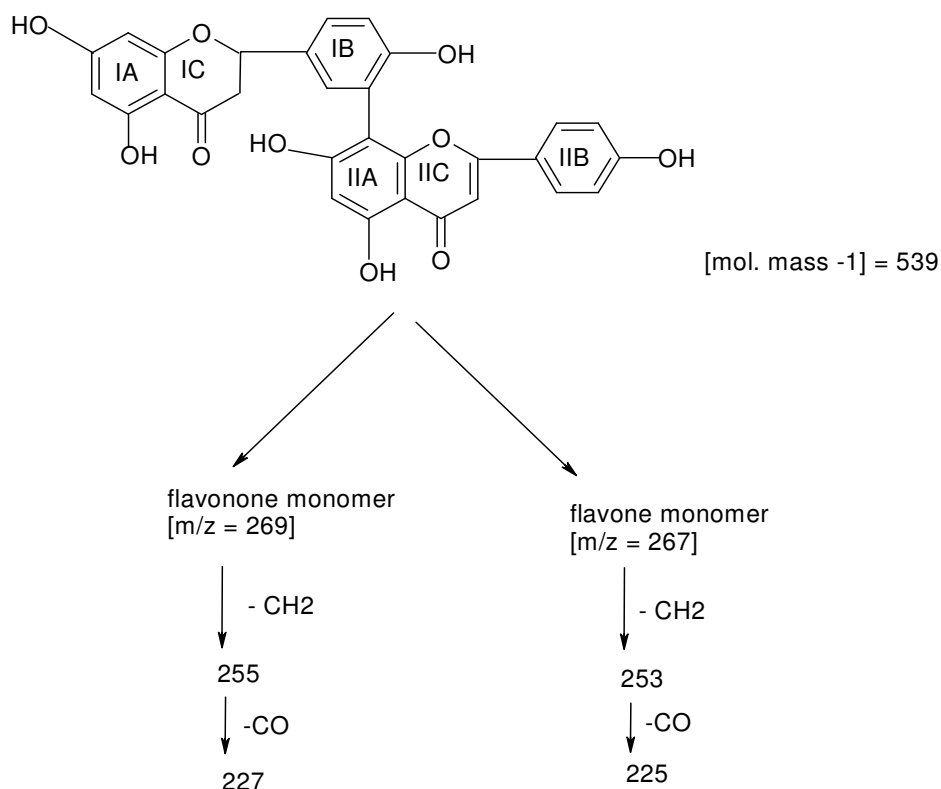
Compound BSRL-2 was isolated in its aglycone form as the absence of glycoside derivative is confirmed by the complete absence of glucosyl signals in the chemical shift range of 4–5 ppm. Although H-3 have related chemical shift to H-6 and H-8, signal due to H-3 is readily distinguished from the H-6 and H-8 signal because it occurred as a singlet at 6.74 ppm compared to the signal from H-6 and H-8 which consistently occur as doublet. This flavonoid type is clearly differentiated from the monohydroxylated form because the C-6 proton in ring A of the monohydroxylated form always occurred as a singlet instead of the doublet observed in the isolated compound. The protons in the A-ring often appear more downfield than the ring protons and it shows some characteristic signal based on the substitution pattern of the ring that makes its identification confirmatory.

The occurrence of the signals of H-2' and H-6' at 8.12 ppm and 8.04ppm was affected by the nature of substitution on the C-ring. The observed resonances differentiated the isolated compound from other classes of flavonoids with different oxidation level on the C-ring such as chalcones, aurones, dihydroflavonols, isoflavones and flavonols. While the coupling constant of these classes of flavonoids is similar for the C-2 and C-6, their chemical shift signals differ by the substitution pattern on the C-ring. Different spectral patterns are observed where there are dual substitutions at the 3<sup>rd</sup> and 4<sup>th</sup> position on the C-ring. The observation of the characteristic H-2, H-6, H-3, H-5 doublet signals clearly differentiate the isolated compounds from the doubly substituted flavonoids at the 3<sup>rd</sup> and 4<sup>th</sup> position which often give multiple signals. The H-3 proton of compound A gave a very clear singlet at 6.72ppm due to the absence of neighbouring protons. The clear singlet distinguished it from the doublet signals from H-2, H-6, H-3 and H-5 signals at similar resonance. This is also differentiated from the isoflavones class with C-2 substitution which occurred further downfield because it's in a beta position to the ketone group at the fourth position of carbon; it is also differentiated

from the chalcones which exist as a doublet due to the double bond between C-2 and C-3. The  $^1\text{H}$  NMR spectra and MS of compound D2 support the structure proposed as shown below. The data obtained correlate with data obtained on previous isolation of the compound 2, 3 dihydro amentoflavone (Chen et al, 2005, Markham et al, 1987 and 1990, Das et al 2005 and Ahmad et al 1981).



**Figure 6.2.2a** Proposed structure 2, 3-dihydro amentoflavone.



**Figure 6.2.2b** Major fragments of proposed 2.3-dihydro-amentoflavone.

BSRL-1  
PROTON CDC13

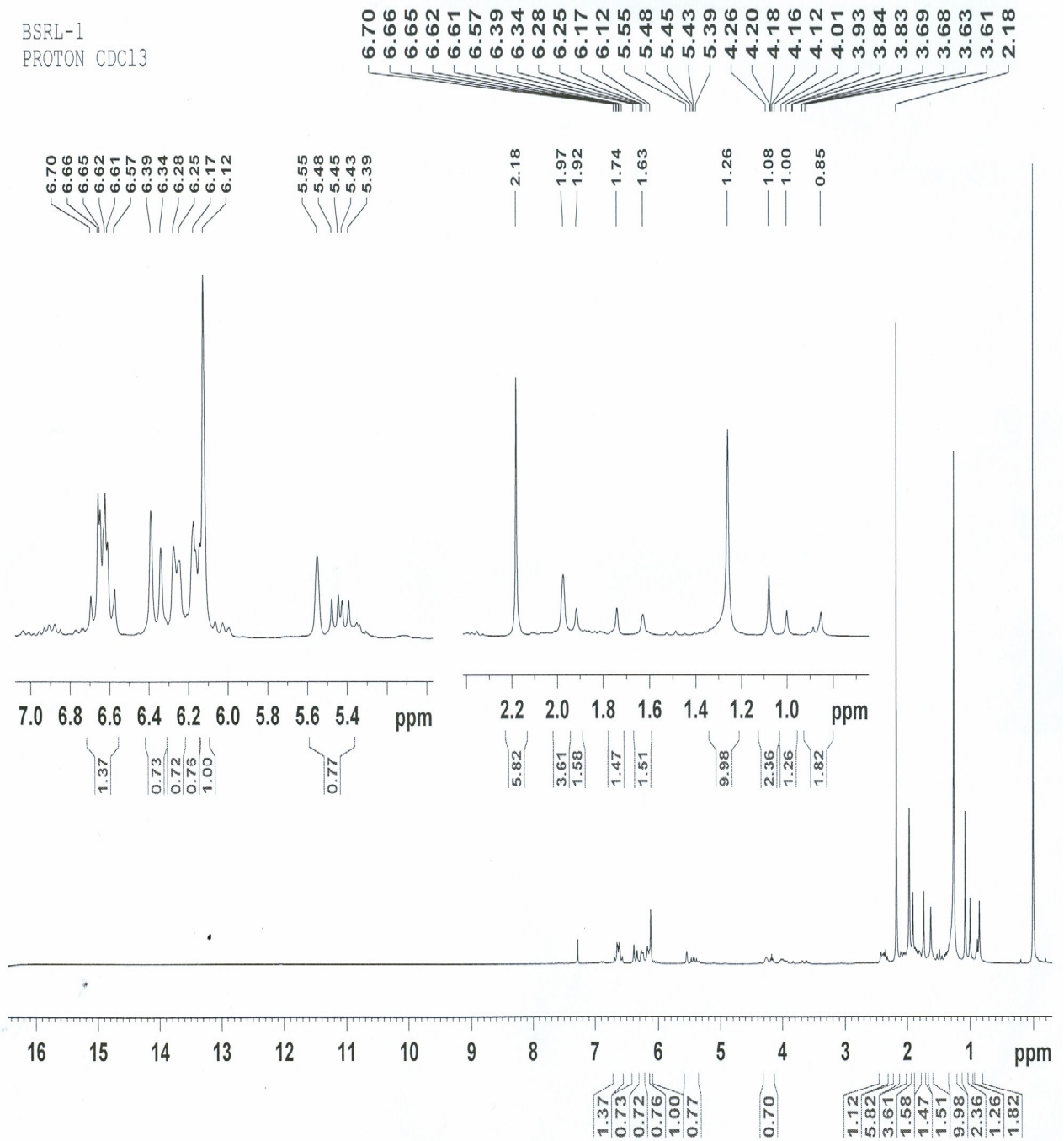


Figure 6.2.1a  $^1\text{H}$  NMR spectrum of Compound 2.

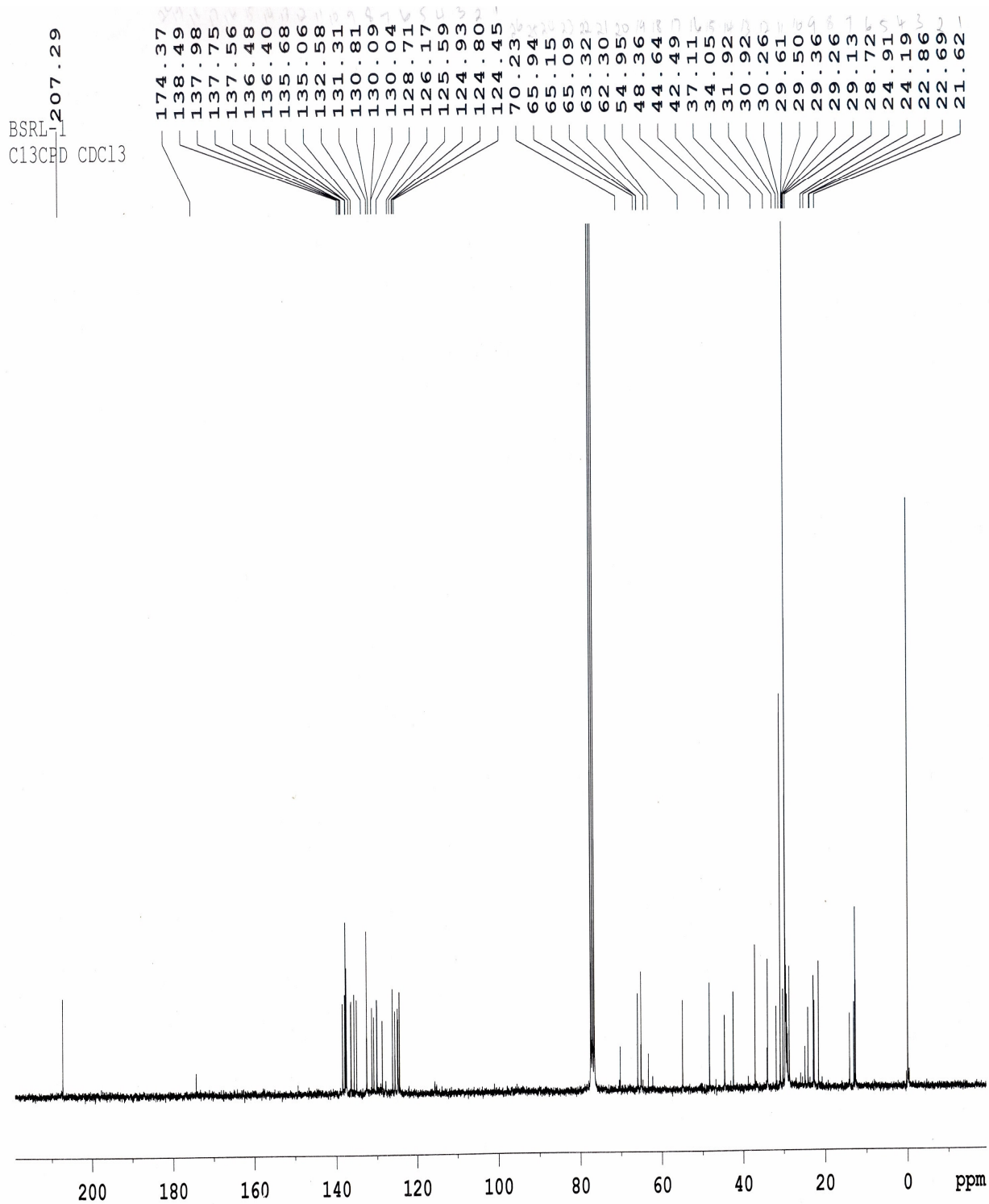


Figure 6.2.1b  $^{13}\text{C}$  NMR spectrum of Compound 1.

BSRL-1  
C13DEPT135 CDC13 {C:\Stumbo} stumbo 1

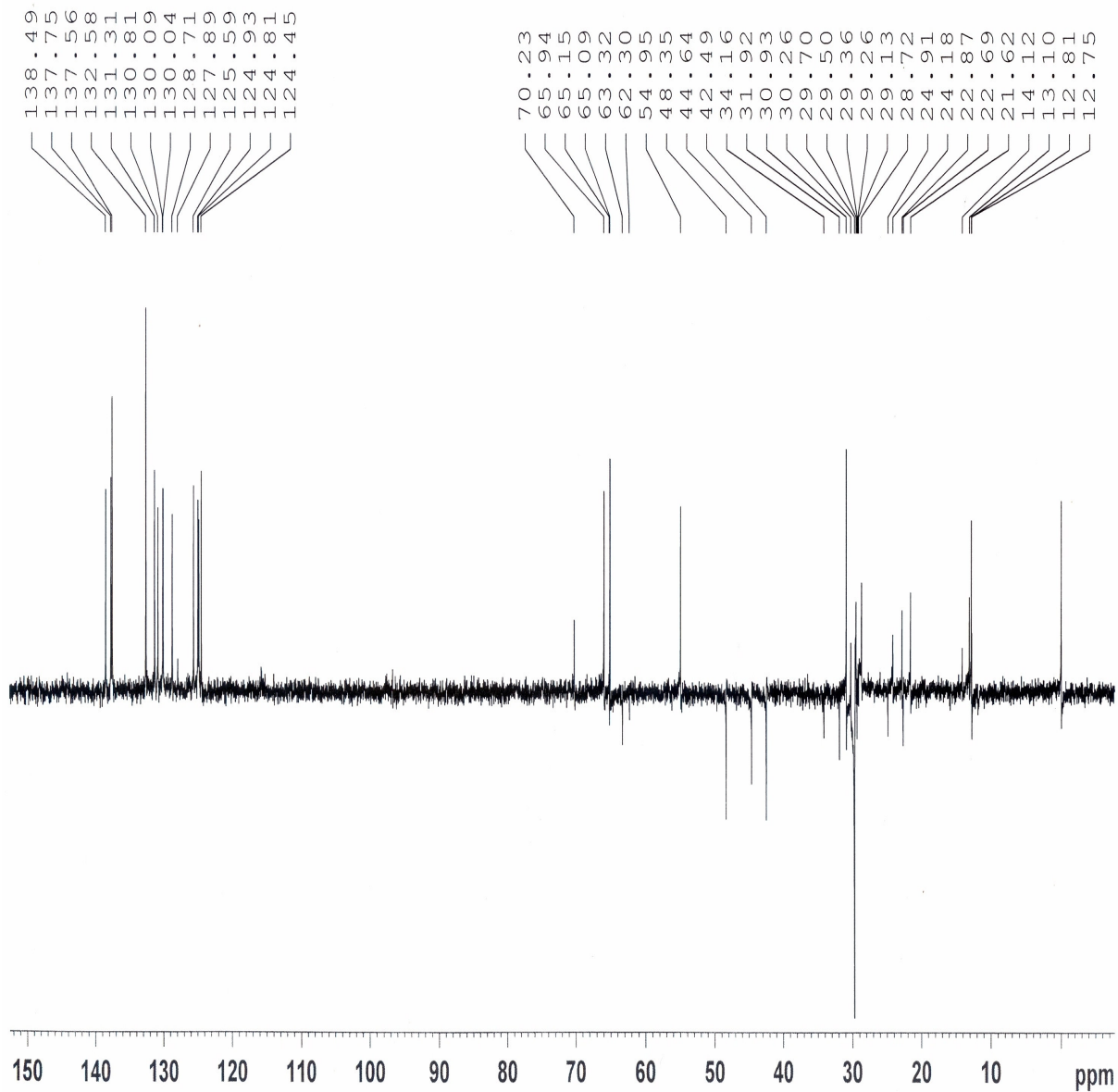


Figure 6.2.1c  $^{13}\text{C}$  NMR DEPT spectrum of Compound 1.

3SRL-1  
1HQCQP CDC13

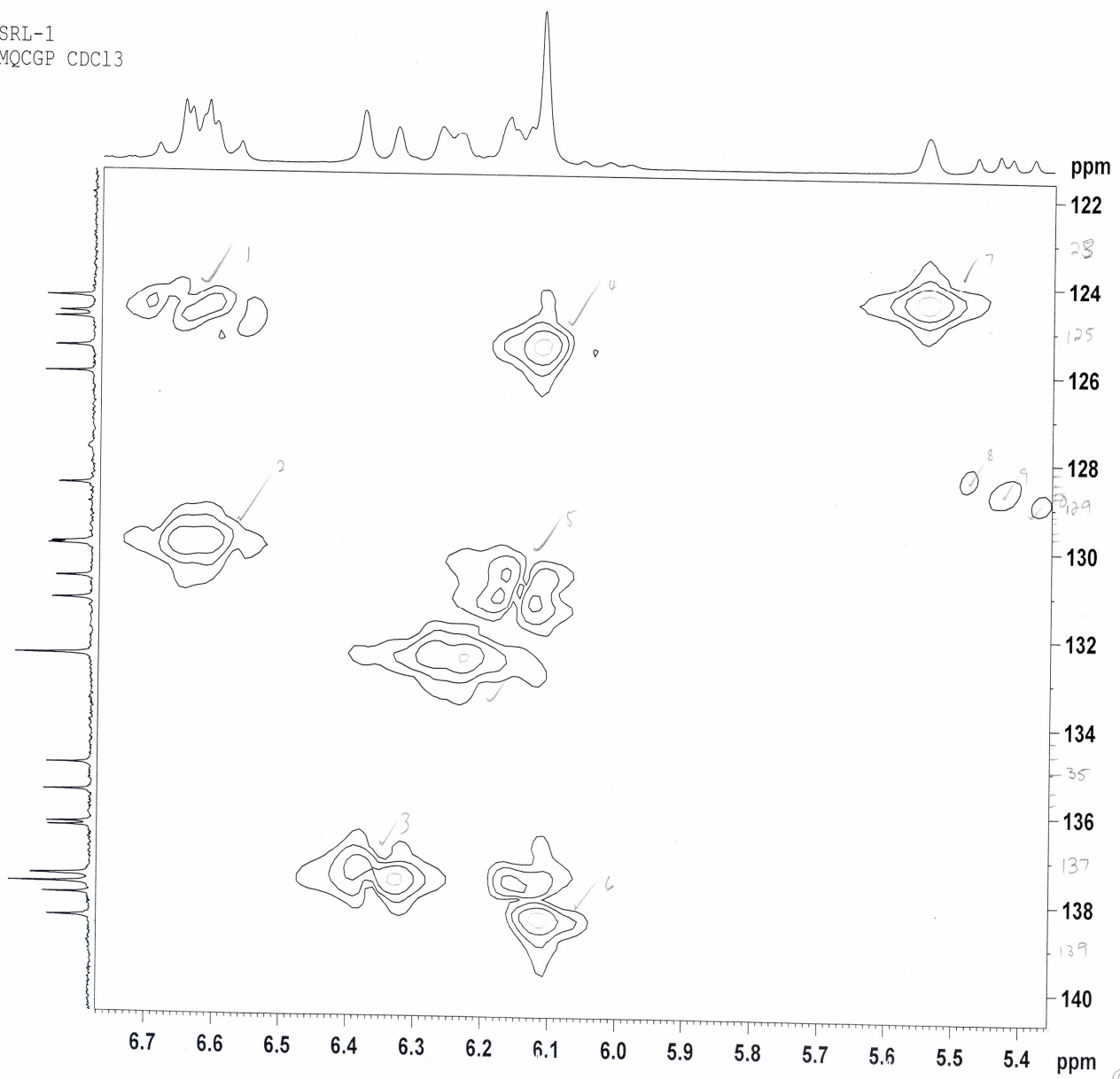
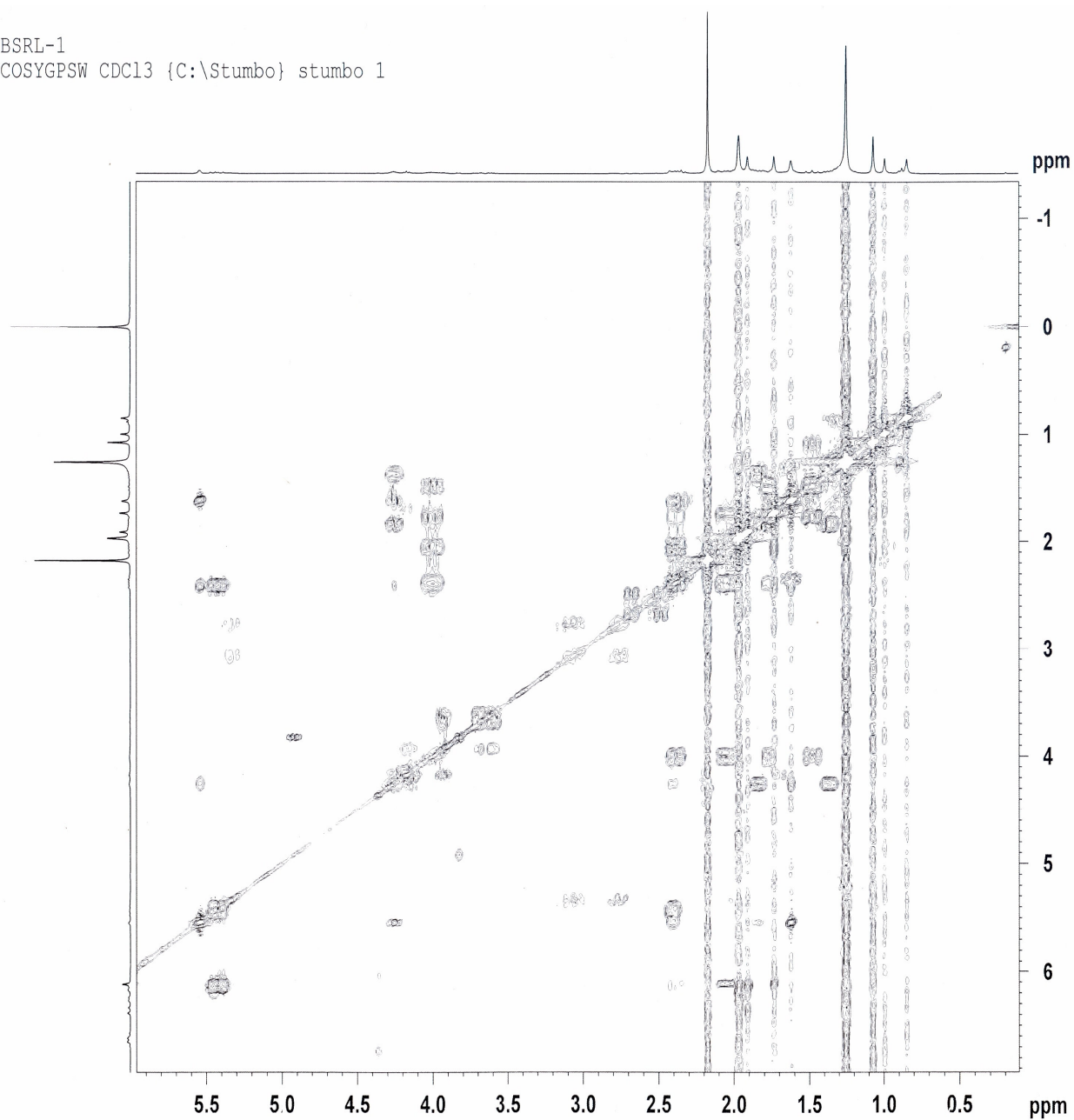


Figure 6.2.1d HMQCQP spectrum of Compound 1.



BSRL-1  
COSYGPSW CDC13 {C:\Stumbo} stumbo 1



**Figure 6.2.1e** COSY spectrum of Compound 1.

BSRL-1 (B. Samuel)  
HMBCGPND Acetone

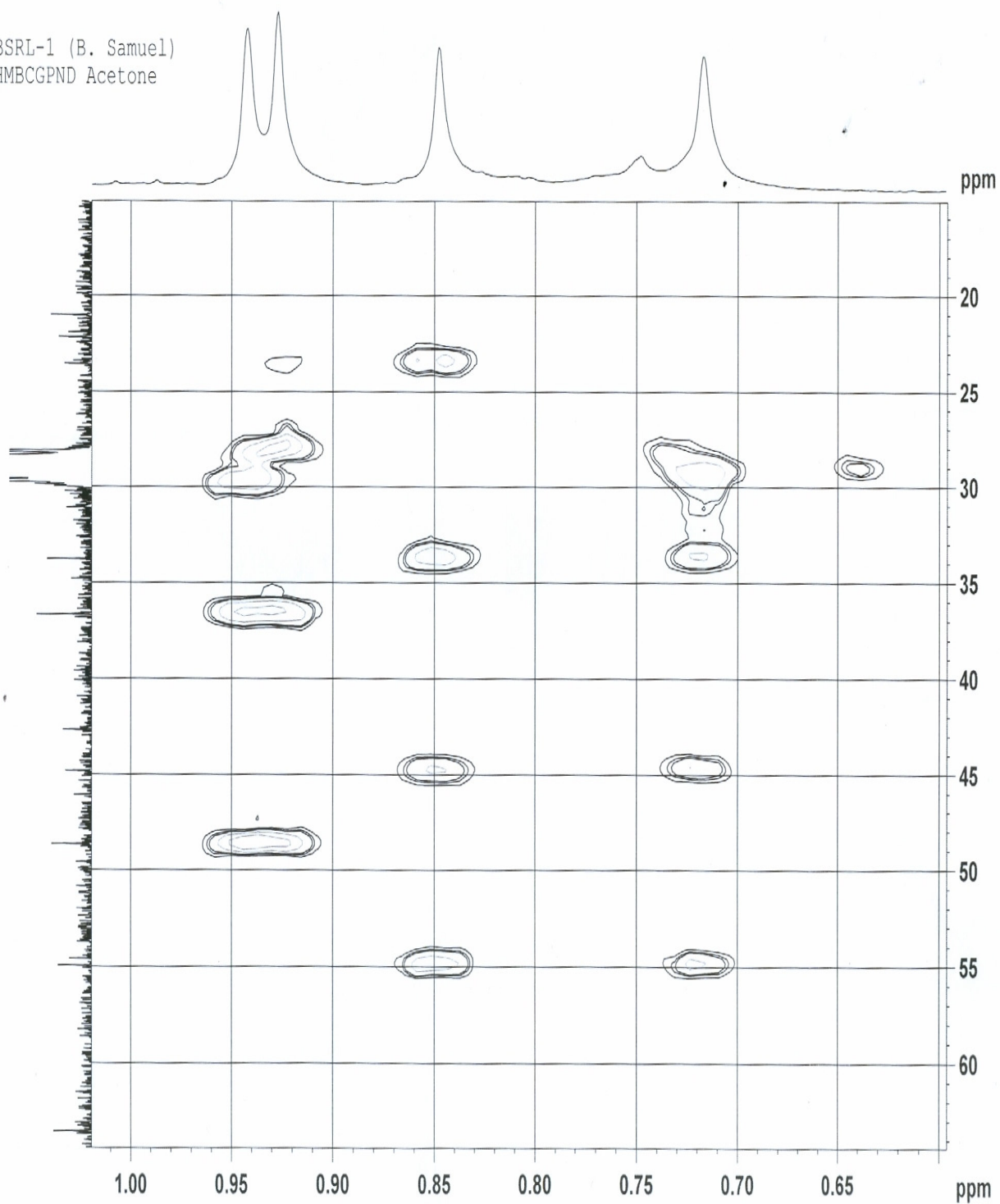
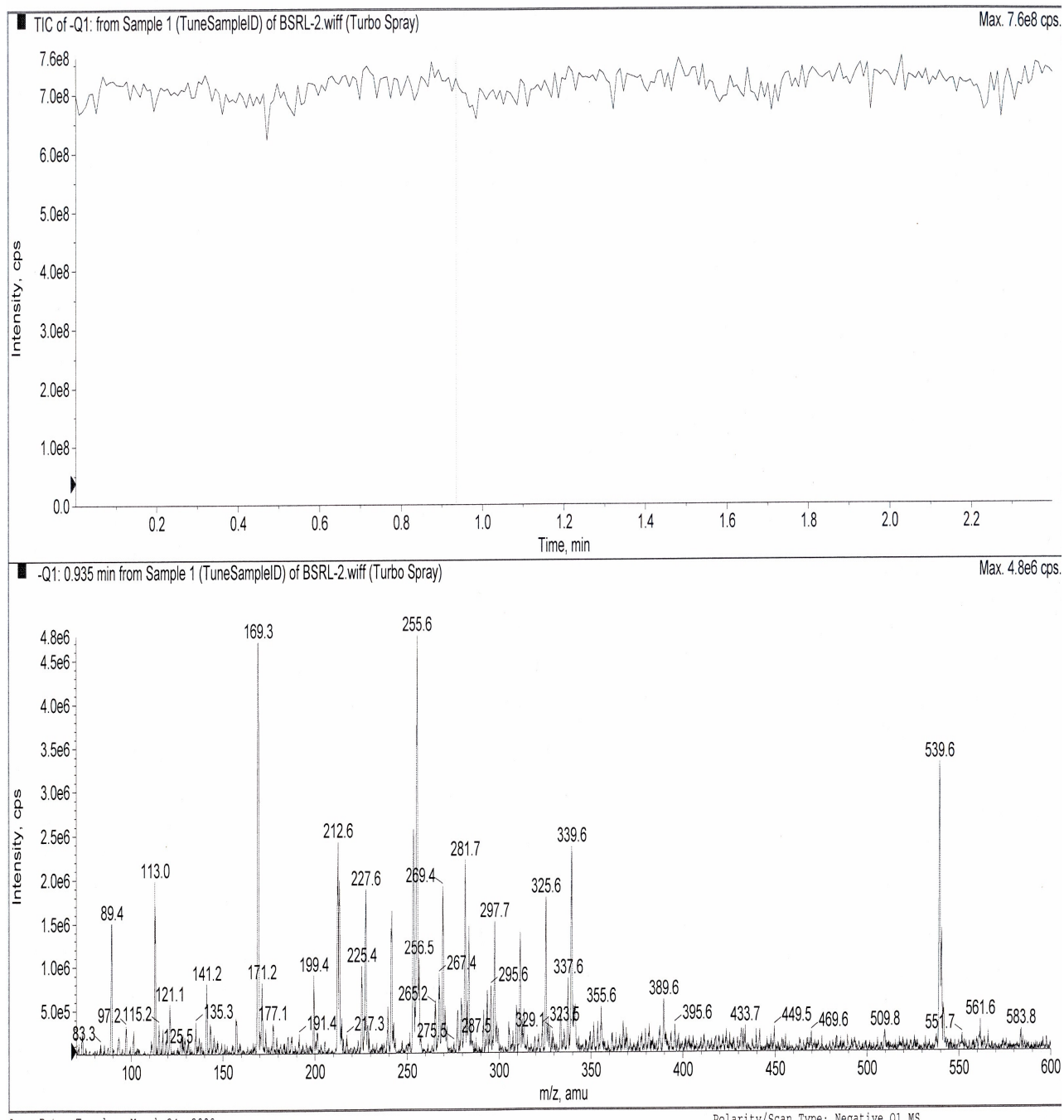


Figure 6.2.2f HMBCGP spectrum of Compound 1.



**Figure 6.2.2a** MS spectrum of Compound 2.

BSRL-2 in acetone (B Samuel)

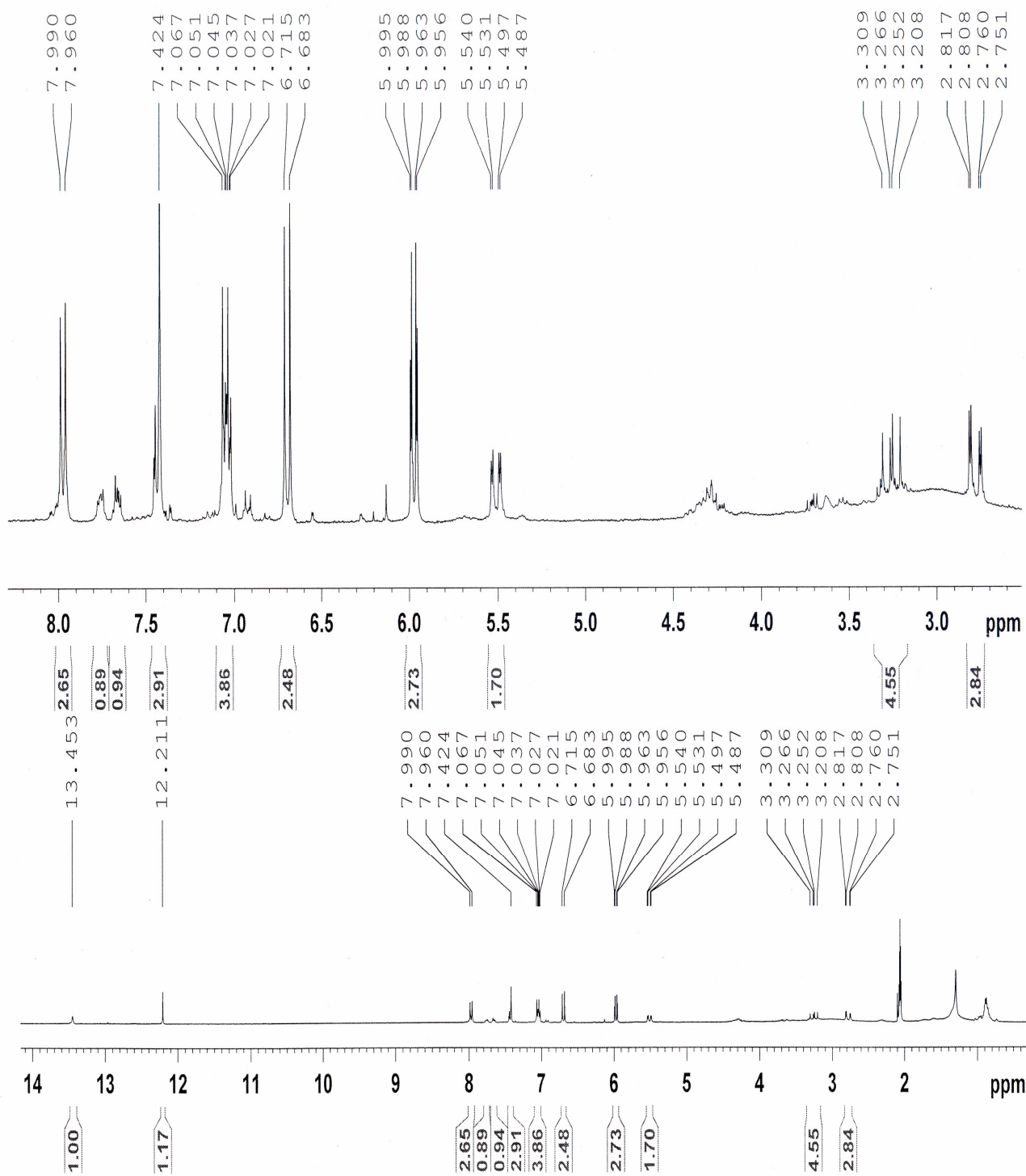


Figure 6.2.2b  $^1\text{H}$  NMR spectrum of Compound 2.

## CHAPTER 7

### 7. Antibacterial activity and cytotoxicity of isolated compounds

#### 7.1 Introduction

During isolation some compounds might undergo chemical modifications because of heat, photooxidation or the effect of organic solvents used. These modifications might increase or decrease the activity of the isolated compounds, hence it is important to conduct quantitative biological activity studies of the isolated compounds to investigate whether activity has been retained or lost during isolation. Also, activity of the isolated compounds against different microbes determines the specificity of the isolated compounds against various microorganisms. In many cases the biological activities of compounds isolated by phytochemists have not been determined.

The idea that herbal drugs are necessarily safe and free from side effects is false. Plants contain hundreds of constituents and some of them are toxic, such as the highly cytotoxic anti-cancer plant-derived drugs, as well as digitalis and the pyrrolizidine alkaloids (Calixto, 2000). In plant bioactivity studies, it is important to screen the isolated compounds for cytotoxicity against various cell lines to gain an indication of potential toxicity. Further investigation of the compounds prior to potential drug development, including acute and chronic toxicity and other *in vivo* toxicity trials, need to be undertaken.

## 7.2. Quantification of antibacterial activity of isolated compounds

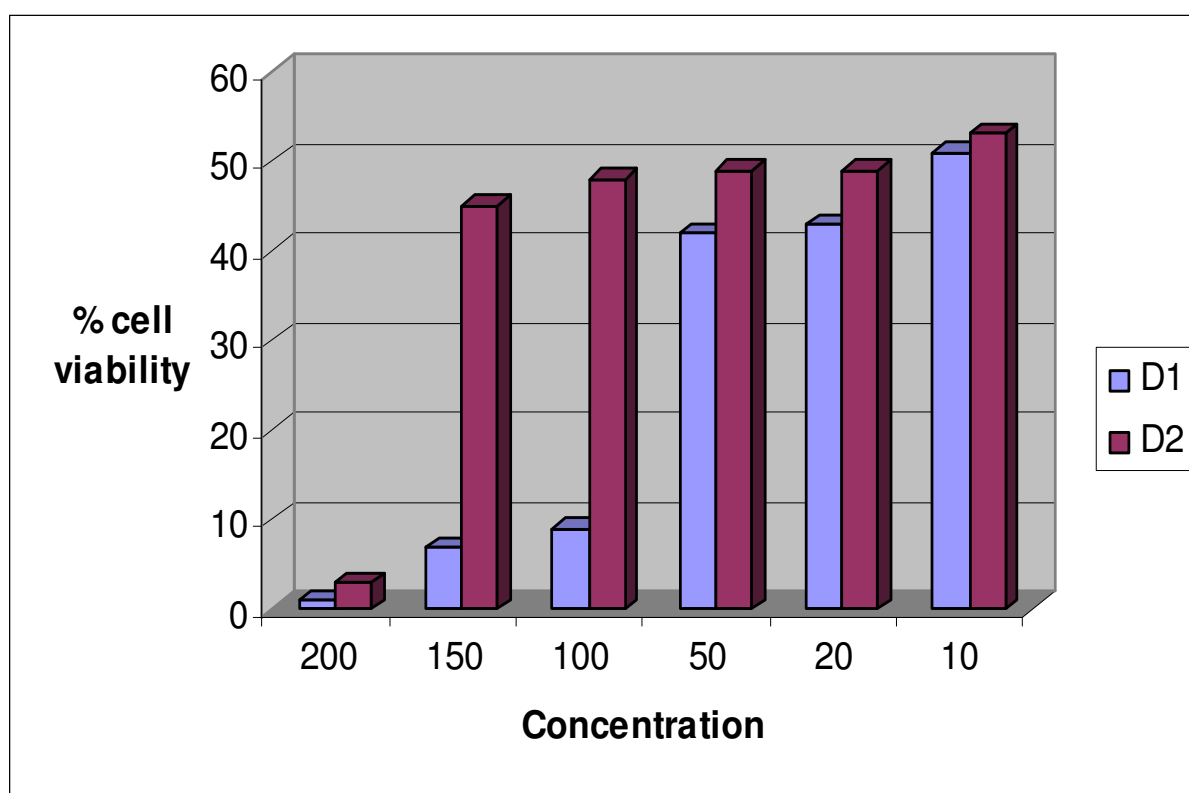
To compare the sensitivity of the microorganisms from the isolated compounds, minimum inhibitory concentration (MIC) values were determined for two isolated compounds. MIC is the lowest concentration of test sample expressed in mg/ml that leads to an inhibition of the growth i.e. at the lowest concentration that gave a decrease in colour. There were substantial differences between the MICs for the different compounds shown in Table 5.6. The minimum inhibitory concentration values for isolated compounds ranged between 0.02 to 1.19 mg/ml. Compound D1 was more active against *P. aeruginosa* with the lowest MIC value of 0.06 mg/ml and compound D2 against *S. aureus* and *P. aeruginosa* with the lowest MIC value of 0.02 mg/ml.

**Table 7.2:** MIC values in mg/ml of the isolated compounds against bacterial species determined in triplicate with standard deviation of. *S. aureus*, *E. faecalis*, *P. aeruginosa* and *E. coli*.

Isolated compounds	Lutein(D1) in mg/ml	2,3-dihydro amentoflavone(D2) in mg/ml	Gentamicin mg/ml
<i>S. aureus</i>	0.16	0.02	0.02
<i>E. faecalis</i>	0.16	0.03	0.02
<i>P. aeruginosa</i>	0.06	0.02	0.02
<i>E. coli</i>	0.09	0.19	0.03

### 7.3 Cytotoxicity of isolated compounds.

The cytotoxicity activity was determined as described in section 3.5.5. The MTT assay indicated that the LC<sub>50</sub> of 2, 3 dihydroxy amentoflavone to Vero cells was 9.4 µg/ml and the LC<sub>50</sub> of lutein was 9.8 µg/ml. The carotenoids (lutein) are known from literature to some measure of toxicity (Leal et al, 1998) and biflavonoids have recently been shown to possess significant cytotoxicity activity (Das et al, 2005). Both these compounds are therefore relatively toxic and may be dangerous to use with the possible exception as a tropical medication. The results give credence to the external use of *R. leptodictya* for the treatment of wounds.



**Figure 7.3** Cytotoxicity of compounds D1 (lutein) and D2 (2.3 dihydro amentoflavone) against Vero cells.

## CHAPTER 8

### 8. Conclusions

The aim of the study was to isolate and characterize antibacterial compounds from *R. leptodictya* and to determine whether the traditional use is justified. During this study, the dried leaves and twigs were extracted by serial extraction using seven extractants of increasing polarity. Dichloromethane (DCM) was the best extracting solvent amongst the seven solvents because it generally extracted the highest quantity of plant material compared to the other solvents, followed by methanol (MeOH), acetone (Act), chloroform (Chl) with, water (Wat) , ethyl acetate (Eth), and hexane (Hex) extracting the lowest quantity.

The acetone extract had the most antibacterial compounds followed by ethyl acetate, hexane, chloroform, methanol and water extracts. This suggests that the antibacterial compounds are of intermediate polarity. The serial microplate dilution method results showed that the acetone extract had the best antibacterial activity with a minimal inhibitory concentration (MIC) of 0.04 mg/ml against *P. aeruginosa*. The acetone extract also had the lowest average MIC value of 0.36 mg/ml against four bacterial species (*S. aureus*, *P. aeruginosa*, *E. coli* and *E. faecalis*) The highest number of antibacterial compounds (6) separated by bioautography was of the acetone extracts against of *S. aureus*. The ethyl acetate extracts had five clear lines of inhibition against *S. aureus*.

The acetone extract was chosen for isolation of active compounds using solvent-solvent extraction as the first step to attempt isolation which resulted in seven fractions namely butanol, water, hexane, carbon tetrachloride, chloroform, ethyl acetate and methanol. The



carbon tetrachloride fraction has the most compounds active against *S. aureus*. This fraction was fractionated by using Silica gel column chromatography which resulted in two compounds D1 and D2. Two isolated compounds were subjected to spectroscopic studies. <sup>13</sup>C and <sup>1</sup>HNMR spectroscopy and MS data led to the identification of the compounds D1 as 2,3-dihydro-amentoflavone and D2 as Lutein. These compounds are common secondary metabolites present in many plant species (Swamy et al, 2006 and Roy et al, 1987 and Aman et al, 2005 and Moss, 1976). The MICs of the two isolated compounds varied from 0.02 to 0.19 against four bacterial species. The 2, 3-dihydro amentoflavone had excellent activity of 20 µg/ml against *S. aureus* and *P. aeruginosa*.

The cytotoxicity of the isolated compounds was investigated using the tetrazolium-based colorimetric assay (MTT) on Vero monkey kidney cells and the hemagglutination assay on formaldehyde-fixed erythrocytes (RBCs). Both compounds were relatively toxic with LC<sub>50</sub> values of below 10 µg/ml. The compounds were therefore at least two times more toxic against mammal cells than against the bacteria. Even though these active compounds were toxic towards the cell lines some chemical modification might provide more effective constituents with lower toxic.

This is the first report of isolation of antibacterial compound from *R. leptodictya*, but there are several more antibacterial compounds that still need to be isolated and characterized.

The results confirm the traditional use of *R. leptodictya* extracts to treat wounds, but the toxicity of the isolated compounds and the crude extracts should be considered as a warning against the internal use of extracts of *R. leptodictya*. Today the world is faced with devastating ailments such as malaria, cancer, diabetes and tuberculosis, and biological resources such as plants might provide solution to this misery.

## CHAPTER 9

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