Pharmacological properties of members of the Sterculiaceae

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DECLARATION

I hereby declare that the experimental work described in this thesis was conducted in the Research Centre for Plant Growth and Development, School of Botany and Zoology, University of Natal Pietermaritzburg from January 1999 to September 2002, under the supervision of Professor J. van Staden and Professor A. K. Jäger.

These studies are a result of my own investigations, except where acknowledgment of other work is specifically indicated in the text and have not been submitted in any other form to another University.

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I declare the above statement to be true.

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ABSTRACT

There is a resurgence of interest in many countries in medicinal plants and their curative properties (HARBORNE & BAXTER, 1993). Little work has previously been conducted on the Sterculiaceae species, especially those located within South Africa. This was a perfect opportunity to broaden the available information on the medicinal properties and chemical constituents of this family, within KwaZulu-Natal.

Of the 50 genera of the Sterculiaceae family, six are located in South Africa: *Cola, Dombeya, Hermannia, Melhania*, *Sterculia* and *Waltheria*.

Seven Sterculiaceae species were chosen for investigation. They varied in growth type and use in traditional medicine. These species included: *Dombeya rotundifolia, D. burgessiae, D. cymosa, Cola natalensis, C. greenwayi, Hermannia depressa* and *Sterculia murex*. Plant material used in the study was collected from a variety of areas, all within KwaZulu-Natal or the Northern Province. There were two collection sites for *D. rotundifolia*, from differing habitats, and results were compared.

The material was screened pharmacologically for anti-bacterial activity using the disc-diffusion assay and Minimal Inhibitory Assay (MIC), and for anti-inflammatory activity using the COX-1 assay.

Only *D. rotundifolia* and *C. natalensis* were tested for anti-bacterial activity using the disc-diffusion assay as the disc-diffusion asay was found to show inconsistencies in the results obtained. Bacteria used included: *Escherichia coli* and *Klebsiella pneumoniae* being Gram-negative, and *Micrococcus luteus, Staphylococcus aureus* and *Staphylococcus epidermidis* being Gram-positive. *D. rotundifolia* exhibited activity, both anti-bacterial and bacteriostatic, in the

leaf, twig and bark extracts from both collection sites. Only the water extract obtained from the leaf material of *C.natalensis* exhibited slight anti-bacterial activity against *S. epidermidis*. Minimal inhibitory concentration (MIC) values were determined using a microdilution assay (25 mg ml^{-1} serially diluted 50 % to 0.195 mg ml^{-1}). Bacteria used in the screening were: *B. subtilis, E. coli, K. pneumoniae* and *S. aureus*. None of the water extracts showed any anti-bacterial activity. Good MIC values were exhibited by *D. cymosa* ethanolic leaf extracts, *C. greenwayi* leaf ethyl acetate extracts especially against *K. pneumoniae* (0.78 mg ml^{-1}) and *S. aureus* (0.39 mg ml^{-1}) and *H. depressa* ethanol and ethyl acetate leaf, stem and root extracts. *D. burgessiae* and *S. murex* showed low activity, with insignificant MIC values.

D. rotundifolia plant material yielded the highest anti-inflammatory activity of all the plant species, with the extracts from the Umgeni Valley Nature Reserve(UVNR) showing the best results. The lowest activity was recorded in the aqueous bark extracts (5% inhibition)and the highest from the ethanolic leaf extract (97% inhibition). *D. cymosa* extracts showed high activity in ethanolic leaf and twig extracts with low activity in all the other extracts. *D. burgessiae, C. greenwayi* and *S. murex* extracts showed high activity in both ethanolic and dichloromethane extracts from leaf and twig material. Activity occurred in the dichloromethane extracts of *H. depressa* obtained from the stem (78%) and root (81%) extracts. *C. natalensis* extracts showed insignificant activity.

Plant material was phytochemically screened for alkaloids, saponins, tannins, cardiac glycosides and cyanogenic glycosides. No alkaloids were detected using pH-partitioning and no cyanogenic glycosides were observed (TLC sandwich method) in any of the extracts of the seven species screened. Using the gelatin salt-block test, tannins were found to be present in the leaf and twig material of *D. rotundifolia*, the leaf material of *C. greenwayi* and the leaf, stem and root material of *H depressa*. The froth test indicated that saponins were

present in the leaf and twig material of *D. rotundifolia* and leaf, root and stem material of *H. depressa*. The haemolysis test indicated the presence of saponins in the *D. rotundifolia bark* material. Screening for cardiac glycosides detected cardienolides in the leaf, twig and bark material of *D. rotundifolia*, and bufadienolides were detected in *D. rotundifolia*, *D. cymosa, D. burgessiae* and *S. murex*.

Five species screened were selected for isolation of active anti-bacterial compounds: *D. rotundifolia, D. burgessiae, D. cymosa, C. greenwayi* and *H. depressa*. Material was extracted by Soxhlet and isolation techniques employed were VLC, TLC separation, Sephadex LH-20 column chromatography and HPLC techniques. The isolated compounds were analysed by NMR and GC-MS. All isolated compounds were fatty acids: Palmitic acid, Myristic acid, Lauric acid, Stearic acid, Acetic acid as welll as stearyl alcohol, eicosane and octadecane

The aqueous eaf extract of H. Depressa exuded a thick mucilage. The production of this mucilage from the *H. depressa* aqueous extract may have medicinal or commercial value. A technique to separate the mucilaginous extract from the leaf material was devised. After extraction, the extract was screened to determine its sugar content through gas chromatography. It was screened for its pharmacological properties: antibacterial and anti-inflammatory activity. The hydrolysing effect of ∝-amylase and HCl on the extract was determined to find its potential use as a bulking agent for use as an appetite suppressant, laxative or against the effects of diarrhoea. It was concluded that the extract is not likely to break down easily in the human digestive system and may be effective against the three listed ailments.

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LIST OF ABBREVIATIONS

Approx.	Approximately
ATCC	American Type Culture Collection
CD_3OD	Deuteromethanol
COX-1	Cycloxygenase 1 Assay
GC-MS	Gas Chromatography-Mass Spectroscopy
Extract A	*H. depressa* aqueous extract - slightly viscous
Extract B	*H. depressa* aqueous extract - mucilaginous slime
HPLC	High Pressure Liquid Chromatography
INT	p-Iodonitrotetrazolium Violet
MH	Mueller-Hinton
MIC	Minimal Inhibition Concentration
NMR	Nuclear Magnetic Resonance
NSAIDS	Non-Steroidal Anti-Inflammatory Drugs
TFA	Trifluoroacetic Acid
TLC	Thin Layer Chromatography
UV light	Ultra Violet Light
UVNR	Umgeni Valley Nature Reserve
VLC	Vacuum Liquid Chromatography

CHAPTER 1

GENERAL INTRODUCTION

1.1 HISTORY OF ETHNOBOTANY

Until the present century, most drugs were originally discovered from folk knowledge that was disseminated throughout indigenous cultures, either orally or through written form such as herbals. Such folk knowledge was of two kinds: specialist knowledge held by a few individuals within the community, and generalist knowledge available to all members of a community (HOSTETTMANN, MARSTEN, MAILLARD & HAMBURGER, 1995).

In the early renaissance there was an explosion of interest in herbals, most of which were based on the work of Dioscorides with incremental improvements made from the author's own knowledge. The first herbal written in the Anglo-Saxon world was the eleventh-century codex known as the *Herbarium of Apuleius Platonicus*. The earliest printed English herbal was an anonymous quarto of 1525, printed by Richard Banckes (BALICK & COX, 1996). A year later a translation of a French herbal was published by Peter Treversi, and in 1538 William Turner published *Libellus de re Herbaria Novus*. In 1551 Henry F. Lyte published a translation of Rembert Dodoens's herbal *Stirpium Historiae Pemptades Sex,* which had achieved renown on the Continent because of its encyclopaedic scope and superb plates of flowers. But the most popular of all sixteenth-century herbals was that of John Gerard, published in 1597. It is one of the few books to remain in print for over 400 years and is one of the most important books on plants ever published in the English language (BALICK & COX, 1996).

1.2 ETHNOBOTANY AND WESTERNISED MEDICINE

Although traditional medicine case is judged 'not proven' by Westernised science, some cultures, notably in India and China, have evolved a philosophy of disease and medicine which is very sophisticated and can explain the use of particular plants. HOUGHTON & RAMAN (1998) state that it is rather arrogant for Western scientists to claim that a preparation is worthless if it has a long history of seemingly successful use. It is generally accepted that as many as two-thirds of the people in developing countries rely on plants as sources of drugs (FARNSWORTH, 1984), in South Africa it is estimated that 80% of the people rely on traditional healing (JÄGER, HUTCHINGS & VAN STADEN, 1996). It is clear that, whereas traditional healers recognise Western medicine and refer their patients to it, the reverse is generally not true (SINDIGA, NYAIGOTTI-CHACHA & KANUNAH, 1995). Many of the techniques or plants used by traditional healers are not accepted by State Authorities or health system. With ethnobotany this situation is slowly changing as the biological activity of many plants are being investigated.

Frequently an inyanga's (Zulu traditional healer) pathological, anatomical and physiological knowledge of the human body is very low. Despite the fact that inyangas are so uninformed as to the causes and nature of diseases, they are conversant enough with their symptoms. Their great rule of pathology is: 'As many symptoms, so many diseases', therefore they tend to treat each symptom individually (BRYANT, 1966). However, commonly with one disease there are many related symptoms. An example would be an ear infection. The symptoms range from sore, fever to loss of balance, all very different symptoms, but all related and by treating the infection results in improvement of balance control. Another of the main reasons why a number of Westernised societies find it hard to accept traditional medicine is because much of it depends on some form of magic (IWU, 1993).

An excerpt found in SINDIGA, NYAIGOTTI-CHACHA & KANUNAH (1995) helps explain this factor even further: 'So we see these medicine men have to be good and effective psychologists to justify their existence. They work by instilling confidence in the sick. A patient believes in his imminent recovery when the medicine man gives him the assurance that he will get better, and almost invariably the sick person does get better. This confidence is founded upon a false premise that because a medicine man is an authority in the art of curing the sick, whenever he speaks, he certainly cannot go wrong.'

The prime advantage of traditional medicine, however, is that it is there, an immediate, existing source of health care for people where they live. It may not be as 'good' as what may be considered ideal, but, in the absence of better alternative health care, there should be a focus on improving on what is available. Added to its many other advantages is its relative cheapness; on the average, folk medicine is cheaper than modern medicine (IWU, 1993).

There are however, some general problems with traditional medicine. The following list was stated in SINDIGA, NYAIGOTTI-CHACHA & KANUNAH, 1995: Traditional medicine does not keep up with scientific and technological advancement. The methods, techniques, ingredients and even training are often kept secret. It is difficult to diagnose chronic illness in time. The rational use of traditional medicine is not well defined; it relies on mysticism and intangible forces, for example witchcraft. Some aspects are based on spiritual and moral principles which are difficult to explicate. It lacks 'measured' doses of drugs; sometimes the side effects of a combination of herbal medicines used it not known. It has an alleged low quality of care because of lack of regulatory mechanisms including control and licensing. It often keeps no written registers of patients hence it is difficult to evaluate, and lastly it experiences loss of knowledge or errors occasioned by large numbers of herbs and other pharmacopoeia; and the diseases to be treated are many.

1.3 PLANTS AND THEIR USES IN TRADITIONAL MEDICINE

It is estimated that 265,000 flowering species grace the earth. Of these, less than 0.5 % have been studied exhaustively for their chemical composition and medicinal value (COX & BALICK, 1994). Traditional medicine is widespread within South Africa. In 1966, A. T. BRYANT registered some 777 different plants in his Zulu-English Dictionary, of which 225 of these have some medicinal use or property. The number of plants known to be used in traditional medicine today has grown through better communication and understanding with the traditional healers. Since plants may contain thousands or even tens of thousands of metabolites, there is currently a resurgence of interest in the plant kingdom as a possible source of new lead compounds for introduction into therapeutical screening programs (HOSTETTMANN, MARSTEN, MAILLARD & HAMBURGER, 1995). Modern studies in chemotaxonomy show that chemical compounds are often characteristic of groups of plants. Thus it is possible that active principles found in one species may be present in other members of the genus or family (HUTCHINGS, 1992). Any species of living organisms displays a variation within many of its important features due to differences in its genetic characteristics, the environmental conditions under which it is grown and the period in its life history when collection took place (HOUGHTON & RAMAN, 1998).

HUTCHINGS (1992) strongly stresses that conclusions reached on the availability and functions of chemical compounds in plants are tentative and the following factors should be noted: Chemical compounds found in a species may not be present in all other members of the genus; chemical compounds known in a species can vary, even within the population, and also with season and stage of development; chemical compounds found in one part of the plant may not be present in all other parts of the plant. Active principles found in plant extracts may not be present in the medicines prepared from the same species;

active principles tested on animals do not necessarily produce the same effects on humans and human responses to medicines vary with the individual.

Traditional medicines are prepared and administered in a variety of ways. Many are similar to Westernised administration, but in a crude manner. They may be by cold infusions (isiChonco), hot infusions (imFudumezelo) and decoctions (imPeko). Treatment is either taken orally, by poultices, lotions, smearing, rubbing into incisions, vapour baths, sweating baths, clyster and emetics (BRYANT, 1966).

1.4 DEPLETION OF RESOURCES AND CONSERVATION DUE TO THE 'MUTHI' TRADE IN KWAZULU-NATAL

The demand for common, fast growing medicinal species is easily met, particularly where only leaves or fruits are used. This category of plant has, therefore, never been of concern to conservation biologists or to herbalists and traders. The impact of the trade on the equally popular, but slow growing or scarce species, is an entirely different matter, particularly where roots, bark, bulb, or whole plants are processed (CUNNINGHAM, 1991).

In Durban in 1915, herbal medicines were sold mainly by herbalists and diviners at the market called *eMatsheni*. Initially, most of the sellers were men, and traditional medicines, both animal and plants, were marketed in small quantities. Today, about 500 informal settlers, primarily women with no specialist training as traditional medicine practitioners, bulk the Durban area (CUNNINGHAM, 1991). About three and a half tons of bark and nearly 200 000 bulbs are traded at Durban's huge traditional medicine markets each week. All material is harvested from the wild. Furthermore, what traders can't sell, they simply throw away (STREAK, 1995). This is a very disturbing fact when it is realised that over 900 indigenous plants are used in traditional healing in

KwaZulu-Natal (SHERRIFFS, 1995).

Plants that face extinction in the wild include wild ginger (*Siphonochilus aethiopicus*), black stinkwood *(Ocotea bullata*), pepperbark (*Warburgia salutaris*), assegai trees (*Curtisia dentata*) and cow-eye flower or mpila. These plants are used to treat colds and flu, malaria and headaches and are often given as emetics (SHERRIFFS, 1995). Most of the trees are ring barked or whole plants are removed from the ground. This extensive exploitation of indigenous plants has led to much concern, and conservation of these species has therefore become very important. But, conservation may not be enough, even though many of the plants are protected by law, the collectors rely on collecting from the wild as a source of income, and are therefore willing to take the risk of being caught. Another idea, which is in practice in KwaZulu-Natal is teaching the collectors and Sangomas basic horticultural skills, to enable them to grow their own plants. Here, the plants have to be sold at low enough prices to take the pressure off the indigenous species in the wild. To take the pressure off wild stocks, concerned people are looking at new and unusual places to grow the plants, including roadside verges and back gardens (SHERRIFFS, 1995).

1.5 THE ETHNOBOTANICAL APPROACH TO DRUG DISCOVERY

In South Africa certain steps are taken before a plant is chosen to be ethnopharmacologically studied. The following steps are adhered to by the Research Centre for Plant Growth and Development at the University of Natal, Pietermaritzburg: Data concerning plants that are used for various ailments; which part of the plant is used; how the drug is prepared, are collected from traditional healers. The plant material is then collected. All the material is botanically identified. A voucher specimen for each species is kept in the University Herbarium. The plant material is screened in a bioassay system that

is specific for the disease the plants are reputedly used for. Plants that contain biological activity in the initial screening then undergo further investigation to isolate the active compounds. The information that is obtained is given back to the traditional healers.

In order to arrive at useful compounds in the shortest possible time, careful selection of plant material is obviously very important. Random collection is one method but it is more judicious to base the selection on certain criteria. Plants used in traditional medicine are more likely to provide pharmacologically active compounds. If a shrub or a tree shows no signs of being attacked by pests and where the leaves have not been eaten nor show any discolourations due to the presence of some microorganism, there is a good chance that some metabolites are present which act as insecticides or antimicrobial agents (HOSTETTMANN, MARSTEN, MAILLARD & HAMBURGER, 1995).

CHAPTER 2

LITERATURE REVIEW

2.1 THE FAMILY STERCULIACEAE AND ITS DISTRIBUTION

The family Sterculiaceae, often referred to as the chestnut family (VAN WYK, 1974) is found throughout the world (PALMER & PITMAN, 1961). It is quite a large family with approximately 1 200 species (50 genera) which occurs in tropical and subtropical regions and mainly includes trees and shrubs (VAN WYK, 1974). However, some of the species are herbaceous (CODD, 1951). Six genera are located in South Africa: *Cola, Dombeya, Hermannia, Melhania*, *Sterculia* and *Waltheria.* In South Africa several well known trees belong to the genera *Dombeya* and *Sterculia (*CODD, 1951, PALMER & PITMAN, 1961).

Table 1 is an extensive literature review outlining the characteristics and habitats of a number of Sterculiaceae species, broken down into the South African and then other African species.

Table 1: Characteristics and habitats of South African and African Sterculiaceae species

Genus and species	Characteristics	Habitat
Cola Schott & Endl. South African Species:		
greenwayi Brenan (Hairy cola)	- Usually a small understorey tree (5-7 m, but may reach 25 m). Bark grey-brown, smooth. Stem in large specimens may become fluted. Leaves elliptic to oblanceolate, dark green above and paler green below. Flowers 5 mm - 10 mm in diameter, coloured dark rusty brown by the soft dense hairs, in tight clusters along the stem (Coates Palgrave, 1977).	- Occurring in evergreen forest and sand forest (Coates Palgrave, 1977).
natalensis Oliv. (Common cola)	- Small tree (8 - 10 m). Trunk frequently crooked, rarely exceeding 25 cm in diameter. Bark grey, smooth, peeling in small flakes in larger specimens, young branches with hairs, but these are soon lost (Coates Palgrave, 1977). Leaves alternate and simple. Flowers solitary or in small fascicles in the axils of the upper leaves (Von Breitenbach, 1965).	- A common species, often somewhat gregarious on rocky ridges, in the coastal forests of the Transkei and Natal (Von Breitenbach, 1965)

Genus and species	Characteristics	Habitat
Cola Schott & Endl. Cont..		
Other species:		
acuminata (Pal.) Schott & Endl.	- Slender, 6 - 12 m, sparsely foliaged mostly at the branch tips, sometimes having tiny hooked spines on the twigs. The five follicles are spread out at right angles, are rough, russet or greenish brown, and many may have up to 14 or 15 seeds (Morton, 1992).	- Grows wild on the mountains of Angola (Morton, 1992).
nitida (Beauv.) Schott & Endl.	- Stout trunked, sometimes butressed, its long stemmed leaves are abovate or elongated, and its clustered, ill-scented, five-parted flowers are white and ivory with purple lines or dark red dots at the base. The fruit consists of a whorl of four to five, very rough, beaked, leathery, or woody pods borne on a single, recurved stalk (Morton, 1992).	- Believed to be native to the Ivory Coast, but has spread throughout the west coast of Africa (Morton, 1992).

Genus and species	Characteristics	Habitat
Dombeya Cav. South African Species:		
autumnalis Verdoorn (Autumn dombeya)	- Shrub/small tree (5 m). Flowers are small, produced in late summer and autumn among leaves (Coates Palgrave, 1977).	- Occurring on rocky hillsides and in riverine fringes (Coates Palgrave, 1977).
burgessiae Gerr. Ex Harv. (Pink dombeya)	- Shrub, 2-4 m tall, usually branching low down. Branches densely villous when young, becoming glabrescent with a brown bark with age. Leaves alternate and simple. Inflorescences axillary, many flowered (Von Breitenbach, 1965). Pink and white flowers (National Botanical Gardens, 1980).	- Occurring at forest margins, or in woodland where there is above-average humidity and same degree of shade, in KwaZulu-Natal, Swaziland and the former Eastern and North-Eastern Transvaal, extending to tropical East Africa (Von Breitenbach, 1965).
buettneri K. Schum	-Shrub (Okwari et al., 2000)	- Southern parts of West Africa sub-region (Okwari et al., 2000)
cymosa Harv. (Natal dombeya)	- Shrub/small slender tree, up to 8 m tall (Coates Palgrave, 1977). Bark whitish. Leaves alternate and simple. Inflorescences of axillary panicles, small flowers (Gibson, 1975).	- Inhabiting coastal shrub, riparian vegetation, and wooded kloofs, from the eastern Cape Province, through KwaZulu-Natal, to Mpumalanga (Von Breitenbach, 1965).
kirkii Mast. (River dombeya)	- Much branched shrub or small tree, up to 9 m tall. Bark light grey, smooth. Leaves alternate and simple, up to 13 cm long and 10 cm broad. Inflorescences of axillary, many-flowered panicles (Von Breitenbach, 1965, Coates Palgrave, 1977).	- Inhabiting riparian thickets, up to 1000 m altitude, in the former north-eastern Transvaal, and extending throughout eastern tropical Africa (Von Breitenbach, 1965).
pulchra N.E.Br. (Silver wild pear)	- Shrub, 2-3 m tall. Leaves alternate and simple (Von Breitenbach, 1965)- Produces creamy white flowers which are rarely pink (Fabian & Germishuizen, 1997).	- Wooded river valleys, along stream banks (Coates Palgrave, 1977), in the mountains of Swaziland and Mpumalanga, at altitudes between 1000 and 1400 m (Von Breitenbach, 1965).

Genus and species	Characteristics	Habitat
Dombeya Cav. Cont...		
Rotundifolia (Hochst.) Planch. (Common wild pear)	- Single-stemmed deciduous tree, 5-6 m tall, with a moderate, irregular-shaped canopy (Immelman et al., 1973). Young tree has a smooth, grey bark, but as it gets older, the bark becomes rough and turns brownish-black (Immelman et al.,1973). It is deeply fissured into irregular, long blocks (Thomas & Grant,1998) and is gnarled and corky (Van Gogh & Anderson, 1988) in the older trunks. Leaves simple and alternate on older twigs, while spirally arranged on young twigs (Thomas & Grant, 1998) . Margins of leaves are irregularly and roundly toothed (Thomas & Grant, 1998), having three or more veins arising from the base (Coates Palgrave et al., 1985). Upper leaf surface is dark green, with paler undersurface (Van Gogh & Anderson, 1988; Thomas & Grant, 1998). Both sides have star-shaped hairs (Immelman et al., 1973). White or light pink star-shaped flowers (Thomas & Grant, 1998) appear in bunches in early spring before the leaves (Palmer & Pitman, 1961). Fruit is a hairy capsule, which develops within the dried-out flower (Immelman et al., 1973).	- Occuring in woodland over a wide range of altitudes in the former Transvaal, and KwaZulu-Natal (Coates Palgrave et al., 1985), occurs more abundantly in the warmer, drier parts (Immelman et al., 1973). It is often found on rocky hillsides, where it occurs in association with other, savanna woodland trees and shrubs (Immelman et al., 1973). With its thick bark, this tree is fire-resistant and can grow in exposed positions in lower altitude grassland. It is also conspicuous growing singly on drier, rocky slopes but also occurs in sheltered, woody slopes (Thomas & Grant,1998).
tiliacea Planch. (Forest dombeya)	- Shrub, 3-6 m tall, up to 30 cm in stem-diameter. Bark grey-brown, very thin, smooth. Leaves alternate, simple. Inflorescences of 1-8 flowered, chymes (Von Breitenbach, 1965).	- Occurring at forest edges (Gibson, 1975). and in secondary shrub, from coast, up to 1000 m altitude. From eastern Cape province to KwaZulu-Natal (Von Breitenbach, 1965).

Genus and species	Characteristics	Habitat
Hermannia L. South African Species:		
auricoma (Szyszy.) K. Schum (golden haired hermannia)	-Stems prostrate. Covered with glands and rough hairs. Leaves erect, finely toothed (Pooley, 1998).	-Occurring in grassland (Pooley, 1998).
depressa N. E. Br. (creeping red hermannia)	- Prostrate herb. Sparsely hairy, slightly glandular. Leaves 40 x 25 mm, bluntly toothed. Flowers in slender inflorescences just above leaves (Pooley, 1998).	- Occurring in grassland, on edge of marshes, from the Cape to Zimbabwe (Pooley, 1998).
coccocarpa K. Schum.	- Much branched shrublet (300 mm). Plant hairless to glandular hairy. Leaves margins toothed, reddish, stalks short,. Flowers occurring in two's, in terminal inflorescences, stem slender, flowers purplish blue, stalks long, slender (Pooley, 1998).	- Occurring in grassland (Pooley, 1998).
cristata H. Bol. FP (crested hermannia)	- Grows up to 300 mm tall. Flowers are red orange (Pooley, 1998).	- Occurring in rocky grasslands, up to 1900 m altitude. Found in the Eastern Cape through to the Northern Province (Pooley, 1998).
gerrardii Harv. (Gerrard's yellow hermannia)	- Coarse prostrate herb (900 mm). Stems simple or branched, roughly hairy. Leaves hairy, margins slightly toothed, stalks short. Flowers in branched inflorescences, flowers pale yellow to orange. Flowers from March to May (Pooley, 1998).	- Occurring on rocky, grassy slopes, 1200 - 2250 m altitude (Pooley, 1998).
grandistipula (Buchinger ex Hochst.) K. Schum. (yellow granny bonnets)	- Shrublet up to 300 mm. Woody rootstock. Stems annual, velvety. Leaves densely, softly hairy, margins toothed, stalks short, stipules large. Flowers, nodding, in pairs, stem erect, flowers yellow (Pooley, 1998).	- Occurring in open grassland, from the Cape to Mozambique (Pooley, 1998).

Genus and species	Characteristics	Habitat
Hermannia L. *cont.*		
hyssopifolia L.	- Erect vigate branched shrub 15 cm - 1.25 m with slender woody stems roughly and densely stellate-hairy. Leaves simple, stalked, oblong-elliptic, broader in the upper-half, finely stellate hairy on both surfaces, toothed at apex only. Inflorescence a bracteate cyme occurring in clusters at the end of the branches and branchlets. Petals yellow to pale greenish (Fox & Norwood Young, 1988).	- Widely distributed in Fynbos from the Cape Peninsula to Port Elizabeth (Fox & Norwood Young, 1988).
sandersonii Harv.	- Long growing shrublet. Woody rootstock. Leaves variable, sparsely hairy, velvety yellowish beneath, stipules about 7 x 3 mm. Flowers found in terminal clusters, very hairy, petals recurved in upper half (Pooley, 1998).	- Occurring in grasslands (Pooley, 1998).
woodii Schinz	- Prostrate herb. Flowers on long stalks, dull pink, red, cream or yellow (Pooley, 1998).	- Occurring in grasslands, 1300 - 1200 m altitude (Pooley, 1998).

Genus and species	Characteristics	Habitat
Melhania Forssk		
South African species:		
didyma Eckl & Zeyh.	Broad leaves, 25 - 40 mm, hairy above, margins shallowly toothed (Pooley, 1998).	
prostrata DC.	- Perennial shrublet, 0.2 - 0.6 m. Stems branched from near base, grey velvety with reddish brown dots. Leaves thinly hairy to hairless above, grey velvety below. Flowers pale yellow, scented at night (Pooley, 1998).	- Occurring in open woodland and grassland (Pooley, 1998).
suluensis Gerstner	- Weak shrublet, 600 - 900 mm. New growth covered in pale brownish hairs. Stems annual. Leaves thin, finely haired, silver grey beneath, margins shallowly toothed. Flowers lemon yellow in colour (Pooley, 1998).	- Occurring on rocky slopes, in bushveld (Pooley, 1998).

Genus and species	Characteristics	Habitat
Sterculia L. South African species:		
africana (Lour.) Flori (African star-chestnut)	- Small to medium-sized tree, 10-25 m tall, with a stout trunk up to 1 m in diameter. Bark brownish, yellowish or whitish, peeling in papery flakes to expose the green inner layers. Leaves congested at the ends of the branches, alternate and simple. Inflorescences of clustered, subterminal panicles (Von Breitenbach, 1965, Coates Palgrave, 1977).	- Occurring in hot and dry lowlands, usually below 1000 m altitude, in Namibia, extending throughout tropical eastern Africa as far as Ethiopia and the Sudan, also in southern Arabia. (Von Breitenbach, 1965).
alexandri Harv. (Cape star-chestnut)	- Small tree, usually 3 -4 m tall, with a thick, usually decumbent stem which is often underground when growing in the open. Bark smooth, silvery, with regularly scattered lenticels. Leaves congested near the ends of the branchlets. Inflorescences of axillary, few-flowered racemes. Flowers pale greenish cream with a few flecks of red or yellow with a rich claret-coloured centre. Flowering season very variable (Von Breitenbach, 1965; Coates Palgrave, 1977).	- Restricted to a few localities in the south-eastern Cape Province, where it occurs on sandy soil in forest margins, scrub, and on open slopes of valleys and kloofs (Von Breitenbach, 1965).
foetida L.	- Tree, deciduous in cold season, with whorled horizontal branches. Red and yellow flowers (Mujumdar et al., 2000).	- Indian subcontinent, Eastern Tropical Africa (Mujumdar et al., 2000)

Genus and species	Characteristics	Habitat
Sterculia L. South African species cont.:		
murex Hemsl. (Lowveld chestnut)	- Small tree, up to 10 m high. Bark rough, grey or blackish. Leaves alternate. Inflorescences racemose, borne at the ends of stout side shoots . Flowering in September and October. (Von Breitenbach, 1965; Coates Palgrave, 1977).	- Inhabiting dry and rocky situations in the lowveld of Swaziland and the former eastern and north-eastern Transvaal, at altitudes between 800 and 1200 m (Von Breitenbach, 1965).
quinqueloba (Garcke) K. Schum. (Large- leaved star-shestnut)	- usually a thickset tree 5-12 m tall. Bark cream to pinkish-brown, smooth and shiny. Leaves crowded near the ends of branches, large, 5-lobed, dark yellowish green above, greyish to yellowish below. Flowers yellow, 5 mm in diameter, in terminal, many-flowered, branched heads. Wood reddish-brown (Coates Palgrave, 1977).	- Occurring at medium to low altitudes, on rock koppies and hill slopes, in deciduous and dry bush (Coates Palgrave, 1977).
rogersii N. E. Br (Common star-chestnut)	- Small tree, usually up to 5 m tall, with a thick, often bottle shaped trunk, 30-60 cm in diameter at the base and tapering rapidly. Bark smooth, shining, greyish-brown, peeling in papery flakes. Branches rather slender. Leaves alternate and solitary, or clustered on short side-shoots. Flowers yellowish-green with reddish guide-lines within (Von Breitenbach, 1965).	- A characteristic species of dry woodland and scrub, usually on rocky hill-sides, in the lowlands of KwaZulu-Natal, Swaziland, the former northern Transvaal, and also Mozambique and Zimbabwe (Von Breitenbach, 1965).

2.2 MEDICINAL USES AND CHEMICAL CONSTITUENTS OF SPECIES OF THE FAMILY STERCULIACEAE

To a certain extent the African members of the family Sterculiaceae serve as sources of medicine, fibre, firewood and timber suitable for furniture, as well as decorative plants (VAN WYK, 1974). Probably the most important member economically is the American cacao tree (*Theobroma cacao*), which is the source of commercial cocoa and chocolate (CODD, 1951).

The continued use of plants as food, as a source of beverages and for their medicinal properties depends on knowledge of the chemical constituents that are present (HARBORNE & BAXTER, 1993).Table 2 represents a detailed list of known medicinal properties and chemical constituents of species from the family Sterculiaceae

Table 2: Medicinal and other human uses and chemical constituents of South African and other Sterculiaceae species

Genus and species	Medicinal and other human uses	Chemical constituents
Cola Schott & Endl.		
South African Species:		
greenwayi Brenan	- Used in traditional medicine (Pooley, 1993).	
natalensis Oliv.	- No mention of use in traditional medicine	

Genus and species	Medicinal and other human uses	Chemical constituents
Cola Schott & Endl. Other species: *acuminata* (Pal.) Schott & Endl. *nitida* (Beauv.) Schott & Endl.		-Stimulant Kola nuts - contain purines, caffeine, theobromine.(Maillard et al., 1986) tannins (Watt & Breyer-Brandwijk, 1962; Hutchings et al., 1996). - Two phenolic substances kolatin & kolatein, catechols, -(-)epicatechol & kolanin & a red anthocyanin pigment (Hutchings et al., 1996).
acuminata (Pal.) Schott & Endl.	- Used medicinally in Angola (Watt & Breyer-Brandwijk, 1962). - Fruits used as a stimulant and topical analgesic (Iwu, 1993).	- Contains no alkaloids (Raffauf, 1996). Aqueous extract of plant gives negative antibiotic tests (Watt & Breyer-Brandwijk, 1962). - Nut consists of tiratable acidity, sugars, 90% general phenol compounds, flavonoid phenols and phenolic acids. Contains 439 - 483 mg/ dm^3 caffeine (Laisisi et al., 1990).

Genus and species	Medicinal and other human uses	Chemical constituents
Cola Schott & Endl.		
Other species cont.:		
nitida (Beauv.) Schott & Endl.	- Stem bark used to treat arthritis and rhematism (Ebana et al., 1991). Fruits and leaves used as stimulant and in healing rituals (Iwu, 1993).	- Contains no: Tannins, phlobatannins, anthraquinones, anthranoids, hydroxymethyl anthraquinones, glucides, saponins and flavanoids. Consists of: Polyphenols, alkaloids, cardiac glycosides and reducing compounds (Ebana et al., 1991).
cordifolia R. Br.	- Produces gum. Local source of 'cola nut'. In West Africa seed, containing caffeine, is used as leprosy remedy (Watt & Breyer -Brandwijk, 1962).	

Genus and species	Medicinal and other human uses	Chemical constituents
Dombeya Cav		
South African Species:		
buettneri K. Schum.	- Aqueous extract of leaves for gastrointestinal disorders (Okwari et al., 2000).	- Polyphenols, cardiac glycosides, alkaloids, tannins, flavonoids and anthrquinones (Okwari, 1999).
burgessiae Gerr. Ex Harv.	- The leaf decoction is drunk as an antimalarial, while the crushed leaves are applied over the affected area against leprosy. Roots used for stomach complaints (Chhabra et al., 1993).	- Contains no alkaloids (Raffauf, 1996). Leaves contain polyuronoids and steroids/triterpenoids (Chhabra et al., 1984)
cymosa Harv.	- Roots used in traditional medicine (Hutchings et al., 1996).	- Contains no alkaloids (Raffauf, 1996).
kirkii Mast.		- Contains alkaloids (Raffauf, 1996).
quinqueseta (Del.) Exell	- The rootbark decoction is drunk against schistosomiasis (Chhabra et al., 1993).Roots are used for stomach aches (Kokwaro, 1976).	- Rootbark contains no alkaloids, flavonoids, saponins and tannins (Chhabra & Uiso, 1990).

Genus and species	Medicinal and other human uses	Chemical constituents
Dombeya Cav cont. South African Species:		
rotundifolia (Hochst) Planch.	- Inner bark used to treat heart problems and nausea in pregnant women (Thomas & Grant, 1998). Decoctions of bark used in delayed labour (Van Wyk et al., 1997) and procuring abortion (Watt & Breyer-Brandwijk, 1962). Infusions of bark or wood used for treatment of intestinal ulcers, headaches, stomach complaints (Coates Palgrave et al., 1985), haemorroids and diarrhoea (Watt & Breyer-Brandwijk, 1962; Van Wyk et al., 1997). Roots used as a colic remedy (Venter & Venter, 1996), and are made into a tonic (Watt & Breyer-Brandwijk, 1962; Coates Palgrave et al., 1985). They are taken as enemas for dyspepsia and sharp pains in the stomach (Hutchings et al., 1996). Leaves rubbed on abscesses as a counter irritant (Watt & Breyer-Brandwijk, 1962).	- Contains no alkaloids (Raffauf, 1996). Has anti-bacterial activity (McGaw, 2000).
tiliacea (Endl.) Planch.		- Yield sterols and extracts have given negative antibiotic tests (Watt & Breyer-Brandwijk, 1962). - 3-dimethyl sulfoniopropoinate (Paquet et al., 1995).

Genus and species	Medicinal and other human uses	Chemical constituents
Dombeya		
Other species:		
calantha K. Schum.		- Flavonoid: Diosmetin-7-glucuronide found in flowers (Subramanian et al., 1973). Flavonoid: kaempferol 3-glycoside found in flowers (Nair & Subramanian, 1962).
cayeuxii André		- Contains no alkaloids (Raffauf, 1996).
claessensii De Wild.		- Contains no alkaloids (Raffauf, 1996).
wallichii (Lindl.) K. Schum.		- Contains no alkaloids (Raffauf, 1996).
shupangae K. Schum.	- Roots and stem used to treat venereal diseases (Iwu, 1993).Root decoction is drunk in the case of abdominal pains, irregular menses and to cause abortion (Chhabra et al., 1993). Leaves are used to treat wounds and prevent abortion (Kokwaro, 1976).	- Leaves give negative results in antifungal and antigonococcal tests (Sawhney et al., 1978a,b).

Genus and species	Medicinal and other human uses	Chemical constituents
Hermannia L. South African species:		
depressa N. E. Br.	- Zulu emetic. Leaf sap in water used for stomach ache (has purgative and diapharetic effect). Plant decoctions taken for coughs. Plant used in mixtures for diarrhoea (Watt & Breyer-Brandwijk, 1962; Hutchings et al., 1996). Used to treat diarrhoea and coughs (Pooley, 1998)	- Contains no alkaloids (Raffauf, 1996).
grandistipula (Buchinger ex Hochst.) K. Schum.	- Leaf infusions sprinkled on eggs to prevent dogs from eating them (Hutchings et al., 1996).	
betonicaefolia E. & Z.	- Root and leaf infusion drunk by Europeans for treatment of respiratory diseases, especially asthma (Watt & Breyer-Brandwijk, 1962).	
candicans Ait.	- Decoction of root used for dysuria (Watt & Breyer-Brandwijk, 1962).	
coccocarpa K. Schum.	- Crushed root used as a plaster for wounds and for treatment of burns (Watt & Breyer-Brandwijk, 1962).	

Genus and species	Medicinal and other human uses	Chemical constituents
Hermannia L cont. South African Species:		
cuneifolia Jacq.	- Europeans apply an infusion or decoction to sores and takes the preparation internally (Watt & Breyer-Brandwijk, 1962).	
evodiodes O.Ktze	- African remedy for burns (Watt & Breyer-Brandwijk, 1962).	Contains no alkaloids (Raffauf, 1996).
geniculata E. & Z.	- Remedy for heart burn and other ailments. For relieving flatulence in pregnant women. Used as cough remedy (Watt & Breyer-Brandwijk, 1962).	Contains no alkaloids (Raffauf, 1996).
hyssopifolia L.	- Decoction of root is an old fashioned household remedy for fits. An ointment from plant and Lobostemon fruiticosus and Psoralea decumbens is an old remedy for 'roos' (erysipelas or eczema) (Watt & Breyer-Brandwijk, 1962). Make tea with aromatic properties (Fox & Norwood Young, 1988).	Contains no alkaloids (Raffauf, 1996).
Hermannia (Mahernia micrantha)	- Used in South Africa as a remedy for colic and flatulence (Watt & Breyer-Brandwijk, 1962).	

Genus and species	Medicinal and other human uses	Chemical constituents
Melhania Forssk		
South African species:		
acuminata Mast.		- Contains no alkaloids (Raffauf, 1996).
didyma Eckl & Zeyh.		- Contains no alkaloids (Raffauf, 1996).
forbesii Planch. ex Mast	- Leaf used for barrenness in women by administration of a cold infusion (Watt & Breyer-Brandwijk, 1962).	- Contains no alkaloids (Raffauf, 1996).
prostrata DC.		- Contains no alkaloids (Raffauf, 1996).
randii Bak.f.		- Contains no alkaloids (Raffauf, 1996).
rehmannii Szyszyl		- Contains alkaloids (Raffauf, 1996).

Genus and species	Medicinal and other human uses	Chemical constituents
Sterculia L.		
South African species:		
africana (Lour.) Fiori	- Decoction of roots is drunk treat backaches, vertigo and hernia (Chhabra et al., 1993). Roots, stembark and leaves are used to treat fever and influenza (Kokwaro, 1976).	- Sterculic acid in seed (Hegnauer, 1973).
murex Hemsl.		- Light petroleum extracts 17% of oil from the seed, the residual meal containing a trace of alkaloidal material and a small amount of cyanogenetic glucosides (Watt & Breyer-Brandwijk, 1962).
*quinqueloba (*Garcke) K. Schum		- Contains no alkaloids (Raffauf, 1996).
rogersii N. E. Br		- Contains no alkaloids (Raffauf, 1996).

Genus and species	Medicinal and other human uses	Chemical constituents
Sterculia L.		
Other species:		
alata Roxb.		- Acid: sterculynic acid (Jevans & Hopkins, 1968; Glasby, 1991). Contains alkaloids (Raffauf, 1996).
appendiculata K. Schum.	- Used as purgative for relief of abdominal pains (Watt & Breyer-Brandwijk, 1962). Stembark decoction is drunk against diabetes (Chhabra et al., 1993), snake bites (Haerdi, 1964) abdominal pains and cerebral palsy (Kokwaro, 1976). Roots treat diarrhoea, leaves treat cardiac pains (Chhabra et al., 1993).	- Stem bark contains alkaloids, flavonoids and tannins (Chhabra & Uiso, 1990)
cinerea A. Rich.	- Yields clear pinkish tragacanth- like gum, sometimes used as an adulterantol gum-arabic (same way as karaya gum) (Watt & Breyer-Brandwijk, 1962).	
colorata Roxb.		- Flavonoids: 6-glucuronosyloxykluteolin, 5,7,3', 4'-tetrahydroxy-6-0-β-D-glucuronylflavone (Nair et al., 1976; Glasby, 1991). Contains no alkaloids (Raffauf, 1996).

Genus and species	Medicinal and other human uses	Chemical constituents
Sterculia *L.* cont.		
Other species:		
foetida L.	- Gum used in medicine (Mujumdar et al., 2000) Leaves used as aperient, diuretic and insect repellent (Chopra et al., 1992).	- β-sitoserol (Desai et al., 1976). Roots contain lupeol, n-triacontanol, stigmasterol and β-stoserol-3-O-β-b-glucopyranoside). Has high anti-inflammatory activity (Mujumdar et al., 2000). Anthocyanin: cyanidin 3-0-glucoside. Flavonoids: isoscutellarin, luteolin 6-0-β-D-glucopyranoside procyanidin β-D-glucopyranoside (Glasby, 1991). Sterculic acid (Harborne & Baxter, 1993) , malvalic acid (Badami et al., 1980) ,dihydrosterculic acid (Hofmann et al., 1954).
pallens Wall. ex Hochr.		- Acids: malvalic acid and sterculic acid (Glasby, 1991). Flavanoids: Quercetin, quercetin 3-0 glucoside and quercetin 3-diglucoside (Ranganathan & Nagarajan, 1980).
pruriens (Aubl.)		- Cyanogenic compounds (Schults & Raffauf, 1990).Contains hydrocyanic acid (Worthley & Schott, 1969).

Genus and species	Medicinal and other human uses	Chemical constituents
***Sterculia* L.** cont.		
Other species:		
tragacantha Lindl.		- Allantoin found in bark (Prista et al., 1960). Malvalic, sterculic and dihydrosterculic aacid Also Palmitic, oleic and linoleic acids (Miralles et al., 1993).
rhynchocarpa K. Schum	- Decoction of bark used for stomach troubles (Watt & Breyer-Brandwijk, 1962). Shoots, bark, seeds are used to treat vernifuge, syphilis, inflammation, digestive disorders. Used externally as poultice for boils, whitlow (Iwu, 1993).	
urens Roxb.	- Used as a laxative, aphrodisiac, the fine powder is used in dentistry as dental adhesive. Also used as a thickener and suspending agent in lotions, creams and hair-setting preparations (Leung & Foster, 1996).	- Gum karaya: partially acetylated polysaccharide, a galacturonor hamnan, containing about 8% acetyl groups and 37% uronic acid residues (Harborne & Baxter, 1993). Contains no alkaloids (Raffauf, 1996). Main constituent of resin is a phlobatannin (Sreedharan Pillai & Madharan Pillai, 1954).

Genus and species	Medicinal and other human uses	Chemical constituents
Waltheria .L		
South African species:		
indica L.	- Women use decoction of root for barrenness. Chew raw root or drink decoction for internal haemorrhages. Used as febrifuge (Petrus, 1989). Used as antisyphilitic (Haerdi, 1964). Decoction used in skin diseases and cleansing wounds. (Watt & Breyer-Brandwijk, 1962). Eyeaches (Kokwaro, 1976). Purgative, emollient, abortifacient (plant), anti-tussive (dry powder of plant), anti-rabies, anti-syphilitic (Petrus, 1989).	- Plant has given positive tests for flavonoids, sterols and tannins. Powdered plant is astringent and yields mucilage, tannin and sugar - no alkaloids (Watt & Breyer-Brandwijk, 1962). Peptide alkaloids called X, Y, Y' AND Z have been isolated from roots (Pais et al., 1963, 1968). Anthocyanins, pelargonidin and cyanidin glycosides have been isolated from petals (Ogbede et al., 1986). Flowers have antifungal activity (Sawhney et al., 1978b). Kaempferol, kaemfeerol-3-0-β-D-galactoside, herbacetin, herbacitin-8-0-β-D-glucuronide, glossypetin, glossypetin-8-0-β-D-glucuronide, 2''-0-β-D-glucosylvitexin, caffeic acid (Petrus, 1989).
Other species:		
americana L.	- Used by Hawaiians in traditional medicine (Abbott & Shimazu, 1985).	- No diarrhoeic activity (Zavala et al., 1998).

2.3 AIMS OF THE STUDY

The aim of this study was to compile an in-depth pharmacological and phytochemical investigation of species from the family Sterculiaceae, that are located within KwaZulu-Natal.

After an extensive literature review, it was clearly evident how little work had previously been conducted on the Sterculiaceae within southern Africa, thus making this an ideal project to broaden the available information on the medicinal properties and chemical constituents of this family, within KwaZulu-Natal.

This project involved the overall biological screening of seven Sterculiaceae species (Plate 1):

Dombeya rotundifolia
Dombeya burgessiae
Cola greenwayi
Sterculia murex
Dombeya cymosa
Hermannia depressa
Cola natalensis

Extracts were made from various plant parts (bark, twigs, roots, stems and leaves) with three solvents, namely ethyl acetate, ethanol and water. These extracts were tested for activity by pharmacological screening for anti-bacterial and anti-inflammatory activity, as well as phytochemical screening for cardiac glycosides, cyanogenic glycosides, tannins, saponins and alkaloids.

Species showing good anti-bacterial activity were subsequently selected for possible isolation of compounds. Isolation techniques employed were VLC, preparative TLC, Sephadex LH-20 column chromatography and HPLC. The species included: *D. rotundifolia*, *D. burgessiae*, *D. cymosa*, and *H. depressa*

leaf material; and *C. greenwayi* twig material.

H. depressa aqueous extract was investigated. The leaf material produced a mucilage. The mucilage was tested for polysaccharide content and breakdown caused by α-amylase, HCl and bacteria.

TLC fingerprints of the extracts from the seven Sterculiaceae species were determined using white light, UV light (254 nm and 366 nm) and staining with anisaldehyde spray reagent.

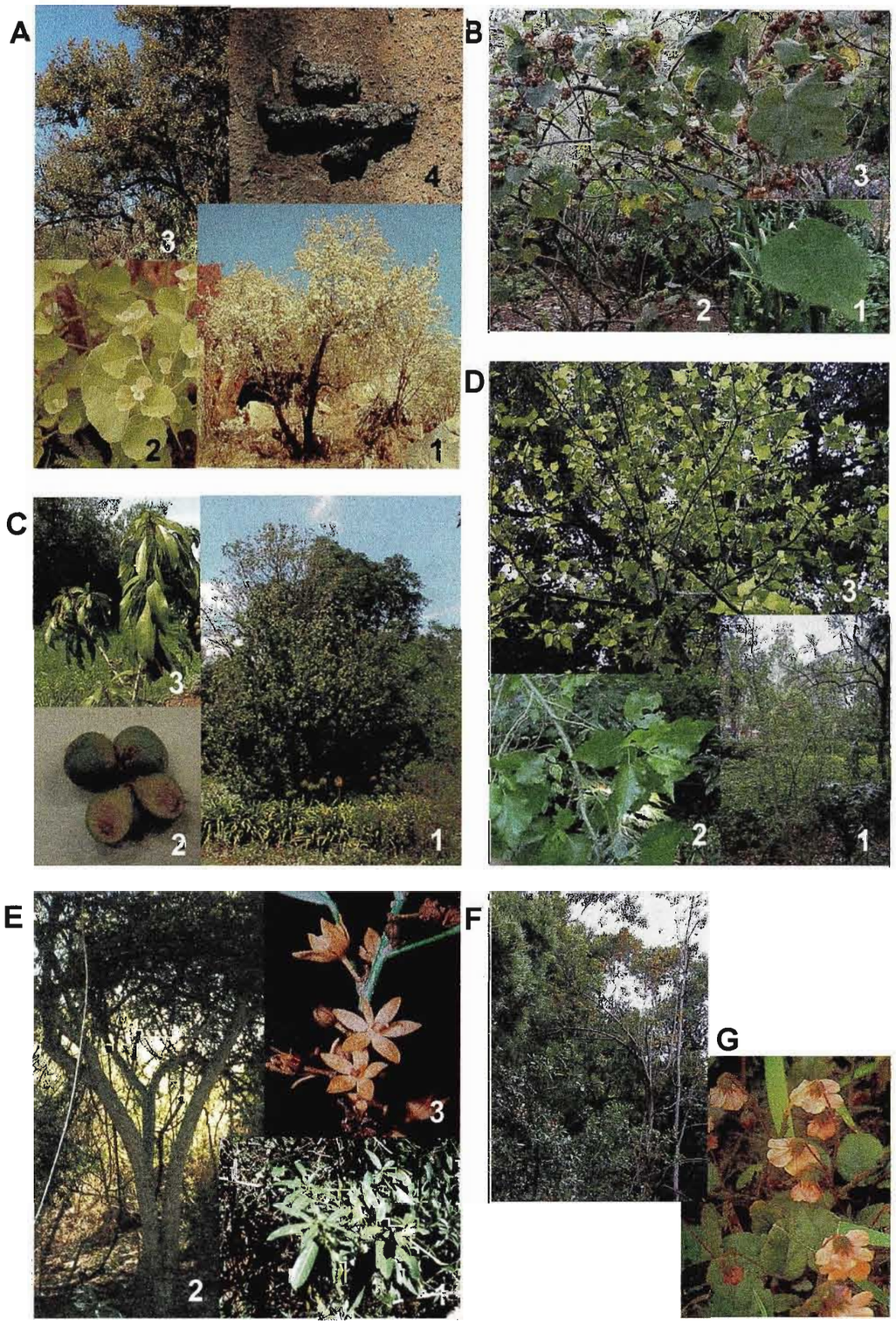


Plate 1 Species screened in this study.

(A) *Dombeya rotundifolia* (1) tree with flowers in spring, (2) young shoots and leaves, (3) tree with leaves in autumn and (4) outer bark from trunk. (B) *Dombeya burgessiae* (1) leaf, (2) and (3) shrub with drying flowers and developing fruit. (C) *Cola greenwayi* (1) tree, (2) fruit and (3) leaves on branches. (D) *Dombeya cymosa* (1) tree, (2) leaves and (3) branches full of leaves. (E) *Cola natalensis* (1) leaves, (2) tree, and (3) flowers. (F) *S. murex* and (G) *H. depressa*.

CHAPTER 3

PLANT COLLECTION

3.1 PLANT COLLECTION

Plant material of the seven species selected for this study was collected from varying areas of KwaZulu-Natal (Fig. 1) (Table 3). All collection sites were inland, with the exception of *C. natalensis*, which was collected on the north coast. There were two collection sites of *D. rotundifolia*, one being situated outside KwaZulu-Natal, in the Limpopo Province.

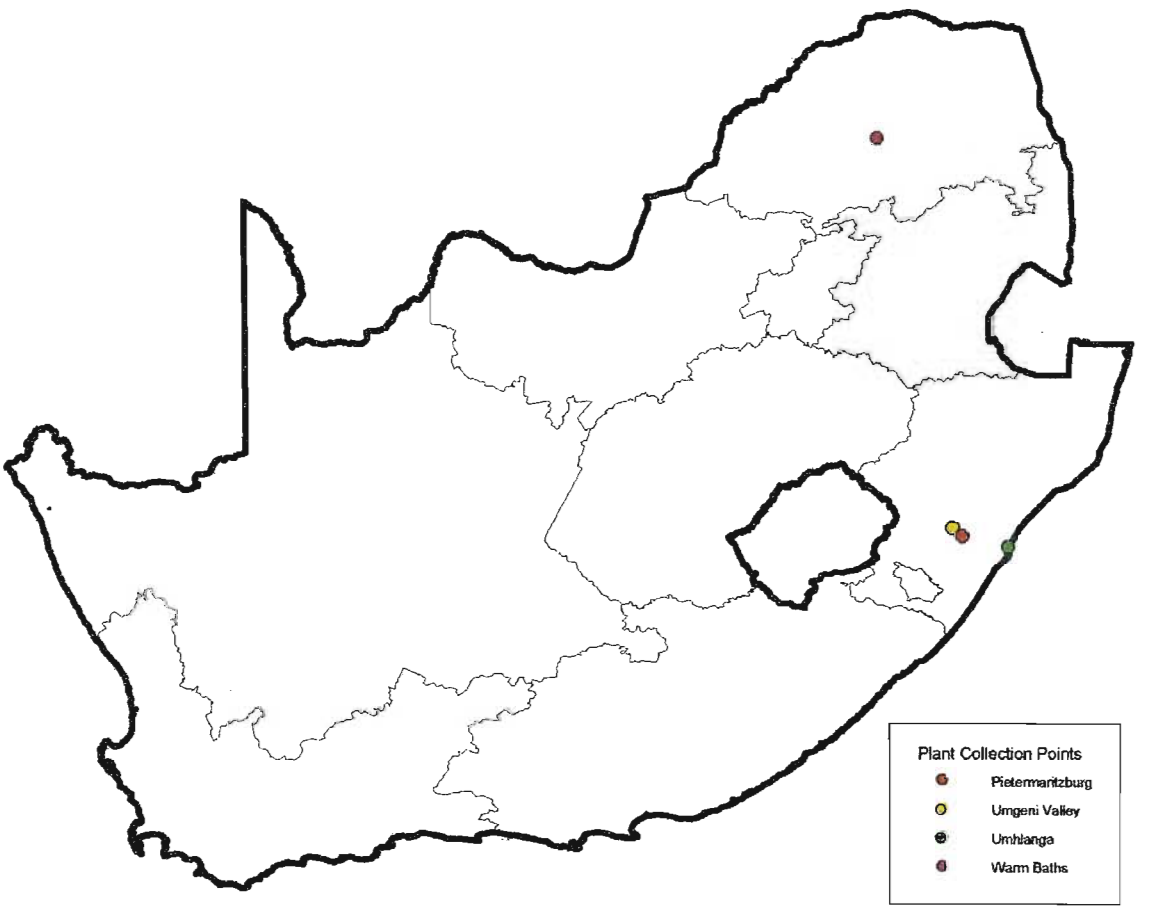


Fig. 1: Sites where Sterculiaceae species were collected for this study

Table 3: Plant species collected for screening, their herbaria numbers and collection sites

Plant species	Collection site	Herbarium voucher number	Plant parts used
Dombeya rotundifolia	1. Umgeni Valley Nature Reserve, Howick 2. Nature farm, Monato, in the Droogekloof area in the Limpopo Province (approximately 30km from Warmbaths)	REID 1 NU REID 2 NU	Leaves, twigs and bark. Leaves, twigs and bark.
Dombeya cymosa	Botanical Gardens, PMB	REID 3 NU	Leaves and twigs.
Dombeya burgessiae	Botanical Gardens, PMB	REID 4 NU	Leaves and twigs.
Cola natalensis	Hawaan forest, on the outskirts of Umhlanga, KwaZulu-Natal.	REID 5 NU	Leaves and twigs.
Cola greenwayi	Botanical Gardens, PMB	REID 6 NU	Leaves and twigs.
Hermannia depressa	Open grassland between Denison Residence and Agriculture faculty, UNP	REID 7 NU	Leaves, stems and roots
Sterculia murex	Botanical Gardens, PMB	REID 8 NU	Leaves and twigs.

Two sites were chosen for the collection of the *D. rotundifolia* material. The differences and similarities between the collection sites are recorded in Table 4.

Table 4: Comparison of the two collection sites for *Dombeya rotundifolia* material

Umgeni Valley Nature Reserve	Monato Farm (Warmbaths)
- Mean annual rainfall 950 - 1100 mm	- Mean annual rainfall: 500 - 650 mm
- Vegetation type: lowveld bushveld	- Vegetation type: mixed bushveld
- Altitude: 1000 - 1500 m	- Altitude: 1500 - 1700 m
- The trees were about 3 m tall	- Due to the larger distance between the trees, the trees were able to grow to about 4 - 6 m in height
- Leaves were a dark green in colour	- The leaves were a lighter green, some with signs of yellowing. This was probably due to the more acidic nature of the soil
- There were a number of trees growing next to each other	- There were a number of trees from which the plant material was collected, but they were scattered over the hillside

3.2 DESCRIPTION OF PLANT PARTS USED

Plant material used for each species was collected from a number of trees or shrubs situated within the same vicinity. Explanation of the terminology used to describe the parts of the plant used is given below:

'Leaves' - A combination of young and old leaves, small and large leaves of each plant was used. In the case of *H. depressa*, all the leaves from each plant were used.

'Twigs' - The small, thin, young woody branches to which the leaves had been attached. Twigs were only obtained from the tree species.

'Stems' - The only 'stems' used were from *H. depressa*. This constituted the remaining aerial parts of the shrublets after the removal of the leaves.

'Bark' - Bark was only collected from *D. rotundifolia*. Outer, gnarled bark from the trunk and older branches was collected as it peeled away easily from the stem.

'Roots' - Roots were only collected from *H. depressa*. The whole root section of each plant was used.

Plant material was dried in an oven at 50°C for 72 h and ground to a homogenous powder and stored in honey jars in the dark at room temperature until further processing (Plate 2).

Plant material was extracted using a number of methods (Plate 3), depending on quantities of extracts required for the different screening and isolation procedures.

Plate 2 Steps followed in collection, drying and storage of plant material. (A) Plant material (1) was collected from the relevant species from (2) Monato nature farm in Limpopo Province, (3) National Botanical Gardens in Pietermaritzburg, (4) Umgeni Valley Nature Reserve in Howick and (5) Hawaan Forest on the outskirts of the coastal resort town of Umhlanga. (B) Material was taken to the laboratory where it was separated into different plant parts and placed in labeled brown paper bags. (C) The brown paper bags were placed in an oven in the garden laboratory and the plant material was dried at 50°C for 72 h. (D) The dried plant material was ground in a Waring blender in the laboratory and placed in labeled honey jars. (E) The honey jars were stored in a cupboard, in the dark, until the plant material was required. (F) A voucher specimen of plant material collected was identified and is housed in the University of Natal Herbarium (NU).

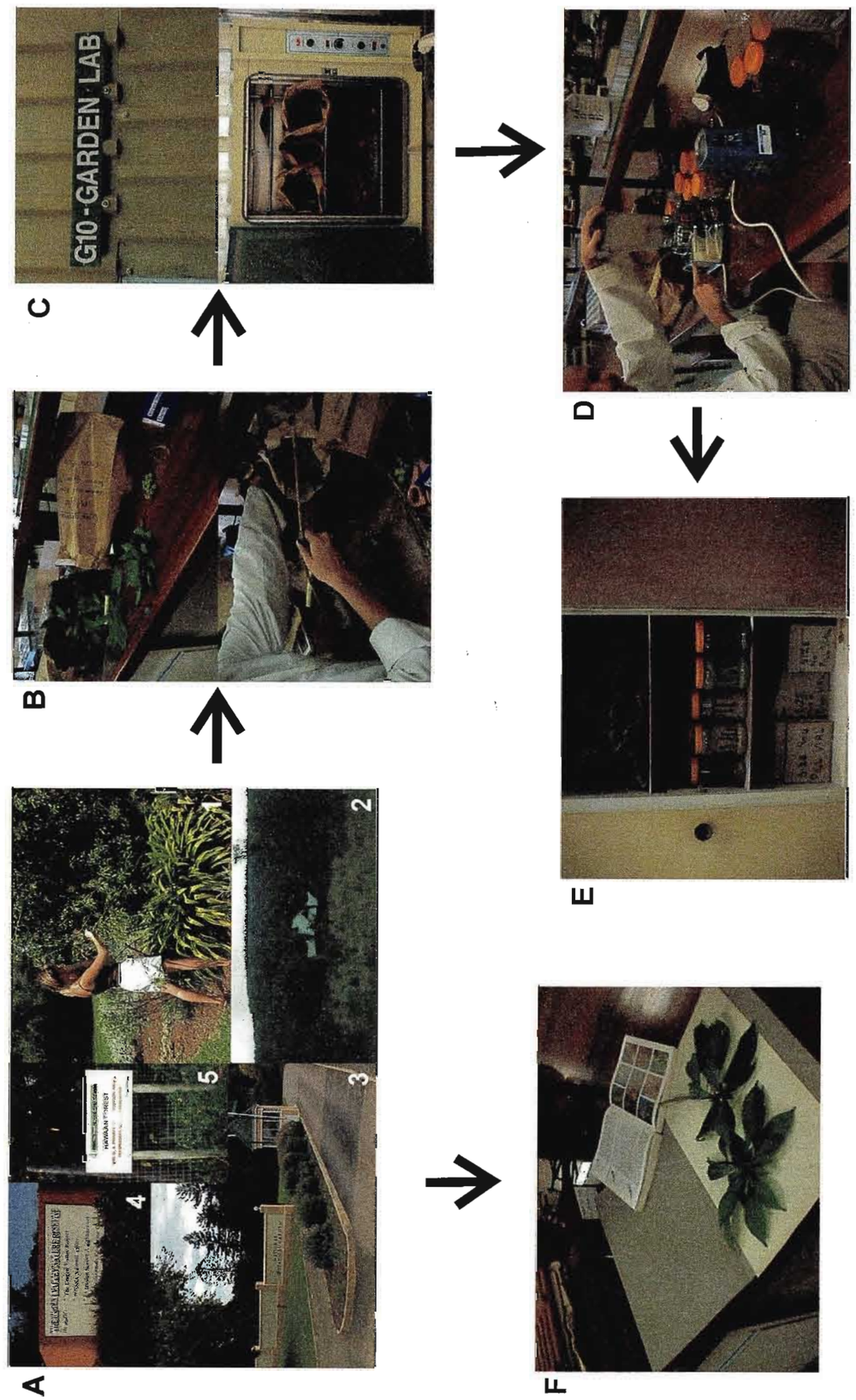
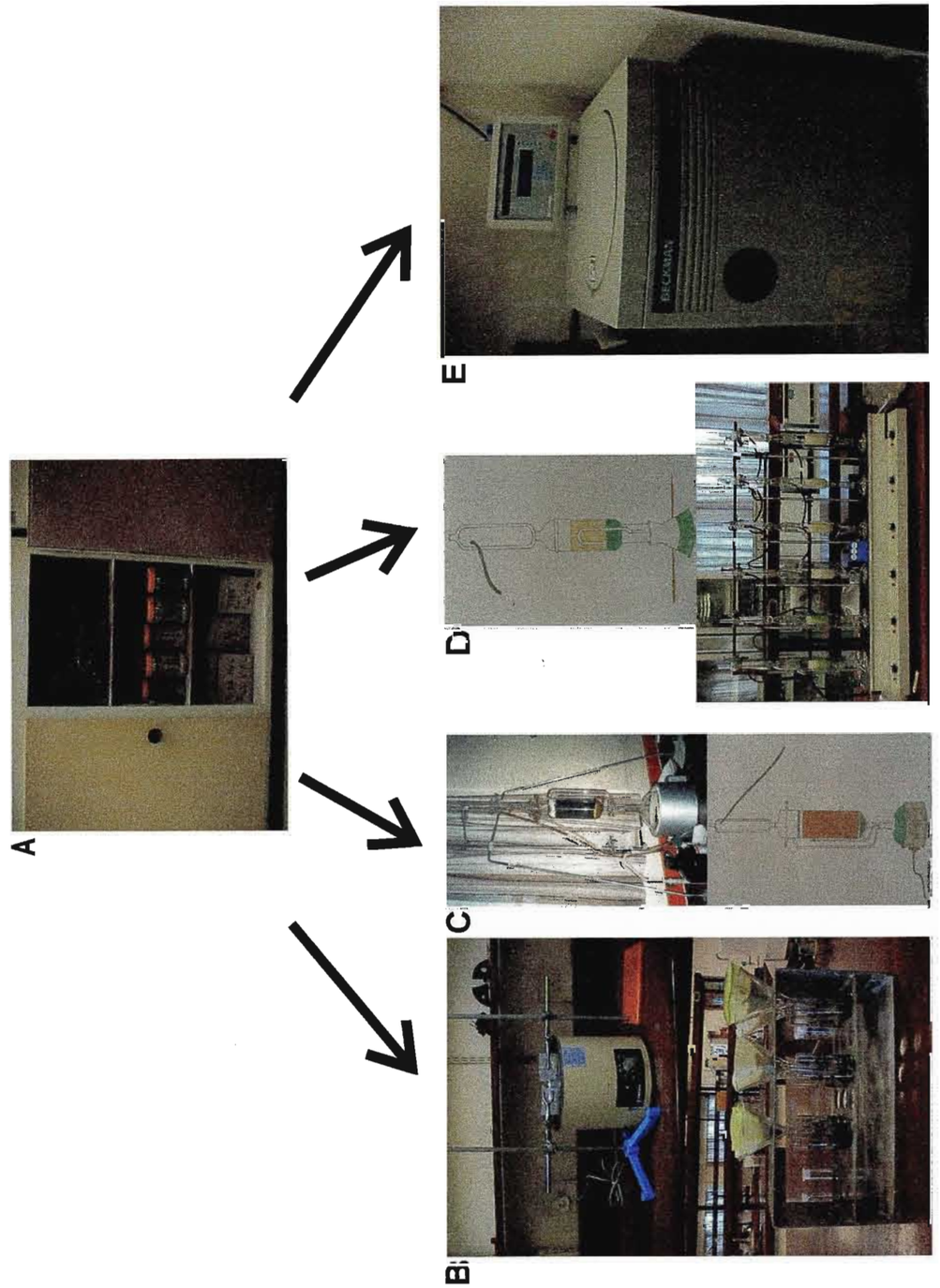


Plate 3 Extraction of plant material. (A) Material was obtained from storage and a variety of extraction techniques were employed. (B) Sonication and filter gravity extraction of small quantities of plant material required for pharmacological and phytochemical screening, (C) large Soxhlet for bulk extraction of *D. rotundifolia* plant material for isolation of biologically active compounds, (D) small Soxhlet for extraction of *D. cymosa*, *D. burgessiae, C. greenwayi* and *H. depressa* plant material for the isolation of biologically active compounds, and (E) centrifugation for separation of aqueous *H. depressa* extract from leaf material.



CHAPTER 4

PHARMACOLOGICAL SCREENING

4.1 INTRODUCTION

There are two main ways to screen and evaluate plant extracts for useful pharmacological activities (RASOANAIRO & RATSIMAMANGA-URVERG,1993): (1) Pharmacological approaches which utilize different animal models also referred to as *in vivo / in situ* tests and *in vitro* models, (2) clinical approaches which are not a clinical evaluation of a pharmacologically defined compound but rather a clinical assay of a plant extract, having a history of traditional uses, after appropriate toxicological investigation.

Plant material was screened pharmacologically for anti-bacterial and anti-inflammatory activity.

Bacteria are responsible for a variety of common illnesses. Inhibition of the various bacteria, by plant extracts, would be beneficial in the study for treatments of a number of ailments. The *in vitro* anti-bacterial assays used in the screening were the disc-diffusion assay (RASOANAIRO & RATSIMAMANGA-URVERG, 1993) and the minimal inhibitory concentration assay (MIC) (ELOFF, 1998). These assays are used to determine the degree of susceptibility of the bacterial strains to the plant extracts.

In the disc-diffusion assay the anti-bacterial activity was determined by the formation of clear zones of inhibition around filter paper discs, lying on bacterial rich agar, loaded with plant extracts which exhibited anti-bacterial activity. This method works by the active plant extracts diffusing into the surrounding

bacteria rich agar, killing and inhibiting further bacterial growth.

The MIC assay involves the serial dilution by 50 % of the plant extracts in a microtiter plate tested against a constant bacterial concentration to determine the minimum concentration of the plant extract required to exhibit inhibition of bacterial growth. This is an effective and accurate method requiring minimal quantities of extract. Results may be difficult to interpret in dense coloured extracts.

Plant extracts were screened for anti-inflammatory activity by screening for prostaglandin-synthesis inhibition activity using the COX-1 assay. Inflammation is a response to injury, amongst other factors, and involves the action of prostaglandins. Prostaglandins are involved in the complex processes of inflammation and are responsible for the sensation of pain (JÄGER, HUTCHINGS & VAN STADEN, 1996). If the production of prostaglandins can be blocked or reduced one has a potential cure. Prostaglandins are biosynthesised from phospholipids in the cell membrane. The biosynthesis can be blocked in two phases. Firstly, at the phospholipase A_2 level. This way is effective, but it has severe side effects. The second phase is at the cyclooxygenase level. Most over-the-counter painkillers inhibit cyclooxygenase (COX). COX exists in two forms: COX-1 and COX-2, both having different roles. To test for anti-inflammatory activity during this study the COX-1 assay was employed (JÄGER, HUTCHINGS & VAN STADEN, 1996). COX-1 is found constitutively expressed in tissue such as stomach, kidney and platelets. The enzyme is responsible for the production of prostanoids that maintain mucocal blood flow, promote mucous secretion, inhibit neutrophil adherence, maintain renal blood flow an dpromote platelet aggregation (WALLACE & CHIN, 1997). In this assay, plant extracts with anti-inflammatory activity inhibit arachidonic acid conversion into prostaglandins resulting in less prostaglandins being synthesized. True anti-inflammatory testing involves the use of animal models,

To indicate bacterial growth 40 µl of 0.2 mg ml^{-1} p-iodonitrotetrazolium violet (INT) was added to each well and the plates were incubated at 37°C for 30 min, and then observed. Bacterial growth in the wells is indicated by a red colour. A totally clear well indicates inhibition by the test substance.

4.2.2 Anti-inflammatory Activity

Extraction of Plant Material

Two separate samples of 1 g of the dried plant material were extracted with 10 ml water and ethanol respectively. Four grams of plant material were extracted with 40 ml dichloromethane. Pill vials, containing plant extracts were placed in a Julabo ultrasound bath (25°C) for 30 min. The extracts were separated from the plant material using a Büchner funnel. The extracts were left to dry overnight in a fume hood and their weight determined.

Cyclooxygenase Assay

The bioassay was performed according to JÄGER, HUTCHINGS & VAN STADEN (1996), using the Prostaglandin-synthesis inhibitor assay (COX -1). Dried plant material was required for this experiment. Two reps were made for each extract so an average could be determined. Ten µl of stock enzyme solution (Sheep seminal vesicle microsomes stored at -70°C) were suspended in 90 µl Tris buffer. Twenty five µl of this solution were then suspended in 975 µl Tris buffer to give 0.25% of the stock. Two thousand µl of cofactor solution (0.3 mg ml^{-1} L - adrenalin and 0.3 mg ml^{-1} reduced glutathione in 0.1 M Tris buffer, pH 8.2) and 400 µl of enzyme solution were mixed and incubated on ice for 15 min. Sixty µl of enzyme/ cofactor solution were added to 20 µl of solvent/ test solution (20 µl aqueous extracts; 25 µl ethanol extracts + 17.5 µl water; 25 µl ethyl acetate extracts + 17.5 µl water) or standard solution (2.5 µl ethanolic

immediately over base plates forming a homogenous top layer. A concentration of plant extract (1 mg) was applied to 6 mm diameter filter paper discs (Whatman No. 3), which were then placed on the surface of the inoculated agar medium. Filter paper discs of neomycin were prepared, as a positive control. The neomycin was diluted to 200 µg ml^{-1}, and 10 µl were pipetted onto each disc. Each petri-dish contained four paper discs with plant extract and a disc with the neomycin control. The plates were incubated at 37°C for 18 h. A clear zone of inhibition formed around discs containing plant extracts which have anti-bacterial activity. Anti-bacterial activity was expressed as the ratio of the inhibition zone (mm) produced by the plant extract to the inhibition zone caused by the neomycin control (RABE & VAN STADEN, 1997).

Minimal Inhibition Concentration (MIC) Assay

This method is the scaling down of the serial dilution technique (ELOFF, 1998), using 96-well microplates to determine the MIC's of extracts. Five ml cultures of four bacterial strains: Two Gram-positive: *Bacillus subtilis* (ATCC 6051), *Staphylococcus aureus* (ATCC 12600) and two Gram-negative: *Escherichia coli* (ATCC 11775), *Klebsiella pneumoniae* (ATCC 13883), were prepared and placed in a waterbath overnight at 37°C. The following morning, the overnight cultures were diluted with sterile MH broth (1/100). The test solution were serially diluted 50% with water. One hundred µl sterile water were added to each well excluding that of the neomycin (control). One hundred µl extract were added to well A (4 replicas), and mixed well with the water. One hundred µl of well A were added to well B, mixed well, and then 100 µl of well B was added to well C. This serial dilution continued up to well H. One hundred µl of well H were discarded. One hundred µl of each bacterial culture were added to the wells of each replica. The microplates were covered and incubated overnight at 37°C.

To indicate bacterial growth 40 µl of 0.2 mg ml^{-1} p-iodonitrotetrazolium violet (INT) was added to each well and the plates were incubated at 37°C for 30 min, and then observed. Bacterial growth in the wells is indicated by a red colour. A totally clear well indicates inhibition by the test substance.

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indomethacin solution + 17.5 µl water). Twenty µl ^{14}C - arachidonic acid (19 Ci/mole, 30 Mm) were added and the assay mixture then incubated at 37°C for 8 min. The reaction was terminated by adding 10 µl 2N HCl. The enzyme activity of background samples were inactivated prior to adding ^{14}C-arachidonic acid, and kept on ice. The ^{14}C-labeled prostaglandins synthesized during the assay was separated from unmetabolized arachidonic acid by column chromatography. Silica gel in eluent 1 (hexane: dioxane: acetic acid 350: 150: 1) was packed in Pasteur pipettes to a height of 3 cm. One ml of eluent 1 was added to each of the assay mixtures, which were then applied to the columns. The arachidonic acid was eluted from the columns with a further 4 ml of eluent 1 and discarded. The labeled prostaglandins were then eluted with 3 ml of eluent 2 (ethyl acetate:methanol 425:75) into scintillation vials. Four ml scintillation cocktail (Beckman Ready Solve) were added to the eluent. After 30 min the radioactivity of the samples was counted using a Beckman LS3801 scintillation counter. The percentage inhibition of the test solutions was obtained by analyzing the amount of radioactivity present relative to the radioactivity in the solvent blank (McGAW, JÄGER & VAN STADEN, 1997). The following equation was used:

$$1 - \left[\frac{DMP_{sample} - DMP_{background}}{DMP_{untreated} - DMP_{background}} \right] \div 100$$

4.3 RESULTS AND DISCUSSION

Anti-bacterial Activity

Only *D. rotundifolia* (Table 5) and *C. natalensis* plant extracts were screened in the disc-diffusion assay. Anti-bacterial activity was expressed as a ratio of the inhibition zone of the extract (100 mg ml^{-1}) to the inhibition zone of the reference (neomycin 500 µg ml^{-1}).

Only the aqueous extract obtained from the dried leaf material of *C. natalensis* exhibited slight anti-bacterial activity (0.08) against *S. epidermidis*. Thus *C. natalensis* plant material had low or no activity against the bacterial strains screened.

The ethyl acetate, ethanol and water extracts of *D. rotundifolia* from both collection sites showed varying amounts of anti-bacterial activity (Table 5).

For leaf material from both collection sites, aqueous extracts showed anti-bacterial activity against *S. epidermidis* (0.23 and 0.08) (Plate 4 A), extractions with ethyl acetate showed anti-bacterial activity against *K. pneumoniae* (0.19 and 0.13) and extractions with ethanol were bacteriostatic against *M. luteus* (0.83 and 0.71) (Plate 4 A). Plant extracts are considered to be bacteriostatic when they are able to inhibit the growth of the bacteria, but not able to kill them (ABERCROMBIE, HICKMAN, JOHNSON & THAIN, 1990). Bacteriostatic activity is differentiated from bacteriocidal activity by the presence of an opaque inhibition zone rather than a clear zone, indicating antibacterial activity was inhibited to an extent.

Aqueous twig extracts from both collection sites showed anti-bacterial activity against *S. epidermidis* (0.23 and 0.23) (Plate 4 B), extractions with ethyl

acetate showed anti-bacterial activity against *K. pneumoniae* (0.14 and 0.07) and extractions with ethanol were bacteriostatic against *S. aureus* (1.22 and 1.22). The aqueous extract obtained from the twig material from UVNR showed anti-bacterial activity against *B. subtilis* (0.12), and the water extract obtained from Monato showed anti-bacterial activity against *M. luteus* (0.21) (Plate 4 B). The ethyl acetate extract from Monato showed anti-bacterial activity against *B. subtilis* (0.07) and *E. coli* (0.07). The ethanol extract exhibited anti-bacterial activity against *M. luteus* (0.89).

Aqueous bark extracts collected from both sites showed anti-bacterial activity against *K. pneumoniae* (0.13 and 0.13), while extractions with ethanol showed anti-bacterial activity against *K. pneumoniae* (0.25 and 0.08). There was anti-bacterial activity detected with the ethyl acetate extracts. The aqueous extract from bark material from Monato showed anti-bacterial activity against *E. coli* (0.08). The ethyl acetate extract from the UVNR showed anti-bacterial activity against *S. aureus* (0.07) and *S. epidermidis* (0.14), there was no anti-bacterial activity from the ethyl acetate extract from Monato. The ethanol extract exhibited anti-bacterial activity against *B. subtilis* (0.10).

It can be concluded that the extracts from both collection sites show overall similarities in their inhibition of the bacterial strains. *K. pneumoniae* was inhibited by four of the nine extracts from each of the two collection sites. *K. pneumoniae* is a Gram-negative bacterium, a group which is considered to be more resistant to inhibition. The presence of anti-bacterial activity in *D. rotundifolia* is in line with its use in traditional medicine (Table 8). *D. rotundifolia* is used to treat diarrhoea (COATES PALGRAVE,1977; VAN WYK, VAN OUDTSHOORN & GERICKE, 1997).

Anti-bacterial activity was reported to be present in leaf material from *D. rotundifolia* (McGAW, 2000). Ethanol, water and hexane extracts of the leaf

material were screened. Ethanol and water extracts had anti-bacterial activity against *B. subtilis* (0.40 and 0.40) and *S. aureus* (0.33 and 0.36). These results did not hold for the results obtained in this study.

The disc-diffusion assay was resolved to be an inaccurate technique and it resulted in too many inconsistencies. Much of the extract applied to the filter paper discs was not absorbed, therefore the concentration of extract per filter paper disc varied. If filter paper discs were not placed flat on the agar containing bacteria, less inhibition would be noted and the results could not be considered accurate. A more accurate method was employed. This method was advantages as it detected anti-bacterial activity and the MIC values within one assay. The disc-diffusion assay indicated the lack of anti-bacterial activity in extracts obtained from *C. natalensis* and therefore the determination of MIC values was not attempted.

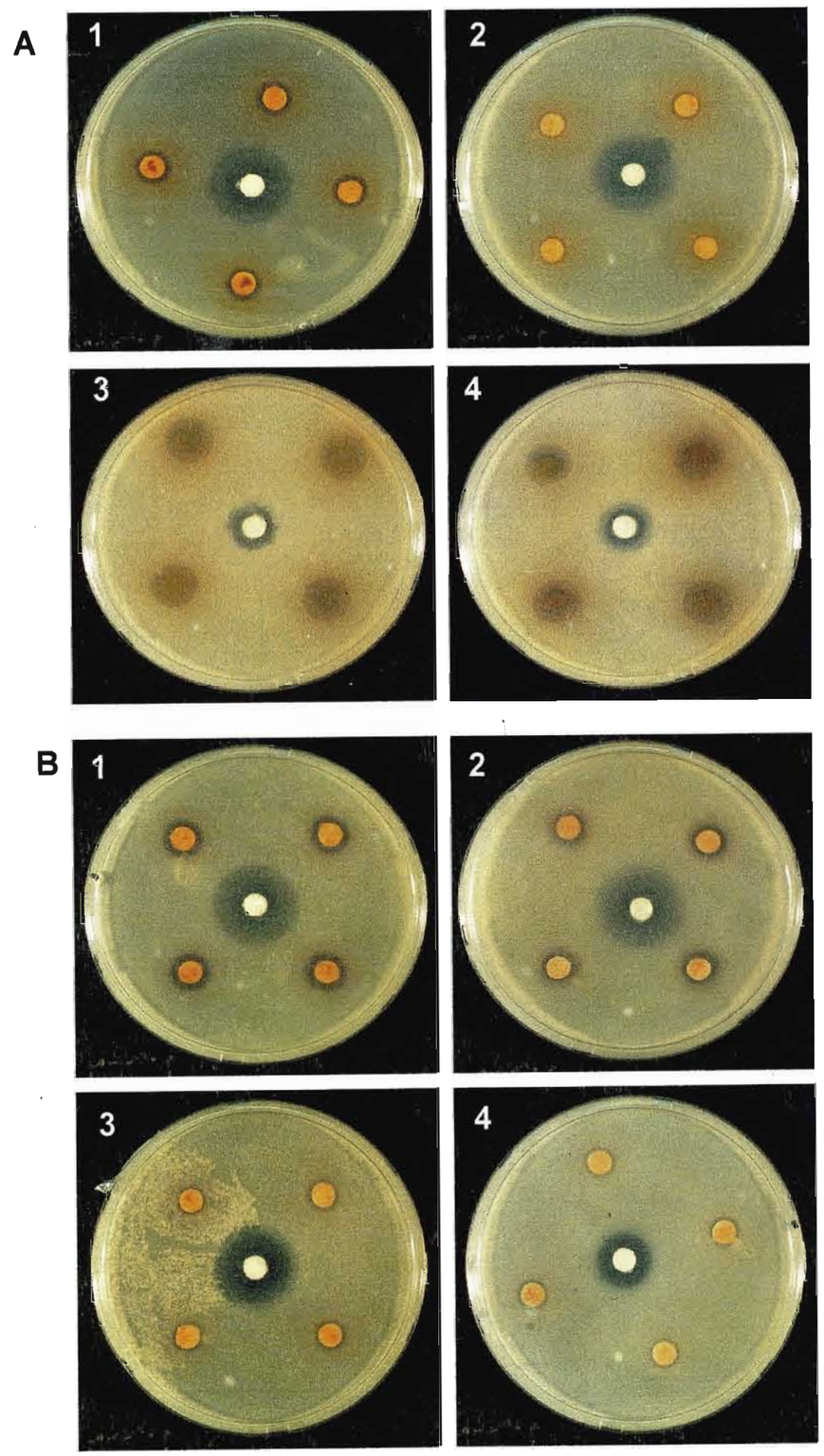
Table 5: Anti-bacterial activity[a] of extracts of *D. rotundifolia* using the disc-diffusion assay

Collection site	Plant part analysed	Extraction Solvent	Bacteria tested[b]					
			Gram-positive				Gram-negative	
			B.s.	*M.l.*	*S.a.*	*S.e.*	*E.c.*	*K.p.*
Umgeni Valley Nature Reserve	Leaves	Water	0	0	0	0.23	0	0
		Ethyl acetate	0	0	0	0	0	0.19
		Ethanol	0	0.83[c]	0	0	0	0
	Twigs	Water	0.12	0	0	0.23	0	0
		Ethyl acetate	0	0	0	0	0	0.14
		Ethanol	0	0	1.22[c]	0	0	0
	Bark	Water	0	0	0	0	0	0.13
		Ethyl acetate	0	0	0.07	0.14	0	0
		Ethanol	0.10	0	0	0	0	0.25
Monato	Leaves	Water	0	0	0	0.08	0	0
		Ethyl acetate	0	0	0	0	0	0.13
		Ethanol	0	0.71[c]	0	0	0	0
	Twigs	Water	0	0.21	0	0.23	0	0
		Ethyl acetate	0.07	0	0	1.22[c]	0	0.07	0.07
		Ethanol	0	0.89	0	0	0	0
	Bark	Water	0	0	0	0	0.08	0.13
		Ethyl acetate	0	0	0	0	0	0
		Ethanol	0	0		0	0	0.08

a - Anti-bacterial activity is expressed as a ratio of the inhibition zone of the extract (100 mg ml^{-1}) to the inhibition zone of the reference (neomycin 500 µg ml^{-1}).

b - Bacteria: *B.s.*, *Bacillus subtilis; E.c.*, *Escherichia coli; K.p.*, *Klebsiella pneumoniae; M.l.*, *Micrococcus luteus*; *S.a.*, *Staphylococcus aureus ; S.e.*,*Staphylococcus epidermidis*.c - Bacteriostatic

Plate 4 Anti-bacterial activity was detected using the disc-diffusion assay for *D. rotundifolia* extracts. Anti-bacterial activity was expressed as a ratio of the inhibition zone of the extract to the inhibition zone of the Neomycin control. (A) Leaf material from (1) UVNR and (2) Monato aqueous extracts showed activity against *S. epidermidis* (0.23 and 0.08), (3) UVNR and (4) Monato ethanolic extracts were bacteriostatic against *M. luteus* (0.83 and 0.71). (B) Twig material from (1) UVNR and (2) Monato aqueous extracts showed activity against *S. epidermidis* (0.23 and 0.23), (3) UVNR aqueous extracts showed activity against *B. subtilis* (0.12), and (4) Monato aqueous extract showed activity against *M. luteus* (0.21)



MIC values of extracts from the Sterculiaceae species are shown in Table 6.

Table 6: The minimum inhibitory concentration (MIC) of plant extracts obtained from the Sterculiaceae species investigated

Plant species	Plant part	Extract	Bacteria used (MIC(mg ml^{-1}))			
			B.s.	*E.c.*	*K.p.*	*S.a.*
D. rotundifolia: - Monato	Leaf	H_2O	–	–	–	–
		Ethyl acetate	–	–	–	12.5
		Ethanol	–	–	–	–
	Twig	H_2O	–	–	–	–
		Ethyl acetate	12.5	–	6.25	12.5
		Ethanol	–	–	–	–
	Bark	H_2O	–	–	–	–
		Ethyl acetate	–	–	–	–
		Ethanol	12.5	–	–	12.5
- UVNR	Leaf	H_2O	–	–	–	–
		Ethyl acetate	–	–	–	6.25
		Ethanol	–	–	12.5	–
	Twig	H_2O	–	–	–	–
		Ethyl acetate	12.5	–	6.25	12.5
		Ethanol	–	–	–	12.5
	Bark	H_2O	–	–	–	–
		Ethyl acetate	6.25	–	–	–
		Ethanol	–	–	–	–
D. cymosa	Leaf	H_2O	–	–	–	–
		Ethyl acetate	12.5	12.5	12.5	12.5
		Ethanol	1.56	1.56	0.78	0.195
	Twig	H_2O	–	–	–	–
		Ethyl acetate	12.5	12.5	6.25	12.5
		Ethanol	–	–	–	12.5

Plant species	Plant part	Extract	Bacteria used (MIC(mg ml^{-1}))			
			B.s.	*E.c.*	*K.p.*	*S.a.*
D. burgessiae	Leaf	H_2O	–	–	–	–
		Ethyl acetate	12.5	12.5	12.5	12.5
		Ethanol	–	–	–	–
C. greenwayi	Leaf	H_2O	–	–	–	–
		Ethyl acetate	6.25	6.25	0.78	0.39
		Ethanol	12.5	12.5	12.5	12.5
	Twig	H_2O	–	–	–	–
		Ethyl acetate	6.25	6.25	12.5	12.5
		Ethanol	12.5	12.5	12.5	12.5
H. depressa	Leaf	H_2O	–	–	–	–
		Ethyl acetate	1.56	0.78	0.78	0.78
		Ethanol	1.56	3.125	3.125	3.125
	Stem	H_2O	–	–	–	–
		Ethyl acetate	0.78	DNT	DNT	3.125
		Ethanol	0.39	3.125	3.125	0.78
	Root	H_2O	–	–	–	–
		Ethyl acetate	0.78	3.125	0.78	3.125
		Ethanol	0.195	1.56	3.125	3.125
S. murex	Twig	H_2O	–	–	–	–
		Ethyl acetate	12.5	12.5	12.5	–
		Ethanol	12.5	6.25	12.5	12.5
Neomycin			1.56×10^{-2}	6.25×10^{-2}	1.56×10^{-2}	3.1×10^{-2}

Bacteria: *B.s., Bacillus subtilis; E.c., Escherichia coli; K.p., Klebsiella pneumoniae;* S.a., *Staphylococcus aureus*
'-': Extracts exhibit no apparent inhibition of bacteria
DNT: Did not test

No aqueous extracts showed any anti-bacterial activity (Table 6). If there is activity present in these extracts, then the concentrations of the extracts must be increased, but these values would be too low or probably only bacteriostatic if an aqueous preparation was used in traditional medicine. If the aqueous extracts do exhibit any bacterial inhibition it would be at a concentration greater than 25 mg ml^{-1}. It is also important for traditional healers to know that the

aqueous extracts are ineffective in treatment against bacterial infections at low concentrations. Organic solvents should be used in the extraction process.

The results obtained from the disc-diffusion assay (Table 5) and the MIC assay (Table 6) for extracts from *D. rotundifolia* plant material indicated some similarities. However, there was no distinct correlation between the two antibacterial assays employed. These results again did not agree with the results reported by McGAW (2000). The MIC values were modest when compared to those of *D. cymosa* and *C. greenwayi* (Plate 5). *D. cymosa* plant extracts showed the best MIC values for the ethanolic leaf extracts, where the extract showed activity against the four bacterial strains used. The ethyl acetate leaf and twig extracts showed only slight activity. The roots of *D. cymosa* are said to be used in traditional medicine (Table 8) (HUTCHINGS, HAXTON SCOTT, LEWIS & CUNNINGHAM, 1996), but leaf extracts would be more effective in the treatment of bacterial infections due to their lower MIC values. *D. burgessiae* showed slight activity in the ethyl acetate leaf extracts. *C. greenwayi* is said to be used in traditional medicine (HUTCHINGS, HAXTON SCOTT, LEWIS & CUNNINGHAM, 1996), but it's use was not specified. The MIC values in the leaf ethyl acetate extracts were good, especially against *K. pneumoniae* (0.78 mg ml^{-1}) and *S. aureus* (0.39 mg ml^{-1}). *H. depressa* ethanolic and ethyl acetate leaf, stem and root extracts exhibited very good MIC values (Plate 5). *H. depressa* extracts would have a noticeable affect against bacterial infections in traditional medicines due to their high anti-bacterial activity. *S. murex* showed low anti-bacterial activity, indicated by high MIC values, in the ethanolic and ethyl acetate twig extracts.

The determined anti-bacterial activity could be due to the presence of a variety of compounds. Some saponins are said to contain anti-fungal and anti-microbial activity (HARBORNE & BAXTER, 1993). Similarly, tannins are known to have anti-viral, anti-tumour and possibly anti-bacterial activity. The plant

material screened during this study was therefore also screened for saponins and tannins as anti-bacterial activity may be caused by these compounds (Chapter 5).

It is commonly accepted that a major reason why living organisms produce biologically active compounds is that they form part of the survival strategy in a competitive environment. Complex chemical interactions are often involved in attraction of pollinators and mating partners or the deterrence and repulsion of predators or infecting agents. The production of such compounds is particularly important where the organism is static and has little ability to move from a hostile situation. It is therefore possible that organisms in these situations may produce particularly interesting bioactive compounds (HOUGHTON & RAMAN, 1998).

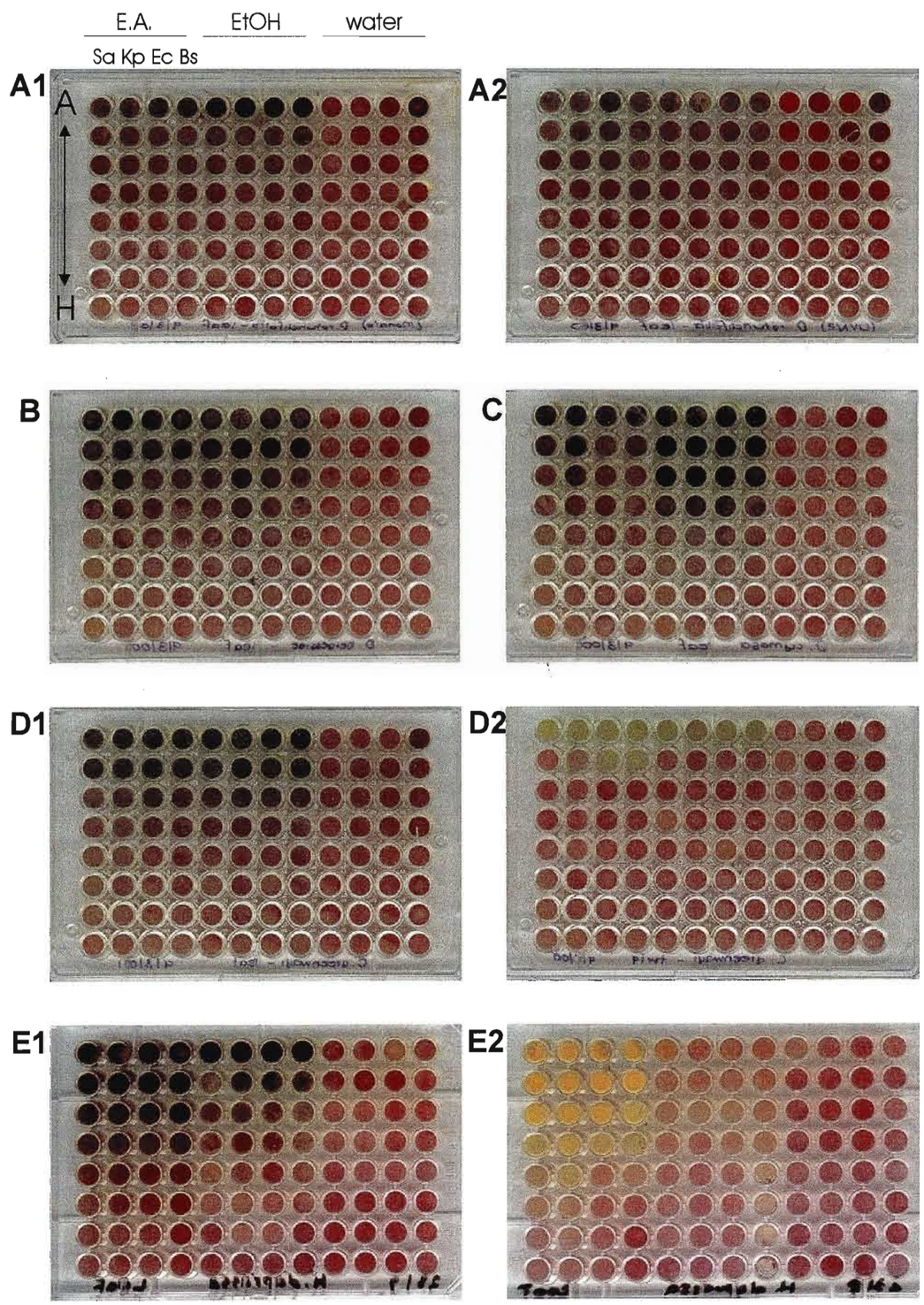


Plate 5 Anti-bacterial activity as determined using the minimal inhibition concentration (MIC) assay. (A) *D. rotundifolia* leaf extracts from (1) Monato and (2) UVNR, (B) *D. burgessiae* leaf extract, (C) *D. cymosa* leaf extract, (D) *C. greenwayi* (1) leaf and (2) twig extracts, and (E) *H.depressa* (1) leaf and (2) root extracts.(Bs - *B. subtilis*, Ec - *E. coli*, Kp - *K. pneumonia*, Sa - *S. aureus*) (EtOH - ethanol, E.A. - ethyl acetate, water).

Anti-inflammatory Activity

Table 7: The average percentage inhibition of cyclooxygenase (COX -1) by Sterculiaceae species investigated

Plant species analysed	Inhibition (%)														
	Leaf			Twig			Bark			Stem			Root		
	H_2O	EtOH	CH_2Cl_2	H_2O	EtOH	CH_2Cl_2	H_2O	EtOH	CH_2Cl_2	H_2O	EtOH	CH_2Cl_2	H_2O	EtOH	CH_2Cl_2
D. rotundifolia:															
- Monato	55	**90**	**89**	**76**	**88**	**79**	5	**87**	**80**	-	-	-	-	-	-
- UVNR	**79**	**97**	**95**	65	**87**	**94**	5	**87**	**93**	-	-	-	-	-	-
D. cymosa	39	**78**	**70**	14	**84**	39	-	-	-	-	-	-	-	-	-
D. burgessiae	28	**76**	**81**	28	**89**	**87**	-	-	-	-	-	-	-	-	-
C. natalensis	14	46	52	8	44	32	-	-	-	-	-	-	-	-	-
C. greenwayi	52	**75**	**78**	7	**74**	65	-	-	-	-	-	-	-	-	-
H. depressa	60	59	62	-	-	-	-	-	-	51	55	**78**	58	58	**81**
S. murex	41	**85**	**87**	18	**72**	**97**	-	-	-	-	-	-	-	-	-
Indomethacin		68													

- Indicates plant parts not screened

The results in Table 7 indicate a pattern of lower anti-inflammatory activity in the aqueous extracts. Both the ethanol and dichloromethane extracts exhibited activity, with the ethanol extracts generally giving the highest activity.

D. rotundifolia exhibited the highest activity of all the plant species analyzed. The results were very similar when comparing the two collection sites, with the extracts from the UVNR showing slightly higher activity. The lowest activity was recorded in the aqueous bark extracts (5%). The highest overall activity was obtained from the ethanolic leaf extract (97%) from the UVNR collection site. The presence of anti-inflammatory activity in *D. rotundifolia* is in line with its use in traditional medicine (Table 8). Infusions are used in the treatment of intestinal ulcers, headaches and stomach complaints (COATES PALGRAVE, 1977; VAN WYK, VAN OUDTSHOORN & GERICKE, 1997).

D. cymosa extracts showed high activity in ethanolic leaf (78%) and twig (84%) extracts. Activity was low for all the other extracts. *D. burgessiae* extracts showed high activity in both ethanolic and dichloromethane extracts from leaf (76 & 81%) and twig (89 & 87%) material. For *C. greenwayi S. murex* and *C. natalensis* extracts showed very low activity. *H. depressa* water extracts showed the lowest activity. Noticeable activity occurred in the dichloromethane extracts obtained from the stem (78%) and root (81%) extracts. All other activity detected was considered as too low.

The suggested high anti-inflammatory activity in plant material containing tannins may be false-positive. Tannins are phenolics which destroy enzymes inhibiting their action. Plant material was screened for tannins in Chapter 5. The COX-1 assay uses enzyme solution of sheep seminal vesicle microsomes.

Table 8: A summary of results obtained from the investigation of seven Sterculiaceae species for their anti-bacterial and anti-inflammatory activity compared to their mentioned use in traditional medicine

Species Investigated	Anti-bacterial activity (MIC assay)	Anti-inflammatory activity (COX-1)	Related Uses in Traditional Medicine
D. rotundifolia	Fair inhibition by leaf and twig ethyl acetate and ethanol bark extracts.	Very good inhibition by EtOH and CH_2Cl_2 leaf, twig and bark extracts (76 – 97%).	Treatment of intestinal ulcers, stomach ache, diarrhoea, wounds, headaches and colic.
D. cymosa	Fair inhibition by ethyl acetate leaf and twig extracts. Very good inhibition by EtOH leaf extracts.	Good inhibition by EtOH and CH_2Cl_2 leaf and extracts (70 – 84%).	Roots used, but not specified.
D. burgessiae	Fair inhibition by leaf ethyl acetate extracts against all bacteria.	Very good inhibition by EtOH and CH_2Cl_2 leaf and twig extracts (76 – 89%).	Treatment of malaria, wounds and stomach ache.
C. natalensis	No relevant activity.	Very low inhibition.	No mentioned use
C. greenwayi	Good inhibition by leaf ethyl acetate extracts, fair inhibition by leaf EtOH extracts and Twig ethyl acetate and EtOH extracts.	Good inhibition by EtOH (75%), CH_2Cl_2 (78%) and EtOH twig (74%) extracts.	Mentioned use, but not specified.
H. depressa	Very good inhibition by leaf, stem and root ethyl acetate and EtOH extracts.	Good inhibition by CH_2Cl_2 stem (78%) and root (81%) extracts.	Treatment of coughs, diarrhoea and stomach ache.
S. murex	Fair inhibition by ethyl acetate and EtOH extracts	Good inhibition by EtOH and CH_2Cl_2 leaf and twig extracts (72 – 97%).	Not specified.

EtOH – Ethanol

CH_2Cl_2 – Dichloromethane

References for mentioned uses in traditional medicine are located in Chapter 2, Table 2

4.4 SUMMARY

- Seven selected Sterculiaceae species were screened pharmacologically for anti-bacterial (disc-diffusion method and MIC assay) and anti-inflammatory (COX-1).
  - Anti-bacterial activity:

    MIC values of plant extracts was detemined against four bacterial strains: Two Gram-positive: *Bacillus subtilis, Staphylococcus aureus* and two Gram-negative: *Escherichia coli, Klebsiella pneumoniae.* Activity was exhibited by six of the species screened: *D. rotundifolia, D. cymosa, D. burgessiae, C. greenwayi, H. depressa* and *S. murex*. No inhibition of bacteria was noted in the aqueous extracts.
  - Good anti-inflammatory activity was noted in ethanol and dichloromethane extracts from *D. rotundifolia* (79 – 97%), *D. cymosa* (78 – 84%), *D. burgessiae* (76 – 87%), *C. greenwayi* (65 – 78%) and *S. murex* (72 – 97%). Activity may be false-positive due to the possible presence of tannins. All aqueous extracts exhibited low activity.

- *D. rotundifolia* plant material was obtained from two collection sites: UVNR and Monato. Results were compared. There were distinct similarities in the anti-bacterial and anti-inflammatory properties of the two collection sites.

- *C. natalensis* has no mentioned use in traditional medicine. Very low anti-inflammatory activity and only a very low ratio of 0.08 bacterial inhibition in the aqueous leaf extract against *S. epidermidis* was noted.

CHAPTER 5

PHYTOCHEMICAL SCREENING

5.1 INTRODUCTION

In this Chapter all the plant species were phytochemically screened for cardiac glycosides, cyanogenic glycosides, saponins, tannins and alkaloids.

'Glycoside' is a general term covering a wide range of substances whose common feature is that they consist of at least one sugar molecule linked via its anomeric carbon to another moiety. Glycosides are relatively polar due to the presence of one or more sugars in the molecule (HOUGHTON & RAMAN, 1998). Cardiac glycosides of plant origin remain chief drugs in the treatment of heart related ailments. All of the organs, or parts thereof may contain cardiac glycosides, but except for a few cases, the concentrations are low (lower than 1%) (BRUNETON, 1995). Cardiac glycosides are made up of both cardienolides and bufadienolides, which are two related groups of C_{23} and C_{24} steroids respectively (HARBORNE & BAXTER, 1993; BRUNETON, 1995). Some Sterculiaceae species are said to contain cardienolides (TREASE & EVANS, 1983).

Cardienolide glycosides are defined by their aglycone structure (HOUGHTON & RAMAN, 1998). They contain a tetracyclic steroidal ring system which is substituted in the 17-position by an μ, β-unsaturated *y*-lactone ring (HARBORNE & BAXTER, 1993). They usually occur in plants as glycosides, with a sugar attachment commonly at the 3-hydroxyl group or, less frequently, at the 1-hydroxyl, 2-hydroxyl position (HARBORNE & BAXTER, 1993). They are 'heart poisons' which, when used in small doses, are extremely valuable

clinically for controlling congestive heart failure.

Bufadienolides can be clearly distinguished from cardienolides by the fact the lactone ring at C_{17} in the steroid nucleus is six-membered rather than five-membered. They occur, like the cardienolides, as *O*-glycosides but can also be found in the free state (HARBORNE & BAXTER, 1993).

Many plants synthesize compounds which are capable of liberating hydrogen cyanide upon hydrolysis (SIEGLER, 1977). This ability has been recognised for centuries in such plants as apricots, peaches, almonds, and other important food plants. There are two chemical types of hydrogen cyanide producing substances (SIEGLER, 1977), namely cyanogenic glycosides and cyanolipids. Plant material in this study was screened for cyanogenic glycosides. Both are derivatives of ∝-hydroxynitriles and both liberate a carbonyl component and hydrogen cyanide when the sugar or the fatty acid moiety respectively is removed (SIEGLER, 1977). Cyanogenic glycosides are known to occur in at least 800 species of plants representing 70 - 80 families. The highest concentrations are found in the leaves, but it may be found in the roots, seeds and other plant tissues. The isolation of cyanogenic glycosides is extremely difficult therefore only two compounds are available commercially: amygdalin and linamarin (SIEGLER, 1977). Amygdalin was used as a positive control in this study.

Saponins are a group of plant glycosides in which water-soluble sugars are attached to a lipophilic steroid (C_{27}) or triterpenoid (C_{30}) moiety. This hydrophobic/hydrophilic asymmetry means that they have the ability to lower surface tension, and are soap-like. They form foams in aqueous solutions (Froth test) and cause haemolysis of blood erythrocytes (Haemolysis test). They are toxic to cold-blooded animals but generally not to warm-blooded animals. They are widely distributed in the flowering plants, having been

identified in more that 100 plant families (HARBORNE & BAXTER, 1993; BRUNETON, 1995). Saponins are toxic to insects and molluscs and some of the most useful natural agents for controlling schistosomiasis snails are saponin in nature. Antifungal activity is present in some saponins, and some saponins may aid plants such as ivy and oats to resist microbial infection (HARBORNE & BAXTER, 1993).

Tannins occur widely in vascular plants, their occurrence in the angiosperms being particularly associated with woody tissues. By definition they have the ability to react with protein, forming water-insoluble copolymers (HARBORNE & BAXTER, 1993). Most tannins that have been purified and studied are biologically active. Chemically, there are two main types of tannin: condensed tannins and hydrolysable tannins. Condensed tannins have been used in medicine to aid the healing of wounds and burns. When applied to the skin, they produce an impervious layer under which the healing process can take place. They are also thought to have some protective value against toxins when taken internally. Hydrolysable tannins are of pharmacological interest because of their antiviral and antitumour properties (HARBORNE & BAXTER, 1993).

Knowledge of the toxicity and properties of plants containing alkaloids dates back to ancient times: opium, cocoa, belladonna, Colchicum and Cinchona having all been used for centuries (BRUNETON, 1995). However, only recently has the concept of an 'alkaloid' been introduced. Alkaloids generally consist of a nitrogen atom in their chemical structures as part of a heterocyclic ring (HARBORNE & BAXTER, 1993; BRUNETON, 1995). Their major occurrence is in the flowering plants, with 40% of all plant families having at least one alkaloid-bearing member. Their distribution is uneven: they may be universal in some families, and rare in others. They can be present throughout the plant or, alternatively, restricted to certain tissues such as the root or bark. The concentration can vary from a small fraction of less than 0.1 % to as much as

12% by dry weight (HARBORNE & BAXTER, 1993). Alkaloids are principally of interest to humans because of their medicinal properties, and many are widely used as drugs. Some are poisonous and others have hallucinogenic effects (HARBORNE & BAXTER, 1993). Alkaloids are detected in plant extracts by a number of well known colour tests. Some are general, like those used in this study, Dragendorff and Mayer, while others are more specific (HARBORNE & BAXTER, 1993).

5.2 MATERIALS AND METHODS

5.2.1 Cardiac Glycosides

One g of dried plant material was added to 30 ml 53% ethanol containing 3% lead acetate (JÄGER & VAN STADEN, 1995). The mixture was boiled for 15 min. It was allowed to cool before filtering and then acidified with acetic acid before partitioning three times against 15 ml dichloromethane. The dichloromethane extracts were filtered over anhydrous sodium sulphate and taken to dryness under vacuum. The resulting residue was resuspended in 1 ml dichloromethane:methanol (1:1).

Two-Deoxy-sugars/Keller-Killiani Test

Five hundred µl of suspended residue were taken to dryness and resuspended in 2 ml glacial acetic acid, containing 2.2 mM $FeCl_3$ (EVANS, 1989). The solution was layered on top of 2 ml H_2SO_4. If a reddish-brown-purple ring appeared at the interphase, and the upper layer slowly became blueish-green, then two-deoxy-sugars are present.

Unsaturated Lactone Ring Tests

Fifty µl of the extracts were applied to TLC plates (Merck Silica 60). The TLC plates were developed in ethyl acetate: methanol: water (81:11:8). The plates were sprayed with Kedde's reagent which indicate the presence of the *y*-lactone ring of cardienolides by pink/blue violet spots (WAGNER, BLADT & ZGAINSKY, 1984; JÄGER & VAN STADEN 1995). A second TLC plate was sprayed with chloramine T-trichloroacetic acid reagent (WAGNER, BLADT & ZGAINSKY, 1984) and heated for 10 min at 100°C, before being viewed under ultraviolet light at 366 nm for yellow-orange zones, indicating bufadienolides. A third TLC plate was sprayed with antimony (III) chloride reagent (WAGNER, BLADT & ZGAINSKY, 1984) and heated at 100°C for 6 min before being observed under ultraviolet light at 366 nm for yellow to yellow-brown spots, again indicating bufadienolides.

5.2.2 Cyanogenic Glycosides

TLC Sandwich Method

Fresh plant material (about 500 mg) was cut into small pieces and extracted with 5 ml of 80% methanol by boiling for five min. The extract was filtered through Whatman No. 1 filter paper. One tenth of the extract was placed onto a TLC plate (as well as 5 µg of amygdalin, which was applied as the standard). The TLC plate was developed using acetate:acetone:chloroform:methanol: water (40:30:12:10:8). The plate was dried and the spots visible in white light, under 254 nm and 366 nm, were marked. A piece of synthetic net fabric and a sheet of sodium picrate paper was placed on the TLC plate, with a glass plate on top. The edges were taped with scotch tape. The 'sandwich' was placed in an oven at 35°C for about 1 h. Coloured spots on the picrate paper indicate the presence of cyanogenic glucosides in the plant material.

5.2.3 Saponins

Froth Test

Dried, ground plant material (100 mg) was placed into a pill-vial. To this pill-vial 10 ml of distilled water was added. A lid was placed on the pill-vial and it was vigorously shaken for 30 sec. The pill-vial was observed over a 30 min period. A 'honey comb' froth occurring above the surface of the liquid after 30 min indicates the presence of saponins in the plant material.

Haemolysis Test

Citrated cattle blood was added to Columbia Blood Agar Base (Oxoid CM 331) giving a highly nutritious medium capable of supporting a wide variety of organisms.

Thirty-nine grams Columba Blood Agar Base were suspended in 1 l distilled water and brought to boil allowing the medium to dissolve completely. The solution was autoclaved at 121°C for 15 min and cooled in a water bath set at 50°C. Sodium citrate (20%) was added to a bleeding bottle, before it was autoclaved. Three ml of the solution were sufficient for 100 ml of whole blood. Aseptically, 50 – 70 ml sterile citrated sheep or cattle blood was added to the Columbia Blood Agar solution. Twelve ml of the blood agar were dispensed into sterile petri-dishes (9 cm).

A portion of blood agar (approx.1cm in diameter) was removed from 5 equidistant areas of the blood agar plates. A heated Pasteur pipette was used to seal off the agar at the bottom of each well (FONG, TIN-WA, FARNSWORTH, 1974) (Fig. 2).

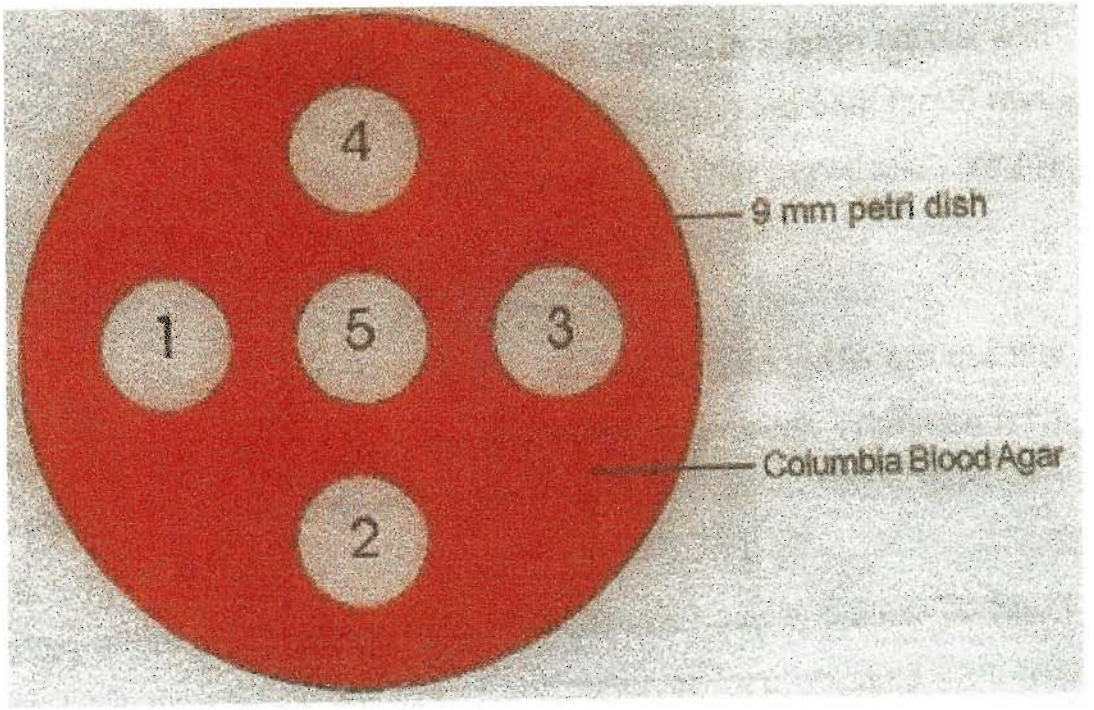


Fig. 2: Diagram showing the layout of the five 'cups' used in the haemolysis test for saponins. (1) - (3) Plant extract; (4) Water control; (5) *Saponaria officinalis* control

With the use of a micro-pipette, equal amounts of the extract were added to three of the five cups. Distilled water was added to the fourth cup as a negative control. Fresh root material of *Saponaria officinalis* was boiled in water for 10 min, over a Bunsen burner (0.1 g ml^{-1}). The cooled extract was added to the fifth cup (centre) as a positive control. The plate was left to stand overnight at room temperature on a sterile surface, in the dark. Development of clear zones of haemolysis indicate the presence of saponins.

5.2.4 Tannins

The test used to screen the plant material for tannins was the gelatin-salt block test (FONG, TIN-WA & FARNSWORTH, 1974, DUNCAN, 1998). Ground, dried plant material (500 mg) was placed in a large pill vial with distilled water (5 ml)

which was sonicated in an ultrasound bath for 30 min. The extracts were filtered through Whatman No. 1 filter paper into new pill vials. To the new pill vials, 2 drops of 10% NaCl solution was added to 'salt out' any non-tannin compounds. The extracts were filtered through Whatman No.1 filter paper and placed into new pill vials. Four drops of the extract were placed into glass plates placed on black cardboard. To this, 1 drop of 1% gelatin solution was added, and observed for precipitate. One drop 1% gelatin solution and 1 drop 10% NaCl was added to one well and again it was observed for precipitate. Finally, 1 drop of 10% ferric chloride solution was added, and the extract was observed for colour change.

Precipitation is indicative of the presence of tannins. Tannins precipitate proteins (gelatin). Sodium chloride enhances the 'salting out' of the protein-tannin complex. If precipitation is observed only with the salt solution (i.e. the control), a false-positive is indicated. Positive tests are confirmed by the addition of ferric chloride solution to the extract and should result in a blue, blue-black, green or blue-green colour and precipitate.

5.2.5 Alkaloids

pH partitioning for alkaloids was performed according to BRIMER, LORENTZEN & WAGNER SMITT (1989). Two g of dried, powdered plant material were extracted with 20 ml 96% ethanol for 30 min, while constantly stirring using a magnetic stirrer. The extract were filtered. Fifteen ml of water was added to the filtrate, and the ethanol was removed by boiling it off over a period of 1 h. The remaining extract was filtered, and taken to a basic pH with a few drops of 2N NaOH, and then partitioned against 10 ml dichloromethane. The organic phase was filtered over anhydrous sodium sulphate and partitioned against 2 ml 0.1 HCl. The HCl phase was divided into two equal parts. Dragendorff's reagent (WAGNER, BLADT & ZGAINSKY, 1984) was added to

one part, and Mayer's reagent (WAGNER, BLADT & ZGAINSKY, 1984) to the other. Observations were made for the development of a red-orange precipitate on the addition of Dragendorff's reagent, and a white precipitate on the addition of Mayer's reagent.

5.3 RESULTS AND DISCUSSION

The seven Sterculiaceae species indicated the presence of numerous phytochemical compounds. Phytochemical compounds are known to have biological or toxic properties making them influential in the abilities of the plants in traditional medicine. Cardiac glycosides are used in controlling congestive heart failure. The cardiac glycosides, cardienolides are known to occur in many Sterculiaceae species (TREASE & EVANS, 1983). Cyanogenic glycosides are poisonous to humans in high doses, saponins contain anti-fungal and anti-microbial activity and tannins have wound and burn healing properties as well as anti-viral and anti-tumour activity (SIEGLER, 1977; HARBORNE & BAXTER, 1993). The suggested high anti-inflammatory activity in plant material containing tannins may be false-positive as tannins are phenolics which destroy enzymes inhibiting their action. The presence of alkaloids was already suggested present in the plant material of the five of the seven species screened. However, the reference RAFFAUF (1992) which stated this information did not verify which parts of the plants lacked alkaloids and it did not give any references to where this information was obtained.

5.3.1 Cardiac Glycosides

Table 9: Results obtained from two cardiac glycoside tests: Keller-Killiani test and the unsaturated lactone tests

Collection site	Plant part analysed	Keller-Killianii test	Unsaturated lactone ring tests		
			Kedde's reagent	Chloramine T- trichloroacetic acid	Antimony (III) chloride reagent
D. rotundifolia					
Umgeni Valley	Leaves	✓	1 pink zone	----------	----------
Nature Reserve	Twigs	✓	1 pink zone	Yellow bands	----------
	Bark	✓	1 pink zone	Yellow bands	----------
Monato	Leaves	✓	/	Yellow, orange bands	----------
	Twigs	✓	1 pink zone	Orange yellow bands	Yellow and grey bands
	Bark	✓	1 pink zone	Yellow bands	Yellow bands
D. cymosa	Leaves	✓	/	----------	Yellow band visible
	Twigs		/	----------	----------
D. burgessiae	Leaves	✓	/	----------	Yellow and green band visible.
	Twigs	✓	/	----------	Yellow band visible

C. natalensis	Leaves	✓	/	----------	----------
	Twigs	✓	/	----------	----------
C. greenwayi	Leaves	✓	/	----------	A number of grey and violet bands
	Twigs	?	/	----------	Very faint violet band
H. depressa	Leaves	?	/	----------	Violet and grey bands
	Stems	?	/	----------	Violet bands
	Roots	?	/	----------	Very light violet band
S. murex	Leaves	?	/	----------	A few yellow bands
	Twig	?	/	----------	----------

✓ - Positive results obtained ? - Results inconclusive

/ - No cardienolides present ---------- - No corresponding colour zones present

Two-Deoxy-Sugars/ Keller-Killianii Test

A negative control was set up where no extract was added to the glacial acetic acid (containing 2.2 mM $FeCl_3$) phase. Visible brown rings formed at the interphase of all *D. rotundifolia* extracts (Plate 6) (Table 9). The brown rings were more evident in the leaf extracts from both sites. They immediately formed with the addition of the glacial acetic acid solution and extract onto the H_2SO_4. The leaf extract from the material from Monato was more concentrated than that obtained from the leaf material from UVNR. The brown ring was therefore more noticeable in the latter. Brown rings formed in the bark and twig extracts to a lesser extent. They were each approximately 2 mm thick and showed the same intensity of brown colour. Twenty four h after the experiment was conducted, the brown rings for all the extracts had increased in thickness while the glacial acetic acid phase containing 2.2 mM $FeCl_3$ and the extracts had become clearer.

The twig and leaf extracts of *D. burgessiae* and the leaf extracts of *D. cymosa* and *C. greenwayi* contained 2-deoxy sugars. The brown rings, at the interphase, were initially 1 mm thick, after 3 h their thickness had increased to 3 mm (Plate 6) (Table 9). Directly after the experiment, no noticeable brown rings were visible in the *S. murex* leaf and twig extracts, or the *D. cymosa* leaf extract, but after about 1 h faint brown rings had formed. In the leaf extract of *C. natalensis* a dark drown ring (3 mm thick) appeared at the interface while a lighter brown ring (1 mm thick) formed with the twig extract. Initially no brown rings formed with the *H. depressa* extracts at the interface of any of the three plant parts screened. After 30 min brown rings began to form while the control remained clear. The formation of the brown rings may be due to the presence of 2-deoxy sugars or it may be due to a chemical reaction taking place between the extracts and the sulphuric acid. Further tests were therefore important.
Due to the presence of the brown rings at the interphase in a number of the

extracts, suggesting the presence of 2-deoxy-sugars in the plant material the extracts were tested for unsaturated lactone rings.

Unsaturated Lactone Ring Tests

After spraying with Kedde's reagent (Plate 6) (Table 9) pink zones appeared on the TLC plate in the leaf, twig and bark extracts of *D. rotundifolia* from UVNR. Twig and bark extracts from Monato showed pink zones with identical R_f values to those from UVNR (0.71 and 0.86 respectively) indicating that similar cardiac glycosides are present in material from both collection sites. Pink zones suggest the presence of cardienolides. Unexpectedly, no pink zones appeared in the leaf extract from Monato. No pink zones appeared in leaf and twig extracts of *D. cymosa, D. burgessiae, C. natalensis, C. greenwayi*, *H. depressa* and *S. murex,* and leaf, stem and root extracts of *H. depressa* indicating cardienolides were not present in these species. TREASE & EVANS (1983) mention some Sterculiaceae have been shown to contain cardienolides. This was found true for *D. rotundifolia.*

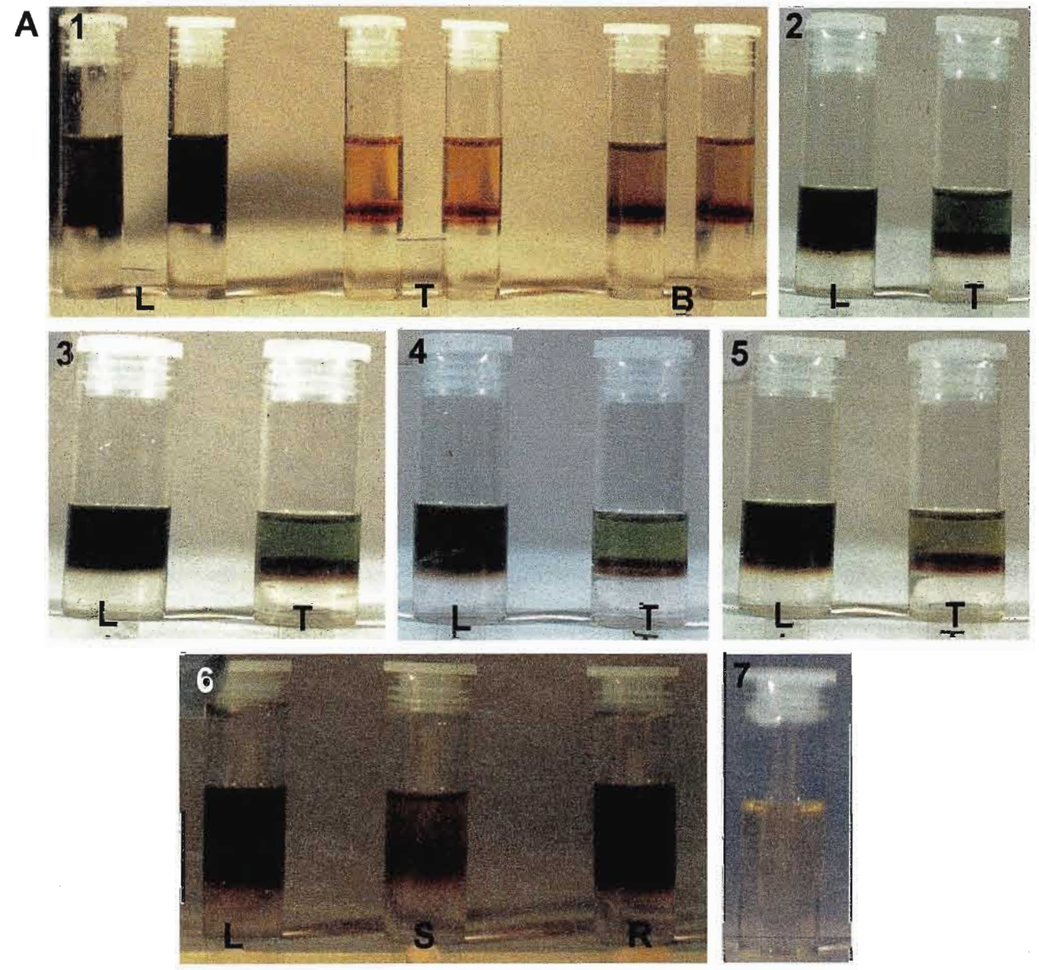
Chloramine T-trichloroacetic acid reagent was viewed under ultraviolet light at 366 nm for yellow-orange zones. Identical compounds were extracted from corresponding plant material from the two localities of *D. rotundifolia* (Plate 8) (Table 9), however, there was a higher quantity from the Monato extracts. Yellow bands appeared in the shoot and bark material collected at UVNR. Several yellow-orange bands appeared in the leaf and twig extracts from Monato, and yellow bands in the bark extracts, confirming the presence of bufadienolides in the previously mentioned plant extracts. Blue-green bands appeared in the UVNR leaf extract. *D. cymosa, D. burgessiae, C. natalensis, C. greenwayi*, *H. depressa* and *S. murex* (Plate 8) extracts did not exhibit yellow-orange bands indicating the absence of bufadienolides. All extracts lacked significant bands.

Antimony (III) chloride reagent sprayed TLC plates were observed under UV light at 366 nm for yellow to yellow brown spots. The purple and grey bands visible under 366 nm on the TLC plate, were visible as light to dark green when viewed in white light. These bands consisted mainly of chlorophyll-type compounds. Similar compounds were extract from corresponding plant material from the two localities of *D. rotundifolia*, with a higher quantity from the Monato extracts. Yellow bands appeared in the twig and bark extracts from Monato, and the leaf extracts from *D. cymosa, D. burgessiae* and *S. murex* (Plate 7) (Table 9). All other extracts exhibited grey and violet bands not associated with the presence of cardiac glycosides.

C. natalensis, which is not used in traditional medicine contained no cardienolides. No bands appeared on spraying of TLC plates with chloramine trichloroacetic acid reagent. Bands appearing after spraying with antimony III chloride reagent were very faint and were therefore considered insignificant.

In small doses cardiac glycosides are extremely valuable medicinally for controlling congestive heart failure (HARBORNE & BAXTER, 1993). Plants containing cardiac glycosides may be beneficial in cardiac treatments, but without a known concentration on intake of prepared extracts, no significant changes could occur due to a too low concentration or it could be fatal due to toxicity. Generally the concentration of cardiac glycosides in plants is very low, lower than 1% (BRUNETON, 1995), so using plant material should normally not result in a fatality. Nevertheless, knowing the concentration is important for proper treatment.

Plate 6 The Sterculiaceae species were screened for two-deoxy-sugars using the (A) Keller-Killiani test . (1) *D. rotundifolia* from UVNR and Monato, (2) *D. cymosa*, (3) *D. burgessiae*, (4) *C. natalensis*, (5) *C. greenwayi*, (6) *H. depressa*, and (7) control. (B) *D. rotundifolia* material from UVNR and Monato was screened for cardienolides by staining with Kedde's reagent. Pink bands indicate positions of cardienolides on TLC plate.
(L - leaf, T - twig, B- bark, S - stem, R - root).



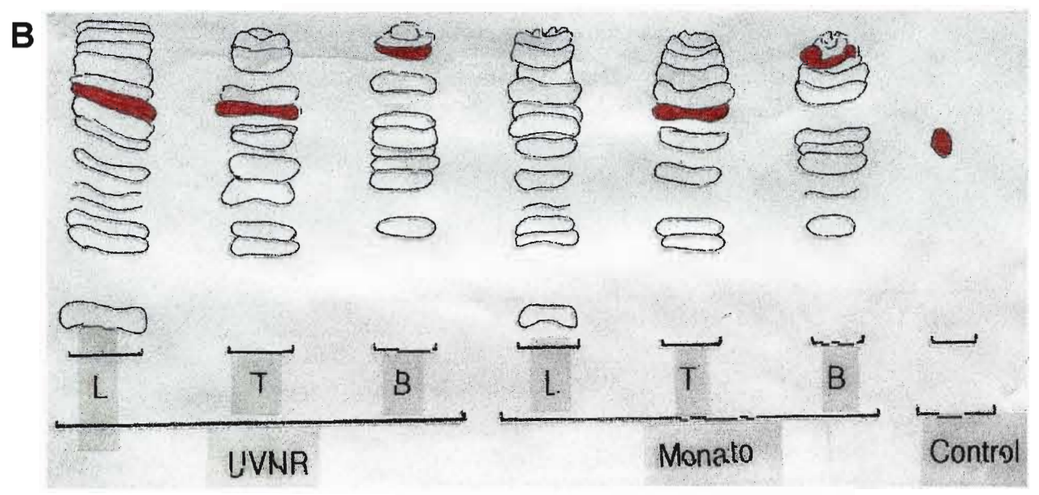
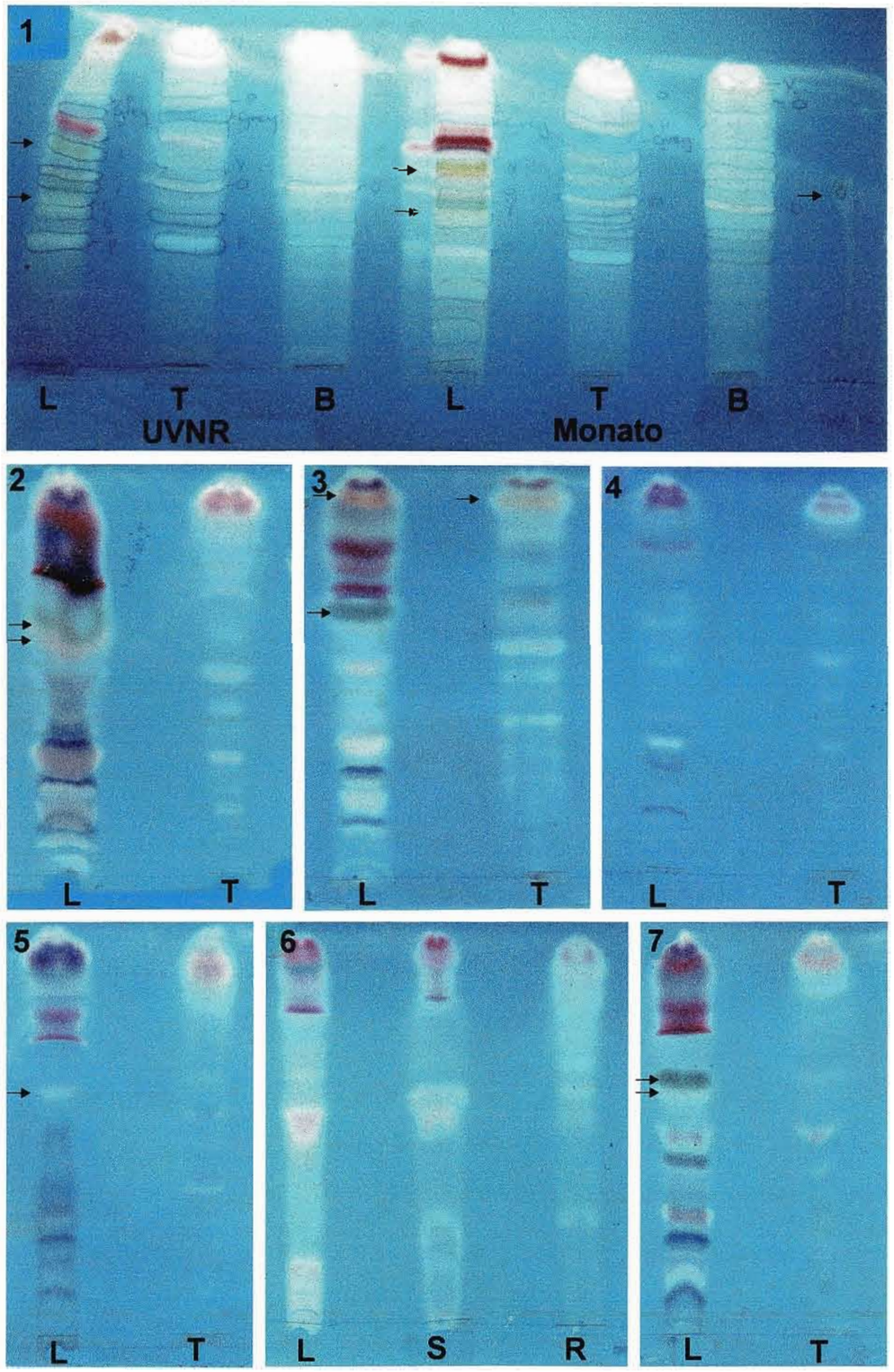


Plate 7 The Sterculiaceae species were screened for bufadienolides by staining with Antimony III Chloride reagent. (1) *D. rotundifolia* from UVNR and Monato, 2) *D. cymosa*, (3) *D. burgessiae*, (4) *C. natalensis*, (5) *C. greenwayi*, (6) *H. depressa*, and (7) *S. murex*. Arrows indicate possible bufadienolides. (L - leaf, T - twig, B- bark, S - stem, R - root).



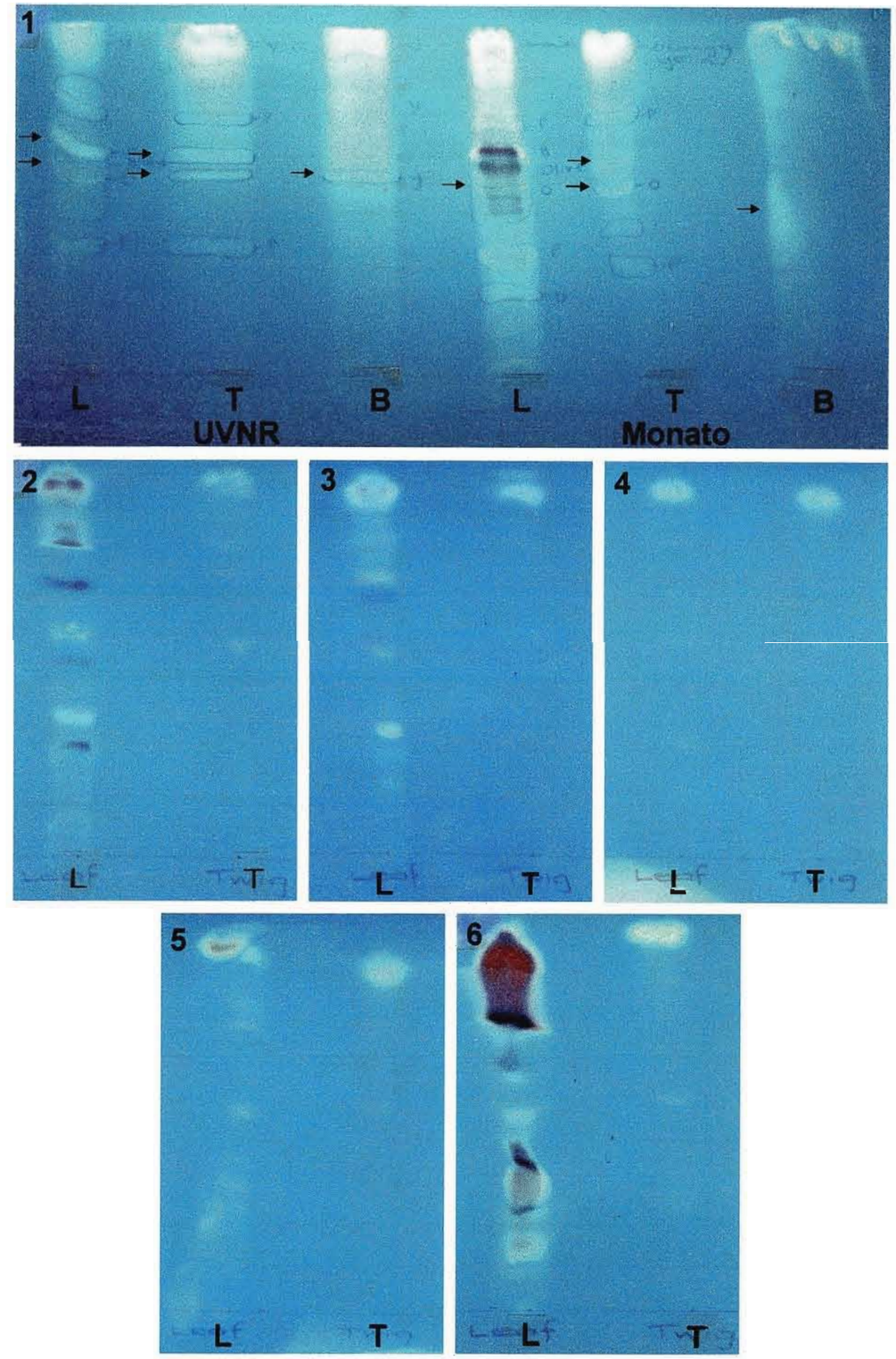


Plate 8 The Sterculiaceae species were screened for bufadienolides by staining with Chloramine T-trichloroacetic acid reagent. (1) *D. rotundifolia* from UVNR and Monato, (2) *D. cymosa*, (3) *D. burgessiae*, 4) *C. natalensis*, (5) *C. greenwayi*, and (6) *S. murex*. Arrows indicate possible bufadienolides.
(L - leaf, T - twig, B - bark).

5.3.2 Cyanogenic Glycosides

TLC Sandwich Method

Fresh material of *D. rotundifolia*, *D. cymosa, D. burgessiae, C. natalensis, C. greenwayi, H. depressa* and *S. murex* were used in the preparation of the extracts used for this experiment. The sodium picrate paper did not develop any colour changes by the termination of the experiment, indicating that cyanogenic glycosides are not present. Absence of cyanogenic glycosides in the seven species screened reduces the possible toxicity of the plants. Light petroleum extracts of the residual meal from the seed of *S. murex* was reported to contain cyanogenic glycosides (WATT & BREYER-BRANDWIJK, 1962). However, seeds were not screened in this study.

5.3.3 Saponins

Saponins are often detected in plant extracts through their haemolytic activity (Haemolysis test) or on the basis of their foam-producing properties (Froth test).

Froth Test

Positive results were only obtained from extracts from *D. rotundifolia* and *H. depressa* material (Table 10) (Plate 9 A). In *D. rotundifolia*, after 30 min there were no bubbles on the reaction surface of the aqueous bark. There were a few bubbles on the reaction surface of the aqueous solutions containing leaf and twig material suggesting the possible presence of saponins. *H. depressa* aqueous solutions showed the presence of bubbles on the reaction surface of all plant material solutions also suggesting possible presence of saponins.

Table 10: Determination of the presence or absence of saponins in *D. rotundifolia* and *H.depressa* using the Froth test

Plant Species	Plant part analysed	Persistence of Froth
D. rotundifolia:		
Umgeni Valley Nature Reserve	Leaves	After 30 min bubbles were present on the reaction surface. They were only visible on the edges.
	Twigs	After 30 min bubbles were present on the reaction surface, but did not cover the entire surface.
	Bark	No bubbles were visible on the reaction surface.
Monato	Leaves	After 30 min bubbles were present over the whole reaction surface.
	Twigs	After 30 min bubbles were present on the reaction surface.
	Bark	No bubbles were visible on the reaction surface
H. depressa	Leaf	After 30 min bubbles were present on the reaction surface but only on the edges.
	Stem	After 30 min bubbles were present on the reaction surface but only on the edges.
	Root	After 30 min bubbles were present on the reaction surface but only on the edges. Most bubbles were visible in the root extract

In the *D. cymosa, D. burgessiae, C. natalensis, C. greenwayi* and *S. murex* aqueous solutions no bubbles persisted on the reaction surface, therefore no saponins appear to be present in the plant material of these species.

Haemolysis Test

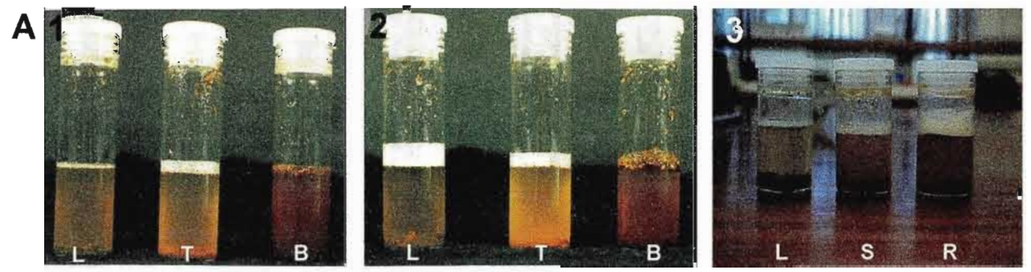
Haemolysis is the rupture of red blood cells with the release of haemoglobin (ABERCROMBIE, HICKMAN, JOHNSON & THAIN, 1990). *Saponaria officinalis* commonly known as soapwort, has long been widely used in household

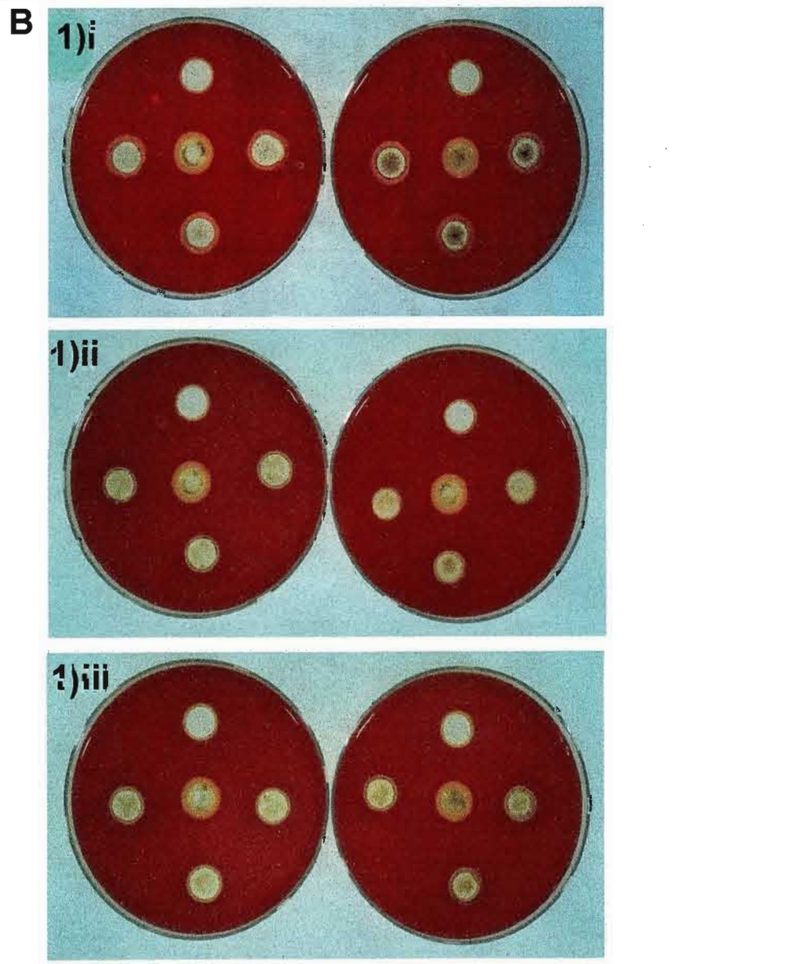
detergents (BRUNETON, 1995). Due to its known abundance of saponins *Saponaria officinalis* was used as the positive control in these experiments.

No clear zones were visible around the wells containing water (negative control) and *S. officinalis* exhibited clear zones after 3 h (positive control). The results of the positive and negative controls indicate the experiment had worked. The leaf and twig extracts of *D. rotundifolia* material from both sites showed no clear zones (Plate 9 B). The bark extracts developed clear zones 16 h after their addition to the blood agar plates. *H. depressa* extracts indicated the presence of saponins in all plant parts when performing the Froth test, however the haemolysis test did not support this (Plate 9 B). The blood agar was not haemolysised by the aqueous extracts as no clear zones formed around the wells containing the extracts.

D. cymosa, D. burgessiae, C. natalensis, C. greenwayi and *S. murex* aqueous extracts were expected to contain no saponins are indicated by the results obtain in the Froth test. This was confirmed by the haemolysis test no clear zones formed around the wells containing the plant extracts from these five species. *Saponaria officinalis* containing 'cups' formed clear zones within a few h.

Saponins are said to have a poisoning ability. They are toxic to cold-blooded animals, but generally not to warm-blooded animals (HARBORNE & BAXTER, 1993). Plants containing saponin-type compounds are therefore not expected to be harmful to humans on ingestion of traditional medicinal plants. Medicinally, they are said to have anti-microbial activity (HARBORNE & BAXTER, 1993).





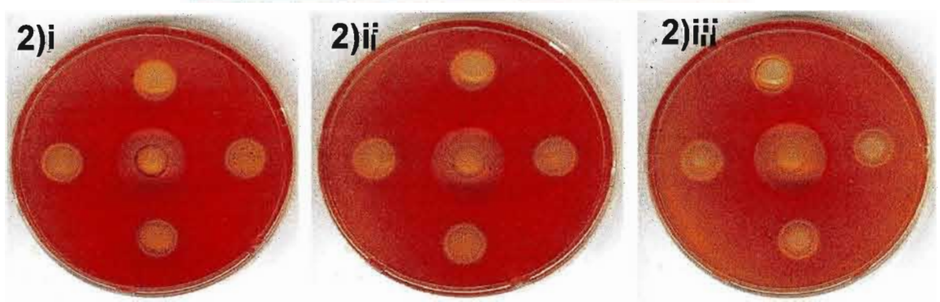


Plate 9 The Sterculiaceae species were screened for saponins using the Froth and Haemolysis tests. (A) Froth test of *D. rotundifolia* aqueous solution from (1) UVNR and (2) Monato aqueous and (3) *H. depressa* aqueous solutions. (B) Haemolysis test results of material from (1) *D. rotundifolia* (i) leaf, (ii) twig and (iii) bark, and (2) *H. depressa* (i) leaf, (ii) stem and (iii) root extracts. (L - leaf, T - twig, B- bark, S - stem, R - root).

5.3.4 Tannins

Table 11: Determination of the presence or absence of tannins in Sterculiaceae species using the gelatin salt-block test

Plant species	Plant part analysed	Four drops extract + 1 drop 1 % gelatin	Four drops extract + 1 drop 1 % gelatin + 1 drop 10 %NaCl	Four drops extract + 1 drop 10 % ferric chloride	Four drops extract (Control)
D. rotundifolia					
Umgeni Valley Nature Reserve	Leaves	Slight off-white precipitate	Slight-off white precipitate	Dark green solution, brown precipitate	Light-yellowy orange colour
	Twigs	Slight off-white precipitate	Slight-off white precipitate	Light green solution, brown precipitate	Light orange colour
	Bark	No precipitate	No precipitate	No colour change	Light-yellowy orange colour
Monato	Leaves	Slight off-white precipitate	Slight off-white precipitate	Dark green solution, brown precipitate	Light-yellowy orange colour
	Twigs	Slight off-white precipitate	Slight off-white precipitate	Light green solution, brown precipitate	Light orange colour
	Bark	No precipitate	No precipitate	No colour change	Light yellowy orange colour

D. cymosa	Leaves	No precipitate	No precipitate	Slight colour change, greeny yellow	Yellowy brown colour
	Twigs	No precipitate	No precipitate	No colour change	Light yellow colour
D. burgessiae	Leaves	No precipitate	No precipitate	Slight colour change, greeny yellow	Yellow colour
	Twigs	No precipitate	No precipitate	No colour change	Very light yellow
C. natalensis	Leaves	No precipitate	No precipitate	No colour change	Light green colour
	Twigs	No precipitate	No precipitate	No colour change	Light orange colour
C. greenwayi	Leaves	White precipitate	White precipitate	Blue-green colour change	Yellowy-brown colour
	Twigs	No precipitate	No precipitate	Slight colour change	Orange- brown colour
H. depressa	Leaves	White precipitate	White precipitate	Green colour	Yellow colour
	Stem	White precipitate	White precipitate	Brown colour	Light yellow colour
	Roots	White precipitate	White precipitate	Dark brown colour	Light browny orange colour
S. murex	Leaves	No precipitate	No precipitate	No colour change	Very light yellow colour
	Twigs	No precipitate	No precipitate	No colour change	Light yellow colour

During the Salting out, the extracts became murky, but there was no sign of definite precipitation within any of the extracts. After filtering the extracts through Whatman No 1, they became clearer.

Colour changes occurred immediately on the addition of the ferric chloride In the gelatine salt-block test and all precipitates formed within seconds of the addition of the various solutions.

The extracts from both collection sites for *D. rotundifolia* yielded similar results (Table 11). The only noticeable difference was in the leaves, where on the addition of ferric chloride to the UVNR extract there was an abundance of precipitate. In the extract from Monato there was noticeably less. Condensed tannins have been used in medicine to aid the healing of wounds and burns. When applied to the skin, they produce an impervious layer under which the healing process can take place (HARBORNE & BAXTER, 1993). The tannins in the leaves of *D. rotundifolia* may be what causes the healing of abscesses (WATT & BREYER-BRANDWIJK, 1962) when used in traditional medicine.

Precipitate formation occurred in the leaf extracts from *C. greenwayi*. There was a noticeable change in extract colour from a yellowy-brown to a dark blue-green with the addition of 10% ferric chloride, indicative of the presence of tannins in the extract. There were slight colour changes in the twig extract from *C. greenwayi* and in the leaf extracts of both *D. cymosa* and *D. burgessiae* but in all these extracts there was no sign of the formation of any precipitate.

Leaf, stem and root extracts from *H. depressa* were positive for tannins. The leaf extract yielded least tannins and the root the most if measured by the degree of colour change and precipitate formed.

It can therefore be suggested that tannins are present in the leaves and twigs of

D. rotundifolia, the leaf extracts of *C. greenwayi*, and the leaf, stem and root extracts of *H. depressa*.

Plant material containing tannins may give false-positive results in assays using enzymes. Tannins are phenolics which destroy enzymes inhibiting their action. Plant material screened for anti-inflammatory activity using the COX-1 assay may indicate false-positive results (Table 7). *D. rotundifolia* leaf and twig (87 - 97 %), *H. depressa* stem (78 %) and root (81 %) and *C. greenwayi* leaf (78 %) material suggest high anti-inflammatory activity, however, these results may be false-positive due to the presence of tannins in the plant material.

5.3.5 Alkaloids

In all the extracts of *D. rotundifolia, C. natalensis, C. greenwayi, D. cymosa, D. burgessiae, S. murex* and *H. depressa* no orange-red precipitate developed with the addition of Dragendorff's reagent and no white precipitate developed with the addition of Mayer's reagent indicating the absence of alkaloids. Alkaloids were reported to be absent from *D. rotundifolia, D. cymosa, D. burgessiae, S. murex* and *H. depressa* (RAFFAUF, 1996). Light petroleum extracts from the residual meal obtained from the seed of *S. murex* contained a trace of alkaloidal material (WATT & BREYER-BRANDWIJK, 1962).

5.4 SUMMARY

- Seven Sterculiaceae species were screened for the following phytochemical compounds, using the test indicated:
  - Cardiac glycosides (two-deoxy-sugars; unsaturated lactone ring test) are used in controlling congestive heart failure (HARBORNE & BAXTER, 1993). *D. rotundifolia* leaf, twig and bark material indicated the presence of cardienolides and bufadienolides. The probable presence of bufadienolides was indicated in the leaf material of *D. burgessiae, D. cymosa* and *S. murex.*
  - Cyanogenic glycosides (sandwich method) are poisonous to humans in high doses. They were lacking in all plant material of the seven species investigated.
  - Saponins (froth test, haemolysis test) contain anti-fungal and anti-microbial activity. Saponins are expected to be present in leaves, twigs and bark of *D. rotundifolia* and the leaves, stems and roots of *H. depressa.*
  - Tannins (gelatin salt-block test) have wound and burn healing properties as well as anti-viral and anti-tumour activity (SIEGLER, 1977; HARBORNE & BAXTER, 1993). Tannins are located in the leaves and twigs of *D. rotundifolia* and the leaves, stems and roots of *H. depressa.*
  - Alkaloids (pH partitioning) were found to be absent in all plant material of the seven species investigated.

- *D. rotundifolia* indicated the same phytocemical compounds present in plant material collected from two different collection sites, differing only in the absence of cardienolides in the Monato leaf material.

CHAPTER 6

ISOLATION OF ANTI-BACTERIAL COMPOUNDS

6.1 INTRODUCTION

Bacterial infections especially in Third World countries are rife. Infectious diseases caused by pathogenic bacteria have increased in terms of annual prevalence of morbidity and mortality. Together with malnutrition and malaria (and more recently AIDS) these diseases are largely responsible for mortality and low life expectancies in most developing countries (RASOANAIVO & RATSIMAMANGA-URVERG, 1993). Readily available, cheap drugs or even easily obtainable local plant extracts, with anti-bacterial activity, are important for the treatment of ailments brought about by bacterial infections. Bacterial infections are highest in informal settlement areas due to poor sanitation and unhygienic conditions.

6.1.1 Bacteria

6.1.1.1 Bacterial Infections

Poor preparation of food aids in infection by bacteria. Using polluted drinking water, gross contamination of environment, residual contamination of washed utensils (could be largely eliminated by use of soap) and personal hygiene are all examples leading to poor food preparation (ROWLAND, 1985). A common ailment brought about by bacterial infections in the human gut is diarrhoea. Proliferation of bacteria in the upper small intestine of man in tropical and subtropical areas is associated with chronic diarrhoea and mal-absorption, especially in severely malnourished children (GRACEY, 1979). Diarrhoea is a prominent clinical feature of childhood malnutrition and is mostly due to gastrointestinal infections and infestations (GRACEY, 1985)

by bacteria, viruses and protozoa (SLEIGH & TIMBURY, 1998). Pollution, in areas of poor sanitation and hygiene can increase 10 to 100 times within days of the onset of rains and persists at these higher levels throughout the rainy season, which is also the period of highest diarrhoeal prevalence (ROWLAND, 1985).

6.1.1.2 Gram-staining

Bacteria are stained with different dyes in order to make them visible under the microscope. The most common method of staining is the Gram method (VLOK & RYKHEER, 1966). Bacteria are divided into two main groups on the basis of the Gram-staining reaction, a division which is reflected in the differences in cell wall structure of the Gram-positive and Gram-negative bacteria (Fig. 3) (GALE, CUNDLIFFE, REYNOLDS, RICHMOND & WARING, 1981).

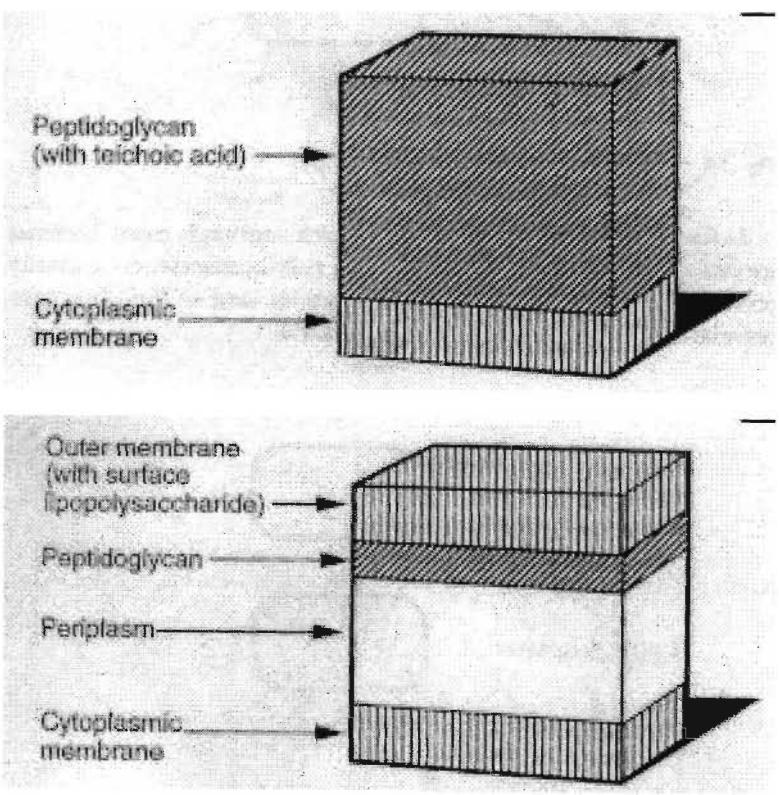


Fig. 3: Diagrams showing the basis of bacteria cell walls A) Gram-negative and B) Gram-positive bacteria (SLEIGH & TIMBURY, 1998)

6.1.1.3 Growth Requirements of Bacteria

Bacteria, like all cells, require nutrients for the maintenance of their metabolism and for cell division. They require a number of essential elements for growth (Table 12) as well as amino acids, purines, pyrimidines and vitamins (SLEIGH & TIMBURY, 1998). Optimal environmental conditions are also essential: water, oxygen, carbon dioxide, temperature and hydrogen ion concentration being the most important requirements (SLEIGH & TIMBURY, 1998).

Table 12: Essential elements required for bacterial growth (SLEIGH & TIMBURY, 1998)

Group	Element	Role
I	Carbon	Required for synthesis of carbohydrate, lipid, protein, nucleic acids
	Hydrogen	
	Oxygen	
	Nitrogen	
	Phosphorus	
	Sulphur	
2	Potassium	Major cation; activates various enzymes
	Calcium	Enzyme cofactor: key role in spore formation
	Magnesium	Multi-enzyme cofactor: stabilizes ribosomes, membranes, nucleic acids; required for enzyme substrate binding
	Iron	Electron carrier in oxidation - reduction reactions; many other functions
3	Copper	Activators and stabilizers of a wide variety of enzymes
	Cobalt	
	Manganese	
	Molybdenum	
	Zinc	

1.Elements required for the synthesis of structural components

2.Elements required for other cellular functions

3.Trace elements

6.1.1.4 Characteristics of Bacteria used in this Study

Table 13: ATCC numbers, Gram-status and growth description of bacterial strains used during this study

Bacterial strain	ATCC Number	Gram (+/-v)	Growth Description
S. aureus	12600	+	Colonies are entire, glistening, circular and smooth.
B. subtilis	6051	+	Colonial variation is observed. One type of colony is flat, irregular-shaped and whitish in colour (opague), the other is smaller, more circular and translucent also flat/rough.
M. luteus	4698	+	Colonies are yellow pigmented
E. coli	11775	-	Colonies are entire, glistening, smooth, translucent.
P. aeruginosa	10145	-	This strain forms two colony types: 90 % rough, slightly spreading and flat; 10 % smooth glistening, entire and
K. pneumonia	13883	-	pulvinate. This strain produces two types of diffusible pigment that may mask each other: fluorescein - green or yellow and pyocyanine - blue. Two colony types: 1) Entire, glistening, smooth, opague, 2 mm in diameter. 2) Smaller, translucent.

6.1.1.4.1 Gram-positive Bacteria

Staphylococcus aureus

S. aureus is a Gram-positive bacterium that is a non-encapsulated, non-motile, non-sporeforming coccus that is arranged in grape-like clusters (SNYDMAN & GORBACH, 1982). *S. aureus* is the main pathogen of the staphylococcus strains and is responsible for pyogenic infections. It inhabits body surfaces (SLEIGH & TIMBURY, 1998) and grows in any tissue in the body (VLOK & RYKHEER, 1966), inhabiting the nose, and less often the skin, throat and gut (SNYDMAN & GORBACH, 1982; SLEIGH & TIMBURY, 1998). On the skin it causes boils, carbuncles and impetigo contagiosa, abscesses, impetigo, sycosis barbae, conjunctivitis, wound infections, deep infections, toxic food poisoning, and skin exfoliation (VLOK & RYKHEER, 1966; SLEIGH & TIMBURY, 1998). The cocci grow to a diameter of 1 m, growing well on ordinary media, at an optimal temperature of 37°C. *S. aureus* is typically golden, but pigmentation varies from orange to white (SLEIGH & TIMBURY, 1998). It is an enterotoxigenic bacteria (GRACEY, 1985).

Staphylococcus epidermidis

S. epidermidis is a Gram-positive bacterium. It is rarely pathogenic and does not produce coagulase or ferment mannitol (SNYDMAN & GORBACH, 1982). It is a universal skin commensal. It inhabits the skin and the gut or upper respiratory tract (SLEIGH & TIMBURY, 1998). *S. epidermidis* grows as white colonies (SLEIGH & TIMBURY, 1998). It is of lower pathogenicity than *S. aureus* but an important pathogen of implanted metal and plastic devices and prostheses (SLEIGH & TIMBURY, 1998).

Bacillus subtilis

Members of the genus *Bacillus* are aerobic, sporing, Gram-positive, chaining bacilli. *B. subtilis* bacteria are small celled, short and thin rod-shaped with rounded ends (VLOK & RYKHEER, 1966; SLEIGH & TIMBURY, 1998).These bacteria cause urinary tract infections and subdural haematoma. They are also associated with a number of allergies. *B. subtilis* has the ability to produce antibiotics against other Gram-positive bacteria (FARRAR & REBOLI, 1981).

6.1.1.4.2 Gram-negative Bacteria

Escherichia coli

Over the past few years enterotoxigenic bacteria (eg: *E. coli*) have become recognised as important causes of infectious diarrhoea (GRACEY, 1985). Bacteria which produce enterotoxins, such as enterotoxigenic *E. coli*, cause intestinal fluid secretion which is stimulated by the enterotoxins and mediated by enzymatic processes within the enterocytes (GRACEY, 1985). *E. coli* is a normal inhabitant of the human and animal intestine. It grows well as large colonies after overnight incubation. The principle diseases caused by *E. coli* are wound infections, especially after lower intestinal tract surgery, peritonis, biliary tract infection, septicaemia, neonatal meningitis and urinary infections usually resulting in diarrhoea (SLEIGH & TIMBURY, 1998).

Klebsiella pneumoniae

K. pneumonia is an enterotoxigenic bacterium (GRACEY, 1985). It lives in the human and animal intestine. It grows well on ordinary media, with colonies which are often, but not always, large and mucoid. *K. pneumoniae*

is also involved in urinary infections, septicaemia and rarely pneumonia (SLEIGH & TIMBURY, 1998).

Pseudomonus aeruginosa

P. aeruginosa is a Gram-negative bacterium that is also enterotoxigenic (GRACEY, 1985). It lives in the human and animal gastrointestinal tract, as well as in water and soil. It grows on routine media over a wide range of temperatures (5°C - 42°C). It is however a strict aerobe. It grows in colonies, and exhibits a fluorescence which has a greenish appearance due to the production of *pyocyanin* (blue-green) and *fluorescein* (yellow). It is an important cause of hospital-acquired infections, causing infections in the urinary tract, wound infections, lower respiratory tract infections and eye infections (SLEIGH & TIMBURY, 1998).

6.1.1.5 Neomycin as a Control

Neomycin is an antibiotic effective against both Gram-positive and Gram-negative bacteria (PAGE, CURTIS, SUTTER, WALKER & HOFFMAN, 1997). It works by binding with 30S and in some cases 50S ribosomal subunits causing miscoding, and inhibits initiation and elongation during protein synthesis (SIGMA CATALOGUE, 2002/2003).

6.1.2 Chromatography, and its Techniques

The key to any successful program involving the investigation of biologically active plant constituents is the availability and choice of chromatographic techniques for the separation of pure substances. Chromatography is one of the most important preparative separation techniques employed in the isolation and purification of plant constituents (HEFTMANN, 1961). It is a widely used experimental technique by which a mixture of compounds can

be separated into its individual components (BETTELHEIM & LANDESBERG, 1991).

Solvents Used in Chromatography

Polarity gives some indication of the distribution of electrical charge across the molecule and is particularly related to the number of electrons and lone-pair electrons in a molecule. The relative size of the less polar region of the molecule is also important, the larger this is the less polar the compound will be compared to related molecules bearing the same functional groups (HOUGHTON & RAMAN, 1998). Solvents can be classified on their polarity (Table 14). The dielectric constant of a solvent is taken as a measure of solvent polarity. It is defined as the ratio of work needed to separate two oppositely charged particles a given distance in a vacuum to the work required to separate them the same distance when they are in a liquid (SCHMID, 1996).

Table 14: Eluotropic series of solvents, arranged in order of increasing polarity

Solvent	Dielectric constant (at 25°C)
Hexane	1.89
Cyclohexane	2.02
Chloroform	4.87
Ethyl acetate	6.02
Glacial acetic acid	6.15
Dichloromethane	9.14
Ethanol	24.30
Methanol	33.60
Water	78.30

Hexane [CH_3 $(CH_2)_4$ CH_3] is very non-polar. There are no double bonds on electronegative atoms. Ethyl acetate has appreciable polarity since both electrons and lone pair electrons are present on the oxygen atom. Water [H_2O] is polar. It is a small molecule and has no lipophilic non-polar portion (HOUGHTON & RAMAN, 1998).

6.1.3 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) is a branch of spectroscopy based on the fact that atomic nuclei oriented by a strong magnetic field absorb radiation at characteristic frequencies (JARDETZKY & ROBERTS, 1981). It differs from mass spectrometry, as it is a non-destroying method. Like electrons, both protons and neutrons have spin. A nucleus that contains an odd number of protons and neutrons (or both) has spin and is magnetically active. Nuclei can be induced to jump from a lower- to a higher- energy spin state by electromagnetic energy of a frequency that matches the energy difference between the two states. The spin responsible for creating the two states is a property of the nucleus of the atom, and this is nuclear magnetic resonance spectroscopy (FOX & WHITESALL, 1997).

6.2 AIMS

Isolation of anti-bacterial compounds involved a variety of techniques, using different equipment. The aim of this Chapter was to isolate the anti-bacterial compounds, while developing an understanding of the techniques.

Isolation of compounds from Sterculiaceae species showing good activity against *S. aureus* in Chapter 4 was attempted. Isolation and purification techniques included vacuum liquid chromatography (VLC), Sephadex LH-20 column chromatography, preparative thin layer chromatography and high pressure liquid chromatography (HPLC).

6.3 GENERAL MATERIALS AND METHODS

6.3.1 Sterculiaceae Species Used for Anti-bacterial Isolation

Analysis of initial anti-bacterial screening determined which species would be selected for isolation of the active compounds. The species used in the isolation procedures were *Dombeya rotundifolia, Dombeya burgessiae, Dombeya cymosa, Cola greenwayi* and *Hermannia depressa* (Table 15).

Table 15: Plant species used in the isolation of active anti-bacterial compounds

Plant species	Plant part	Extracting solvent
D. rotundifolia	leaf	ethyl acetate
D. burgessiae	leaf	ethyl acetate
D. cymosa	leaf	ethanol
C. greenwayi	twig	ethyl acetate
H. depressa	leaf	ethyl acetate

With respect to *D. rotundifolia*, similar results were obtained from plant material collected from both collection sites, UVNR and Monato. Material used in the isolation of compounds was collected from the UVNR in KwaZulu-Natal as this area was more accessible.

6.3.2 Extraction of Plant Material by Soxhlet

Small Soxhlet Apparatus

Plate 3 (D) shows the column assembly of the small Soxhlet apparatus. A small Soxhlet apparatus was used in the extraction of *D. cymosa* (60 g), *D. burgessiae* (80 g), *C. greenwayi* (100 g) and *H. depressa* (116 g) dry

material. Ten g of dried, crushed plant material were placed in a thimble. Solvents (ethanol, ethyl acetate and dichloromethane) (150 ml) were placed in a Buchi flask and heated. The extraction took place over 2 h or until there was little or no further colour being extracted from the plant material. The solvents were evaporated under vacuum and the mass of residues determined.

Large Soxhlet Apparatus

Plate 3 (C) shows the column assembly of the large Soxhlet apparatus. A large Soxhlet apparatus was used in the bulk extraction of plant material. Only *D. rotundifolia* leaf material (250 g) was extracted in this way. Two litres of ethanol were placed into the boiling flask. The extraction continued over two days, with the system being switched off overnight as a safety precaution. The ethanol leaf extract obtained by Soxhlet was evaporated under vacuum. The dried residue was weighed and redissolved in ethanol.

6.3.3 Solvent Ratio Determination for Thin Layer Chromatography

In determining the best solvent ratios, hexane and ethyl acetate were used. The solvents were mixed in the following ratios:

Hexane: ethyl acetate - 1:1 Hexane: ethyl acetate - 1:2

Hexane: ethyl acetate - 2:1 Hexane: ethyl acetate - 4:1

TLC plates were spotted with the relevant extracts and the TLC plates run in the different solvent combinations. The best separation for extracts from *D. rotundifolia, D cymosa, D. burgessiae* and *C. greenwayi* was obtained when using hexane: ethyl acetate (1:1)(v/v), while the best separation for the *H. depressa* extract was a ratio of (4:1)(v/v). These ratios were used in all TLC preparations throughout the study unless otherwise stated. TLC plates used throughout the study were Merck Silica gel $60F_{254}$, 0.25 mm thick, sizes 20 x 20 cm and 20 x 5 cm respectively.

6.3.4 Bioautographic Spray Method

Cultures of *Staphylococcus aureus* were prepared under sterile conditions. *S. aureus* was suspended in 20 ml MH broth, in two autoclaved centrifuge tubes, which were placed in a waterbath (37°C) overnight. A thin layer chromatography (TLC) plate was prepared, run in a solvent solution and the solvent allowed to evaporate.

The overnight culture was centrifuged at 3 000 g for 10 min. The pellets were resuspended with 10 ml fresh MH broth to give an optical density of c.// 0.8. The plate was sprayed in a spray hood, placed in a metal tray on moist Carlton paper and covered with clingwrap. It was incubated under 100% relative humidity at 38ºC for 24 h.

The plate was removed from the oven and allowed to dry slightly. Ten ml (2 mg ml^{-1}) INT solution were sprayed onto the plate. It was incubated at 100% humidity for 6 h at 37ºC. Inhibition of growth was indicated by clear zones on the TLC plate.

6.3.5 Isolation Techniques

6.3.5.1 Vacuum Liquid Chromatography (VLC)

Column Preparation

A large column was used (400 ml). One hundred and forty-five g of silica (Silica gel 60 (0.040 - 0.063 mm) were poured slowly into a column, on top of a already placed filter paper disc and a thin layer of sand. Silica (5 g) was mixed with the dry sample, which was placed neatly on top of the silica in the column. Glass wool was placed on top of the silica and weighed down with a few marbles.

Solvent Gradient Used

Solvents used were hexane and ethyl acetate. The gradient began with 100 % hexane, and ended with 100 % ethyl acetate (Table 16).

Table 16: Gradients of solvents (hexane: ethyl acetate) used in VLC for further purification of samples

Sample	Hexane (ml)	Ethyl acetate (ml)
A	400	0
B	340	60
C	320	80
D	300	100
E	280	120
F	260	140
G	240	160
H	200	200
I	160	240
J	140	260
K	80	320
L	40	360
M	0	400

Addition and Collection of Solvents

With the addition of the solvent (400 ml) into the column, the vacuum was switched on. The solvent was allowed to run through the column, until the 400 ml had been collected in the collection flask. The solvents were removed under vacuum using a Buchi apparatus. The residues were placed

in pill vials and dried overnight. The weight of the extract was determined and the extracts were redissolved (10 mg ml^{-1}) in ethyl acetate.

Anti-bacterial activity in the different fractions collected (A through M) was determined using the bioautographic spray method. Anisaldehyde spray reagent determined the position of additional compounds. TLC plates (20 x 20 cm glass plates, 0.25 mm and 1 mm) spotted with the fractions in 25 µl aliquots (0.25 mg of extract) were run in a solvent solution of hexane: ethyl acetate (1:1) (v/v).

6.3.5.2 Sephadex LH-20 Column Chromatography

Preparation and Running of the Column

The stationary phase used was Sephadex LH-20, and the mobile phase cyclohexane: dichloromethane: methanol (7:4:1)(v/v/v).

The Sephadex was swelled in the solvent mixture. The column was assembled in a cold room (10°C) (This avoids the generation of air bubbles). The amount of sample used was the equivalent of 1 % of the amount of Sephadex LH-20 in the column (5 - 10 g sample was sufficient for 50 -100 g Sephadex LH-20, with a column diameter of 1 - 2 cm). All the swollen Sephadex LH-20 was poured into the column while the tap was open. The column was washed with solvent mixture until the Sephadex LH-20 had settled. The sample, dissolved in the same solvent mixture, was applied as a narrow band to the top of the column, with the tap still open. Fractions (10 ml) were collected. Separation was noted by spotting fractions on TLC plates and running a bioautographic assay to determine the location of anti-bacterial compounds.

6.3.5.3 Preparative Thin Layer Chromatography

TLC plates were strip loaded with active fractions from previous separation with VLC. Extracts were dissolved in 50 mg ml^{-1} of the relevant solvent. The extracts were streaked on glass plates (Merck Silica gel $60F_{254,}$ 20 x 20 cm, 0.25 mm) in 25 µl aliquots, and run in hexane: ethyl acetate (1:1) for all plant extracts, except *H. depressa* which was run in hexane: ethyl acetate (1:4) (v/v).

A small section (2 x 20 cm) of glass was cut from the plates and a bioautographic assay performed on it to indicate the position of anti-bacterial bands.

Bands containing active fractions were scrapped off using a blade. The silica was placed in glass flasks (50 ml) containing 20 ml of the relevant solvent. The flasks were placed in an ultra-sound bath for 15 min before the solvent was separated using a Büchner funnel or gravity filtration through Pasteur pipettes. For gravity filtration glass wool was placed in a pipette acting as a filter, covered by a small quantity of silica (Silica gel 60 (0.040 - 0.063 mm). Up to 5 pipettes were secured together with an elastic band. This procedure was repeated twice to ensure removal of all silica from extract. Extracts were placed in glass pill vials, dried and their weights determined.

Fractions were redissolved at 10 mg ml^{-1} in the relevant solvent. To determine purity, they were spotted onto 2 TLC plates (20 x 20 cm, 0.025cm). The TLC plates were run in a solvent solution of 1:1 hexane: ethyl acetate. A bioautographic assay was used to determine the presence of anti-bacterial compounds on the one plate and anisaldehyde spray reagent used to visualize compounds present.

6.3.5.4 High Pressure Liquid Chromatography (HPLC)

The dried fractions showing anti-bacterial activity, which required further isolation were redissolved in 100 mg ml^{-1} methanol. A Varian model 5000 Liquid Chromatograph HPLC apparatus was used in the isolation of compounds through a column (Phenomenex, 260 x 10 mm, C18, 5 µm). Distilled water was prepared for HPLC by filtering through Millipore^{R} filter paper (0.22 m). A gradient of methanol (HiperSolv^{TM}) and water were the mobile phase. Flow rate was set at 2.5 ml min^{-1}. One hundred µl was injected into the system per run. Detection was at wavelengths of 210 nm, 305 nm, and 400 nm respectively using a Spectrasystem UV3000 HR detector

6.3.6 Preparation of Compounds for NMR

NMR was performed by Mr Martin Watson and Craig Grimmer of the School of Chemistry and Physical Sciences (University of Natal Pietermaritzburg). The compounds were redissolved in a solution of deuteromethanol or chloroform. The ^{13}C-NMR spectrum of the sample dissolved in CD_3OD was recorded at 200 MH_Z using a Kratos MS 80RF double-focussing magnetic sector instrument at 70eV. TMS was used as an internal standard.

6.3.7 Gas Chromatography - Mass Spectroscopy

GC-MS was performed by Mr James Ryan of the School of Chemistry and Physical Sciences (University of Natal Pietermaritzburg) and Brett Parel of the School of Pure and Applied Chemistry (University of Natal Durban). The compounds were redissolved in chloroform. Instrumentation: MAT CH7 A mass spectrometer (Finnigan MAT, Bremen) coupled with Varian type 1700 gas chromatograph; carrier gas: helium; column: optimal (M + N), 25 M x

0.32 mm, layer 0.5 m; injector temperature: 250°C; ionization energy: 70 eV; gradient: 120°C isotherm for 1 min, linear with 10°C min^{-1} to 280°C.

6.4 ANTI-BACTERIAL COMPOUND ISOLATION

6.4.1 *Dombeya rotundifolia*

Materials and Methods

Plate 10 outlines the procedures used for the isolation of anti-bacterial compounds from *D. rotundifolia* leaf material extracted with ethyl acetate.

Dried, ground leaf material (250 g) was extracted in ethyl acetate using a large Soxhlet apparatus. The crude residue (2.56 g) obtained was separated by VLC. Anti-bacterial activity was determined by bioautography. Fractions indicating similar active zones with identical R_f values and colours were combined. Preparative TLC (Merck Silica gel $60F_{254}$, 0.25 mm) ensured purification of isolated compounds. The compounds were analysed by NMR and GC-MS.

Results

Thirteen fractions (A - M) were collected by VLC and analysed for their anti-bacterial activity (Table 17). VLC of the ethyl acetate leaf extract of *D. rotundifolia* (Plate 10 B) showed separation of compounds when viewing after staining with ansialdehyde spray reagent. A bioautographic assay indicated anti-bacterial areas in the form of white zones. The areas of anti-bacterial interest are indicated by a rectangle (Plate 10 B). Other white zones not chosen for isolation contained fractions with insufficient residue.

Table 17: Fractions (A - M) collected from VLC of *D. rotundifolia* ethyl acetate leaf extract, showing relative information including mass of fractions eluted, percentage of total, colour of fraction, presence of activity and which fractions were used in preparative TLC

Fraction	Mass of Fraction (mg)	Percentage of total (%)	Activity against *S. aureus*	Colour of Extract	
A	32	1.25	X	White	
B	329	12.85	X	Mustard yellow	
C	265	10.35	X	Dark olive green	
D	215	8.40	X	Dark olive green	
E	201	7.85	X	Olive green	
F	151	5.90	✓	Olive green	1
G	146	5.70	✓	Yellowy-green	1
H	140	5.47	X	Yellowy-green	
I	124	4.84	X	Olive green	
J	49	1.91	✓	Olive green	2
K	86	3.36	✓	Olive green	2
L	50	1.95	✓	Browny-green	2
M	42	1.64	✓	Browny-green	2
Total	1.83 g	71.48 %			

1 - Fractions (F & G) combined for TLC preparation

2 - Fractions (J, K, L & M) combined for TLC preparation

Fractions F (260 ml hexane: 140 ml ethyl acetate), G (240 ml hexane: 160 ml ethyl acetate), J (140 ml hexane: 260 ml ethyl acetate), K (80 ml hexane: 320 ml ethyl acetate), L (40 ml hexane: 360 ml ethyl acetate), and M (0 ml hexane: 400 ml ethyl acetate) all exhibited anti-bacterial activity (Plate 10 B).

Fractions F and G indicated the presence of anti-bacterial activity at the same R_f value (0.82) and were combined for further isolation by preparative TLC. Similarly, Fractions J, K, L, and M were combined as they exhibited active anti-bacterial compounds at an R_f value of 0.025. Fractions F and G were less polar as they were eluted from the VLC column where hexane concentrations were higher than ethyl acetate concentrations. Hexane, with a dielectric constant of 1.89 (25°C), is a very non-polar solvent as there are no double bonds on electronegative atoms (HOUGHTON & RAMAN, 1998). On the other hand, Fractions J, K, L, and M contained more polar compounds as they exhibit a low R_f value (0.025) and eluted at high concentrations of ethyl acetate.

Preparative TLC ensured further isolation of active compounds. Combined Fraction FG was loaded onto four TLC plates and combined Fraction JKLM onto four plates, one and two zones were obtained from each run, respectively (Table 18) (Plate 10 C).

Table 18: Preparative TLC zones recovered for *D. rotundifolia* VLC fractions

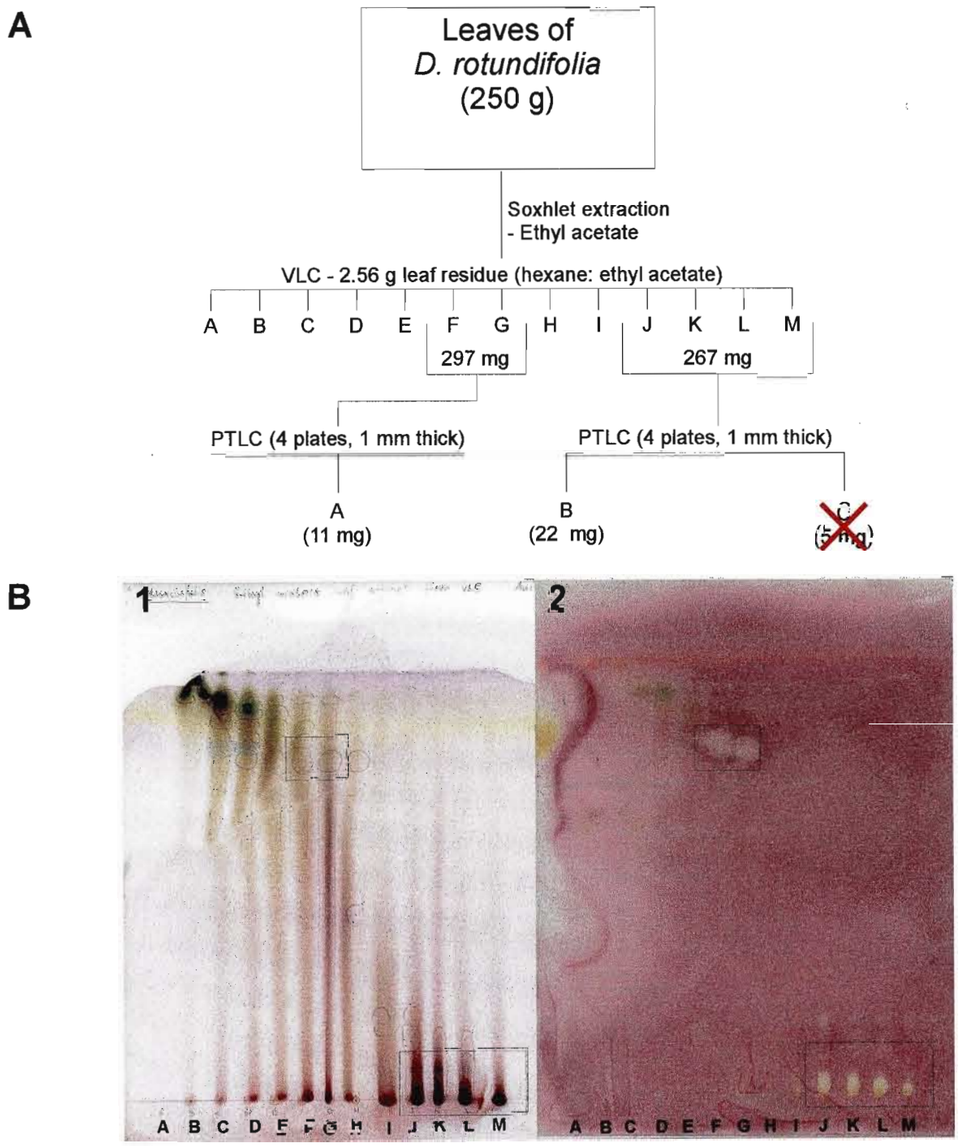
VLC Fraction/s	Scraped zone	Mass of Fraction (mg)	Activity?	Purity	Mass sent for NMR (mg)
1) F & G	A	15	✓	✓	11
2) J, K, L & M	B	35	✓	✓	22

Compound A, obtained from combined Fraction FG (Table 18) showed anti-bacterial activity and reasonable purity of the compound after bioautography and anisaldehyde spray reagent analysis, respectively. Of the two zones scraped from combined Fraction JKLM, only Compound B showed significant activity and purity after analysis. Compound C was discarded.

Compound A (11 mg) and Compound B (22 mg) were analysed by NMR and GC-MS (Appendix A). NMR analysis indicated the compounds were fatty acids, but they were not identifiable. GC-MS indicated the main components of Compound A (Fig. A.1) to be a combination of Myristic acid (95 %) (Fig. A.2), Palmitic acid (93 %) (Fig. A.3) and Stearic acid (78 %) (Fig. A.4). Compound B (Fig. A.5) indicated the presence of Lauric acid (Fig. A.7) MS *m/z* (rel. int.): 73.10 (100), 60.10 (83.54), 55.15 (67.36), 43.10 (68.88), 41.20 (77.35); Myristic acid (Fig. A.8) MS *m/z* (rel. int.): 73.10 (89.46), 60.10 (70.89), 55.10 (72.86), 43.15 (100), 41.20 (77.01) and Palmitic acid (Fig. A.9) MS *m/z* (rel. int.): 73.10 (100), 60.10 (81.96), 55.10 (85.03), 43.15 (99.28), 41.15 (89.41); as well as a high concentration of acetic acid (Fig. A.6) MS *m/z* (rel. int.): 60.10 (43.24), 45.10 (88.41), 43.10 (100), 42.10 (16.77), 29.10 (12.85).

Polarity of the fatty acids could have been affected by a number of factors. The combination of fatty acids the compound was composed of could have effected the polarity resulting in the same fatty acid exhibiting different R_f values. The salt and free acid of the same compound could have been detected due to the pH of the TLC solvent system.

Plate 10 Isolation of anti-bacterial compounds from *D. rotundifolia* leaf extract. (A) Flow chart outlining the isolation procedure followed. (B) VLC fractions (A - M) were spotted on TLC plates and analysed by (1) staining with anisaldehyde spray reagent and (2) bioautography. (C) A representative chromatogram indicates the position of bands recovered by preparative TLC methods indicating fatty acid composition.



6.4.2 *Dombeya burgessiae*

Materials and Methods

Plate 11 outlines the procedures used for the isolation of anti-bacterial compounds from *D. burgessiae* leaf material extracted with ethyl acetate.

Dried, ground leaf material (80 g) was extracted in ethyl acetate using a small Soxhlet apparatus. The crude ethyl acetate residue (2.65 g) obtained was separated by VLC. Anti-bacterial activity was determined by bioautography. Fractions indicating similar active zones were combined. Preparative TLC ensured purification of isolated compounds (Merck Silica gel $60F_{254,}$ 20 x 20 cm, 0.25 mm). One compound was analysed by NMR and GC-MS.

Results

Thirteen fractions (A - M) were collected by VLC and analysed for their anti-bacterial activity (Table 19). VLC of the ethyl acetate leaf extract of *D. burgessiae* (Plate 11 B) verified the separation of compounds when viewing after staining with ansialdehyde spray reagent and a bioautographic assay indicated active zones (white zones on a red background). Black rectangles indicate zones chosen for isolation (Plate 11 B).

Table 19: Fractions (A - M) collected from VLC of *D. burgessiae* ethyl acetate leaf extract, showing relative information including mass of fractions eluted, percentage of total, colour of fraction, presence of activity and which fractions were used in preparative TLC

Fraction	Mass of Fraction (mg)	Percentage of total (%)	Activity against *S. aureus*	Colour of Extract	
A	36	1.36	X	White	
B	256	9.66	X	Yellow green	
C	174	6.57	X	Dark green	
D	127	4.79	X	Dark green	
E	108	4.08	✓	Dark yellowy-brown	1
F	82	3.09	✓	Yellowy-brown	1
G	51	1.92	X	Yellowy-brown	
H	47	1.77	X	Yellow	
I	35	1.32	X	Yellowy-olive green	
J	21	0.79	X	Yellowy-olive green	
K	27	1.02	X	Olive green	
L	19	0.72	X	Olive green	
M	10	0.38	X	Olive green	
Total	0.993 g	37.47 %			

1 - Fractions (E & F) combined for TLC preparation

Fractions E (280 ml hexane: 120 ml ethyl acetate) and F (260 ml hexane: 140 ml ethyl acetate) exhibited anti-bacterial activity (Plate 11 B). The activity exhibited by these fractions indicated the presence of anti-bacterial activity at the same R_f value (0.82) and they were combined for further separation by preparative TLC. The anti-bacterial indicated a non-polar nature as the fractions were eluted in a solvent solution of high hexane

concentration, hexane being a very non-polar solvent. Notably, the active components exhibited the same R_f value and colour as the active components from *D. rotundifolia* ethyl acetate leaf extract. Other indicated anti-bacterial active zones that were not further isolated contained fractions consisting of a number of impurities or insufficient residue.

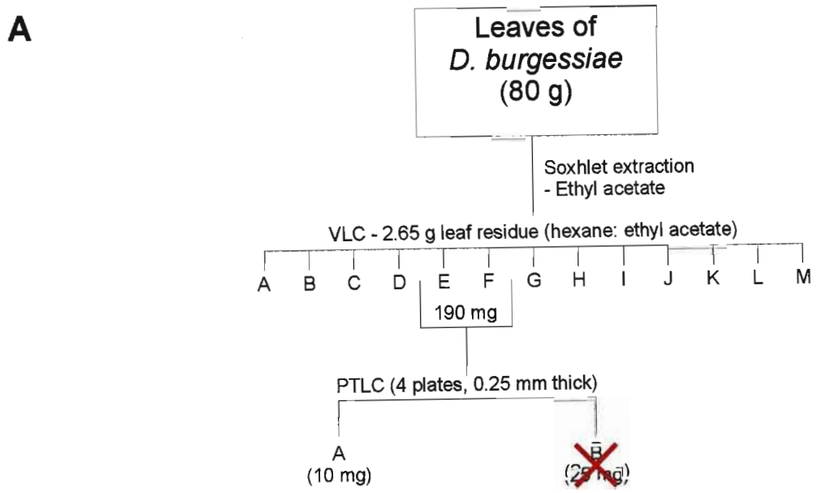
Preparative TLC ensured the isolation of the active compound from combined Fraction EF (Table 20) (Plate 11 C).

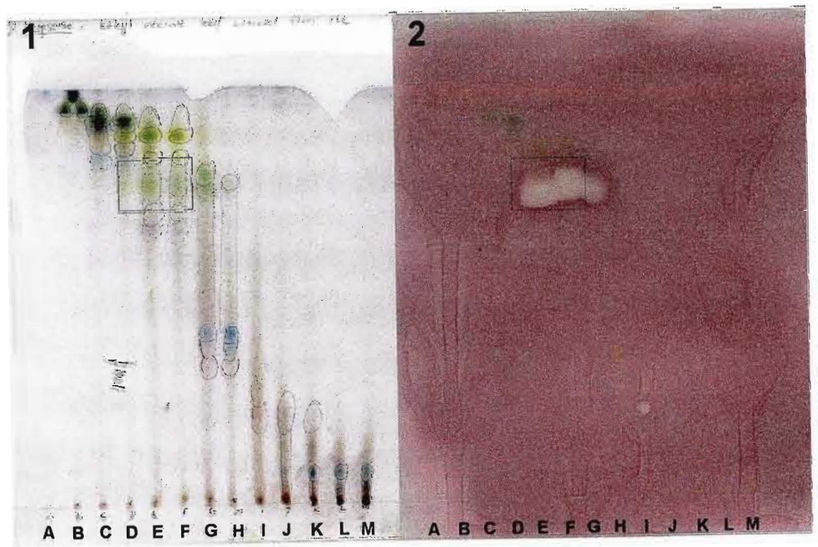
Table 20: Preparative TLC zones recovered for *D. burgessiae* VLC fractions

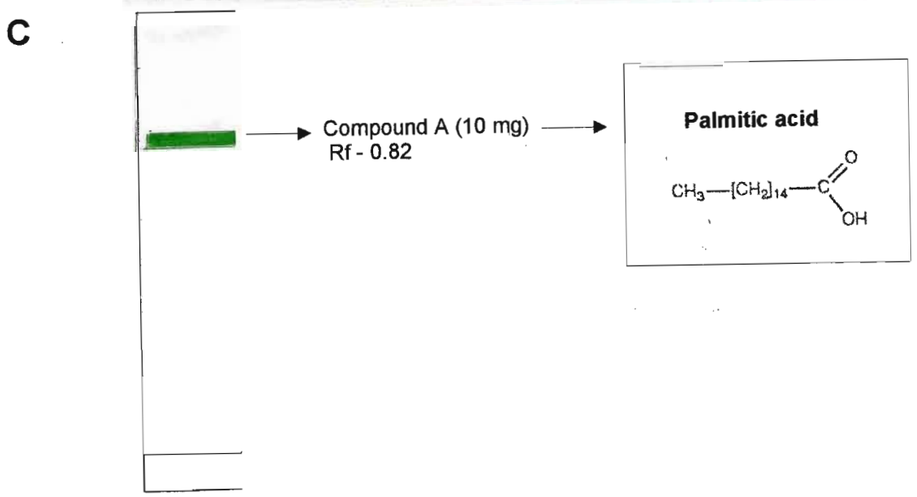
VLC Fraction/s	Scraped Zone	Mass of Fraction (mg)	Activity?	Purity	Mass sent for NMR (mg)
1) E & F	A	34	✓	✓	10

Active Compound A (34 mg) (originally of combined Fraction EF) was obtained from preparative TLC. The compound appeared to be pure by staining with anisaldehyde spray reagent. Compound A (10 mg) from *D. burgessiae* ethyl acetate leaf extract was analysed by NMR and GC-MS. From NMR analysis Compound A was evidently a fatty acid. GC-MS analysis (Appendix A) indicated the main component to be Palmitic acid (Fig. A.11) MS *m/z* (rel. int.): 73.10 (100), 60.10 (85.61), 55.15 (79.77), 43.15 (97.60), 41.15 (86.56).

Plate 11 Isolation of anti-bacterial compounds from *D. burgessiae* leaf extract. (A) Flow chart outlining the isolation procedure followed. (B) VLC fractions (A - M) were spotted on TLC plates and analysed by (1) staining with anisaldehyde spray reagent and (2) bioautography. (C) A representative chromatogram indicates the position of the band recovered by preparative TLC methods indicating fatty acid composition.







6.4.3 *Dombeya cymosa*

Materials and Methods

Plate 12 outlines the procedures used in the isolation of anti-bacterial compounds from *D. cymosa* leaf material extracted with ethanol.

Dried, ground leaf material (60 g) was extracted in ethanol using a small Soxhlet apparatus. The crude ethanol residue (2.65 g) obtained was separated by VLC. Activity was determined by bioautography. Fractions indicating zones with similar activity were combined. A Sephadex LH-20 column was run with the combined fraction (277 mg). A total of 80 fractions were collected from the Sephadex LH-20 column and activity was determined by bioautographic methods. Preparative TLC ensured purification of the isolated compounds (Merck Silica gel $60F_{254,}$ 20 x 20 cm, 0.25 mm) Three compounds were analysed by NMR spectroscopy.

Results

Thirteen fractions (A - M) were collected by VLC (Table 21) and were viewed after staining with anisaldehyde spray reagent. A bioautographic assay indicated anti-bacterial areas and rectangles indicate the zones showing good anti-bacterial activity which were chosen for isolation (Plate 12 B).

Table 21: Fractions (A - M) collected from VLC of *D. cymosa* ethanol leaf extract, showing relative information including mass of fractions eluted, percentage of total, colour of fraction, presence of activity and which fractions were used in preparative TLC

Fraction	Mass of Fraction (mg)	Percentage of total (%)	Activity against *S. aureus*	Colour of Extract	
A	65	2.45	X	White	
B	1080	40.08	X	Orange	
C	278	10.49	X	Dark green	
D	100	3.77	X	Dark green	
E	26	0.98	✓	Dark green	1
F	35	1.32	✓	Dark green	1
G	37	1.37	✓	Dark green	1
H	59	2.23	✓	Dark green	1
I	57	1.15	✓	Dark green	1
J	36	1.35	✓	Green	1
K	37	1.02	✓	Green	1
L	13	0.49	X	Yellowy-green	
M	11	0.42	X	Yellowy-green	
Total	1.797 g	67.12 %			

1 - Fractions (E through to K) combined for separation by Sephadex LH-20

Activity was visible in Fractions E (280 ml hexane: 120 ml ethyl acetate) through to H (200 ml hexane: 200 ml ethyl acetate) (R_f - 0.59), and Fractions H (200 ml hexane: 200 ml ethyl acetate) through to K (80 ml hexane: 320 ml ethyl acetate) (R_f - 0.27). The latter group of fractions exhibited a more polar nature. All active fractions (E - K) were combined and further separated by Sephadex LH-20 column chromatography.

A total of 80 fractions were collected during the running of the Sephadex LH-20 column. Of the 80 fractions collected, 24 exhibited anti-bacterial activity (Plate 12 C). Of these active fractions, 8 were combined and further separated by preparative TLC.

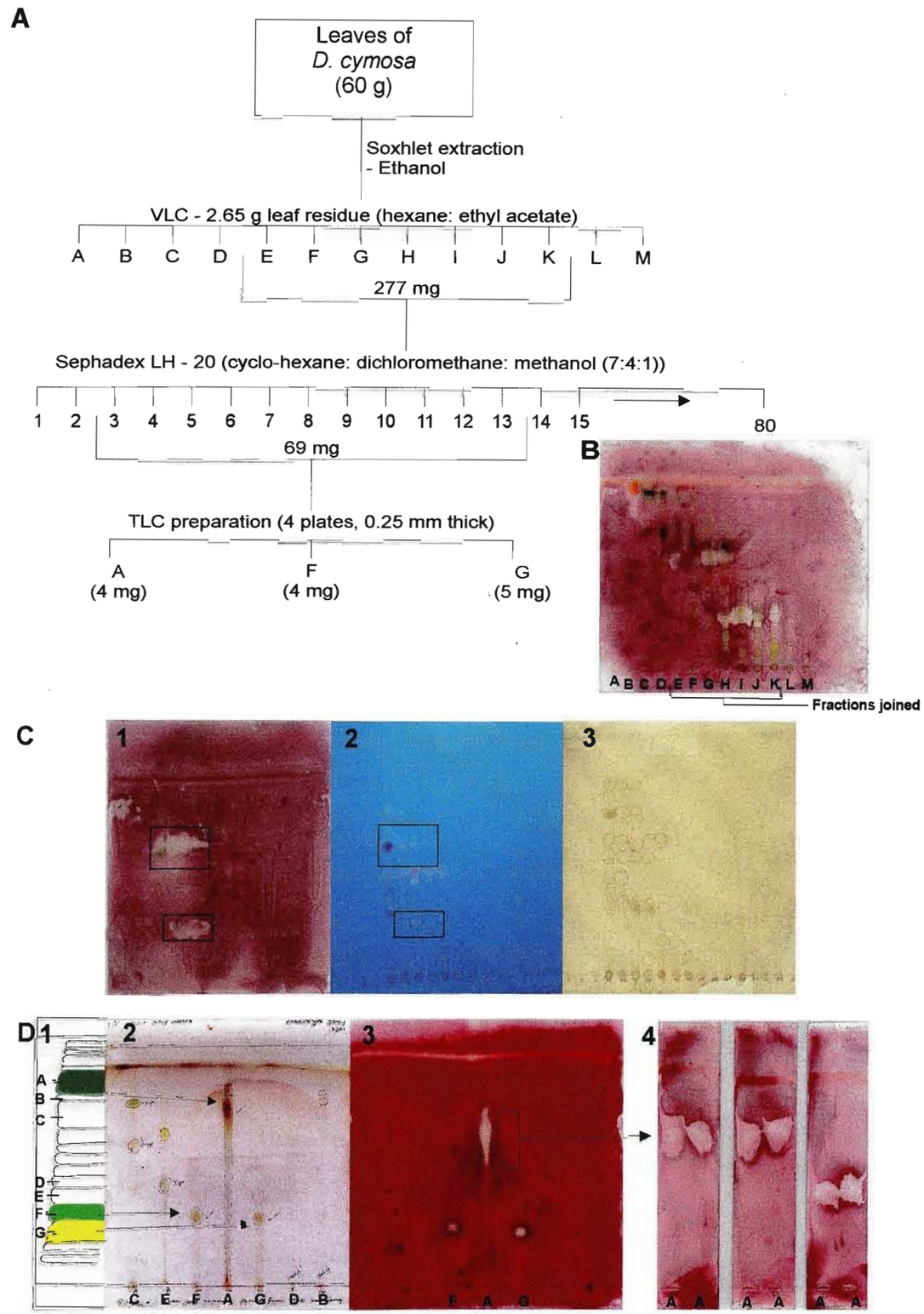
Table 22: Preparative TLC zones recovered for *D. cymosa* VLC fractions

Sephadex LH-20 Fraction	Scraped Zone	Mass of Fraction (mg)	Activity?	Purity	Mass sent for NMR (mg)
1) E - K	A	6	✓	✓	4
	B	4	X	–	-
	C	9	X	–	-
	D	11	X	–	-
	E	15	X	–	-
	F	10	✓	✓	4
	G	13	✓	✓	5

A total of seven zones were recovered for expected anti-bacterial activity (Table 22), labelled alphabetically A through to G. Bioautography showed three of these compounds were active and pure, namely Compound A (R_f - 0.82), Compound F (R_f - 0.29) and Compound G (R_f - 0.21) (Plate 12 D). Compound A appeared as a broad band but after running in a solvent solution gradient it was determined that the band was made up of one compound only (Plate 12 D_4).

From NMR analysis, Compounds A, F and G were all found to be triglycerides, some with double bonds in the esterfying fatty acids. Identification of the compounds was inconclusive.

Plate 12 Isolation of anti-bacterial compounds from *D. cymosa* leaf extract. (A) Flow chart outlining the isolation procedure followed. (B) VLC fractions (A - M) were spotted on TLC plates and analysed by (1) bioautography. (C) Active fractions collected from Sephadex LH-20 column chromatography were spotted on TLC plates and analysed by: (1) bioautography, (2) UV 366 nm light and 3) by staining with anisaldehyde spray reagent. (D) (1) Representative TLC chromatogram indicating colours and zones scrapped by preparative TLC methods, (2) TLC plates were stained with anisaldehyde spray reagent and (3) bioautography indicated position of active compounds while (4) varying solvent gradients determined the purity of Compound A.



6.4.5 *Cola greenwayi*

Materials and Methods

Plate 13 outlines the procedures used in the isolation of anti-bacterial compounds from *C. greenwayi* twig material extracted with ethyl acetate.

Dried, ground leaf material (176 g) was extracted in ethyl acetate using a small Soxhlet apparatus. The crude ethyl acetate residue (2.0 g) obtained was separated by VLC. Activity was determined by bioautography. Fractions indicating similar active zones were combined. Preparative TLC ensured purification of isolated compounds. The chemical structures of these compounds were analysed by NMR and GC-MS.

Results

Thirteen fractions (A - M) were collected by VLC and analysed for their anti-bacterial activity (Table 23) (Plate 13 B).

Table 23: Fractions (A - M) collected from VLC of *C. greenwayi* ethyl acetate leaf extract, showing relative information including mass of fractions eluted, percentage of total, colour of fraction, presence of activity and which fractions were used in preparative TLC.

Fraction	Mass of Fraction (mg)	Percentage of total (%)	Active against *S. aureus*	Colour of Extract	
A	46	2.30	X	White	
B	360	18.00	X	Bright yellow	
C	104	5.20	X	Bottle green	
D	289	14.45	✓	Bottle green	1
E	161	8.05	✓	Dark mustard	1
F	96	4.80	✓	Green	
G	54	2.70	✓	Green	
H	56	2.80	✓	Yellow	2
I	50	2.50	✓	Yellow	2
J	33	1.65	✓	Yellow	3
K	26	1.30	✓	Yellow	
L	14	0.70	✓	Yellow	
M	11	0.55	✓	Yellow	
100% MeOH	655	32.75	✓	Green	
Total	1.955 g	97.75 %			

1 - Fractions (D and E) mixed for TLC preparation

2 - Fractions (H and I) mixed for TLC preparation

3 - Fraction (J) isolated further by TLC preparation

Fractions D (300 ml hexane: 100 ml ethyl acetate) and E (280 ml hexane: 120 ml ethyl acetate), H (200 ml hexane: 200 ml ethyl acetate) and I (160 ml

hexane 240 ml ethyl acetate), and J (140 ml hexane: 260 ml ethyl acetate) all exhibited anti-bacterial activity (Plate 13 B).

Fractions D and E indicated the presence of anti-bacterial activity at the same R_f value (0.48) and were combined for further separation by preparative TLC. Similarly, Fractions H and I showed similar activity at R_f values of 0.59 and 0.13, and were combined. Fraction J exhibited activity at an R_f of 0.47 and further separated by preparative TLC. Ten plates were used for preparative TLC of combined Fraction DE (400 mg), combined Fraction HI (100 mg) used five plates and Fraction J (30 mg) used three plates.

Table 24: Preparative TLC zones recovered for *C. greenwayi* VLC fractions

VLC Fraction/s	Scraped Zone	Mass of Fraction (mg)	Activity?	Purity	Mass sent for NMR (mg)
1) D & E	A	15.0	✓	✓	11.0
2) H & I	B	5.0	✓	✓	3.3
	C	9.0	✓	✓	6.1
3) J	D	4.0	✓	✓	2.8

Compound A (originally from combined Fraction DE), Compounds B and C (originally from combined Fraction HI) and Compound D (originally J) were obtained by preparative TLC (Table 24). The compounds were apparently pure as indicated after staining with anisaldehyde spray reagent (Plate 13 C). Isolation of more anti-bacterial zones was attempted but resulted in too little compound available for identification.

The four compounds were analysed by NMR and GC-MS (Appendix A). GC-MS analysis indicated no corresponding acids with Compound A , the main components of Compound B were (Fig. A.12) Stearyl alcohol (95 %) (Fig. A.13) and Palmitic acid (96 %) (Fig. A.14), Compound C (Fig. A.15) was indicated to contain Eicosane (Fig. A.16) MS *m/z* (rel. int.): 85.15 (49.47), 71.20 (70.67), 57.15 (100), 43.15 (84.64), 41.20 (40.58) and the main components of Compound D (Fig. A.17) were Eicosane (Fig. A.18) MS *m/z* (rel. int.): 85.15 (47.63), 71.20 (70.50), 57.15 (100), 43.15 (80.67), 41.20 (42.73); Myristic acid (Fig. A.19) MS *m/z* (rel. int.): 73.10 (96.41), 60.10 (90.68), 55.20 (78.18), 43.15 (100), 41.10 (92.67) and Palmitic acid (Fig. A.20) MS *m/z* (rel. int.): 73.10 (100), 60.10 (83.19), 55.10 (75.76), 43.15 (98.25), 41.15 (84.85).

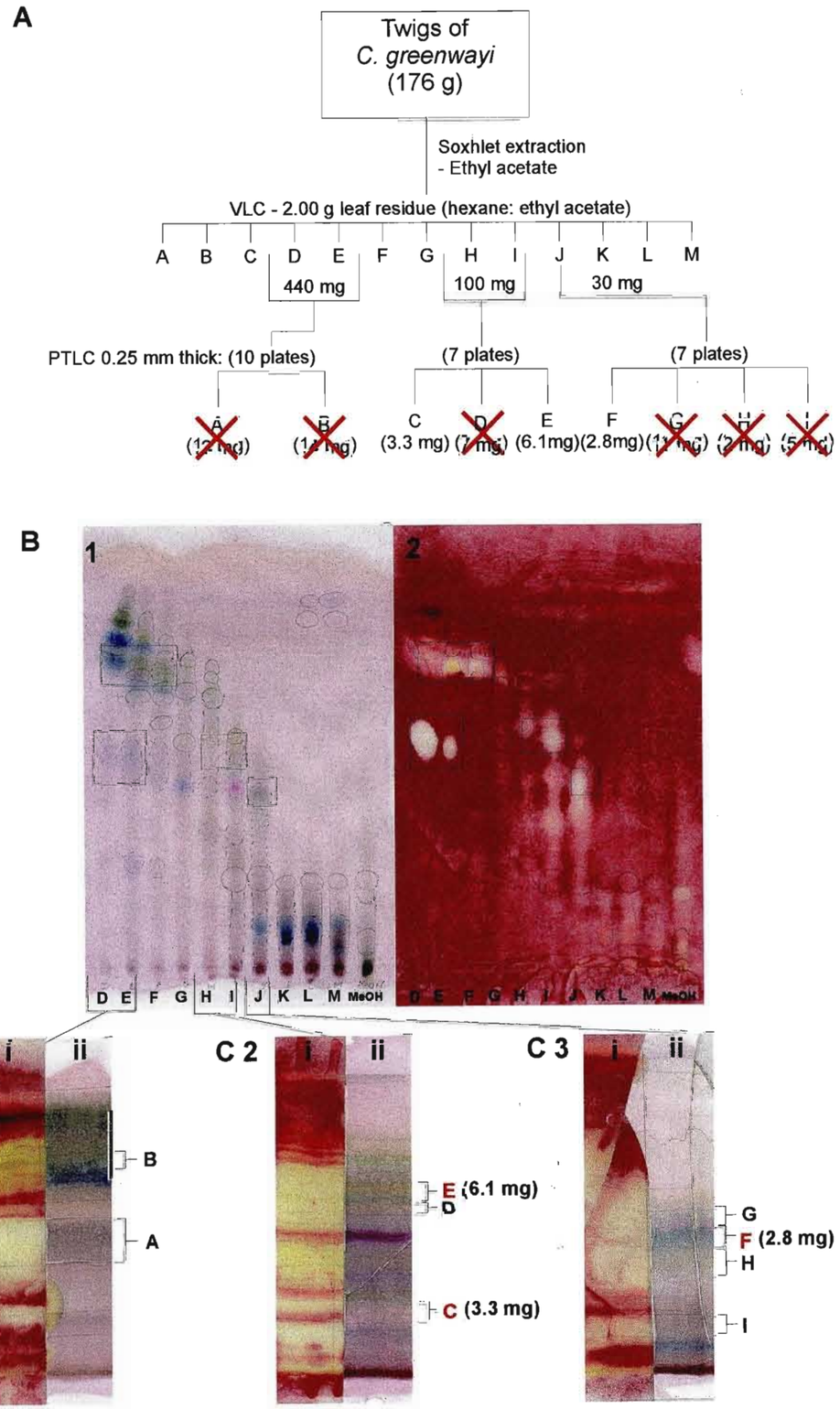


Plate 13 Isolation of anti-bacterial compounds from *C. greenwayi* twig extract. (A) Flow chart outlining the isolation procedure followed. (B) VLC fractions (D - M) were spotted on TLC plates and analysed by (1) staining with anisaldehyde spray reagent and (2) bioautography. (C) Preparative TLC methods were employed for further isolation. TLC chromatograms composed of (1) mixture of VLC Fractions D & E, (2) Fractions H & I and (3) Fraction J were analysed by (i) bioautography and (ii) anisaldeyde spray reagent techniques.

6.4.4 *Hermannia depressa*

Materials and Methods

Plate 14 outlines the procedure used in the isolation of anti-bacterial compounds from *H. depressa* leaf material extracted with ethyl acetate.

Dried, ground leaf material (116 g) was extracted in ethyl acetate using a small Soxhlet apparatus. The crude ethyl acetate residue (5.26 g) obtained was separated by VLC. Activity was determined by bioautography. Preparative TLC allowed for further purification of compounds. HPLC ensured isolation of specific compounds. The identification of compounds was by NMR and GC-MS.

Results

Thirteen fractions (A - M) were collected by VLC and analysed for their anti-bacterial activity (Table 25).

Table 25: Fractions (A - M) collected from VLC of *H. depressa* ethyl acetate leaf extract, showing relative information including mass of fractions eluted, percentage of total, colour of fraction, presence of activity and which fractions were used in preparative TLC.

Fraction	Mass of Fraction (mg)	Percentage of total (%)	Activity against *S. aureus*	Colour of extract	
A	58	1.16	✓	White	1
B	932	18.64	✓	Dark browny-yellow	2
C	408	8.16	X	Very dark green	
D	249	4.98	X	Very dark green	
E	83	1.66	X	Dark green	
F	120	2.40	✓	Dark green	3
G	137	2.74	✓	Dark green	4
H	61	1.22	X	Browny-mustard	
I	41	0,82	X	Browny-mustard	
J	27	0.54	X	Mustard yellow	
K	23	0.46	X	Mustard yellow	
L	35	0.70	X	Mustard yellow	
M	21	0.42	X	Mustard yellow	
Total	2.195 g	43.88 %			

1 - Isolated compound

2 - Fraction B further isolated by TLC preparation

3 - Fraction F further isolated by TLC preparation

4 - Fraction G further isolated by TLC preparation

Compound A (400 ml hexane: 0 ml ethyl acetate)(R_f - 1) was eluted as a pure compound showing anti-bacterial activity, no further isolation was attempted. It was a very non-polar compound as it moved with the solvent

front in 100 % hexane (Plate 14 B). Fractions B, F and G exhibited dense residues with visible active zones. The fractions were impure so further isolation was required using preparative TLC (Table 26).

Table 26: Preparative TLC zones recovered for *H. depressa* VLC fractions

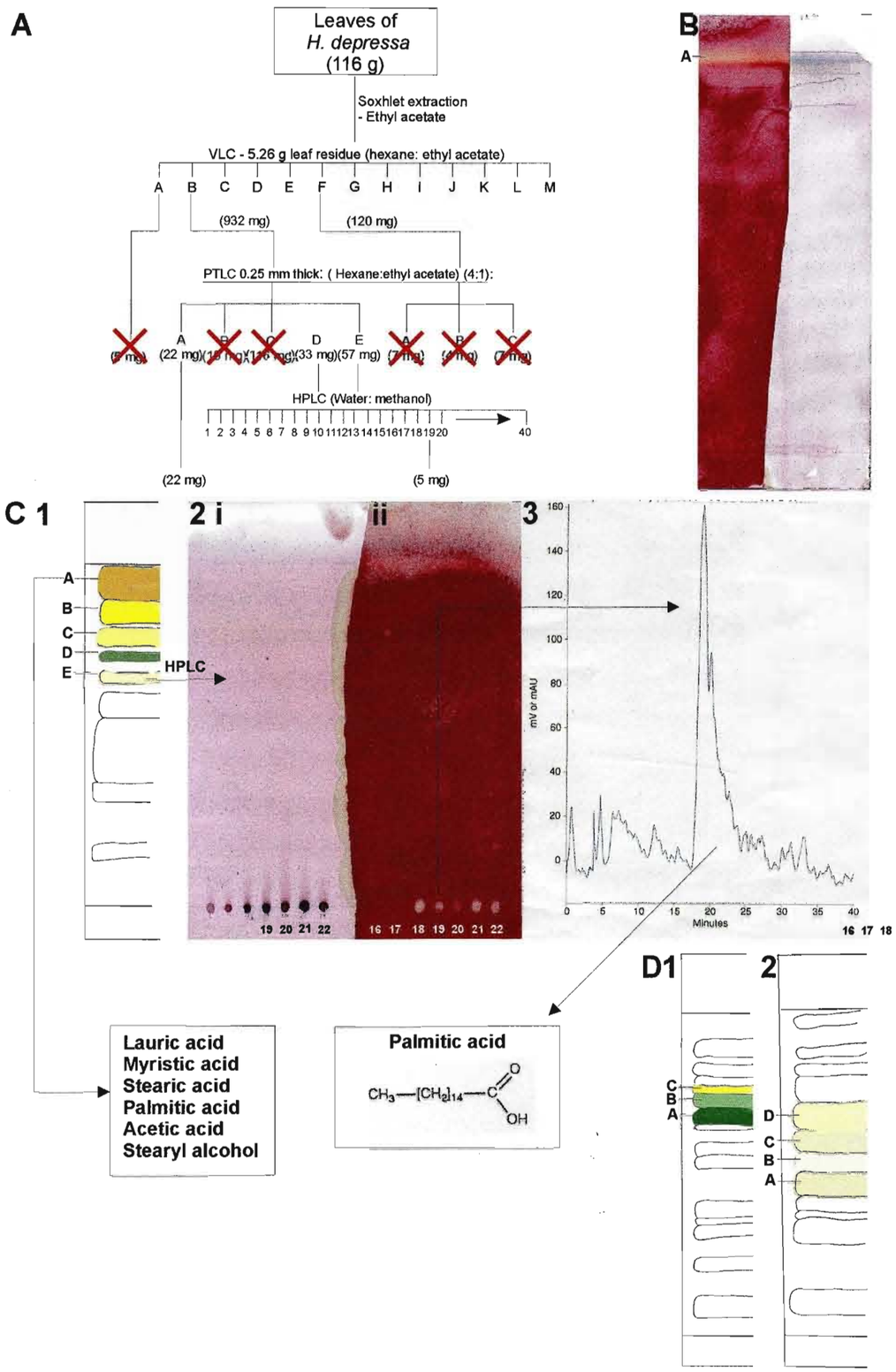
VLC Fraction/s	Scraped Zone	Mass of Fraction (mg)	Activity?	Purity	Mass sent for NMR (mg)/ HPLC
1) A	–	–	✓	✓	5
2) B	A	149	✓	✓	22
	B	15	✓	X	-
	C	116	✓	X	-
	D	33	✓	X	-
	E	57	✓	X	HPLC
3) F	A	7	✓	X	-
	B	4	✓	X	-
	C	7	✓	X	-
4) G	A	8	X	-	-
	B	6	X	-	-
	C	8	X	-	-
	D	8	X	-	-

Compound B (A) (Rf - 0.95) was obtained by preparative TLC and was indicated pure after staining with anisaldehyde spray reagent. *H depressa* Fraction B part E (57 mg) through staining with anisaldehyde spray reagent was resolved to be an impure compound. Further purification was attempted by HPLC. A gradient elution of 50 to 80 % methanol for 15 min followed by 80 - 100 % methanol for 25 min exhibited good separation of the active compound. Fraction 19, eluted between 82 and 85 % methanol facilitated the active fraction (Plate 14 C). A total of 5 mg of this isolated fraction was

collected from 17 HPLC runs for NMR analysis. VLC Fraction F indicated zones of activity, but after recovery, too little residue was available for further isolation as the samples were still impure (Plate 14 D_1). No activity was exhibited from zones recovered from Fraction G.

The three compounds obtained from *H. depressa* ethyl acetate leaf extract were analysed by NMR, indicating that Compound A, Compound B(A) and Compound B(E) to be fatty acids. GC-MS suggested Compound B(A) (Fig. A.21) to be a combination of Acetic acid (Fig. A.22) MS *m/z* (rel. int.):60.05 (45.19), 45.10 (83.33), 43.10 (100), 42.10 (17.73), 29.20 (12.58); Lauric acid (Fig. A.24) MS *m/z* (rel. int.): 73.10 (100), 60.10 (85.91), 55.10 (64.63), 43.20 (73.04), 41.20 (79.17); Stearyl alcohol (Fig. A.25) MS *m/z* (rel. int.): 83.15 (85.39), 69.10 (76.86), 55.15 (100), 43.15 (99.25), 41.15 (78.71); Myristic acid (Fig. A.26) MS *m/z* (rel. int.): 73.10 (100), 60.10 (80.48), 55.10 (65.92), 43.15 (76.94), 41.15 (78.27); Stearic acid (Fig. A.28) MS *m/z* (rel. int.): 73.10 (81.56), 60.10 (67.48), 55.10 (66.30), 43.15 (100), 41.15 (72.33) and Palmitic acid (Fig. A.27) MS *m/z* (rel. int.): 73.10 (99.76), 60.10 (83.51), 55.10 (78.71), 43.15 (100), 41.20 (87.19). For Compound B(E) (Fig. A.29) the main component was Palmitic acid (Fig. A.30) MS *m/z* (rel. int.):73.10 (95.54), 60.10 (87.97), 55.10 (80.75), 43.10 (100), 41.15 (95.79) (Appendix A).

Plate 14 Isolation of anti-bacterial compounds from *H. depressa* leaf extract. (A) Flow chart outlining the isolation procedure followed (B) A TLC chromatogram of VLC Fraction A indicating the position of compound A. (C)(1) A representative TLC chromatogram indicating VLC Fraction B after further isolation by preparative TLC methods. Additional isolation by HPLC techniques was employed: (2) indicates HPLC fractions collected near expected activity (i) after staining with anisaldehyde spray reagent and (ii) bioautography, (3) A representative HPLC chromatogram of the expected anti-bacterial compound. (D) (1) A representative TLC chromatogram of VLC Fraction F indicating colour and position of zones scrapped, and (2) A representative TLC chromatogram of VLC Fraction G indicating colour and position of zones scrapped



6.5 GENERAL DISCUSSION

Use of Sterculiaceae species for Traditional Medicine in Line with Anti-bacterial Activity

Bacterial infections are a common occurrence in the numerous informal settlements located within South Africa where water is obtained from contaminated streams and where good sanitary methods of living are lacking. Traditional healers are relied upon to provide treatment for the common ailments caused by bacterial infections. Scientific research demonstrates the effectiveness of the treatments used and is important in bridging the gap between Westernised medicine and the traditional medicines. Stomach aches and related gut infections as well as throat infections are commonly caused by Gram-positive ailments. These infections are largely not life threatening. Gram-negative bacteria are the typical causes of diarrhoeal and dysenteric diseases. These are more harmful and often associated with death.

The presence of anti-bacterial activity in the species screened is in line with their uses in traditional medicine. *D. rotundifolia* is commonly used in traditional medicine to treat intestinal ulcers, stomach complaints (COATES PALGRAVE, COATES PALGRAVE & COATES PALGRAVE, 1985), haemorroids and diarrhoea (WATT & BRANDWIJK, 1962; VAN WYK, VAN OUDTSHOORN & GERICKE, 1997). *D. burgessiae* shrubs are applied over affected areas in treatment against leprosy and the roots are used for stomach complaints (CHHABRA, MAHUNNAH & MSHIU, 1993). *H. depressa* is used in treatment against stomach ache, coughs and diarrhoea (WATT & BRANDWIJK, 1962; HUTCHINGS, HAXTON-SCOTT, LEWIS & CUNNINGHAM, 1996; POOLEY, 1998). *C. greenwayi* and the roots of *D. cymosa* are used in traditional medicine by the Zulu people of KwaZulu-

Natal (POOLEY, 1993; HUTCHINGS, HAXTON-SCOTT, LEWIS & CUNNINGHAM, 1996).

Plant Fatty Acids

By 1990, GUNSTONE stated that the number of natural plant fatty acids discovered exceeded 1000. The general characteristics of plant fatty acids are that they are unbranched saturated or unsaturated chains with an even number of carbon atoms (GUNSTONE, 1990), however, there are fatty acids which are branched or have an uneven carbon number. Fatty acids occur mainly in plants in a bound form, esterfied to glycerol, as fats or lipids (HADLEY, 1985). The length of the carbon chains vary depending on the functions of the compounds. Short-chain fatty acid are made up of up to eight carbons. They have substantial solubility in water (GURR & JAMES, 1971) and are rapidly digested and absorbed in the intestinal tract. Medium-chain fatty acids consist of eight to 14 carbon atoms, while long-chain fatty acids have 16 and more carbon atoms. Long-chain fatty acids are less soluble in water because of the size of the hydrocarbon chain (GURR & JAMES, 1971).

In TLC, long-chain compounds are slightly less polar than their medium chain analogues (GUNSTONE, 1990). Polyunsaturated compounds are slightly more polar that their more saturated homologues (GUNSTONE, 1990). Normal saturated fatty acids are generally more soluble in chloroform.

Anti-bacterial Compounds from the Five Sterculiaceae Species Investigated

All the active anti-bacterial compounds isolated during this study were fatty acids. This was evident after NMR and GC-MS analysis.

Lauric (Dodecanoic) acid ($CH_3(CH_2)_{10}COOH$) is a saturated acid widely distributed in plants and is a major component of some seed fats (GURR & JAMES, 1971). Myristic (Tetradecanoic) acid ($CH_3(CH_2)_{12}COOH$) is a saturated acid widespread occasionally as a major component (GURR & JAMES, 1971). Stearic (Octadecanoic) acid ($CH_3(CH_2)_{16}COOH$) is less prominent in leaf lipids, but is a major saturated acid in seed fats (GURR & JAMES, 1971; HARBORNE & BAXTER, 1993) and vegetable oils and fats (HARBORNE & BAXTER, 1993). Palmitic (Hexadecanoic) acid ($CH_3(CH_2)_{14}COOH$), a C_{16} acid, is one of the most common major saturated acid in leaf lipids and also occurs in quantity in some seed oils (GURR & JAMES, 1971; HARBORNE & BAXTER, 1993). Palmitic acid has previously been reported to be the major anti-bacterial compound in a mixture of fatty acids from *Diplotaxis harra, Ericaria microcarpa* (HASHEM & SALEH, 1999) and *Pentanisia prunelloides* (YFF, LINDSEY, TAYLOR, ERASMUS & JÄGER, 2002). Acetic acid (Ethanoic) acid occurs as alcohol acetates in many plants and in some plant triglycerides (GURR & JAMES, 1971). Triglycerides or triacylglycerides consist of glycerol triesters of fatty acids therefore one compound may be consist of a number of fatty acids (VOET & VOET, 1990).

Continual redissolving during the purification procedures of the extracts led to contamination by solvents. Diisooctylphthalate was a common contamination in all compounds isolated. It was visible by GC-MS at a retention time of 25.20 min. Diisooctylphthalate is used in the manufacturing of glass pill vials.

It is possible that anti-bacterial activity of some fatty acids during bioautographic screening may be false positive due to the lipophilic compounds having a hydrophobic affect on the aqueous anti-bacterial spray

solution. Anti-bacterial activity of fatty acids in the Sterculiaceae species is not false positive as the MIC assay indicated activity in the extracts.

Anti-bacterial Activity of Fatty Acids

Fatty acids commonly show anti-bacterial properties. This has been the topic of much research. The first known mention of the use of fatty acids as antimicrobial agents appeared in 1899 (KABARA, 1986).Since 1966, KABARA and coworkers have studied the structure-function (antimicrobial) of some 500 natural and synthetic lipophilic compounds. The results from these screening activities indicate strong relationships between lipid structures and their biological activity (KABARA, 1986; MARSHALL & BULLERMAN, 1994) i.e. the anti-bacterial activities of fatty acids are influenced by both fatty acid structure and bacterial species (NIEMAN, 1954; KABARA, 1980).

Unsaturation is an important factor in determining the anti-bacterial activity of fatty acids, activity increase with progressive unsaturation. However, saturated fatty acids can act also as growth inhibitors (NIEMAN, 1954). Activity is greater in saturated, monounsaturated and polyunsaturated fatty acids with long carbon chains especially C_{12}, $C_{16:1}$ and $C_{18:2}$, respectively (MARSHALL & BULLERMAN, 1994). Position and number of double bonds are a greater influence on biological activity of long chain (C_{12}) fatty acids as compared to short chain fatty acids (KABARA, 1980). Unsaturation in contrast to esterfication is less effective with low chain fatty acids as compared to higher chain fatty acids (KABARA, 1986). Fatty acids esterfied to monohydric alcohols became inactive, while fatty acids with polyhydric alcohols were generally more active (KABARA, 1980). *Cis* isomers are more effective than *trans* isomers (MARSHALL & BULLERMAN, 1994).

Gram-positive bacteria are more susceptible to fatty acids than Gram-negative bacteria (MARSHALL & BULLERMAN, 1994). Anti-bacterial effects of minute amounts of fatty acids have been noted with Gram-positive bacteria (NIEMAN, 1954). Gram-negative organisms are affected by very short chain fatty acids (C_6) (KABARA, 1980).

The insertion of the non-polar moieties of the fatty acids into the phospholipid layer of the bacterial cell membrane could explain the anti-bacterial action of fatty acids. This causes a change in membrane permeability, alteration of the activity of some membrane proteins essential for maintenance of cellular functions and uncoupling of the oxidative phosphorylation system (SAITO & TOMIOKA, 1988).

Anti-bacterial Activity of Tannins and Saponins

D. rotundifolia leaves and *H. depressa* leaves contain tannins (Chapter 5, Table 11). Tannins are known to be used for their anti-bacterial activity (HARBORNE & BAXTER, 1993). They have been shown to decrease and inhibit bacterial growth in both Gram-positive and Gram-negative bacteria (DJIPA, DELMÉE & QUENTIN-LECLERCQ, 2000). Most frequently tannin containing plants are used to treat diarrhoea and dysentry (LONGANGA OTSHUDI, VERCRUYSSE & FORIERS, 2000). It may be due to the presence of tannins that the extracts of these plant parts exhibit anti-bacterial activity.

A few of an abundance of examples in the literature where anti-bacterial activity is associated with tannins include: *Machaerium floribundum*, Carob pod extract, *Lotus carniculatus*, Wattle species, (SCALBERT, 1991) *Landolphia owrrience* (EBI & OFOEFULE, 1997), *Ocimum gratissimum*, (OFFIAH & CHIKWENDU, 1999) *Roureopsis obliquifoliolata*, *Cissus*

rubiginosa (LONGANGA OTSHUDI, VERCRUYSSE & FORIERS, 2000) and *Suzygium jambos* (DJIPA, DELMÉE & QUENTIN-LECLERCQ, 2000).

Saponins are also known to have anti-microbial activity (HARBORNE & BAXTER, 1993). *D. rotundifolia* and *H. depressa* leaf extracts contain saponins (Chapter 5, Table 9). Some examples in the literature where anti-bacterial activity is associated with saponins include *Yucca shidigera*, (WALLACE, ARTHAUA & NEWBOLD, 1994), *Landolphia owrrience* (EBI & OFOEFULE, 1997), *Astragalus melanophrurius* (CALIS, TURUKER, TASDEMIR, WRITE, STICHER, LUO & PEZZUTO, 1997) and *Panax ginseng* root extract (FUKUDA, TANAKA & SHAYAMA, 2000).

6.6 SUMMARY

- Five of the seven Sterculiaceae species screened for anti-bacterial activity were chosen for isolation of the active compounds. All species indicated the isolated compounds to be fatty acids.
  - *D. rotundifolia* ethyl acetate leaf extract. Compound A contained a combination of Palmitic acid, Myristic acid and Stearic acid. Compound B contained a combination of Palmitic acid, Myrsitic acid, Lauric acid and Acetic acid.
  - *D. burgessiae* ethyl acetate leaf extract. Compound A was Palmitic acid.
  - *D. cymosa* ethyl acetate leaf extract. Compounds were determined to be fatty acids.
  - *C. greenwayi* ethyl acetate twig extract. Compound B contained a combination of Palmitic acid, Stearyl alcohol and Eicosane. Compound C contained Eicosane. Compounds D contained a combination of Palmitic acid, Myristic acid and eicosane.
  - *H. depressa* ethyl acetate leaf extract. Compound B (A) contained a combination of Palmitic acid, Myristic acid, Stearic

acid, Lauric acid, stearyl alcohol and octadecane. Compound B (E) was Palmitic acid.

- During the fractionation of the crude residue through the VLC column resulted in a very low recovery rate. On addition of 100% methanol as in the case of *C. greenwayi* twig extract, much of the remaining extract was recovered (97.79%). The 100% methanol may improve recovery but the fraction recovered was exceedingly impure and bioautography determined only a small anti-bacterial zone. The 100% methanol fraction was therefore not considered for further purification and discarded. It was expected that overall, in the individual VLC experiments, most of the anti-bacterially active compounds had been recovered.

- Polarity of the isolated compounds was affected by the combination of fatty acids it was composed of, thus resulting in the same fatty acid exhibiting different R_f values.

CHAPTER 7

MUCILAGENOUS *HERMANNIA DEPRESSA* AQUEOUS EXTRACT

7.1 INTRODUCTION

7.1.1 Gums and Mucilages

A common occurrence in a number of the Sterculiaceae species screened during this study was the production of mucilage by the aqueous extracts. Leaf and twig extracts of *D. burgessiae* and *D. cymosa*, leaf extracts of *S. murex*, leaf and stem extracts of *H. depressa* and the unripe fruit extract of *C. greenwayi* all produced a mucilage. However, it was produced most abundantly by the *H. depressa* leaf extract.

7.1.1.1 What is a Gum or Mucilage?

The Collins English Dictionary (1998) defines a gum as any of various sticky substances that exude from certain plants, hardening on exposure to air and dissolving or forming viscous masses in water, while a mucilage is defined as a complex glutinous carbohydrate secreted by certain plants. The Oxford Dictionary of Biology (1996) describes a gum as a variety of substances obtained from plants. Typically they are insoluble in organic solvents but form gelatinous or sticky solutions with water. A mucilage is defined as a gum-like substance. They are hard when dry and slimy when wet.

Most commonly, the term gum as technically employed in industry refers to plant polysaccharides or their derivatives which are dispersible in either cold or hot water to produce viscous mixtures or solutions (WHISTLER, 1959). An effort has been made to distinguish between mucilages and gums on the

basis that gums readily dissolve in water whereas mucilages form slimy masses (TYLER, BRADY & ROBBERS, 1981). However, a classic example of an important industrial gum, karaya gum (*Sterculia urens*), particles imbibe large amounts of water to swell to great size but they do not dissolve or form homogeneous suspensions unless ground extremely fine or subjected to treatment in an autoclave (MANTELL, 1947). Gums are therefore best powdered. If they are unpowdered, addition of water yields a non-homogenous colloidal solution. If finely powdered, each particle swells and the appearance and behaviour of the solution is more like a homogeneous solution (MANTELL, 1947).

Gums are natural plant hydrocolloids that may be classified as anionic or nonionic polysaccharides or salts of polysaccharides. They are translucent, amorphous substances that are frequently produced in higher plants as a protective after injury (TYLER, BRADY & ROBBERS, 1981). Less pure gums tend to exhibit varying colours, from pale yellow to brown.

The gum industry is a very productive one. Gums find diverse applications in pharmacy. They are ingredients in dental and other adhesives and in bulk laxatives (TYLER, BRADY & ROBBERS, 1981). Gum arabic (*Acacia senegalensis*) is the most important polysaccharide gum of commerce. It has been an article of commerce since pre-Christian times (WHISTLER & SMART, 1953). Gum arabic is used as a demulcent, soothes irritations of the mucous membranes in humans and is used as a food emulsifier, stabilizer and thickener (HARBORNE & BAXTER, 1993). Another gum, Gum tragacanth (*Astragalus gummer*) is used as a food emulsifier, stabilizer and as a bulking agent (HARBORNE & BAXTER, 1993). Guar gum is used in Herbex Appetite Control Tablets. This water soluble mucilage turns into gel in the stomach when taken with liquid, naturally reducing hunger and the 'bottomless pit' feeling in the stomach.

7.1.1.2 Sterculiaceae Gums and Mucilages

The Sterculiaceae family consists of a large number of genera encompassing species that produce gums or mucilaginous substances. The various genera exuding these substances range from very small herbs to large trees. The gum from some species, e.g. *Sterculia urens* (karaya gum), are significant industrial gums.

Of the *Cola* species, *C. cordifolia* from west tropical Africa is known to yield a gum (HOWES, 1949). Of the *Brachychiton* species which occur in Australia, *B. diversifolius* trees can exude a bucket of gum from a single tree (SMITH & MONTGOMERY, 1953). The gum is pale yellow, however, it may be very dark in colour and form in lumps. *B. rupestris* is rich in a sweet mucilaginous or gummy juice (HOWES, 1949). Of over a hundred *Sterculia* species, at least a dozen and a half yield gum (HOWES, 1949). Some of the *Sterculia* species known to exude gums are *S. campanulata, S. foetida, S. guttata, S. ornata* and *S. villosa* which are all found in India, *S. barteri*, and *S. cinerea* which are found in Africa, *S. acerifolia, S. diversifolia, S. plantanifolia, S. quadrifica* and *S. rupestris* from Australia, and *S. hypochroa* and *S. thorelii* from Indo-China (SMITH & MONTGOMERY, 1953). Gum exudes from grey, corky bark of the tall tree *S. tragacantha* (Africa) and sometimes from the fruit follicles when punctured by insects. The bark of *S. setigera* (Africa) yields a pale tragacanth-like gum and a watery sap which is refreshing to thirsty travellers. The gum is used to prepare indigo-dyed cloth, dressing fabrics and a slightly granular jelly (HOWES,1949). *S. scaphigera,* known to occur in Indo-China and Thailand yields a gum from the fruit and not from the trunk of the tree (SMITH & MONTGOMERY, 1953). When the fruit is macerated with water, the pericarp or outer shell increases enormously in volume forming a gelatinous mass. The jelly is sweetened and eaten as a delicacy, which the Chinese call 'ta-hai-tsze' and regard as a specific in diarrhoea and dysentery treatment (HOWES,1949). The most

well known and commercially important of all the gums produced from this family is karaya gum, from *S. urens*.

Karaya Gum

Sterculia urens exudes gum known as karaya gum (HOWES, 1949; MORTON, 1977). Sometimes also *S. villosa* and *S. tragacanth* gums are referred to as karaya gum (TYLER, BRADY & ROBBERS, 1981). *Sterculia urens* is a large deciduous tree growing in the dry, elevated areas of North and Central India (SMITH & MONTGOMERY, 1953). They are tall, reaching a height of 8 to 10 m, and their trunks are large with a soft corky structure. The gum exudes naturally or from incisions made to the heartwood. The incisions produce knoblike masses of gum that are collected frequently for nine months. The tree is then allowed to rest for two to three years (MANTELL, 1947; SMITH & MONTGOMERY, 1953; TYLER, BRADY & ROBBERS, 1981). All parts of the tree exude this soft gum when injured (MORTON, 1977). The gum is obtained from the trees throughout the year except the rainy season and the best quality of the gum is obtained during the hot spell (March through to middle June) (MANTELL, 1947) before the monsoon season (GOLDSTEIN & ALTER, 1959). As the weather becomes warmer, gum yield, as well as quality increases (GOLDSTEIN & ALTER, 1959).

Karaya gum is the least soluble of all commercial plant exudates. It absorbs water, swells to several times its original bulk, and forms a discontinuous type of mucilage (TYLER, BRADY, & ROBBERS, 1981). Its viscous nature decreases with storage, especially after being reduced to a powder and in hot, humid environments (MORTON, 1977). It is insoluble in alcohol and alkali (GOLDSTEIN & ALTER, 1959; MORTON, 1977). The gum swells in 60 % alcohol, but it is insoluble in organic solvents (LEUNG & FOSTER, 1996). It absorbs water rapidly (MORTON, 1977) and swells to form viscous

colloidal solutions or dispersions at low concentrations (e.g. 1 %). Higher concentrations (up to 4 %) produce a viscous gel-like paste (MORTON, 1977; LEUNG & FOSTER, 1996).

Karaya gum is an acetylated polysaccharide, (8 % acetyl groups, 37 % uronic acid residues) (MORTON, 1977; HARBORNE & BAXTER, 1993), which is characterized by an acid nature (GOLDSTEIN & ALTER, 1959). It has a high molecular weight (9 500 000), its structure is complicated and has not been determined, but it is expected to have three different types of chains (LEUNG & FOSTER, 1996). Karaya gum consists of an acetylated branched heteropolysaccharide with a high component of D-galacturonic acid (43 % yield) and D-glucuronic acid residues (TYLER, BRADY & ROBBERS, 1981), D-galactose (14 %), L-rhamnose (15 %) (WHISTLER & SMART, 1953) and ketohexose (SMITH & MONTGOMERY, 1953). Dispersions of the gum in water have a pH of 3.6 - 4.4 (SMITH & MONTGOMERY, 1953). It is used medicinally as a laxative (TYLER, BRADY & ROBBERS, 1981, LEUNG & FOSTER, 1996) and for throat inflammation (MORTON, 1977). Water in which the leaves and cut ends of branches are placed becomes thick, like clear jelly. Indian vetinarians use this mucilage as a remedy for pleuropneumonia in cattle (MORTON, 1977). It is used in foods, including curries, making sweet meats, French dressing, cheese spreads, sherbets, ice-cream sticks and meringues. It is also used in the making of certain toiletries, cosmetics, adhesive for dentures (it is resistant to bacterial and enzymatic hydrolysis) (TYLER, BRADY & ROBBERS, 1981), lotions and papermaking (MORTON, 1977).

7.1.1.3 Gums as Bulking Agents

Bulking agents may be ingested for a number of reasons: as an appetite suppressants, laxatives or in the treatment against diarrhoea. They are

usually dried substances that in the presence of water swell to a much greater volume.

7.1.1.3.1 Appetite Suppressant

To eat is important. It is a necessity for survival to obtain daily the required nutrients and vitamins necessary for survival. However, many people overdo it by ingesting more food (especially fats) than is required. Generally these people are obese. One way to overcome overeating would be to suppress the desire to eat. Anorectic, or appetite suppressant drugs, are drugs that suppress the subjective awareness of hunger. They fall into two broad categories: those acting directly on the central nervous system, and those which act primarily on peripheral mechanisms (SILVERSTONE & KYRIAKIDES, 1982). Bulking agents fall into the later category.

Obesity is a health risk. An increase in body weight of 20% or more above desirable weight is considered a health hazard (COUNCIL FOR AGRICULTURAL SCIENCE AND TECHNOLOGY, 1987). It is associated with an increased incidence of cancer of the colon, prostate and rectum in men and breast, cervix, endometrium and ovary in women. Respiratory problems are also common, as is the risks from anaesthesia and surgery, above those of normal weight patients. Obese people are also more prone to osteoarthritis (GILLHAM, PAPACHRISTODOULOU & HYWEL THOMAS, 1997), hypertension, hyperlipidemia, diabetes, gout, cardiovascular disease and gallbladder diseases (KANNEL, 1983).

Types of Bulking Agents used as Appetite Suppressants and Associated Problems

The pharmaceutical industry is a consistent user of methylcellulose. The non-nutritive bulking action of methycellulose is accompanied by demulcent

and surfactant properties and it contains no protein contaminants that could cause allergies. It is not metabolized and is useful where non-nutritive bulk is desired for the treatment of obesity (GREMINGER & SAVAGE, 1959). The idea of filling up the stomach with an inert, non-digestible bulk agent has a refreshing simplicity about it. However, one example given, involving trials of methylcellulose have failed to indicate that it helps obese patients to lose weight by causing them to eat less (SILVERSTONE & KYRIAKIDES, 1982). COLGAN (1994) states that bulking agents and starch blockers that are supposed to reduce food digestion do not work. Studies on women using *Plantago ovata* as an appetite suppressor show these products are useless and counterproductive, because the aim is not only to help patients to lose weight, but also to learn adequate dietary habits that include the consumption of fibre-rich foods (MORO & BASILE, 2000).

7.1.1.3.2 Bulking Agents as a Laxative and Against Diarrhoea

Bulking agents can be used as a laxative or in the treatment against diarrhoea. To have a laxative effect, on ingestion of the bulking agent, it absorbs water allowing it to form a mucilaginous consistency allowing the stool to 'slide' with ease through the ileum. Alternatively, it aids in the treatment of diarrhoea by absorbing excess water which causes attempted hardening of the stool.

Many bulking agents have been offered to the constipated. Constipation can be relieved by the use of a hydrophilic colloid of methylcellulose (GREMINGER & SAVAGE, 1959). It is a bulking agent with little water-holding capacity and has a modest effect on stool weight (THOMPSON, 1989). Due to the fact that methylcellulose in aqueous media can swell to 40 times its dry volume, it is used in the treatment of various intestinal ailments, such as diarrhoea, where bulking action is desirable (GREMINGER & SAVAGE, 1959). Psyllium hydrophilic mucilloid is a powder derived from the

seed coat of the flowering plant, *Plantago ovata* (*Plantago isbaghula*) that is native to India. The seed mucilage of *Plantago ovata* (psyllium) is a mixture of polysaccharides (WHISTLER & SMART, 1953). In the United Kingdom, it is known as 'isbaghula' and has been employed as a laxative in the Orient for a millennium. The modern preparation consists of powdered natural mucilage. When placed in water, the swelling may be 15 times the initial volume. If ingested the mucilage increases the faecal volume and favours defecation. Therefore it is important to drink large quantities of liquid with this product. If not accompanied by liquids, in the gut this gelling action results in firming up diarrhoea stools as it thickens the intestinal contents (THOMPSON, 1989).

The products of certain vegetable gums, such as gum arabic (*Acacia senegalensis*) and karaya (*Sterculia urens*), are available for treatment of constipation. They attract water and form a gel that is resistant to mechanical action. Digestion by colon bacteria is unpredictable, but metabolites of these gums are thought to have a laxative effect (THOMPSON, 1989). As a bulk laxative, gum karaya, is a second to psyllium seeds in importance. The coarse gum particles absorb water and swell 60-100 times their original volume, forming a discontinuous type of mucilage. It is believed that this type of mucilage is very effective as a laxative (GOLDSTEIN & ALTER, 1959) due to its indigestible and non-absorbing properties by the body.

7.1.1.3.3 Digestion, Focussing Primarily on Carbohydrate Digestion

For a plant mucilage to be effective as a bulking agent it is important that it will pass through the human digestive system without breaking down. A major component of mucilages are polysaccharides.

In chemical digestion the large carbohydrates, lipids, protein and nucleic acid molecules in food are split into smaller molecules by hydrolysis. Digestive enzymes produced by the salivary glands, tongue, stomach, pancreas and small intestine catalyse these catabolic reactions (TORTORA & GRABOWSKI, 2000).

Chewing of food and mixing with saliva is the beginning of the hydrolysis of food (CLAYMAN, 1995). Chemically saliva is 99.5 % water and 0.5 % solutes. Two digestive enzymes make up part of these solutes, salivary amylase which acts on starch and lingual lipase which acts on triglycerides. Chloride ions in the saliva activate salivary amylase (TORTORA & GRABOWSKI, 2000) which initiates the breakdown of starch (CLAYMAN, 1995; TORTORA & GRABOWSKI, 2000) by hydrolyses producing -limit dextrins, maltotriose and maltose (GANONG, 1975).

Even though food is usually swallowed too quickly for all starches to be reduced to disaccharides in the mouth, salivary amylase in the swallowed food continues to act on the starches for about another h, at which time stomach acids inactivate it (TORTORA & GRABOWSKI, 2000). Carbohydrate digestion does not occur in the stomach.

The mucosal surface of the stomach is covered by columnar epithelial cells which extend into gastric pits containing glands. The cell type found particularly in the neck region of the gland is the oxyntic (acid forming) or parietal cell, which is responsible for the secretion of HCl (SANFORD, 1982).

The pancreas produces pancreatic juice, which is a clear colourless liquid consisting mostly of water, some salts, sodium bicarbonate and several enzymes. The sodium bicarbonate gives pancreatic juice a slightly alkaline pH (7.1 - 8.2) that buffers acidic gastric juice in chyme, stops the action of

pepsin from the stomach and creates the proper pH for action of digestive enzymes in the small intestine. The enzymes in pancreatic juice include a carbohydrate-digesting enzyme called pancreatic amylase (TORTORA & GRABOWSKI, 2000). In the duodenum, α-amylase, an enzyme produced by the pancreas breaks down starch into maltose, which is a disaccharide (CLAYMAN, 1995). The pancreas produces pancreatic α-amylase, which breaks down starch similarly to salivary amylase (GANONG, 1975).

Small intestine juice is a clear yellow fluid. It contains water and mucus and is slightly alkaline (pH 7.6). Together, pancreatic and intestinal juices provide a liquid medium that aids the absorption of substances from chyme as they come in contact with the microvilli. The absorptive epithelial cells synthesize digestive enzymes, called brush border enzymes. Among the brush-border enzymes are four carbohydrate-digesting enzymes called -dextrinase, maltase, sucrase and lactase (TORTORA & GRABOWSKI, 2000). In the small intestine maltase, sucrase and lactase are enzymes secreted by certain glands in the intestinal wall. They convert disaccharide sugars into monosaccharide sugars (CLAYMAN, 1995). The intestinal mucosa contains the digestive enzymes maltase, lactase, sucrase and isomaltase which break down maltose and maltotriose to form glucose, lactose to form galactose and glucose, sucrose to form fructose and glucose and -limit dextrins to form glucose, respectively (GANONG, 1975).

7.1.1.4 Wound Healing

Many African plants are used traditionally in the treatment of a number of different wounds. The plants are used as first aids, in cleansing and washing of wounds. Other uses include treatment of boils, abscesses, cuts, skin lesions, snake and dog bites, insect stings, bruises, pains, soothing of burns, inflammation, scabies and the dressing of wounds (ONAYADE, ONAYADE & SOFOWARA, 1996). Gums are sticky substances that can

seal plant wounds (THOMPSON, 1989), however, they can also be used in the repair of human wounds. A mucilaginous plant extract with anti-bacterial or anti-inflammatory properties would invariably aid in quicker healing.

7.2 AIMS

The production of mucilage from the *H. depressa* aqueous extract may have medicinal or commercial value. The aim of this part of the study was to investigate the properties of the mucilage and in doing so determine its effective use in traditional medicine. The extract was also investigated to determine similar properties to *S. urens*, a commercially important species from the same family.

7.3 EXTRACTION OF PLANT MATERIAL

Dried, ground leaf material (116 g) was extracted with ethyl acetate (10 g leaf material/ 150 ml ethyl acetate) using a Soxhlet apparatus for 2 h. The leaf material was removed from the Soxhlet and dried in a fume cupboard allowing for evaporation of all ethyl acetate. The leaf material was then placed in distilled water (1l) and allowed to stand overnight. The solution became very viscous. To extract the viscous solution from the leaf material, the solution was centrifuged at 9 000 g for 20 min. Two different extracts were obtained (Plate 15):

1. *H. depressa* extract A (Extract A): A slightly viscous liquid, golden brown in colour (Plate 15 A). This extract was the top layer removed after centrifugation;
2. *H. depressa* extract B (Extract B): A more viscous, mucilaginous slime, dark cream in colour (Plate 15 B).

Extract A dried to become dark, brittle and shiny. As the more viscous extract (Extract B) dried it took on a darker colour and became very hard (Plate 15 B_2). With addition of water to separate this substance once again exhibited a mucilaginous nature. The dry weight of the extract was 7.795 g. During the study, both extracts were studied, however, there was greater focus on the more viscous mucilage (Extract B).

Thin layer chromatography of the two extracts (Extract A and Extract B) was performed. The extracts were loaded onto plates and run in a solvent solution of chloroform, methanol and water in a ratio of 60:35:8 over a distance of 8 cm. The TLC plates were viewed with white light before and after spraying with anisaldehyde spray reagent (Plate 15 C).

7.4 SUGAR ANALYSIS BY GAS CHROMATOGRAPHY (GC) OF AQUEOUS *HERMANNIA DEPRESSA* EXTRACTS

7.4.1 Introduction

The mucilage of *H. depressa* was expected to contain a number of sugars. The smell of the dried *H. depressa* aqueous extract was sweet and caramel-like. On autoclaving the smell became molasses-like.

7.4.2 Materials and Methods

Hydrolysis of Polysaccharides

Plant material (0.5 mg) of Extract B was placed into a glass vial (screw cap). The extract was taken to dryness under nitrogen. Two hundred l 2M trifluoroacetic acid (TFA) were added to the extract. The solution was incubated for 2 h at 100 C in a heating block (The screw cap ensured no leaking/ evaporation of water). The TFA was removed under nitrogen.

Preparation of Sugar Standards for Gas Chromatography

Sugars used: Sucrose (Merck)
D(+) Glucose (Merck)
L(+) Rhamnose (Merck)
D(-) Fructose (Merck)
and D - Glucuronic acid (Sigma)

Sugars were dissolved in 80 % ethanol to a concentration of 1 mg ml^{-1}. Five hundred l were taken to dryness under nitrogen.

Gas Chromatographic Analysis

Two hundred l of a pyridine solution, containing 25 mg ml^{-1} hydroxylamine monohydrochloride, were added to both the extract and sugar preparations and incubated at 40 C for 20 min in a heating block. One hundred l of the pyridine solution were transferred to an Eppendorf tube and dried under nitrogen. Fifty l Sil-A (Sigma catalogue number 139-1) were added to each tube and allowed to react for 15 min at room temperature, before centrifuging in a millifuge for 10 s.

One μl of the supernatant was subjected to gas chromatography (GC) using a Varian 3700 chromatograph fitted with a flame ionisation detector (using helium), The column was 2 m by 6 mm and packed with Chromosorb warp mesh 80 - 100. The carrier gases were nitrogen and air. The injector port was set at 200 C and the detector at 280 C. The column temperature started at 125 C and ramped up to 270 C at a rate of 4 C min^{-1}. The final temperature of 270 C was held for 10 min. A HP 3394A integrator was used to record the retention times.

7.4.3 Results

Table 27: The retention times of the sugar standards

Sugar	Average Retention Time (min)
Sucrose	32.20
D(+) -Glucose	18.42
D(-) Fructose	16.38
L(+) Rhamnose	13.79
D - Glucuronic acid	19.82 (Main peak)

Tentative identification of the sugars from the chromatograms was made possible using standard sugar samples (Table 27) (Appendix B). The retention time of the peaks on the chromatograms produced from the *H. depressa* aqueous extract could then be compared for tentative identification (Table 28).

Table 28: The retention times of the sugar components of the aqueous *H. depressa* extract

Retention Time (min)	Corresponding Sugar
7.17; 8.23; 10.52; 12.43	No corresponding sugars
13.94	L(+) Rhamnose
18.17	D(+) -Glucose
18.60	D(+) -Fructose
20.16	D - Glucuronic acid

The representative chromatograms for the sugar standards and the *H. depressa* extract are located in Appendix B. The extract was injected 10 times to ensure accurate readings. Table 28 indicates the average of the readings obtained. The chromatograms suggest that the *H. depressa* extract

(Extract B) is a polysaccharide composed of L(+) rhamnose, D(+) -glucose and D - glucuronic acid. Karaya gum contains D-galacturonic acid, D-galactose, L-rhamnose (WHISTLER & SMART, 1953) and D-glucuronic acid (TYLER, BRADY & ROBBERS, 1981). Unfortunately, a sample of Karaya gum and galacturonic acid and galactose standards were not available during the identification process.

7.5 PHARMACOLOGICAL SCREENING

7.5.1 Introduction

In informal settlements, the burning of wood or use of paraffin for cooking and heating is common where electricity is not supplied. Burn wounds are therefore prevalent in these areas. Westernized treatments are generally inaccessible and the local people rely on what is available for the treatment of injury as faster healing is desirable. Some communities use leaves of specific plants to cover wounds and protect them from infection and to keep them moist enabling them to heal faster. Using leaf material with pharmacologically active compounds for example anti-bacterial or anti-inflammatory would hasten recovery.

H. depressa aqueous leaf extracts were indicated to have no or very little anti-bacterial and anti-inflammatory activity in Chapter 4, pages 51 and 54, respectively. No anti-bacterial activity was detected while anti-inflammatory activity for aqueous leaf extracts gave 60 % inhibition which is considered low. For this study the aqueous extracts, Extract A and Extract B were screened.

7.5.2 Materials and Methods

Anti-bacterial Screening

Extract A and Extract B were screened for anti-bacterial activity. Extracts were redissolved in distilled water (50 mg ml^{-1}). Minimal inhibition concentration (MIC) was determined using the serial dilution technique devised by ELOFF (1998). Only Gram-positive bacteria were used, namely *Staphylococcus aureus* and *Bacillus subtilis* as outlined in Chapter 4, pp 41 - 42.

Anti-inflammatory Screening

Extract A and Extract B were screened for anti-inflammatory activity. Extracts were redissolved in distilled water (2.5 mg ml^{-1}). The bioassay was performed according to JÄGER, HUTCHINGS & VAN STADEN (1996), using the prostaglandin-synthesis inhibitor assay (COX-1) as outlined in Chapter 4, pp 42 - 43.

7.5.3 Results

Anti-bacterial Screening

Extract A and Extract B showed no activity over the whole dilution range tested. No activity was expected and this was confirmed with the results obtained. In Chapter 4 of this study, aqueous extracts of *H. depressa* leaf material were shown to have no anti-bacterial activity.

Anti-inflammatory Screening

Extract A and Extract B were screened for anti-inflammatory activity (2.5 mg ml^{-1}). Both extracts exhibited low activity (44 % and 31 % respectively) (Table 29).

Table 29: Percentage inhibition of cyclooxygenase (COX-1) by *H. depressa* aqueous extracts

Extract	Inhibition (%)
Extract A	44
Extract B	31
Indomethacin (control)	68

7.6 THE EFFECT OF α-AMYLASE AND HCl ON *HERMANNIA DEPRESSA* AQUEOUS EXTRACT

7.6.1 Introduction

H. depressa aqueous viscous mucilaginous slime (Extract B) exhibits properties of a bulking agent. With addition to water it swells dramatically absorbing large quantities of water. It may have a use as an appetite suppressant, but more readily as a laxative or against diarrhoea. It is important that it has the ability to pass through the digestive system of the human while still exhibiting the mucilaginous nature and its ability to absorb large quantities of water.

The objective of this experiment was to determine the level of mechanical and chemical breakdown of the extract if it should pass through the human digestive system.

7.6.2 Materials and Methods

Enzyme Hydrolysis

A simple method was devised to simulate the effect -amylase would have on the aqueous *H. depressa* extract (Extract B). *H. depressa* extract is composed of polysaccharides as determined previously in this Chapter.

Ground, dry powder (0.5 g) of Extract B was added to a solution of 100 ml distilled H_2O containing 50 l α-amylase in a Schott bottle. The pH was maintained at 6.9 - 7.1. This technique simulates saliva which is 99.5 % water and 0.5 % solutes (TORTORA & GRABOWSKI, 2000). A control was set up, whereby 0.5 g of Extract B was added to 100 ml distilled water.

The heat stable α-amylase solution from *Bacillus globigii* (1,4- -D-Glucan-glucanohydrolase) was used. This is similar to the α-amylase obtained from human saliva.

The Schott bottle was placed in a waterbath at 37°C (internal temperature of human body) and shaken to simulate mechanical digestion for 1 h. Salivary amylase continues to act on the starches for about 1 h after ingestion, at which time the food reaches the stomach and the stomach acids inactivate it (TORTORA & GRABNOWSKI, 2000).

Acid Hydrolysis

The concentration of HCl secreted by parietal cells is about 150 -160 m mol^{-1} (SANFORD, 1982). Extract B (0.4 g), autoclaved and non-autoclaved, were placed in 4 ml 10 % HCl in a pill vial to determine the extent of acid hydrolysis of the extract. The pill vial was placed in an ultrasound bath for 15

min. Whereafter it was allowed to stand. Changes and any breakdown of the extracts were recorded.

7.6.3 Results

Enzyme Hydrolysis

After 1 h, the extract in the control and the experiment exhibited the same characteristics. The small quantity of extract (0.5 g) granules had taken up water and swelled considerably. The 100 ml solutions displayed a mucilaginous consistency (Plate 15 D). No hydrolysis of extract was visibly noted. TLC fingerprints confirmed these results as the control and experiment produced similar chromatograms.

Acid Hydrolysis

Carbohydrates are not hydrolysed in the stomach, however, the release of acids may effect the aqueous extract. The extract was therefore tested using a simple test to simulate the action of HCl in the stomach.

On placing extracts into 10 % HCl the solutions became murky, the autoclaved extract more so than the non-autoclaved solution. This may be due to hydrolysis taking place during the autoclaving process. Within 5 min extracts had swollen to more than five times their original sizes, from water uptake from the solution. The extracts exhibited no signs of hydrolysing in solution. After 4 h the extracts had not broken down, but exhibited a mucilaginous nature due to uptake of water. Acid hydrolysis of the extracts was minimal (Plate 15 E).

7.7 DEGREE OF *HERMANNIA DEPRESSA* AQUEOUS EXTRACT HYDROLYSIS IN THE PRESENCE OF BACTERIA

7.7.1 Introduction

This test was performed to determine if there is a similarity between *H. depressa* mucilage and karaya gum in the ability to resist bacterial breakdown. Karaya gum is resistant to bacterial and enzymatic breakdown (MORTON, 1977; PAGE, CURTIS, SUTTER, WALKER & HOFFMAN, 1997). With resistance to bacterial breakdown the mucilage can be used to cover wounds, keeping them wet and aiding in protection from infection.

Why the Chosen Bacteria

Staphylococcus aureus is usually responsible for sores and boils appearing on human skin. If *H. depressa* extracts are used as a replacement of the vaseline base in ointments, it is important that *S. aureus* does not cause the break down of the extract. It has previously been indicated that the aqueous extract does not exhibit anti-bacterial activity (Table 6, Chapter 4).

Escherichia coli is a naturally occurring bacterial strain in the human digestive system. Even though it occurs in minute quantities, by causing rapid hydrolysis of the aqueous extract the extract would not be appropriate in combatting diarrhoea or constipation.

7.7.2 Materials and Methods

Standard Curve

A standard curve measuring the viscosity of the extracts, which was determined by drops min^{-1}, was prepared (Fig. 4). Samples were prepared

of Extract A and Extract B (autoclaved and non-autoclaved) using a variety of dilution gradients in distilled water, from 0 g ml^{-1} to 150 g ml^{-1}, and placed on a shaker overnight to ensure the extract swelled homogeneously in the water.

Two ml of prepared, suspended extract were placed in a syringe (Promex) where the plunger of the syringe had been removed. The syringe was held vertically in place by a clamp facing downwards. The syringe ensured standardization of drops. No air bubbles were formed ensuring accurate results. The viscosity of the varying sample concentrations was determined by counting the drops min^{-1} eluting from the syringe. Results for all four extracts were recorded and plotted in a line graph (Fig. 4) to determine the standard curves of each extract. The standard curves allowed for comparing the viscosity of extracts after they were infected with bacteria.

Experiment

Autoclaved and non-autoclaved extracts of Extract A and Extract B were used in this experiment. Two replicas were prepared of each, for each bacterial strain. Extracts (250 g) were placed in 5 ml distilled water (50 g ml^{-1}) in a small glass flask (25 ml) and shaken overnight. Overnight cultures of Staphylococcus aureus and *E. coli* were grown in Mueller - Hinton (MH) broth, in a waterbath at 35°C. The following day 200 g bacterial cultures were added to each flask containing 50 g ml^{-1} extract solution, shaken in a waterbath (35°C). Extracts were examined after 5 h, 24 h and 48 h.

7.7.3 Results

Standard Curve and Bacterial Hydrolysis of Extracts

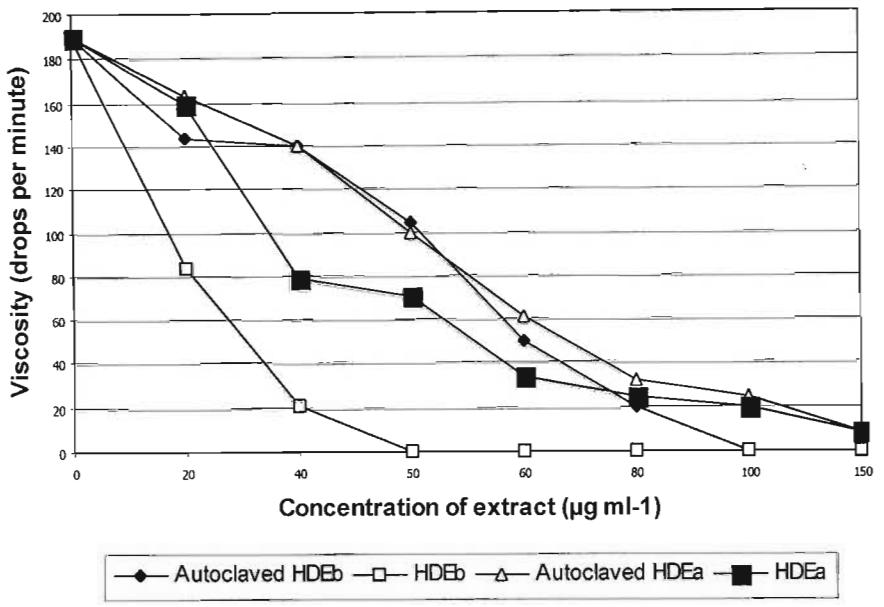


Fig. 4: Standard curve depicting viscosity (drops min^{-1}) for autoclaved and non-autoclaved aqueous *H. depressa* Extract A (HDEa) and Extract B (HDEb)

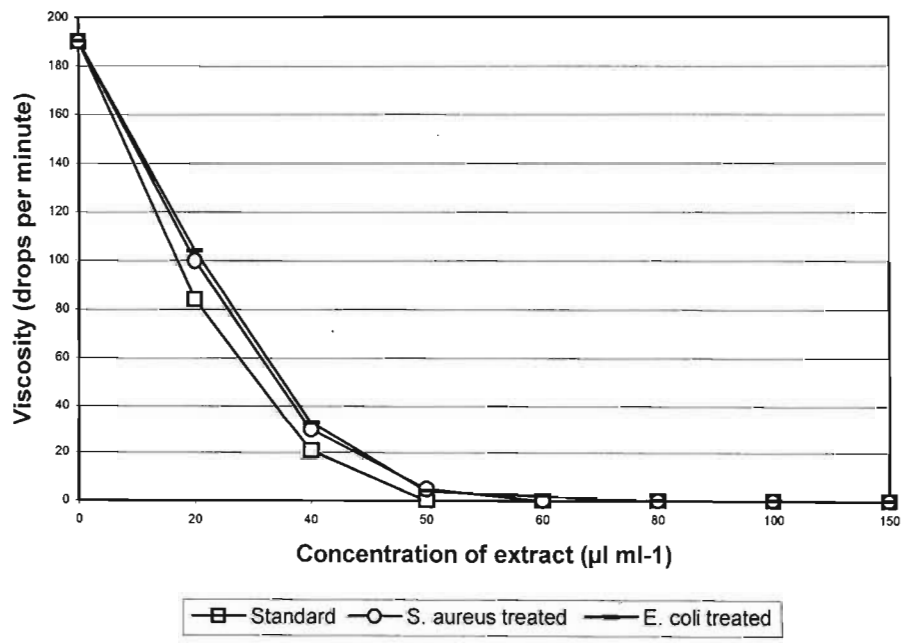


Fig. 5: Viscosity (drops min^{-1}) against a standard curve for non-autoclaved aqueous *H. depressa* Extract B to determine bacterial hydrolysis on the extract after 48 h

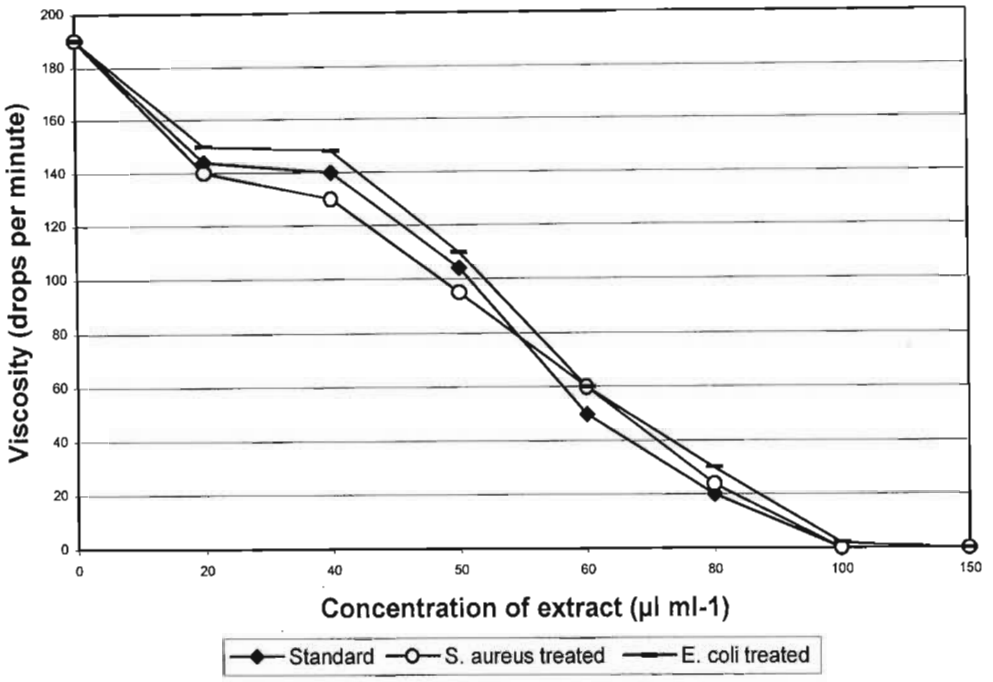


Fig. 6: Viscosity (drops min^{-1}) against a standard curve for autoclaved aqueous *H. depressa* Extract B to determine bacterial hydrolysis on the extract after 48 h

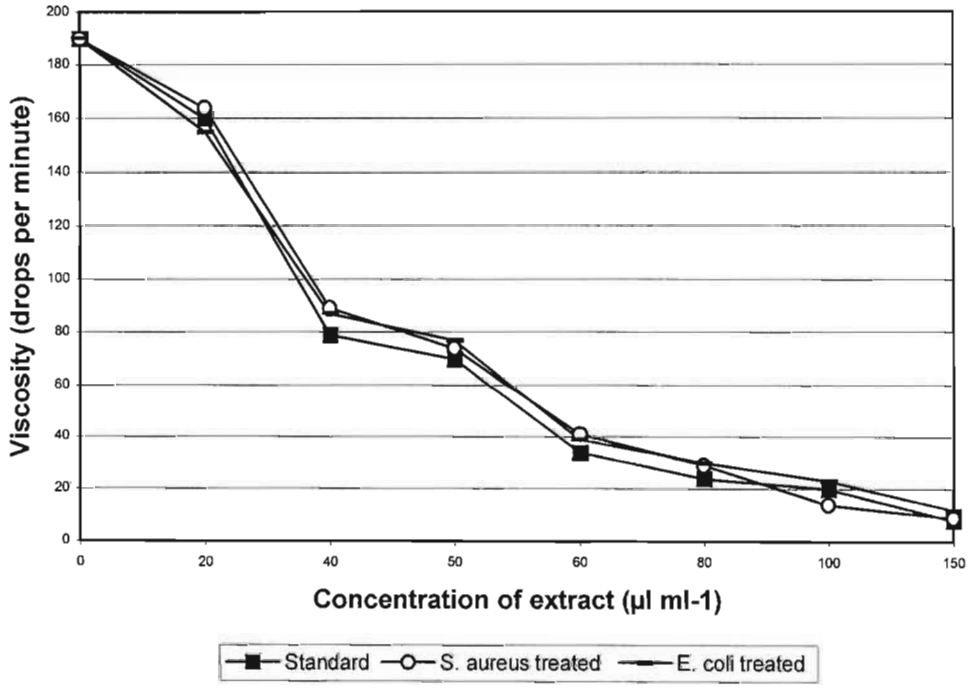


Fig. 7: Viscosity (drops min^{-1}) against a standard curve for non-autoclaved aqueous *H. depressa* Extract A to determine bacterial hydrolysis on the extract after 48 h

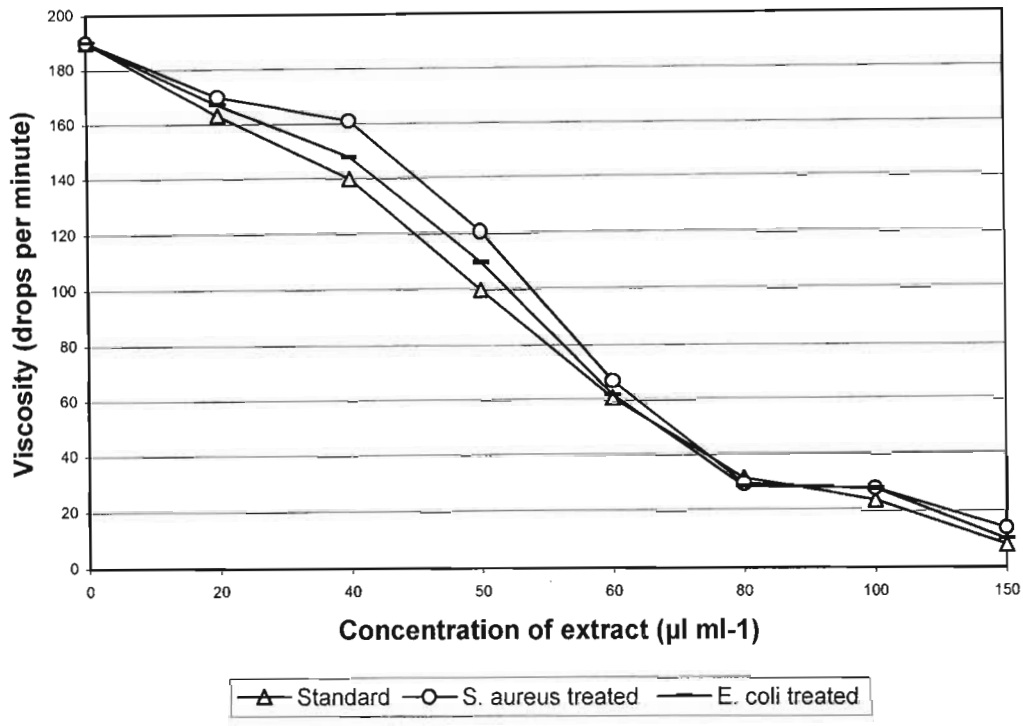


Fig. 8: Viscosity (drops min^{-1}) against a standard curve for autoclaved aqueous *H. depressa* Extract A to determine bacterial hydrolysis on the extract after 48 h

Autoclaving extracts caused a decrease in viscosity of the extracts, the non-autoclaved Extract B extract was more viscous than the autoclaved Extract B extracts and similarly with the Extract A extracts. Karaya gum dissolves when its aqueous dispersions are autoclaved (MANTELL, 1947). Some hydrolysis takes place because the gum occurs as the salt of an acidic polysaccharide and some free acidic groups may exist (WHISTLER & SMART, 1953). This may have occurred in the autoclaved *H. depressa* aqueous extracts resulting in the less viscous nature.

The viscosity of the Extract B extracts is higher than that of Extract A extracts (Fig. 4). The non-autoclaved Extract B extract showed the highest viscosity. At 20 g ml^{-1} the extract was very viscous and at 50 g ml^{-1} no more extract passed from the syringe.

Measuring viscosity of the extracts was a difficult procedure as the extracts swell in water, but do not necessary dissolve in it, therefore many solutions are lumpy and these lumps clog the syringe especially in the more viscous, slime extracts. The extracts were ground to a powder as gums are best powdered. If they are unpowdered, addition of water yields a non-homogenous colloidal solution. If finely powdered, each particle swells and the appearance and behaviour of the solution is more likely to be a homogenous solution (MANTELL, 1947). The extracts were autoclaved in case they contained other bacteria or pathogens that would possibly effect the results.

The extracts were treated with bacterial strains of *S. aureus* and *E. coli* and were examined after 5 h, 24 h and 48 h. Viscosity was recorded after 48 h. Over time there were no changes. The *S. aureus* and *E. coli* bacteria did not hydrolyse the extracts and therefore their viscosity remained constant. This is apparent by comparison with standard curves (Figs 5 - 8). The bacterial-treated extracts generally showed a slightly less viscous nature. This can be attributed to the addition of MH broth to these solutions.

7.8 THE EFFECT OF 60 % ETHANOL ON HERMANNIA DEPRESSA AQUEOUS EXTRACTS

7.8.1 Introduction

A test was performed to determine if there is a similarity between *H depressa* mucilage and Karaya gum as the latter is said to swell in 60 % alcohol (LEUNG & FOSTER, 1996).

7.8.2 Materials and Methods

Dried pieces of Extract A and Extract B extracts were placed in pill vials containing 10 ml of 60 % ethanol and allowed to stand. Changes in the extracts were recorded.

7.8.3 Results

Small bubbles appeared from Extract A. A golden brown pigment was released within seconds of it being added to the 60 % ethanol solution. Within five min the extract had begun to swell, as it absorbed water from the solution. After 10 min the extract was already more than five times its original size. After a period of 18 h the 60% ethanol solution was golden brown and the piece of extract was very brittle. The solution was slightly viscous (Plate 15 F).

Small bubbles appeared from Extract B. After 10 min the solvent solution remained clear. After 18 h, a yellow pigment had been released into the 60 % ethanol solution. The piece of extract remained the same size and was very hard. It did not take in water from the 60 % ethanol solution. The alcohol concentration in the solution inhibited the ability of the extract to absorb water (Plate 16 F). The *H. depressa* extract did not exhibit the same property as Karaya gum as it did not swell in 60 % ethanol.

7.9 GENERAL DISCUSSION

In addition to the mucilage formed by the aqueous extract of *H. depressa* leaf extract; leaf and twig extracts of *D. burgessiae* and *D. cymosa*, leaf extracts of *S. murex*, twig extracts of *H. depressa* and the unripe fruit extract of *C. greenwayi* also exuded mucilaginous aqueous extracts from dried plant material. The mucilage produced by *C. greenwayi* fruit may be the

release of pectins. Many Sterculiaceae species produce either a gum or mucilage. Some of which have important economical value and are also used medicinally. *S. urens*, for example, yields an economically important gum, Karaya gum, which has extensive use in food products, cosmetics and medicine.

Bulking Agent - Appetite Suppressant, Diarrhoeal and Constipation Control

The production of mucilage and the expected ability of the extract to pass through the human digestive system without hydrolysing are in line with its uses in traditional medicine. Leaf sap in water is used for stomach ache, having a purgative effect (WATT & BREYER-BRANDWIJK, 1962), resulting in the relief of constipation. It is also used in a mixture with other plants in the treatment against diarrhoea (HUTCHINGS, HAXTON SCOTT, LEWIS & CUNNINGHAM, 1996).

Sterculiaceae species which are used as bulking agents in appetite suppressing includes karaya gum, which is not digested or absorbed by humans. It is used as a filler and is an ingredient in some weight loss formulations (LEUNG & FOSTER, 1996). In Portuguese Guinea many tribes use the kola nut (*Cola natida*) which is consumed at any social gathering. It reduces the craving for food considerably (MAY & MCLELLAN, 1971). This function may be a property of the fruit acting as a bulking agent.

The idea of requiring a bulking agent in rural Africa may seem extreme if it is to be used as an appetite suppressant. However, mass production and selling of the product on the world market would result in bringing money into the poor rural communities.

However, more effective in the rural areas would be the use of the easily prepared aqueous extract in combat against diarrhoea and constipation, a form of cheap, natural 'drug'. These are common ailments in informal settlements where clean water and good sanitation are lacking. No vaccines are available against the most frequent gastrointestinal infections. The control of these infections requires preventative healthcare measures.
The dried, crushed leaf material could be ingested or soaked in water, dried, ground with a mortar and pestle, then ingested. Local herbalists and 'muthi' shops could prepare the homogeneously ground extract. Mixing with food would ensure it passes easily through the digestive tract.

The bloated feeling that may result after ingestion would be a slight discomfort to endure in order to rid the problems of frequent diarrhoeal and constipation problems. Swallowing bulk extract could cause it to lodge in the esophagus. Attempts to wash it down with water would result in swelling and further obstruction, necessitating surgical removal (THOMPSON, 1989). The problem could be overcome by ensuring patients mix a controlled amount of the fine powdered gum with food.

Wound Healing

The covering of wounds is a necessity which has always been practised by humans (ONAYADE, ONAYADE & SOFOWARA, 1996). It was for a long time the only branch of the healing arts (FISH & DAWSON, 1967). There is a high risk of infection through an open wound. Wounds should not be allowed to dry out, as desiccation kills healthy cells (ONAYADE, ONAYADE & SOFOWARA, 1996). In plants, gums and related compounds effect wound healing by acting as protective agents that cover them (ROSS & BRAIN, 1977). Cleaning of a wound followed by occlusion is important in preventing dirt and exudates from accumulating which may lead to bacterial infection. Healing is enhanced when plants with anti-septic activity are used

(VAN WYK & GERICKE, 2000). *H. depressa* aqueous extracts do not exhibit significant pharmacological activity and would therefore not aid pharmacologically in the healing process of wounds if used to cover them. They may, however, still protect the wound from new infections and keep the wound moist as it heals. Some plants used in dressings contain mucilage, which provides a mechanical barrier and also keeps the wound from drying out (VAN WYK & GERICKE, 2000). *H. depressa* leaves are small (40 x 25 mm) and are inappropriate to wrap around wounds. The mucilage extracted from the leaves can be used as a carrier for topical applications. The application would allow the skin to breath, which is preferable to lipophilic applications which include Vaseline, a common application carrier of ointments.

A number of *Hermannia* species are used in the treatment of wounds. The southern Sotho use the crushed root of *H. coccocarpa* K. Schum as a plaster for wounds and for the treatment of burns. *H. erodiodes* O. Ktze is an African remedy used to treat burns. The Europeans apply an infusion or a decoction of *H. cuneifolia* Jacg. to sores, and the powdered leaves of *H. veronicaefolia* Hochst to suppurating wounds (WATT & BREYER-BRANDWIJK, 1962).

7.10 SUMMARY

- The *H. depressa* aqueous extract can be described as a polysaccharide composed of L(+) rhamnose, D(+) -glucose and D - glucuronic acid, with the dispersions of gum in water having a slightly basic pH of 7.1 - 7.3. The extract does not dissolve or hydrolyse in boiling water. After 1 y in storage the dried, ground extract still absorbs large quantities of water and takes on a mucilaginous nature.

- A number of basic experiments suggested that the extract had no anti-bacterial or anti-inflammatory effects, however, it was not hydrolysed by α-amylase enzyme, HCl or bacteria. It is expected that its passage in the human digestive tract would not affect its mucilaginous properties.

- It is important to determine the toxicity level of the extract for human consumption

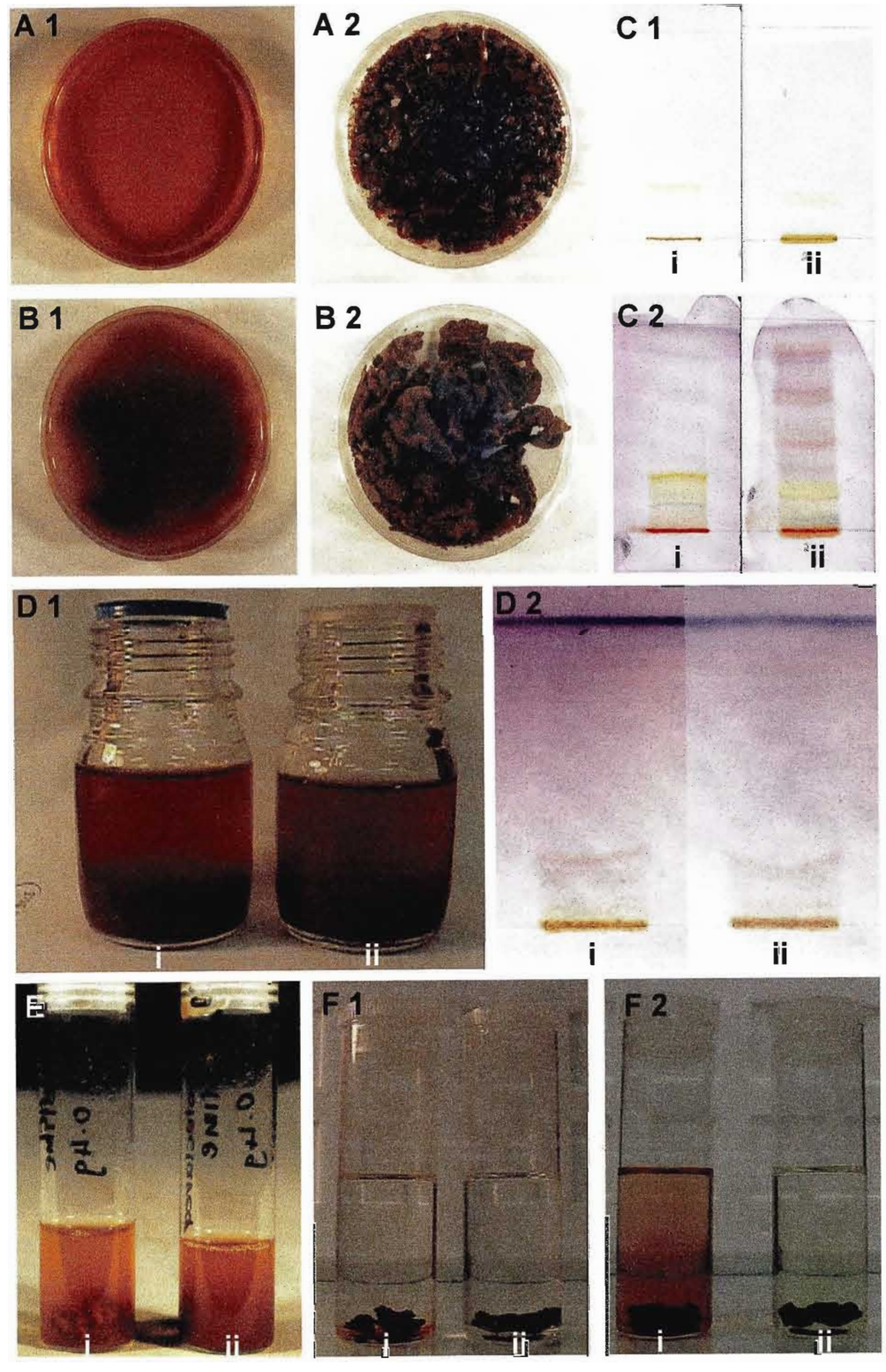


Plate 15 *Hermannia depressa* aqueous leaf extract. (A) Extract A (1) a slight viscous liquid when wet and (2) brittle and darker in colour when dry. (B) Extract B (1) a very viscous, mucilaginous slime when wet and (2) dark and hard when dry. (C) TLC fingerprints of the aqueous extracts (i) Extract A and (ii) Extract B run in a solvent solution of chloroform, methanol, water (60:35:8)(v/v/v), viewed by (1) white light and (2) after staining with anisaldehyde spray reagent. (D) Results of amylase hydrolysis test of Extract B after 1 h in (1) (i) water control and ii) 5 % amylase solution, both indicating much swelling from water uptake and no hydrolysis of extract. These results were confirmed by TLC fingerprints of Extract B after 1 h in (2) (i) water control (ii) 5 % amylase solution. (E) Results of HCl hydrolysis of Extract B after 4 h in (1) 10 % HCl, and (2) water control suggested there was no hydrolysis of the extract. (F) (i) Extract A and (ii) Extract B after (1) 1 h and (2) 18 h in 60 % ethanol solution.

CHAPTER 8

THIN LAYER CHROMATOGRAPHIC FINGERPRINTING

8.1 INTRODUCTION

Thin Layer Chromatographic (TLC) fingerprinting is an efficient way of characterising analytical fingerprints of plant extracts (WAGNER & BLADT, 1996). A photographic atlas is an aid to the identification of plants used in traditional medicine or the identification of a plant species from dried specimens. If toxic plants have been used in a potion taken by a patient, a comparison with TLC fingerprint chromatograms could establish the identity of the toxic plant allowing for quicker, more direct treatment. A chromatogram of a species may vary depending on the area the collection took place and the time of year of harvesting. Plants adapt the ability to function adequately through seasons of extreme heat with abundance of rain to long periods of cold with little rain. A variety of new components may be produced by the plant during each season. It is therefore important to record all collection information.

For TLC fingerprinting results to be employable, they must be carried out under the same conditions, resulting in good colour visibility and clear separation of the different components for identification. Note must be taken of harvesting time of the material used. TLC fingerprints were developed of the seven Sterculiaceae species.

The aim of this study was to establish the fingerprinting chromatograms of each species studied. Focussing on components within the same species obtained using different solvents and on components of different species from the same family.

8.2 MATERIALS AND METHODS

Preparation of Extracts

Plant material (1 g) was extracted in 10 ml water and ethanol and (2 g) 20 ml ethyl acetate (only small quantities of extracts are obtained from ethyl acetate, hence more plant material was required). Plant material was placed in an ultrasound bath for 30 min, filtered and dried. The residues were redissolved at a concentration of 10 mg ml^{-1}.

Loading of Extracts

Extracts were loaded by streaking onto plastic TLC plates (Merck Silica Gel 60 F_{254}, 10 x 10 cm) in 25 µl aliquots, 1 cm band, 1 cm apart (0.5 mg extract). Silica gel is an efficient absorbent for the TLC separation of most drug extracts (TAYLOR, 1999)

TLC Separation

Solvents hexane and ethyl acetate were used in the separation of components as they moved up the TLC plates. Solutions of hexane: ethyl acetate tested were 1:2 (v/v), 1:1 (v/v) and 2:1(v/v). The best separation of the extract components was 1:1(v/v) of the ethanol and ethyl acetate plant extracts. The water extracts showed good separation in solvent solutions of chloroform: methanol: water (60:35:8)(v/v/v). All plates were developed over a distance of 8 cm.

Visualization of TLC Plates

Once the extract had been separated by TLC and the solvents evaporated the

separated components were visualised under visible (white) and ultraviolet light at 254 nm and 366 nm. The TLC plates were then stained with anisaldehyde spray reagent and heated at 110°C for 8 min, allowing for the colour development of various components previously not visible.

Data was recorded by scanning (visible light and anisaldehyde) and taking photographs (UV light) of the different fingerprints of the various species. R_f values determine the locality of the various compounds. This value was calculated by determining the ratio of the distance travelled by the band maximum to the distance travelled by the leading edge of the mobile phase.

Ultra-violet Light

UV light detection is non-destructive (GIBBONS & GRAY, 1998) making it a favourable technique for compound detection. Photographs were taken with a Pentax ME super camera, with a 200 mm lens attached. The F-stop was set at F 11, and the shutter speed was set on B. Photos were taken in a dark room under UV light of 366 nm (long wavelength exposing blue light) and 254 nm (short wavelength exposing pale green light). The shutter release was held down for intervals of 30 sec, 1 min and 1 min 30 sec; and 10 sec, 15 sec and 20 sec respectively. However, not all compounds absorb UV light, so a spray reagent was also used.

Anisaldehyde Staining

This is a destructive method. A common method of rendering colourless substances on chromatograms visible is to spray them with a reagent solution (JORK, FUNK, FISCHER & WIMMER, 1990). Spray reagents, when applied to a layer, produce colours from colourless substances. Some spray reagents are very specific, but many others will react with a broadly based type of compound

(HOUGHTON & RAMAN, 1998). Anisaldehyde-sulphuric acid is a universal reagent for natural products that makes colour differentiation possible (JORK, FUNK, FISCHER, WIMMER, 1990).

The anisaldehyde stain was prepared in a fume hood. Ethanol (465 ml) was cooled in a Schott bottle, on ice. Glacial acetic acid (5 ml), concentrated sulphuric acid (13 ml) and para-anisaldehyde (13 ml) were added to the cold ethanol in the order mentioned, keeping the solution constantly cold. A clean glass pipette was used for each chemical as they activate each other when not in the whole mixture The mixture was stored in the Schott bottle (covered with tin-foil to eliminate all light) in a freezer. The clear mixture turns yellow or pink as it 'goes off'.

The TLC plate was sprayed evenly with a glass sprayer (held 25 cm from the plate) in a fume hood with anisaldehyde spray reagent, ensuring the compounds did not 'run'. The surface of the plate was air-dried for a few seconds and heated at 110°C for 8 min in an oven, until the plate turned pink.

8.3 RESULTS AND DISCUSSION

TLC analysis was used to compare the chemical composition of the crude water, ethanol and ethyl acetate extracts of the seven Sterculiaceae species. This enabled for the comparison of extracts of different plant parts within a species and at a species level (Plates 16 - 20). All plant material was collected in early autumn (March/April). The seasonal profiles of the species were not investigated. The R_f values and colours of the various components were compared. The same substances indicate the same R_f values and colours under the same experimental conditions.

On viewing at UV 366 nm light components were clear and distinct, while at UV

254 nm light many components were undetected. For ethanol and ethyl acetate extracts the UV 254 nm chromatograms were not displayed. Ethanol is a relatively polar solvent. TLC fingerprints of ethanol extracts, especially of leaf material, displayed too many compounds to aid in distinguishing minor differences between plant parts or species. The ethyl acetate extracts liberate more distinguishable compounds, resulting in better TLC fingerprint identification. Leaf extracts universally contained an abundance of components. Notably chlorophyll is present in ethanol and ethyl acetate extracts, indicating its very polar nature. Chlorophyll is evident when viewed under visible light as a variety of green shades, blue after staining with ansialdehyde spray reagent and purple or pink at UV 366 nm light.

Comparison of Extracts Within Species

Ethanol and ethyl acetate extracts from the same species largely separated many similar compounds (Plates 16 - 19), with a few additional compounds visible from either extract. Aqueous extracts (Plate 20) separated compounds different to ethanol and ethyl acetate extracts, and could not be compared with them in relation to R_f values due to developing in different solvent solutions.

Comparison of Extracts Between Species

There were a number of similarities within a genus. A comparison of TLC fingerprints from the three *Dombeya* species screened, *D. rotundifolia, D, burgessiae* and *D. cymosa*, indicated numerous compounds exhibiting similar chemical profiles. This is evident in plates 16 and 17, where solid arrows (right pointing) indicate compounds present in *D. rotundifolia, D. burgessiae* and *D. cymosa*. The non-solid arrows (left pointing) indicate compounds present in *D.burgessiae* and *D. cymosa* only. These compounds exhibit the same R_f values and colours, forming the basis of the qualitative process of TLC.

Differences were observed on a quantitative level by comparing the relative intensities of the spots.

A comparison of TLC fingerprints of the two *Cola* species: *C. natalensis* and *C. greenwayi*, indicated a number of similar components (Plate 18). Solid arrows indicate the common components. However, as revealed in Chapters 4 and 5, these two species of *Cola* exhibit different pharmacological and phytochemical properties. *C. greenwayi* exhibited good anti-inflammatory and anti-bacterial activity, while *C. natalensis* exhibited no or little activity. *C. greenwayi* therefore contains a number of compounds not associated with *C. natalensis*. These compounds are in all likelihood not visible as they are available in very small quantities.

H. depressa TLC fingerprint chromatograms exhibit many compounds not present in any of the other species investigated. *H. depressa* exhibits a different growth form to the other species. It is a herbaceous plant growing in grasslands. This may account for the numerous compounds as it adapts to its lifestyle.

Anti-bacterial Compounds

The symbol 'x' on the plates (Plate 16 - 19) indicates the position of anti-bacterial compounds, identified by bioautography and isolated by bioassay guided fractionation (Chapter 6). Some of the active compounds are not visible on the TLC fingerprint chromatograms as they are not very colourful, even after viewing by UV light and after staining with anisaldehyde spray reagent. This is possibly because their concentration is very low. After further purification the active compounds would be more noticeable.

Saponins and Cardiac Glycosides

The TLC detection of saponins using an anisaldehyde spray reagent is indicated by mainly blue, blue violet and sometimes red and yellow brown zones (WAGNER & BLADT, 1996). Inspection after anisaldehyde staining exhibited yellow, blue violet and red zones in *D. rotundifolia* leaf, twig and bark extracts and *H. depressa* mainly root, but also in leaf and stem extracts, indicating the expected presence of saponins in these species. These results are in line with experiments from Chapter 5. However, specific detection of saponins by TLC is possible with blood reagent only (WAGNER, BLADT & ZGAINSKY, 1984).

The TLC detection of cardiac glycosides by white light is indicated by weak brown, violet and weak blue zones (WAGNER & BLADT, 1984) and under UV 366 nm exhibits blue flourescent, yellow brown or yellow green zones. On inspection it was impossible to point out the position of possible cardiac glycosides on the TLC chromatograms.

8.4 SUMMARY

TLC provides a chromatographic drug fingerprint (WAGNER, BLADT & ZGAINSKI, 1984). TLC fingerprint chromatograms were developed of the seven Sterculiaceae species to achieve a broader chemical knowledge of the species.

- Plant material was collected at the same time of year (March/April). Material was dried and stored under the same conditions and the TLC chromatograms were developed according to a uniform devised technique, making the results obtained employable.

- The presence of similar bands between and within a species was observed and illustrated. Many similar compounds were present within the same genera, notably within the *Dombeya* species.

- The position of anti-bacterial compounds was indicated on the TLC chromatograms. Most were not visible at a low concentration.

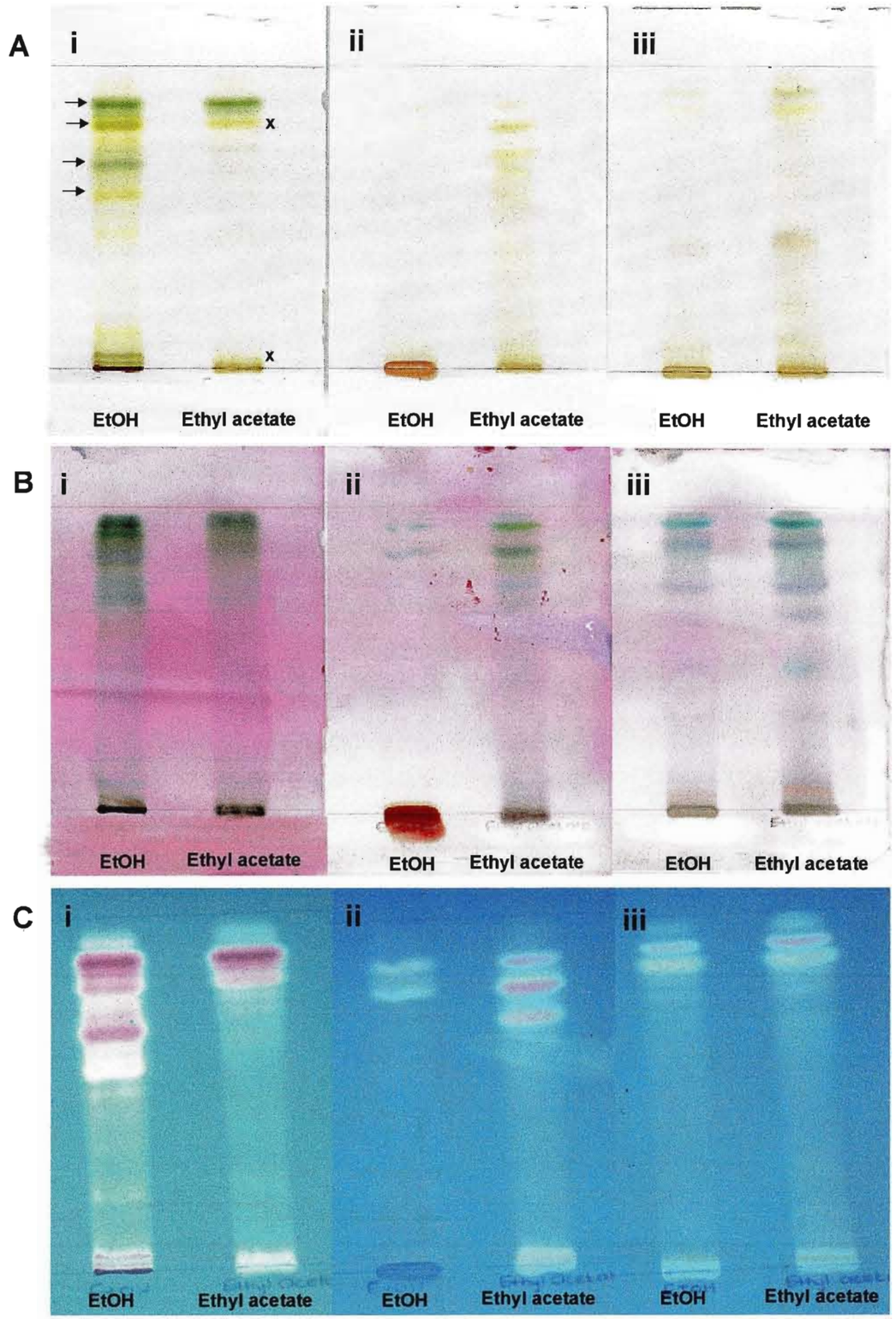


Plate 16 TLC fingerprint chromatograms of *D. rotundifolia* (i) leaf, (ii) twig and (iii) bark material viewed by (A) white light, (B) after anisaldehyde spray reagent staining, and (C) UV 365 nm light. (EtOH - ethanol).

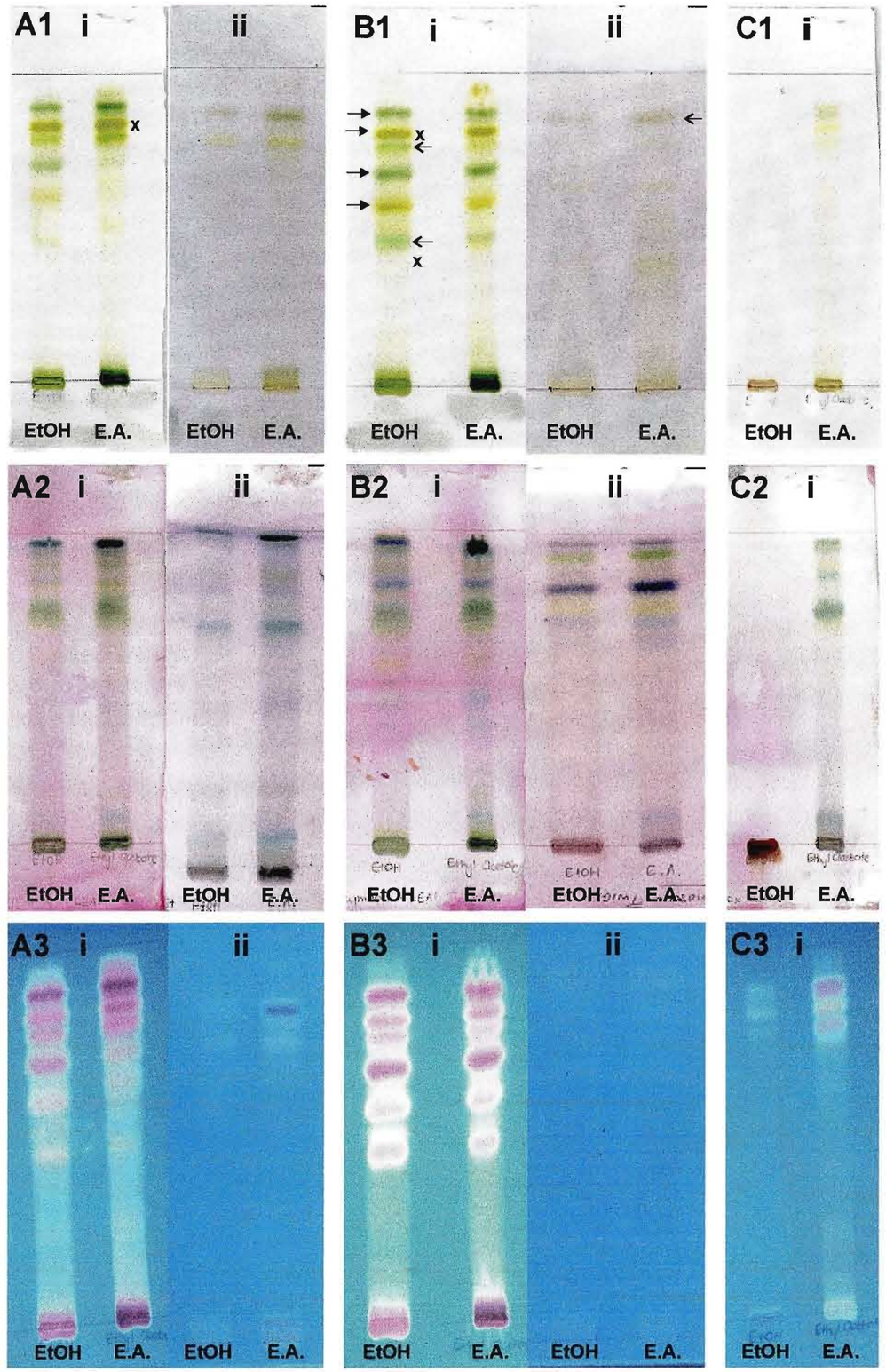
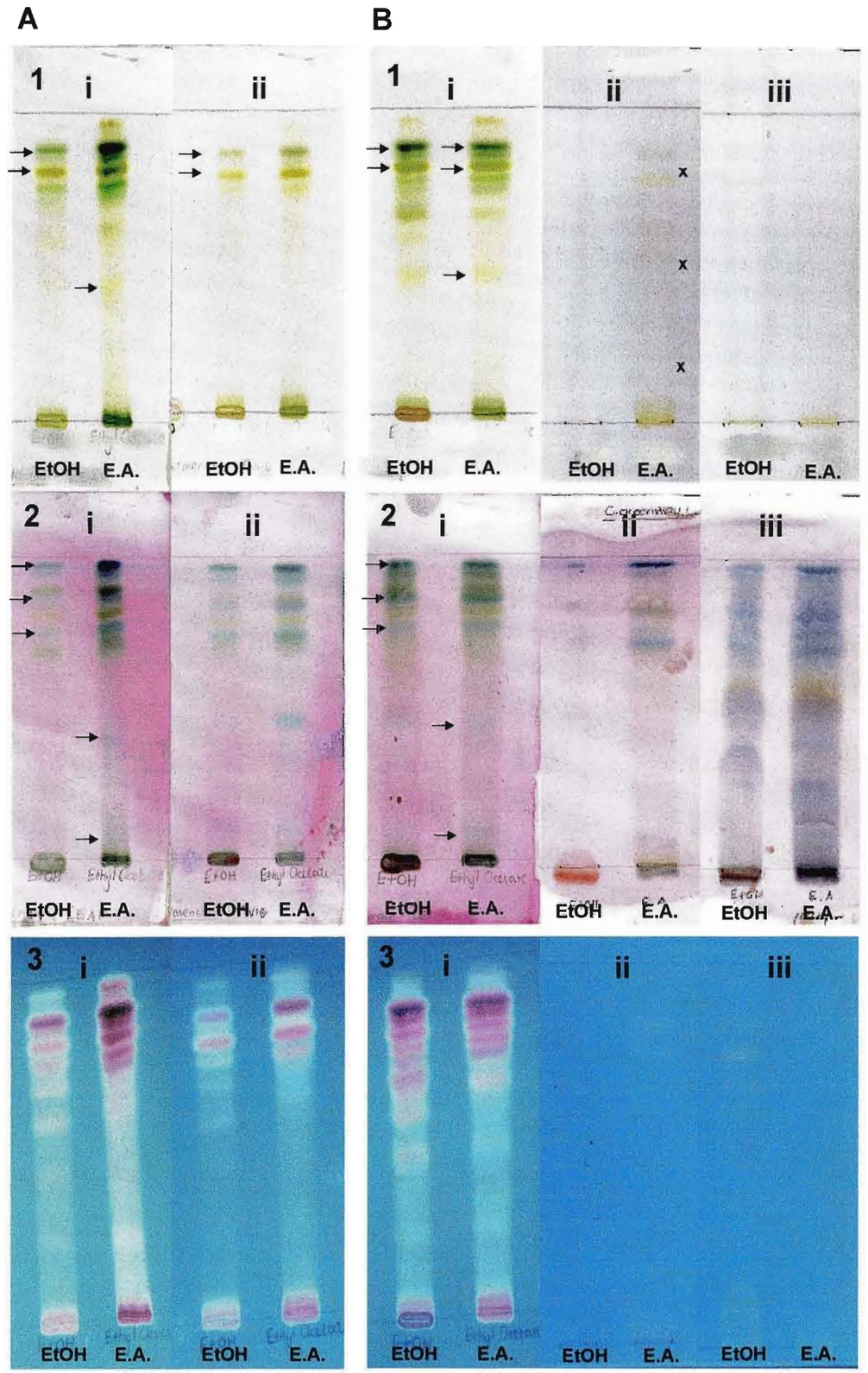


Plate 17 TLC fingerprint chromatograms of (A) *D. burgessiae* and (B) *D. cymosa* (i) leaf and (ii) twig material and (C) *S. murex* (i) twig material viewed by (1) white light, (2) after anisaldehyde spray reagent staining, and (3) UV 365 nm light. (EtOH - ethanol, EA - ethyl acetate).

Plate 18 TLC fingerprint chromatograms of (A) *C natalensis* and (B) *C. greenwayi* (i) leaf, (ii) twig, and (iii) fruit material viewed by (1) white light, (2) after anisaldehyde spray reagent staining, and (3) UV 365 nm light. (EtOH - ethanol, EA - ethyl acetate).



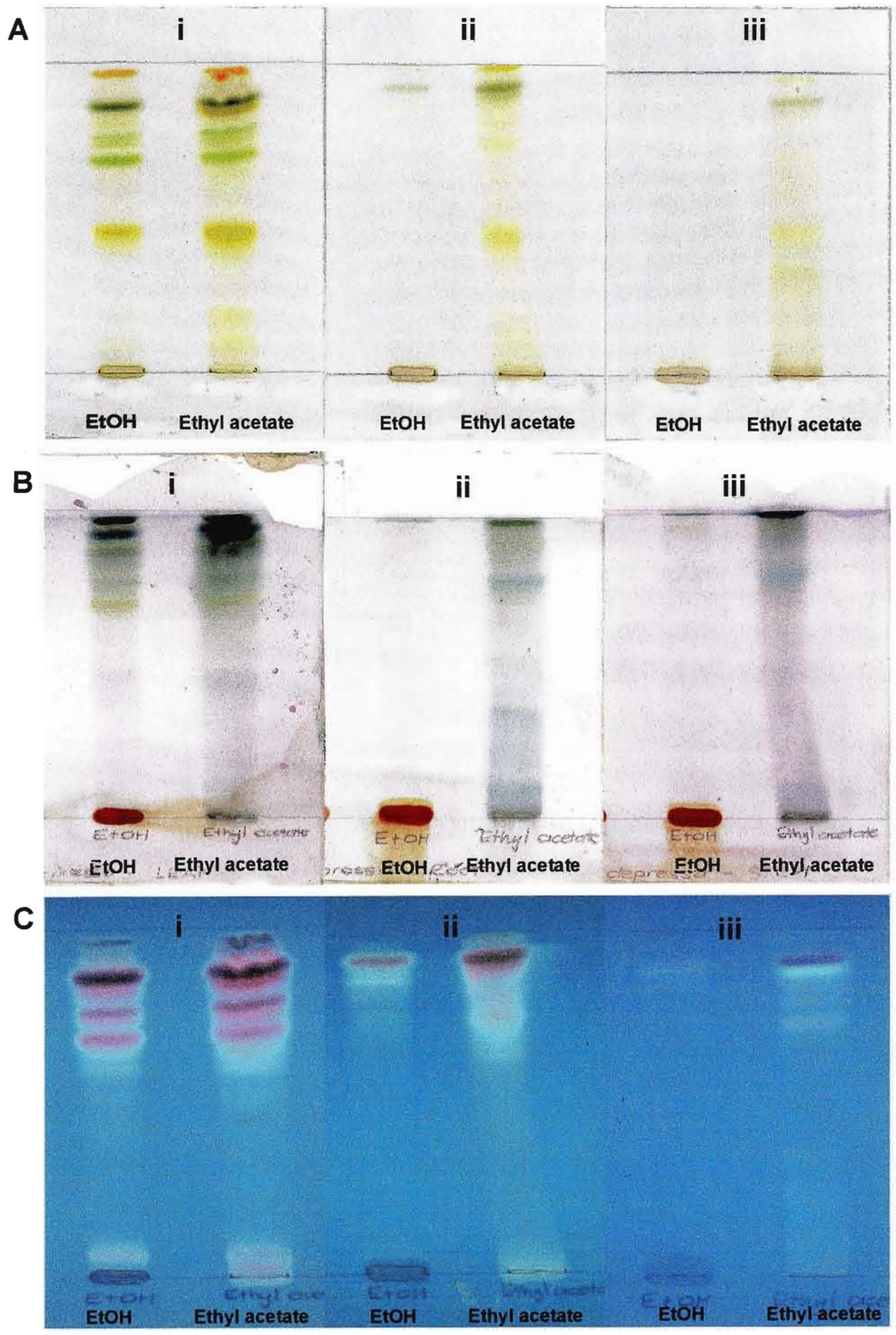


Plate 19 TLC fingerprint chromatograms of *H. depressa* (i) leaf, (ii) stem and (iii) root material viewed by (A) white light, (B) after anisaldehyde spray reagent staining and (C) UV 365 nm light. (EtOH - ethanol).

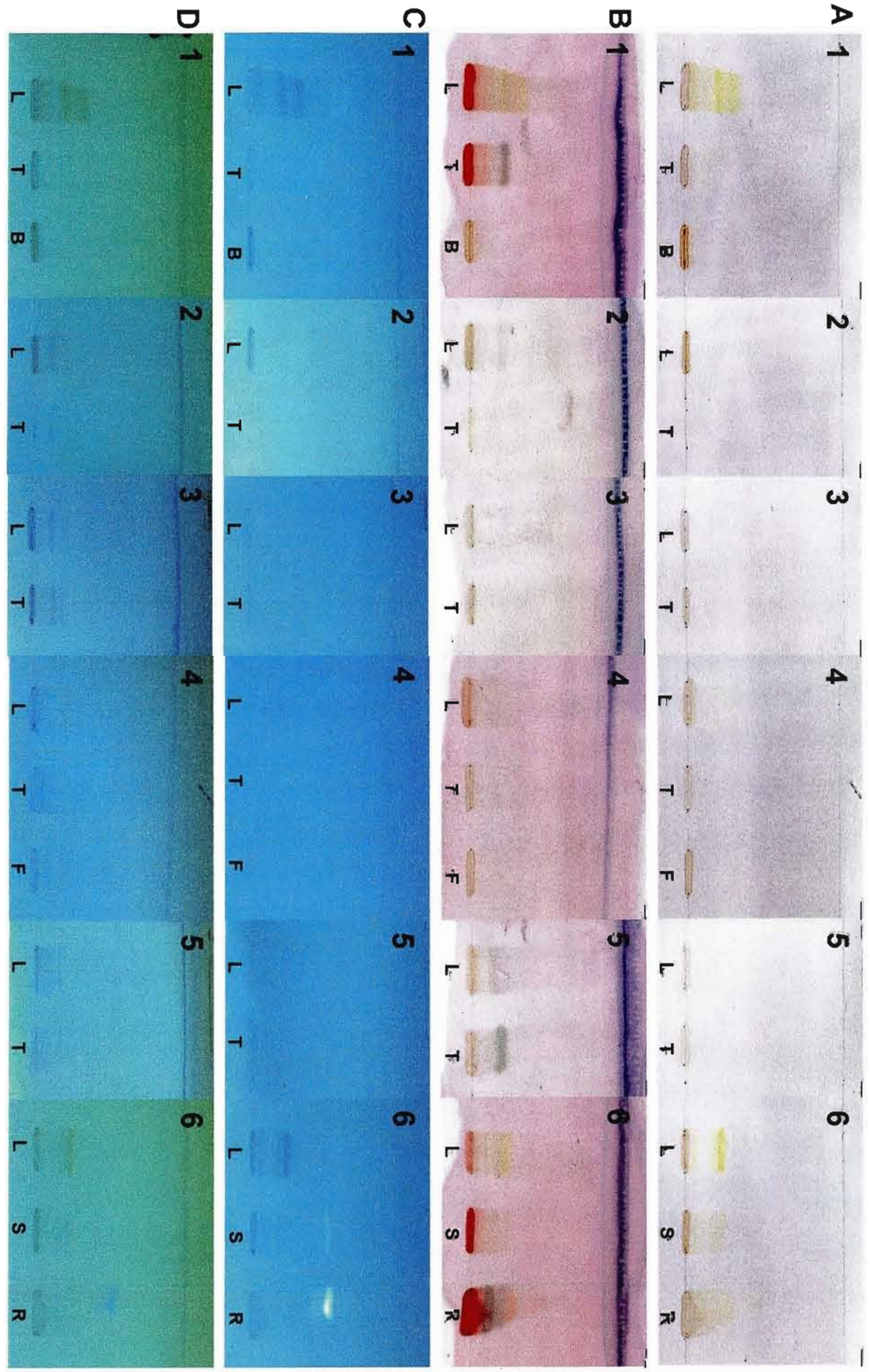


Plate 20 TLC fingerprint chromatograms of water extracts of 1) *D. rotundifolia*, 2) *D. burgessiae*, 3) *D. cymosa*, 4) *C. natalensis*, 5) *C. greenwayi* and 6) *H. depressa* material viewed by (A) white light, (B) after anisaldehyde spray reagent staining, (C) UV 365 nm light, and (D) UV 254 nm light. (L - leaf, T = twig, S - stem, B - bark, F - fruit, R - root).

CHAPTER 9

GENERAL CONCLUSIONS

The use of traditional medicine is widespread within South Africa. Investigation by ethnobotanical screening for chemical constituents and biological activity exhibited by medicinal plants leads to the discovery of new drugs and is important in South Africa in preventing the loss of valuable traditional knowledge which is important for the survival of its culture and traditional beliefs. These studies are important in bridging the gap between traditional and Westernised medicines.

There had been very little previous chemical investigation of South African Sterculiaceae species used in traditional medicine. In this study seven species were biologically investigated, varying in growth type (small herb, shrubs and large trees) and traditional usage, allowing for a broad investigation of the KwaZulu-Natal species of the family Sterculiaceae. Screening revealed good anti-bacterial and anti-inflammatory activity in most of the seven species screened. The presence of numerous phytochemical compounds was also discovered. Many bacteria show a high level of resistance to currently used antibiotics. It is important to focus on the research and development of effective drugs. Five plants containing biological activity in the initial anti-bacterial screening underwent further investigation to isolate the active compounds. The active compounds isolated were all common fatty acids. Biological activity of species corresponded with their use in traditional medicine. *C. natalensis* has no recorded use in traditional medicine and indicated the lack of biological activity. TLC fingerprinting of extracts from the Sterculiaceae was another step undertaken towards increasing the available information on the chemical make-up of the Sterculiaceae species investigated.

Overall it was noted that the pharmacological and phytochemical properties varied between members of the family and within a genus. There were distinct similarities within the same species inhabiting different locations. It was also noted that chemical compounds found in one part of the plant were not always present in other parts of the plant.

Future recommendations related to this research would be to screen the species investigated for more pharmacological activities to generate a greater scope of the active biological diversity. The screening of plant material for anti-inflammatory activity indicated excellent inhibition therefore isolation of these active compounds could give beneficial results.

The investigation of the selected Sterculiaceae species actively used in traditional medicine is an important step forward in drug discovery. This study has resulted in new discoveries and has led to a broader knowledge of the chemistry of KwaZulu-Natal Sterculiaceae species investigated.

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APPENDIX A

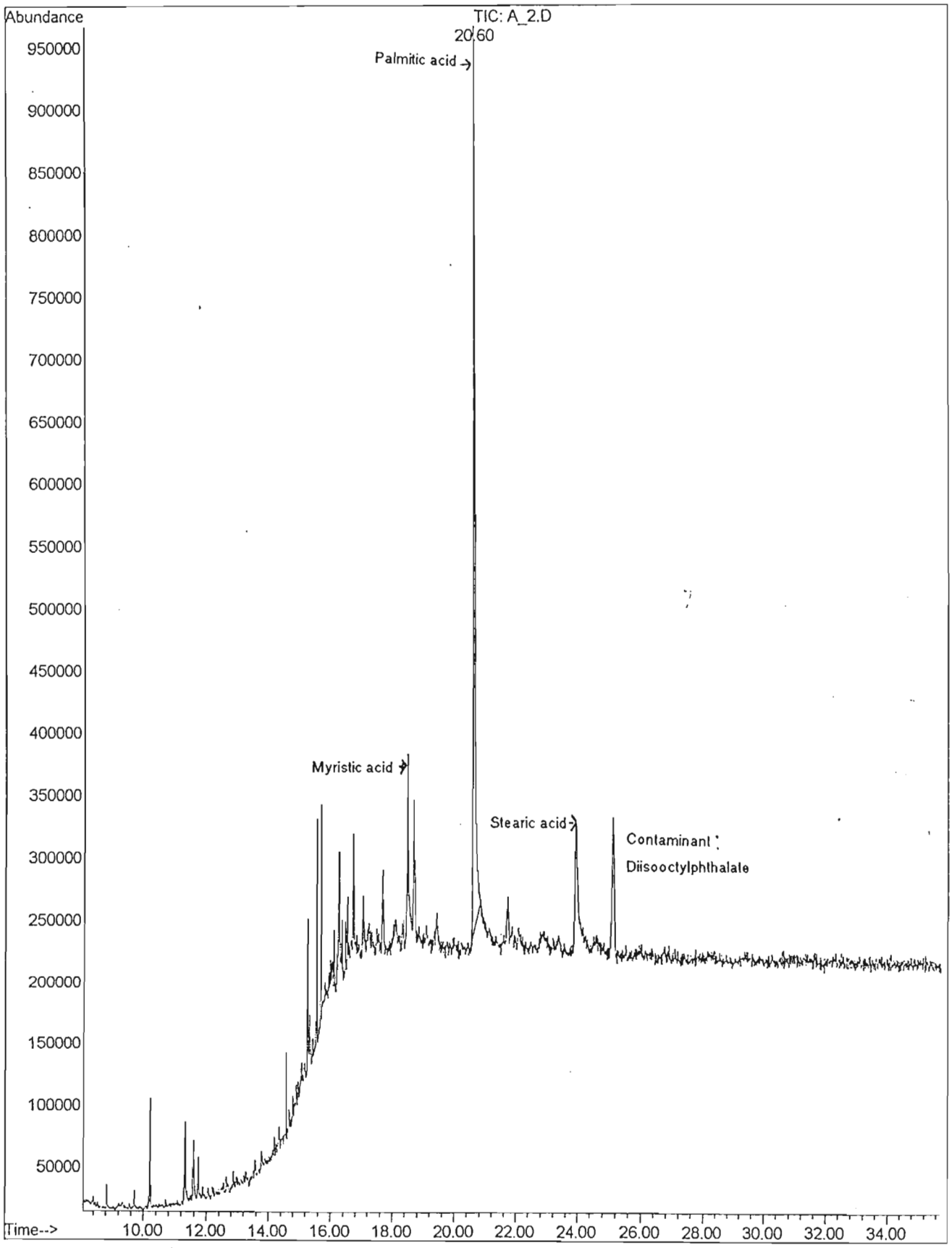

Fig. A.1: *D. rotundifolia* GC-MS spectrum of isolated anti-bacterial Compound A

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Quality : 95
ID : Myristic acid P1035

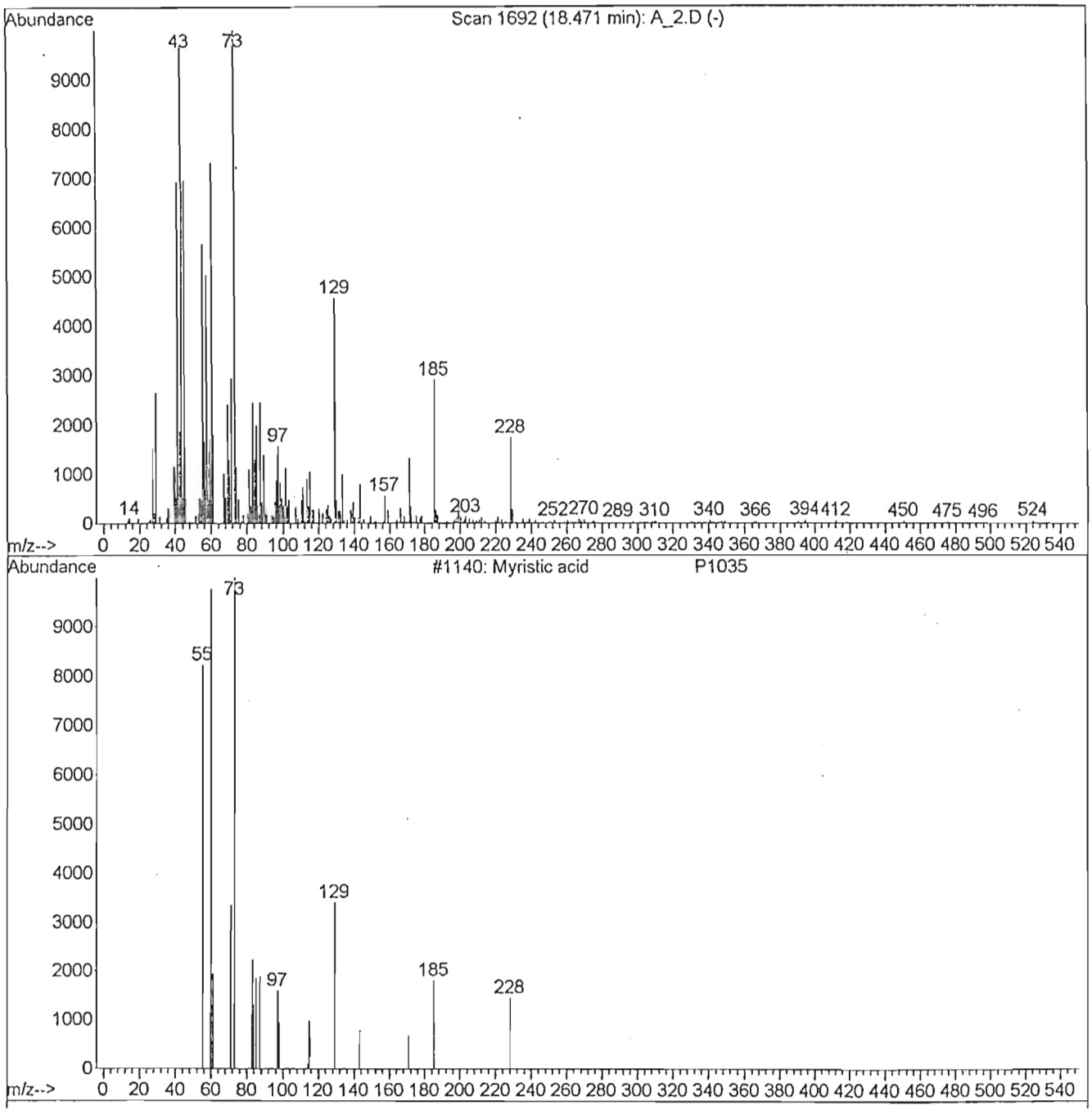

Fig. A.2: GC -MS library search of *D. rotundifolia* Compound A at a retention time of 18.471 min corresponding with Myristic (Tetradecanoic) acid spectrum

```
Library Searched : C:\Database\PMW_TOX2.L
Quality          : 93
ID               : Palmitic acid                    P1210
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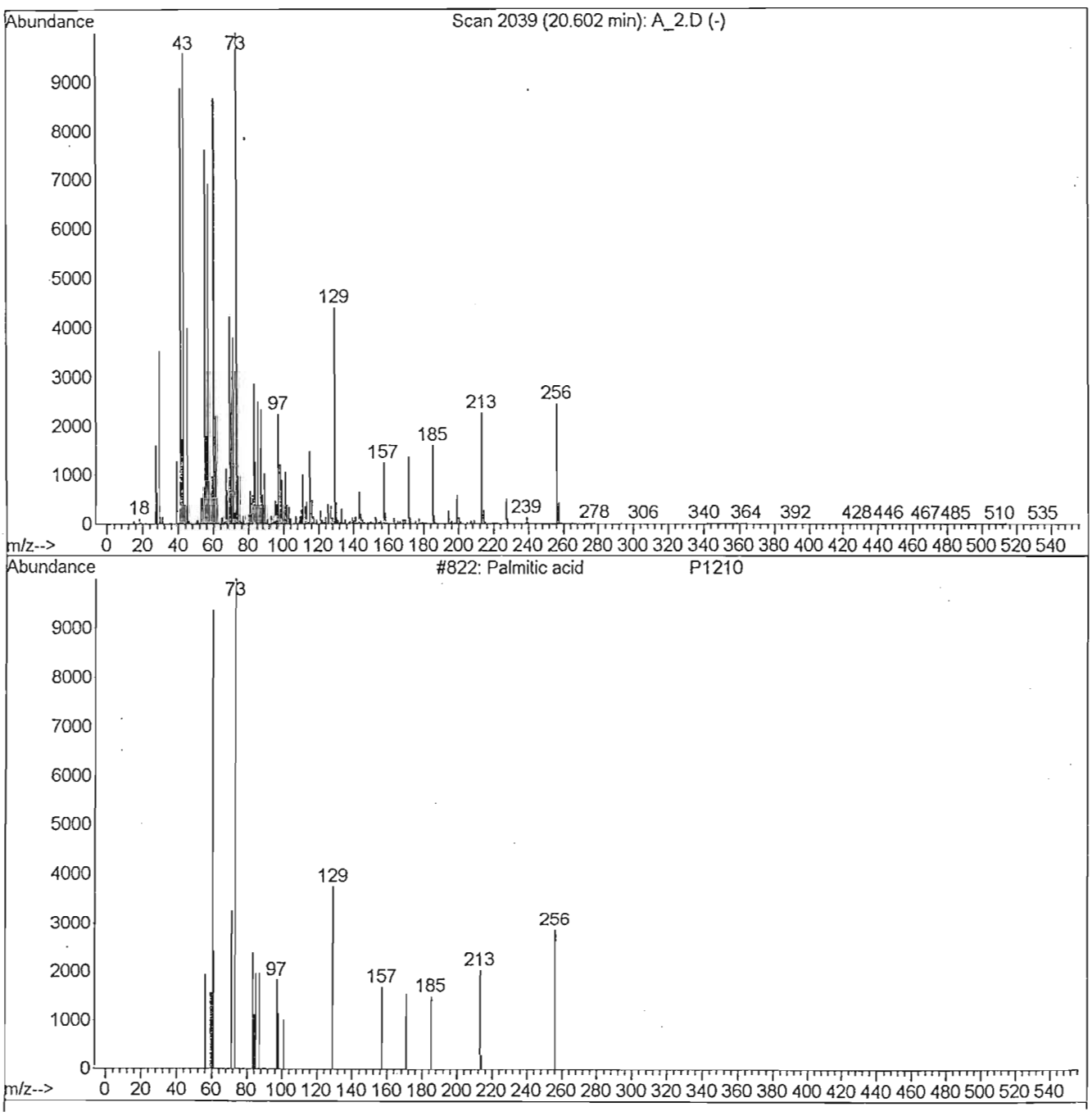


Fig. A.3: GC -MS library search of *D. rotundifolia* Compound A at a retention time of 20.602 min, corresponding with Palmitic (Hexadecanoic) acid spectrum

```
Library Searched : C:\Database\Nist98.l
Quality          : 78
ID               : Octadecanoic acid
```

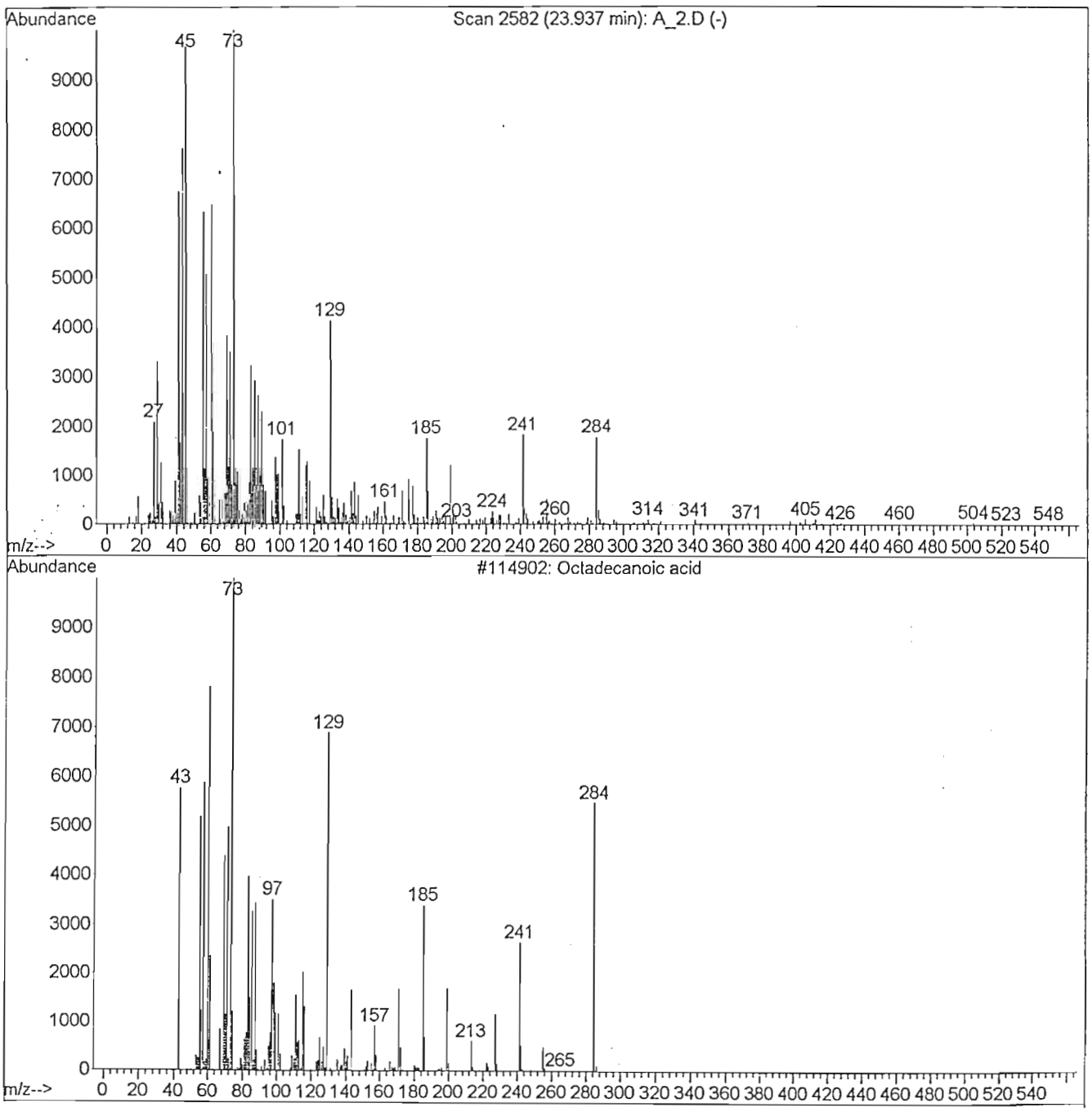


Fig. A.4: GC -MS library search of *D. rotundifolia* Compound A at a retention time of 23.937 min corresponding with Stearic (Octadecanoic) acid spectrum

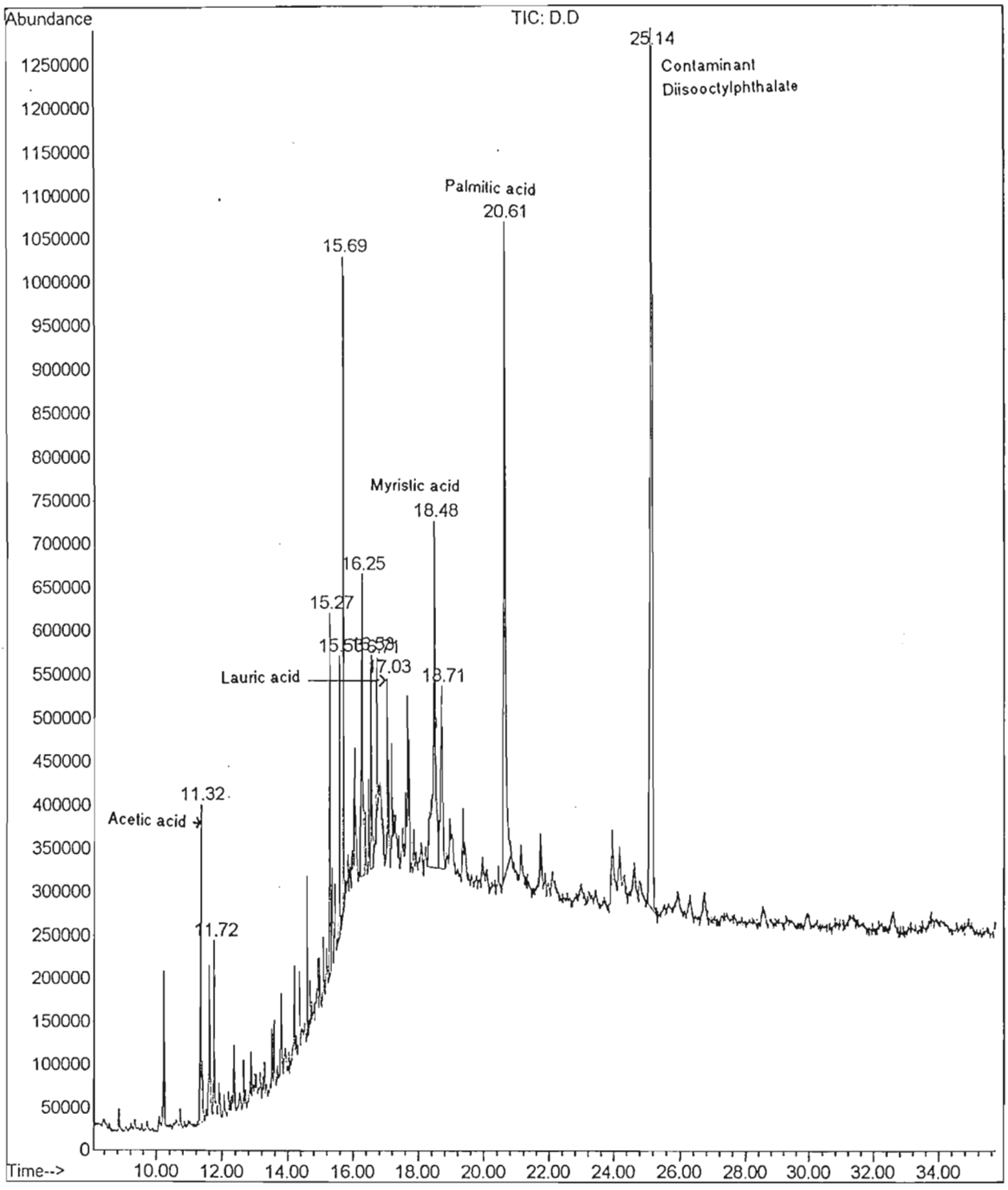
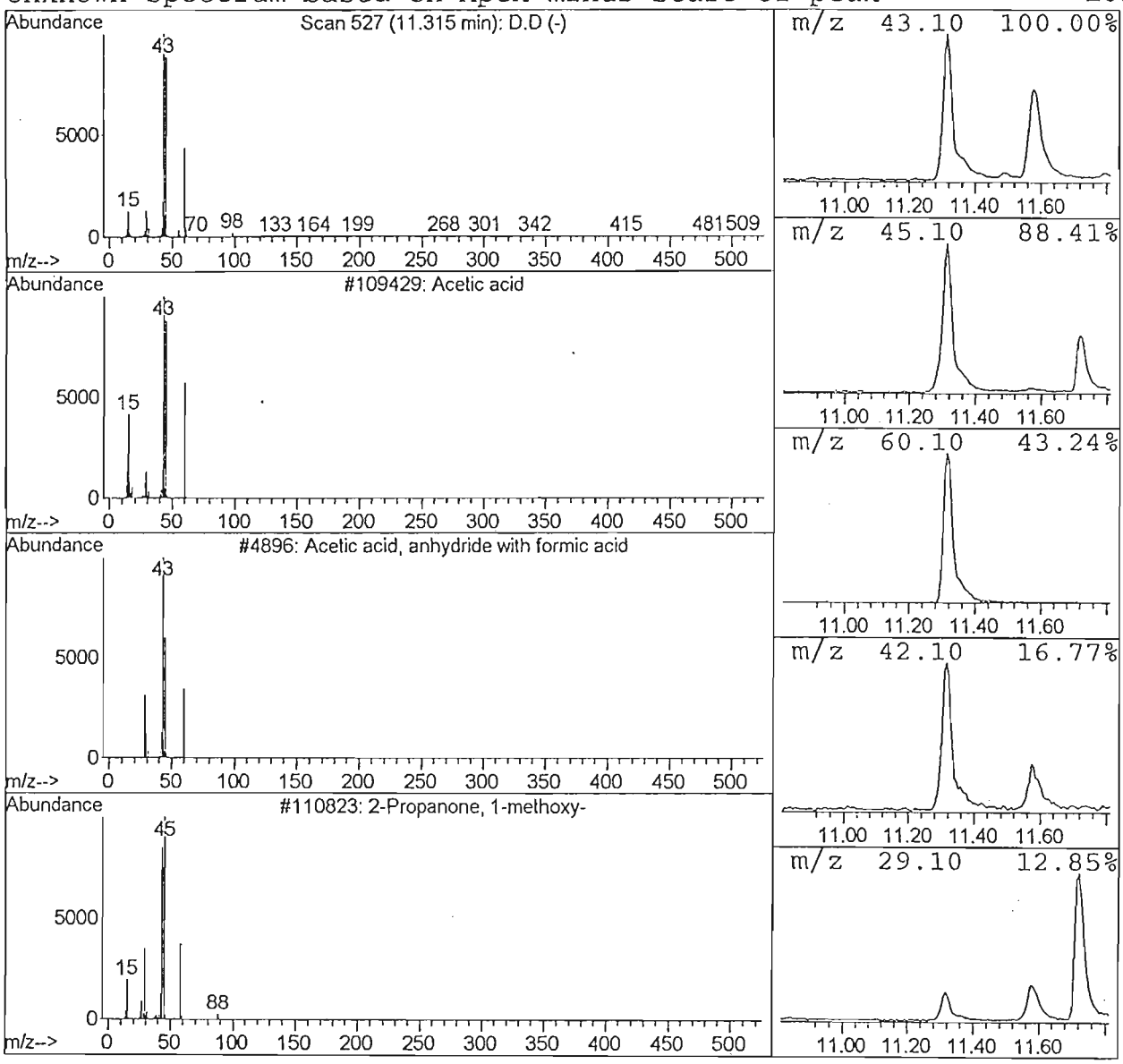


Fig. A.5: *D. rotundifolia* GC-MS spectrum of isolated anti-bacterial Compound B

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex minus start of peak



Peak Number: 1 at 11.32 min Area: 9041574 Area % 5.75

The 3 best hits from each library.		Ref#	CAS#	Qual
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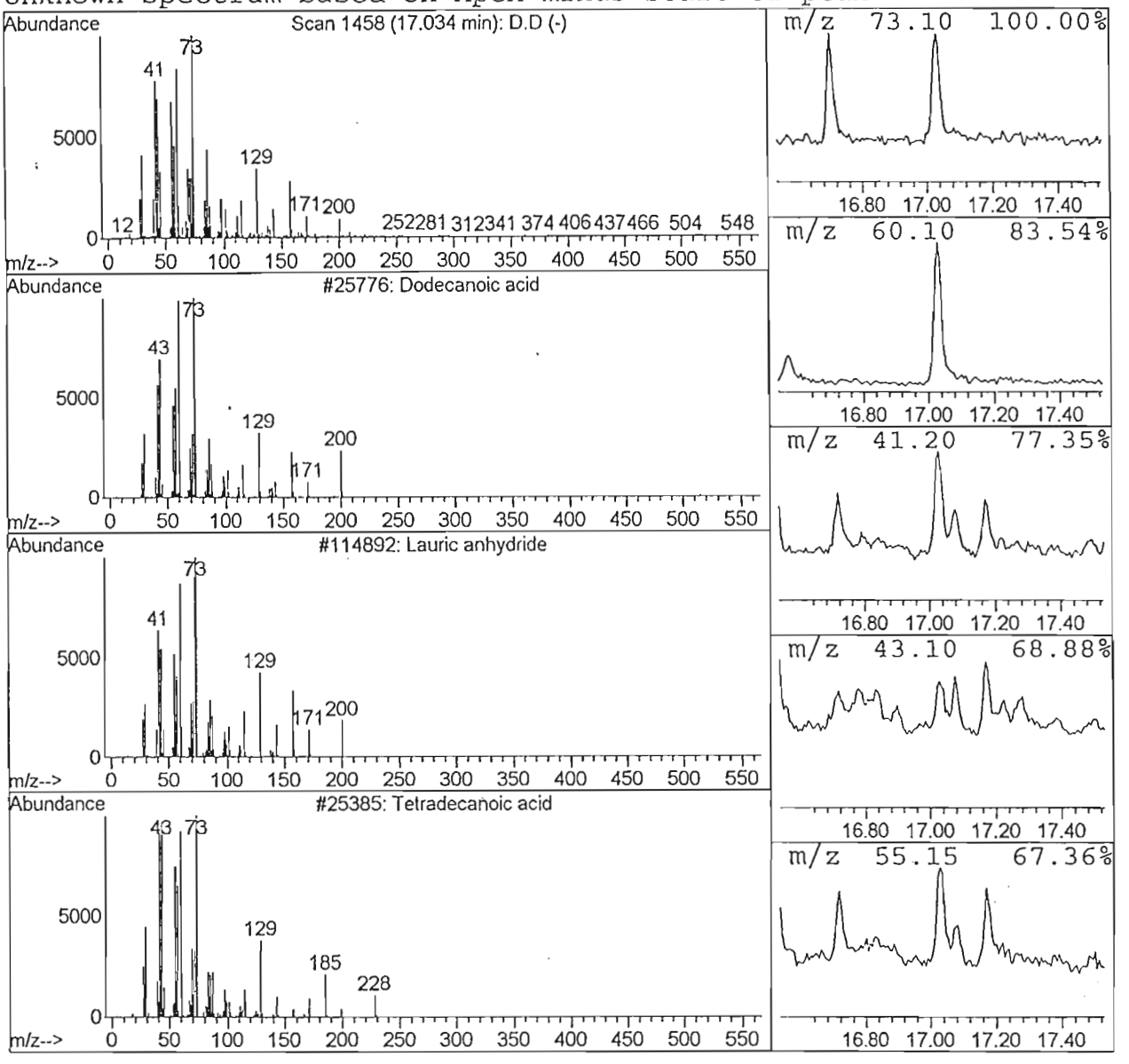
C:\Database\PMW_TOX2.L				
1 Acetic acid	...	1548	000064-19-7	78
2 Isopropanol	...	1546	000067-63-0	4
3 Ethylene glycol monomethylether	...	3779	000109-86-4	1
C:\Database\Nist98.l				
1 Acetic acid		109429	000064-19-7	90
2 Acetic acid, anhydride with form...		4896	002258-42-6	40
3 2-Propanone, 1-methoxy-		110823	005878-19-3	28

Fig. A.6: GC -MS library search of *D. rotundifolia* Compound B at a retention time of 11.315 min corresponding with Acetic (Ethanoic) acid spectrum

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex minus start of peak 204



Peak Number: 9 at 17.03 min Area: 2873171 Area % 1.83

The 3 best hits from each library.		Ref#	CAS#	Qual
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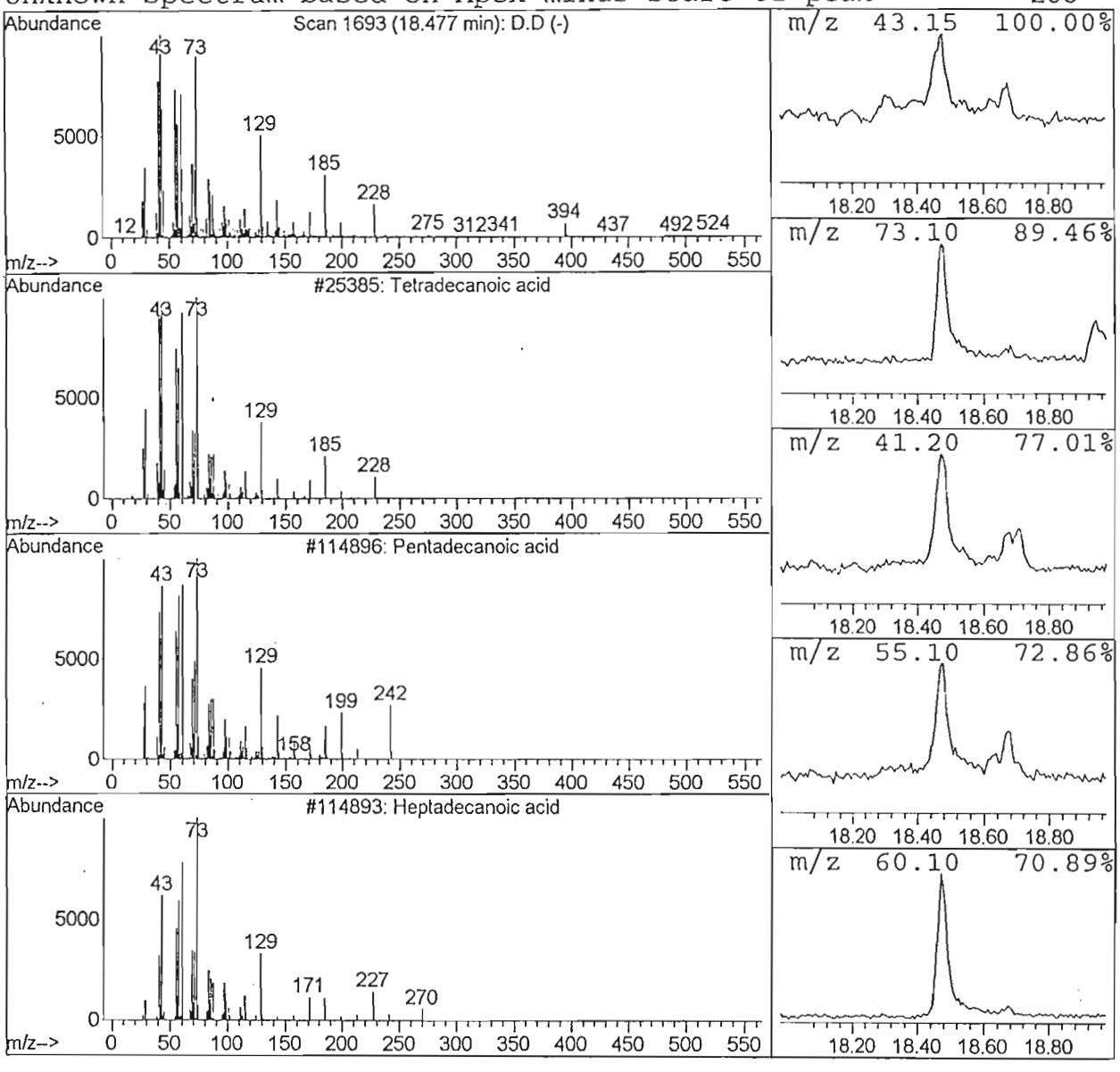
C:\Database\PMW_TOX2.L				
1 Myristic acid	...	1140	000544-63-8	52
2 Acetic acid	...	1548	000064-19-7	4
3 Cyclamate-M AC	...	1229	001124-53-4	1
C:\Database\Nist98.l				
1 Dodecanoic acid		25776	000143-07-7	94
2 Lauric anhydride		114892	000645-66-9	81
3 Tetradecanoic acid		25385	000544-63-8	58

Fig. A. 7: GC -MS library search of *D. rotundifolia* Compound B at a retention time of 17.034 min corresponding with Lauric (Dodecanoic) acid spectrum

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex minus start of peak 205



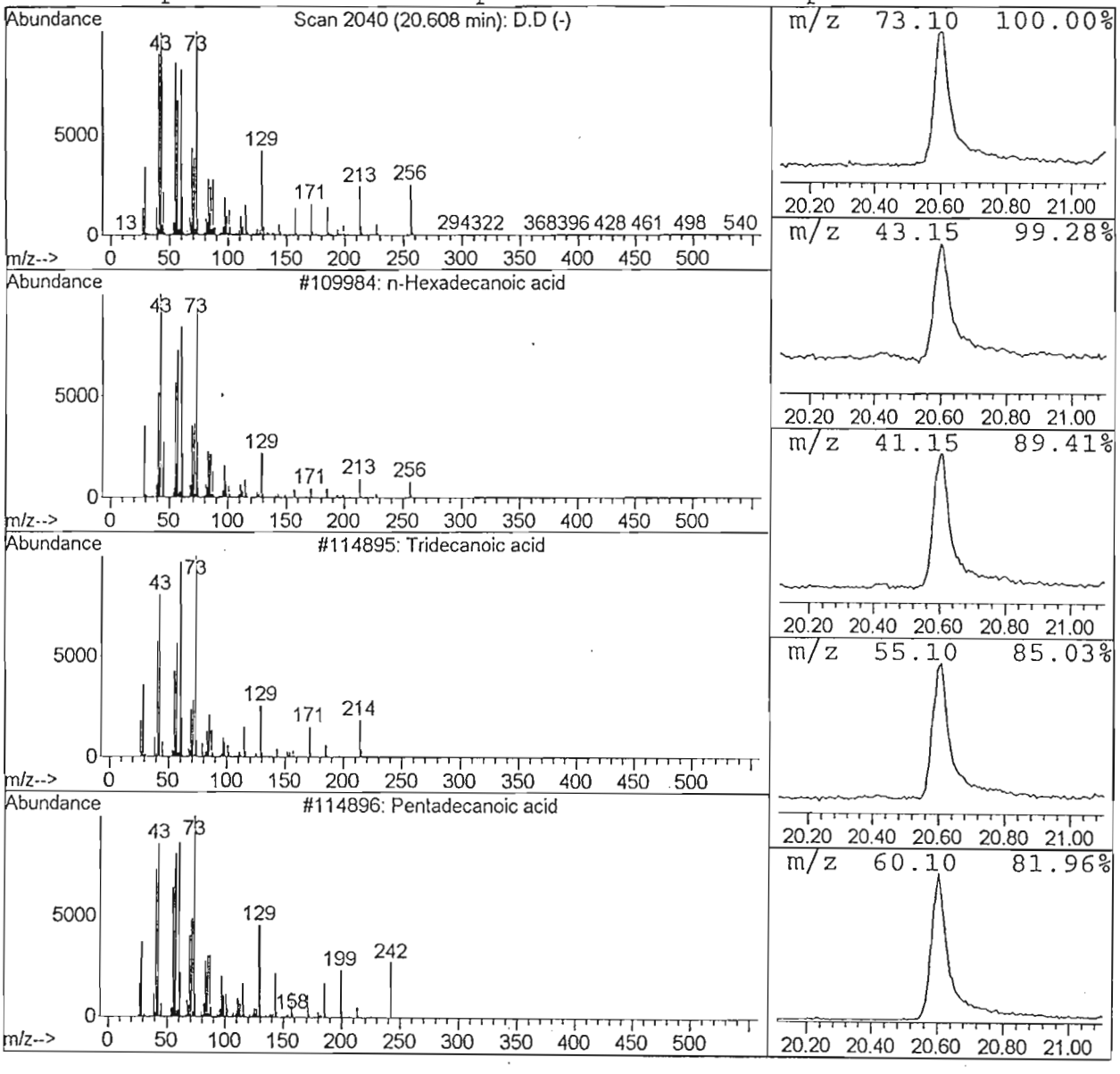
Peak Number: 10 at 18.48 min Area: 20227920 Area % 12.87

The 3 best hits from each library.		Ref#	CAS#	Qual
C:\Database\HPPest.L	No hits were retrieved.			
C:\Database\PMW_TOX2.L				
1 Myristic acid	...	1140	000544-63-8	70
2 Acecarbromal artifact	...	1026	000000-00-0	10
3 Dibutyladipate	...	722	000105-99-7	9
C:\Database\Nist98.l				
1 Tetradecanoic acid		25385	000544-63-8	98
2 Pentadecanoic acid		114896	001002-84-2	70
3 Heptadecanoic acid		114893	000506-12-7	58

Fig. A.8: GC -MS library search of *D. rotundifolia* Compound B at a retention time of 18.477 min corresponding with Myristic (Tetradecanoic) acid spectrum

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex minus start of peak



Peak Number: 12 at 20.61 min Area: 27617924 Area % 17.58

The 3 best hits from each library.		Ref#	CAS#	Qual
C:\Database\HPPest.L	No hits were retrieved.			
C:\Database\PMW_TOX2.L				
1 Palmitic acid	...	822	000057-10-3	46
2 Myristic acid	...	1140	000544-63-8	37
3 Acetic acid	...	1548	000064-19-7	5
C:\Database\Nist98.1				
1 n-Hexadecanoic acid		109984	000057-10-3	96
2 Tridecanoic acid		114895	000638-53-9	90
3 Pentadecanoic acid		114896	001002-84-2	87

Fig. A.9: GC -MS library search of *D. rotundifolia* Compound B at a retention time of 20.608 min corresponding with Palmitic (Hexadecanoic) acid spectrum

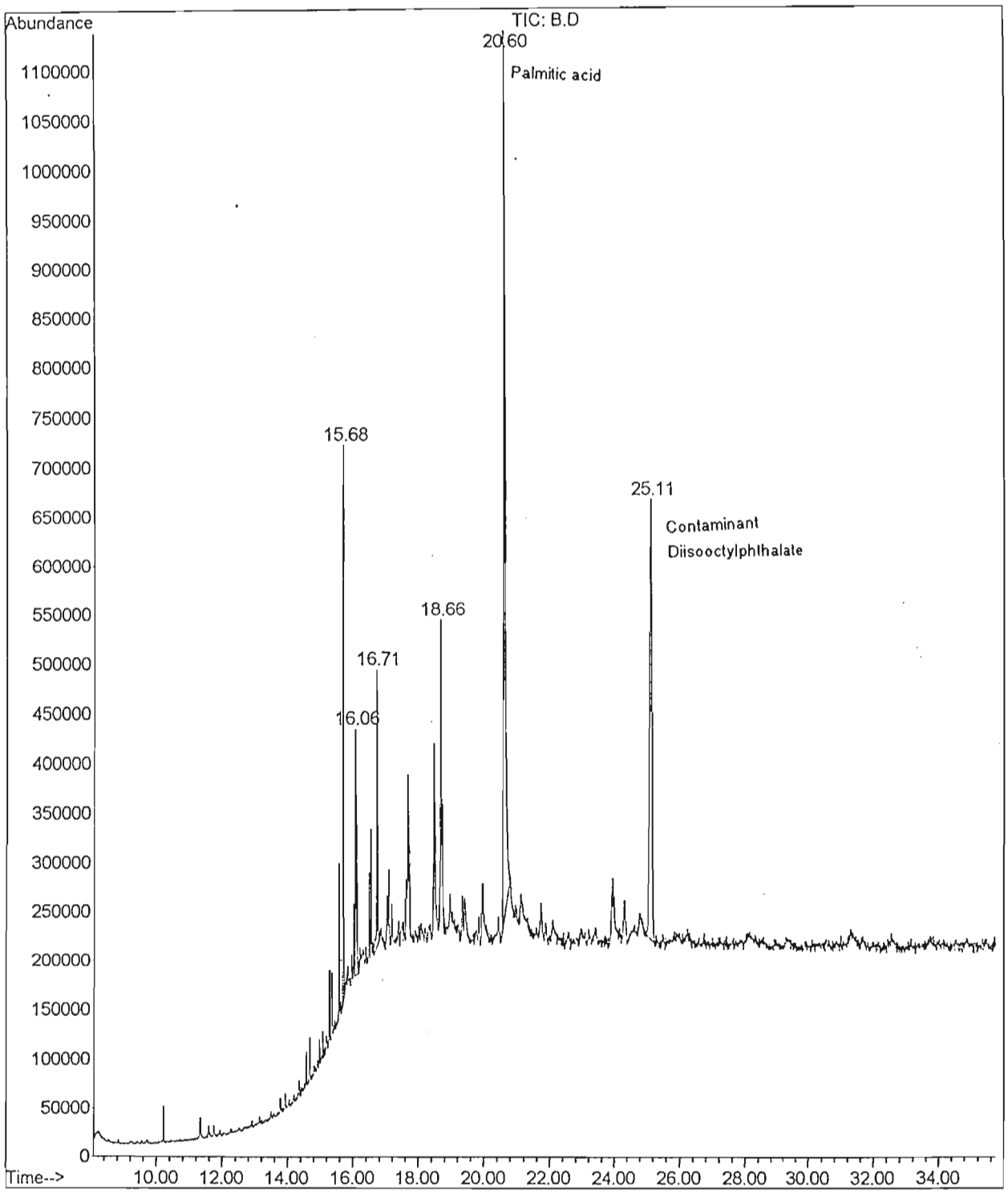
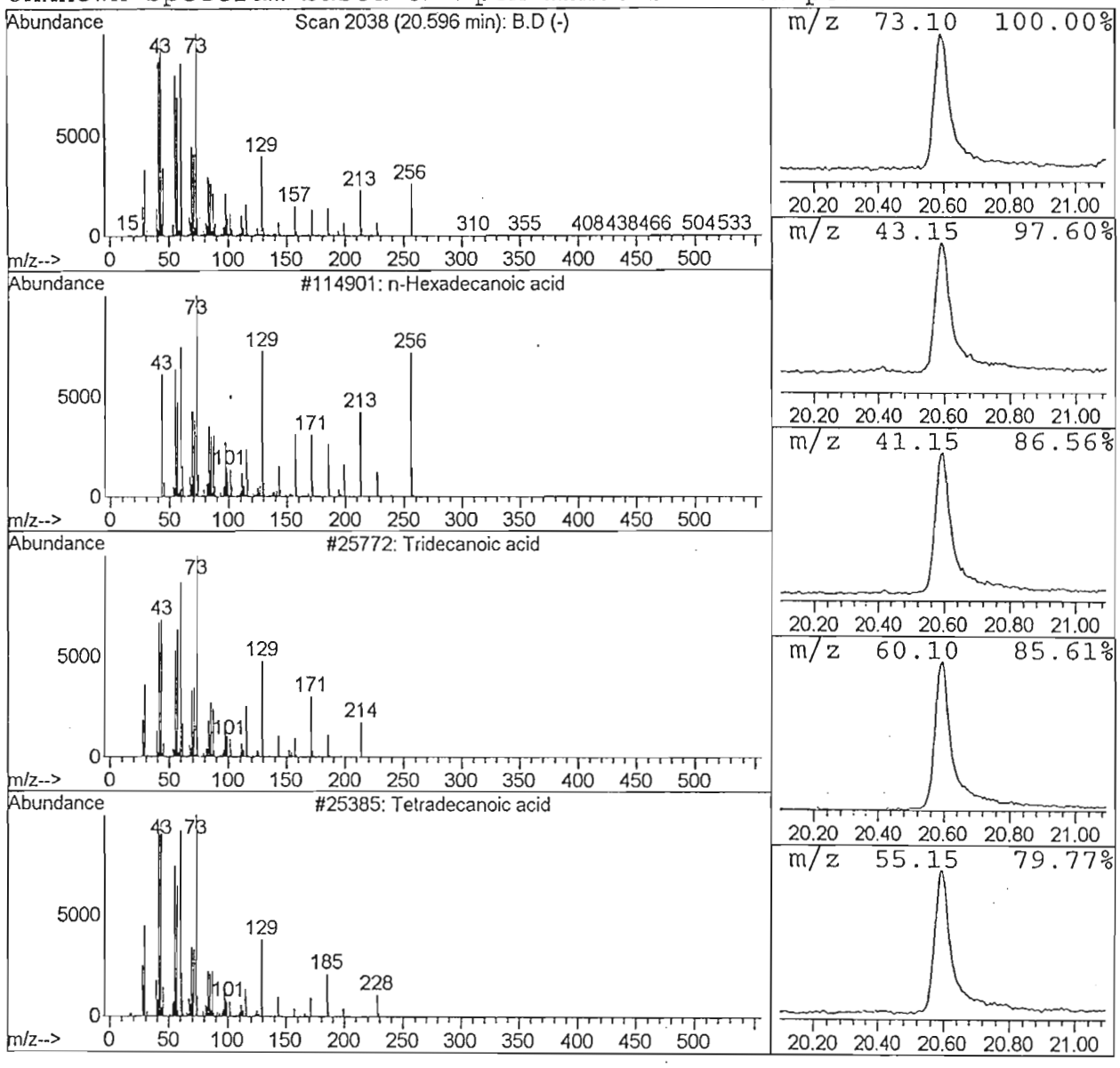


Fig. A.10: *D. burgessiae* GC-MS spectrum of isolated anti-bacterial Compound A

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex minus start of peak 208



Peak Number: 5 at 20.60 min Area: 31266592 Area % 42.70

The 3 best hits from each library.		Ref#	CAS#	Qual
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C:\Database\PMW_TOX2.L				
1 Palmitic acid	...	822	000057-10-3	95
2 Myristic acid	...	1140	000544-63-8	25
3 Acetic acid	...	1548	000064-19-7	4
C:\Database\Nist98.l				
1 n-Hexadecanoic acid		114901	000057-10-3	98
2 Tridecanoic acid		25772	000638-53-9	95
3 Tetradecanoic acid		25385	000544-63-8	93

Fig. A.11: GC -MS library search of *D. burgessiae* Compound A at a retention time of 20.596 min corresponding with Palmitic (Hexadecanoic) acid spectrum

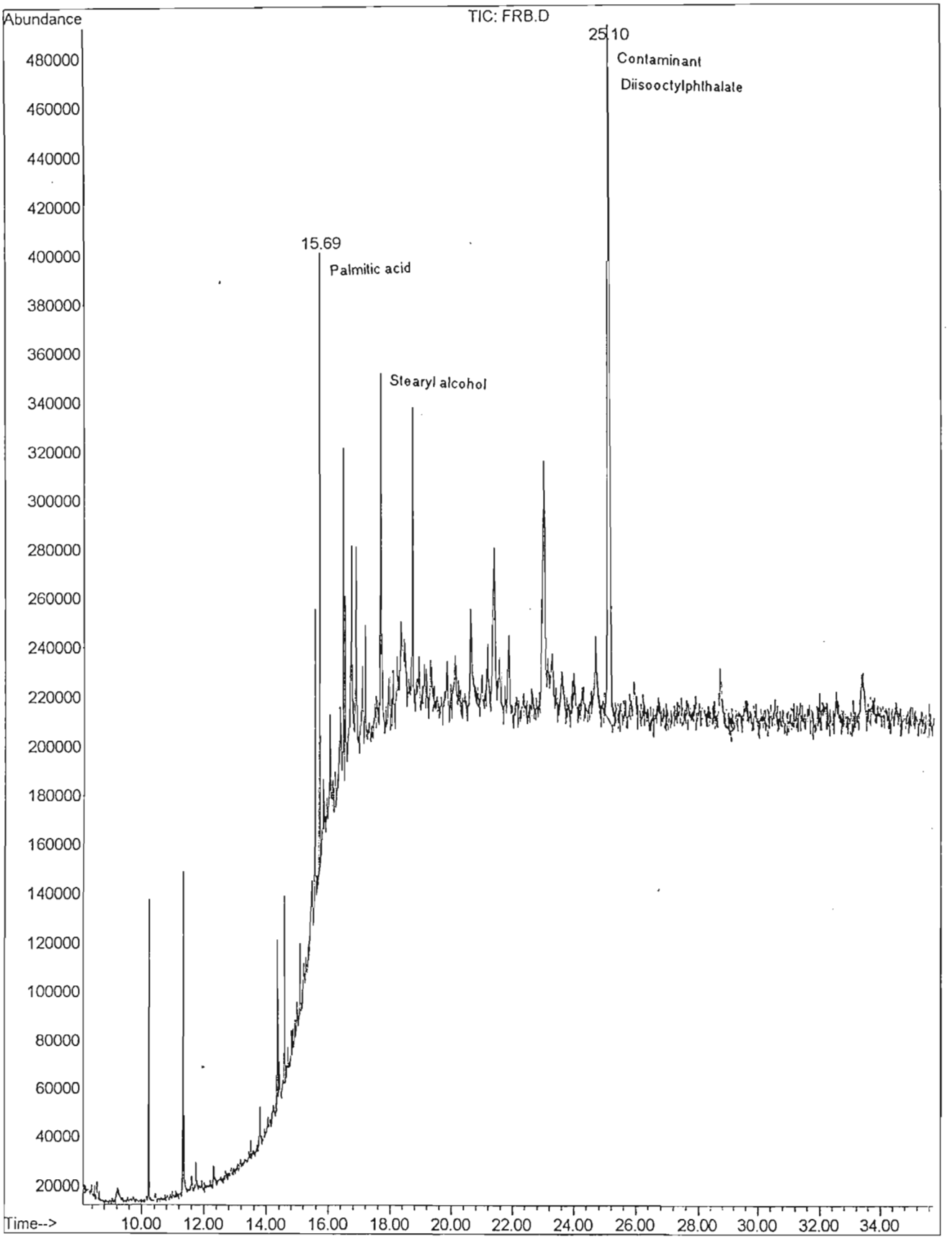


Fig. A.12: *C. greenwayi* GC-MS spectrum of isolated anti-bacterial Compound B

```
Library Searched : C:\Database\PMW_TOX2.L
Quality          : 95
ID               : Stearyl alcohol                    P1298
```

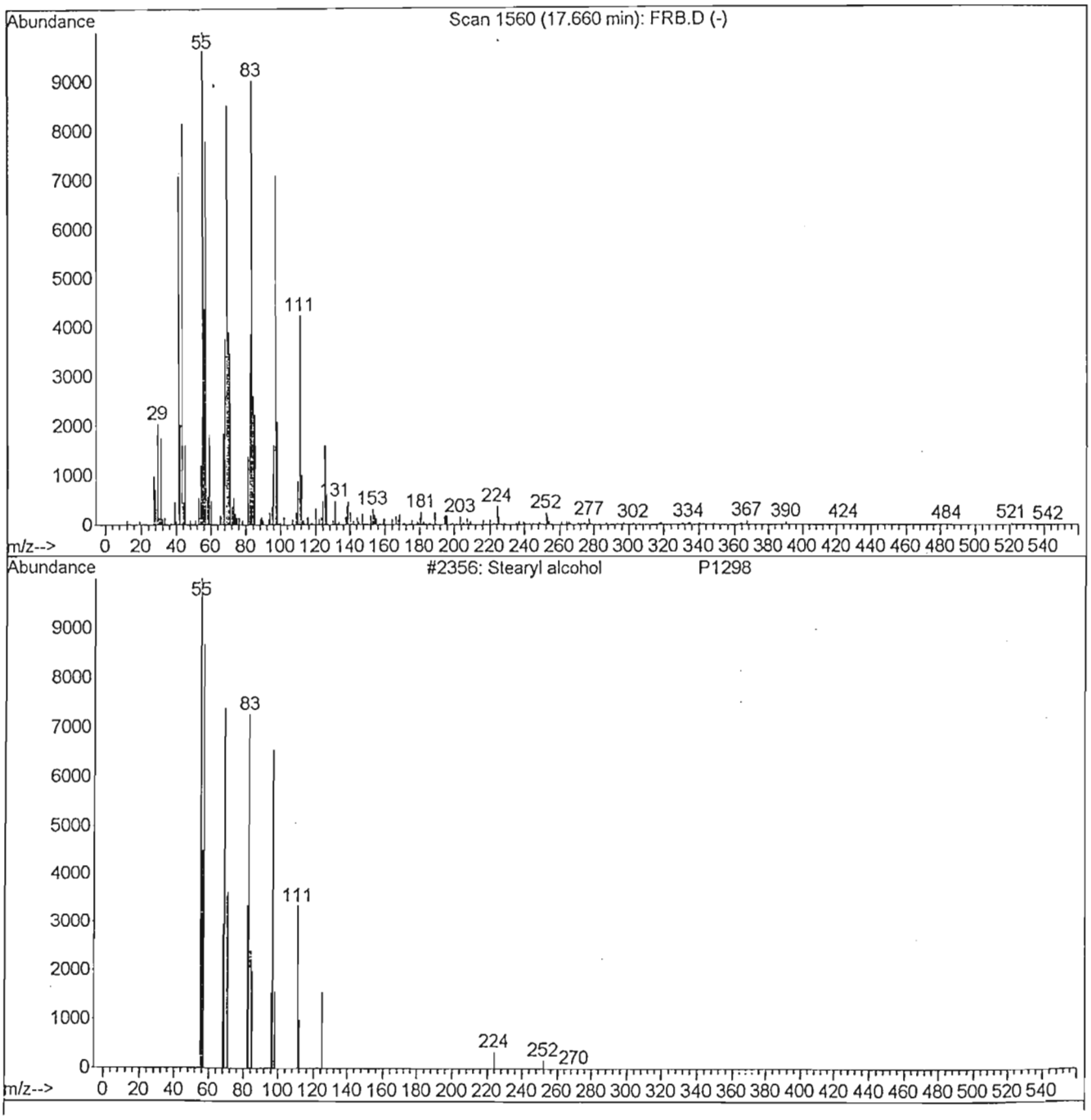


Fig. A. 13: GC -MS library search of *C. greenwayi* Compound B at a retention time of 17.660 min corresponding with Stearyl alcohol spectrum

Library Searched : C:\Database\PMW_TOX2.L
Quality : 96
ID : Palmitic acid ME P1297

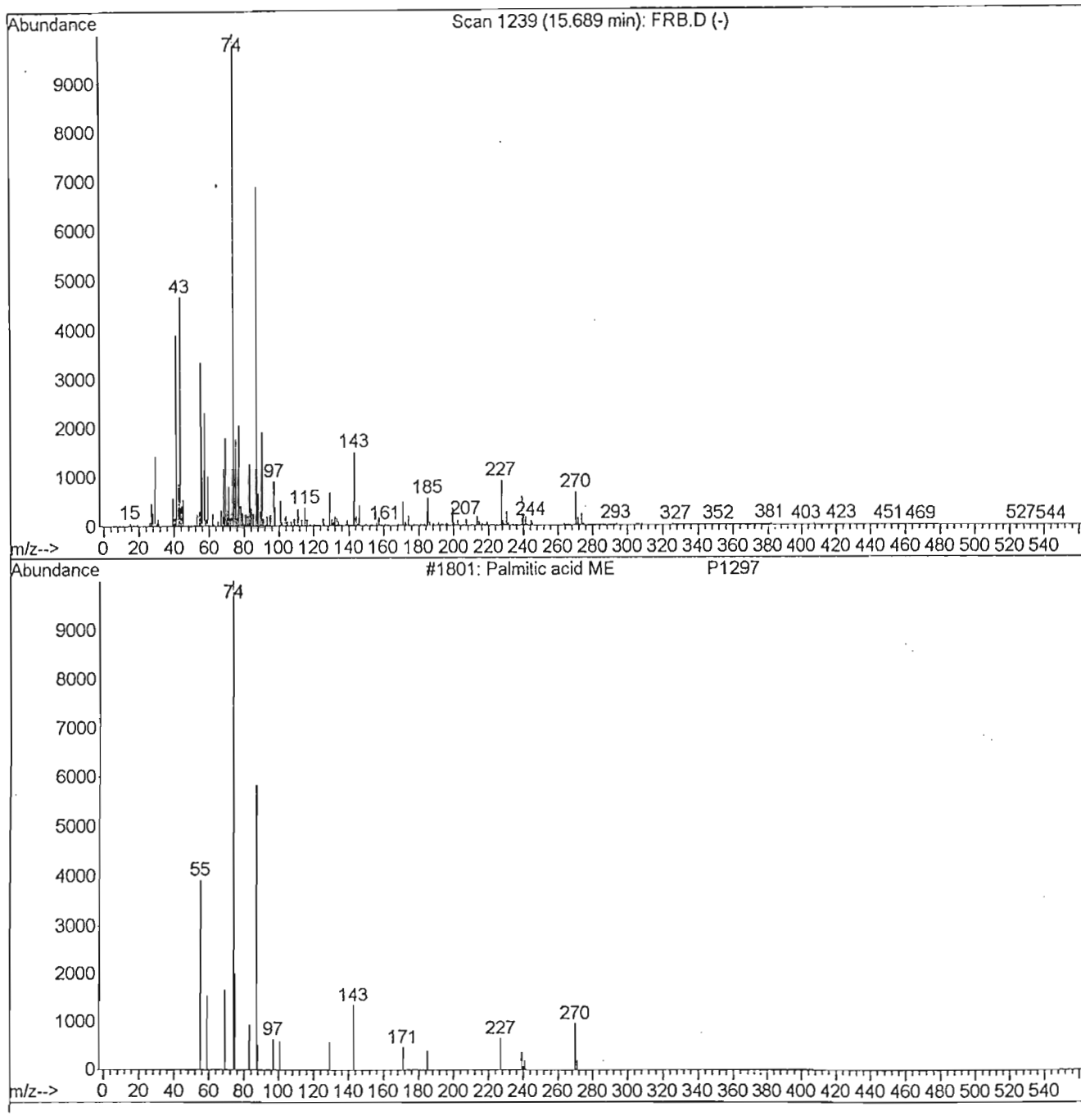


Fig. A.14: GC -MS library search of *C. greenwayi* Compound B at a retention time of 15.689 min corresponding with Palmitic (Hexadecanoic) acid spectrum

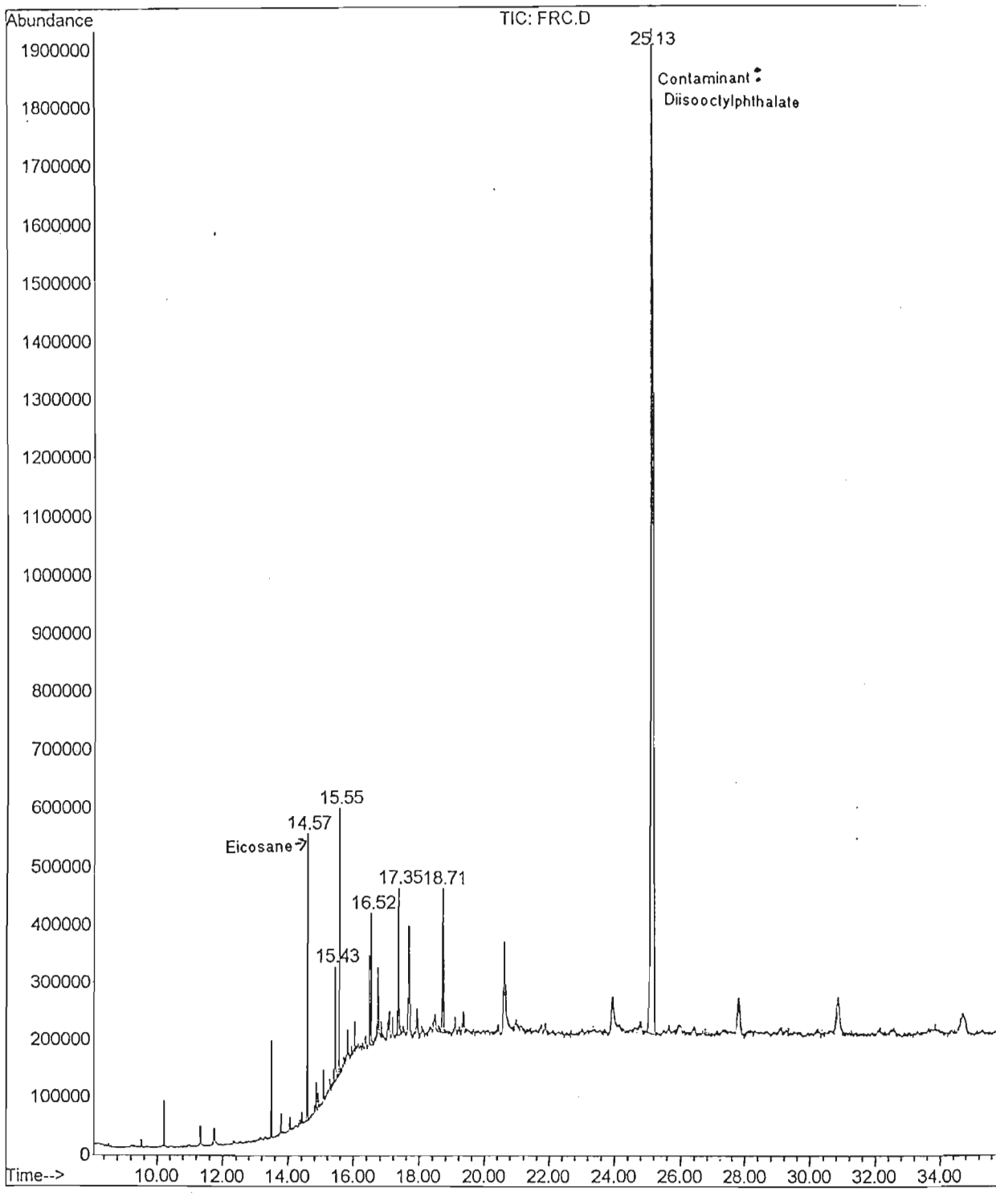
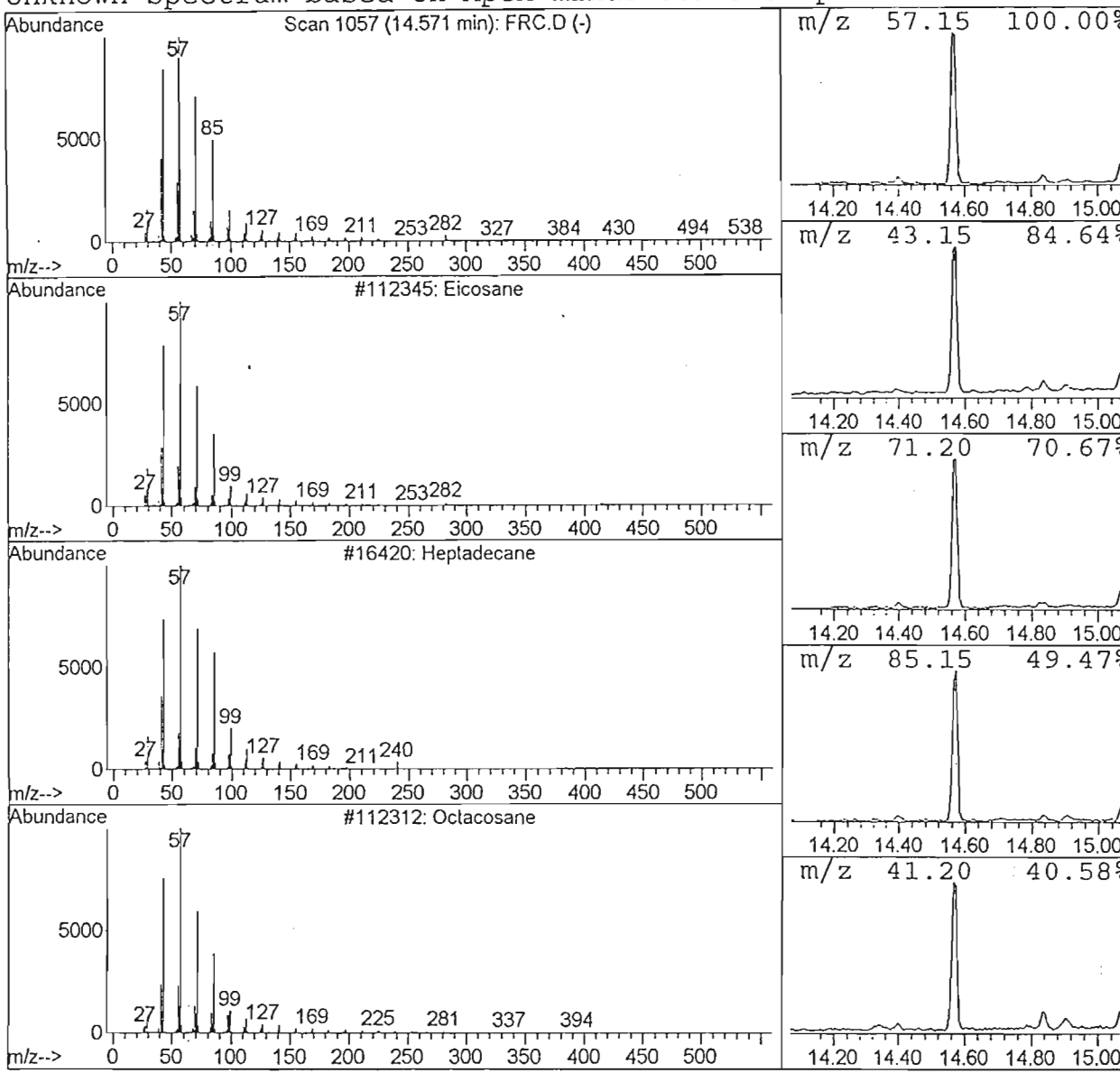


Fig. A.15: *C. greenwayi* GC-MS spectrum of isolated anti-bacterial Compound C

Unknown Spectrum based on Apex minus start of peak



Peak Number: 1 at 14.57 min Area: 5391331 Area % 5.10

The 3 best hits from each library.		Ref#	CAS#	Qual
C:\Database\HPPest.L	No hits were retrieved.			
C:\Database\PMW_TOX2.L				
1 Tricosane	...	2364	000638-67-5	90
2 Nonadecane	...	2363	000629-92-5	90
3 Hexadecane	...	2353	000544-76-3	90
C:\Database\Nist98.l				
1 Eicosane		112345	000112-95-8	98
2 Heptadecane		16420	000629-78-7	97
3 Octacosane		112312	000630-02-4	91

Fig. A. 16: GC -MS library search of *C. greenwayi* Compound C at a retention time of 14.571 min corresponding with Eicosane spectrum

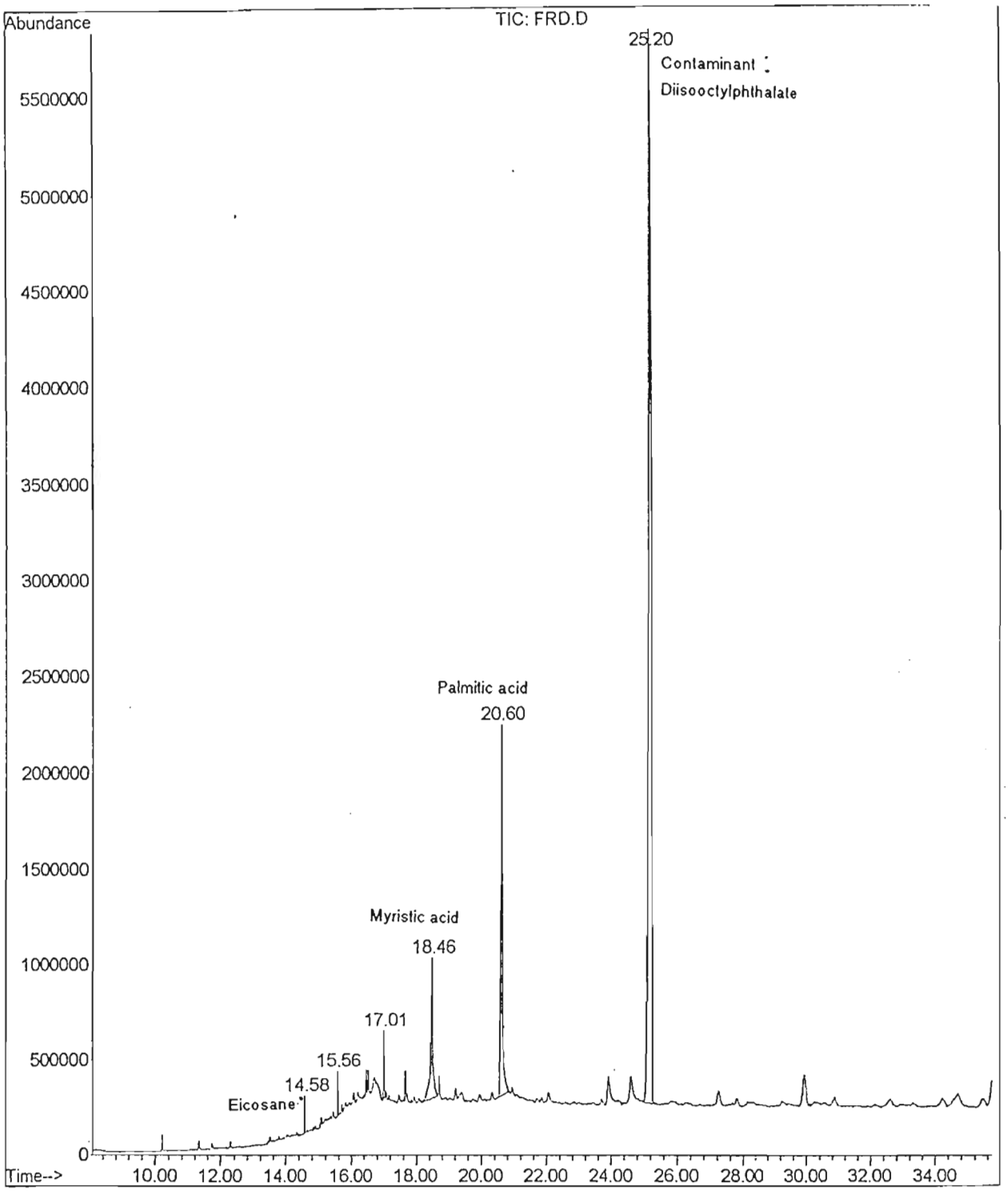
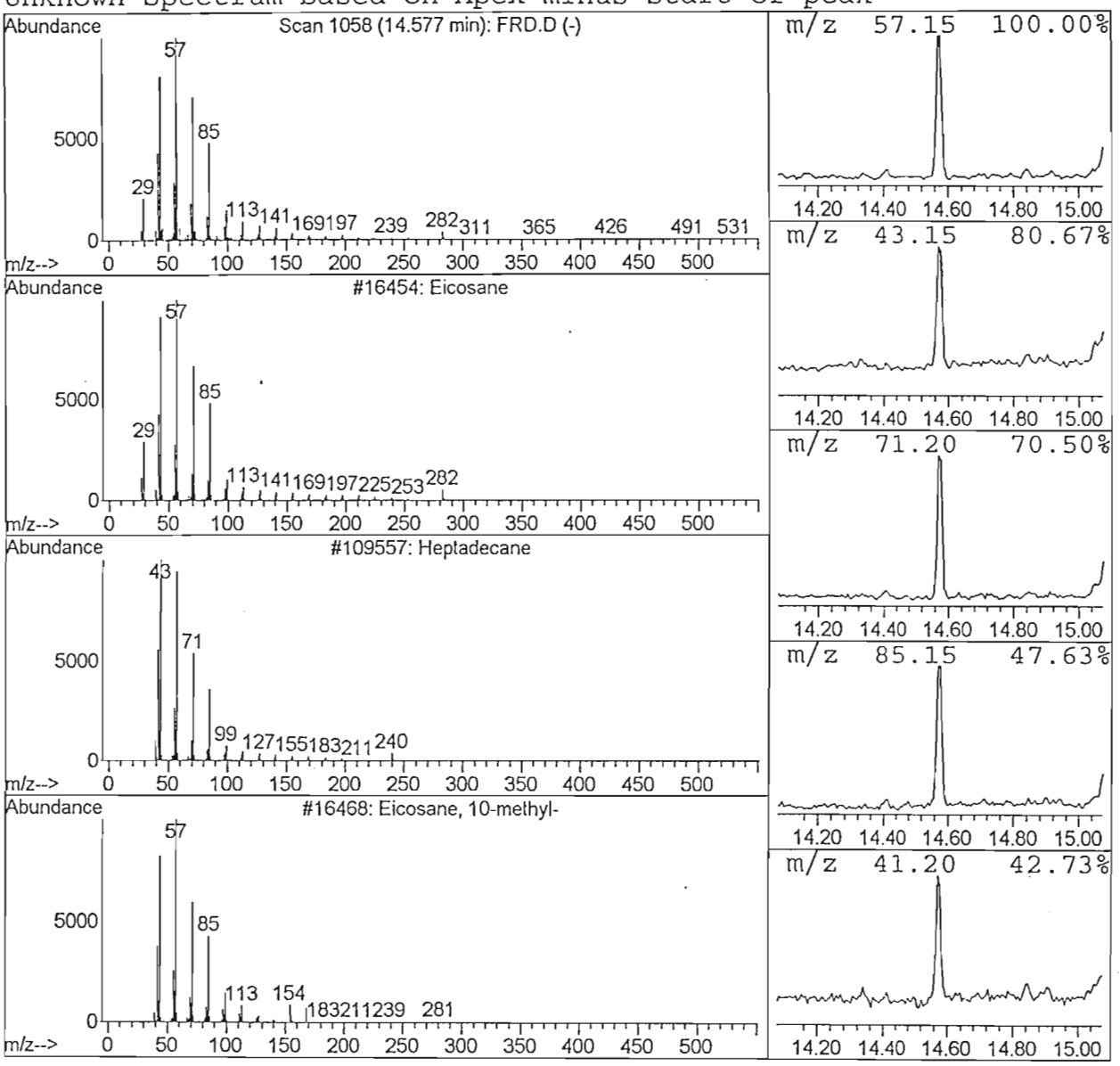


Fig. A.17: *C. greenwayi* GC-MS spectrum of isolated anti-bacterial Compound D

Unknown Spectrum based on Apex minus start of peak



Peak Number: 1 at 14.58 min Area: 2203744 Area % 0.53

The 3 best hits from each library.		Ref#	CAS#	Qual
C:\Database\HPPest.L	No hits were retrieved.			
C:\Database\PMW_TOX2.L				
1 Octacosane	...	3797	000630-02-4	90
2 Hexadecane	...	2353	000544-76-3	86
3 Nonadecane	...	2363	000629-92-5	83
C:\Database\Nist98.l				
1 Eicosane		16454	000112-95-8	97
2 Heptadecane		109557	000629-78-7	94
3 Eicosane, 10-methyl-		16468	054833-23-7	93

Fig. A.18: GC -MS library search of *C. greenwayi* Compound D at a retention time of 14.577 min corresponding with Eicosane spectrum

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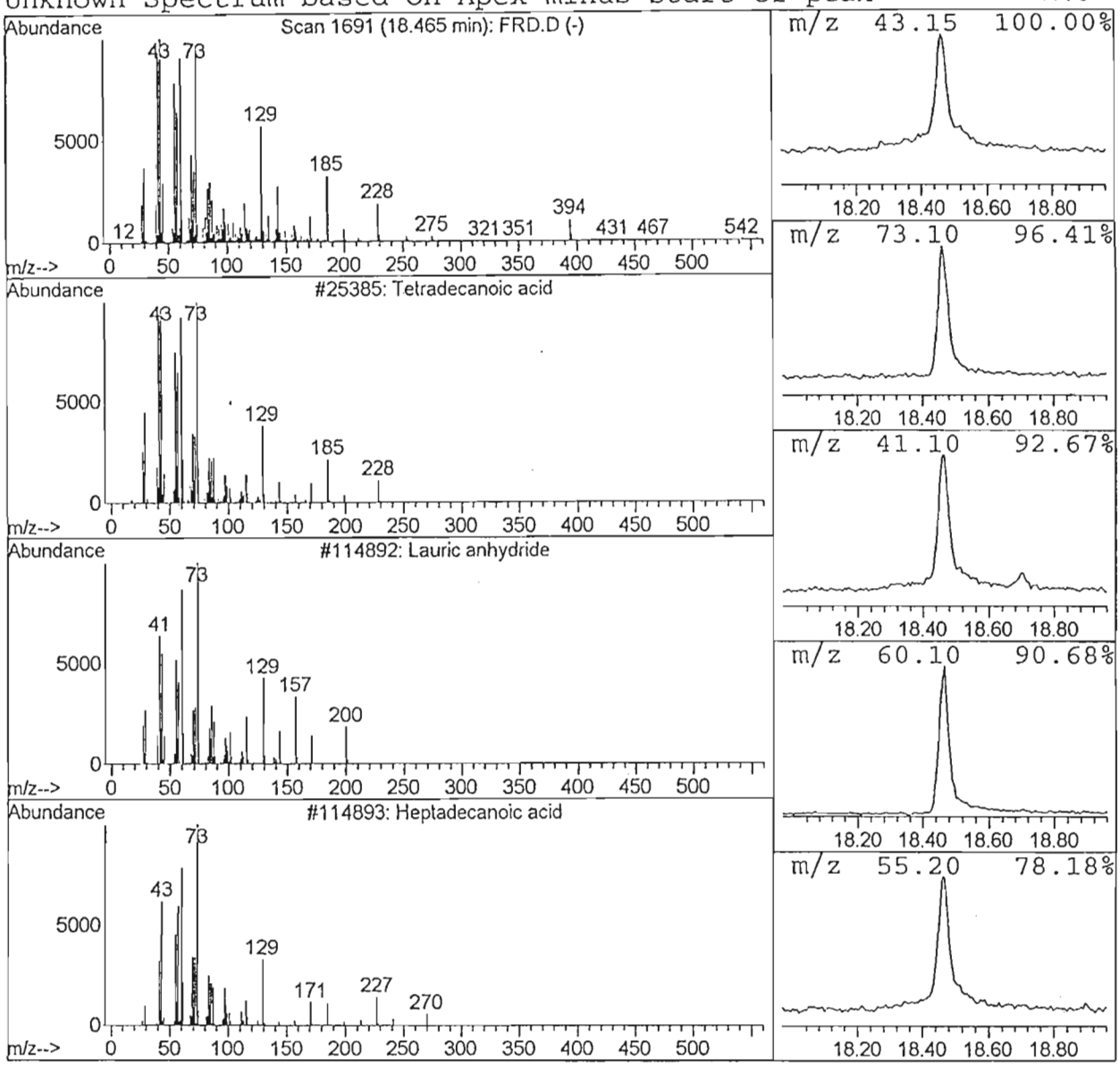
Unknown Spectrum based on Apex minus start of peak 216

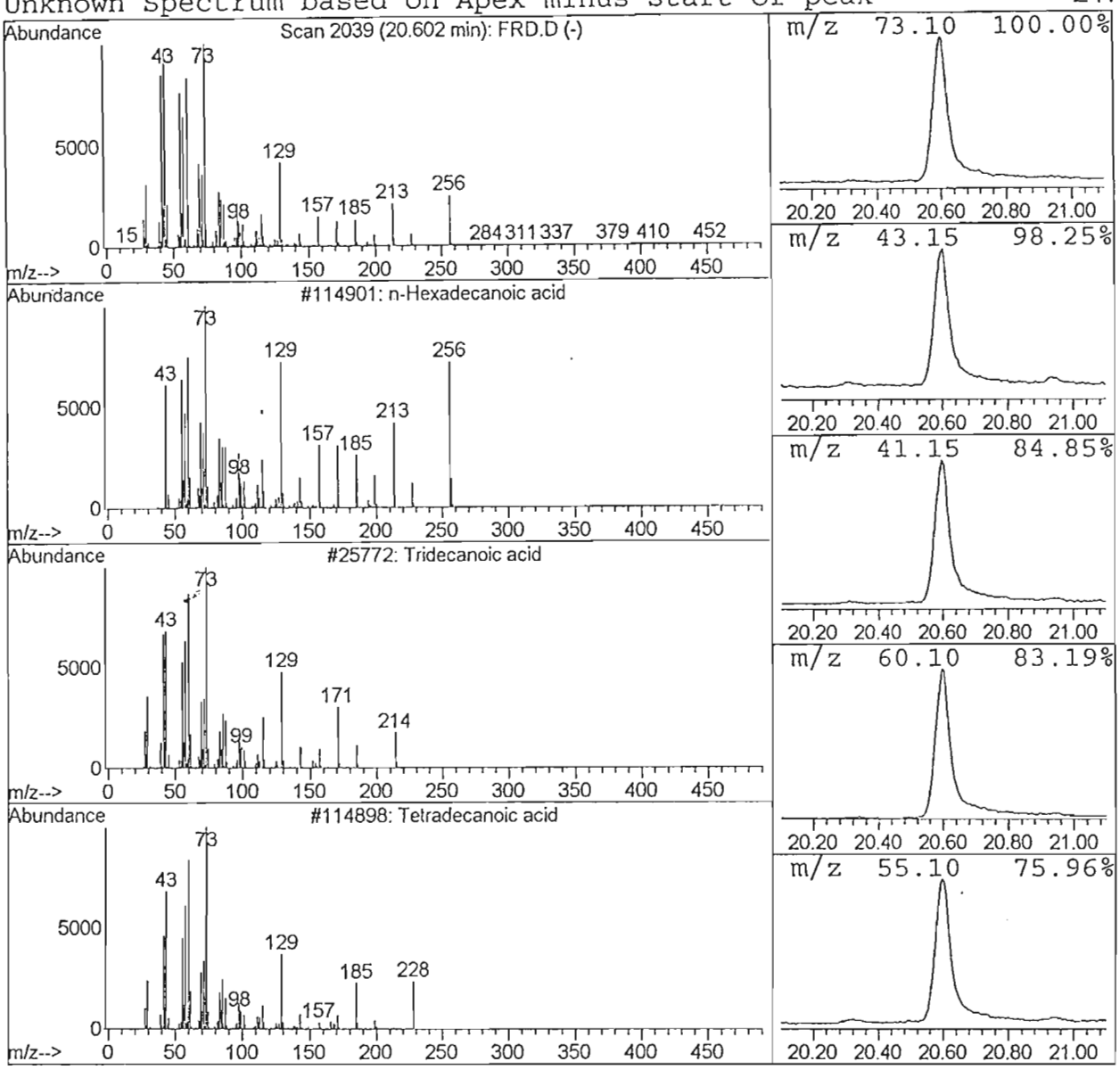


Peak Number: 4 at 18.46 min Area: 32152564 Area % 7.71

The 3 best hits from each library.		Ref#	CAS#	Qual
C:\Database\HPPest.L	No hits were retrieved.			
C:\Database\PMW_TOX2.L				
1 Myristic acid	...	1140	000544-63-8	89
2 Diethylene glycol dipivalate	...	1904	000000-00-0	9
3 Dibutyladipate	...	722	000105-99-7	7
C:\Database\Nist98.l				
1 Tetradecanoic acid		25385	000544-63-8	99
2 Lauric anhydride		114892	000645-66-9	81
3 Heptadecanoic acid		114893	000506-12-7	62

Fig. A.19: GC -MS library search of *C. greenwayi* Compound D at a retention time of 18.465 min corresponding with Myristic (Tetradecanoic) acid spectrum

Unknown Spectrum based on Apex minus start of peak



Peak Number: 5 at 20.60 min Area: 68240659 Area % 16.37

The 3 best hits from each library.		Ref#	CAS#	Qual
C:\Database\HPPest.L	No hits were retrieved.			
C:\Database\PMW_TOX2.L				
1 Palmitic acid	...	822	000057-10-3	97
2 Myristic acid	...	1140	000544-63-8	52
3 Acetic acid	...	1548	000064-19-7	4
C:\Database\Nist98.l				
1 n-Hexadecanoic acid		114901	000057-10-3	99
2 Tridecanoic acid		25772	000638-53-9	95
3 Tetradecanoic acid		114898	000544-63-8	89

Fig. A.20: GC -MS library search of *C. greenwayi* Compound D at a retention time of 20.602 min corresponding with Palmitic (Hexadecanoic) acid spectrum

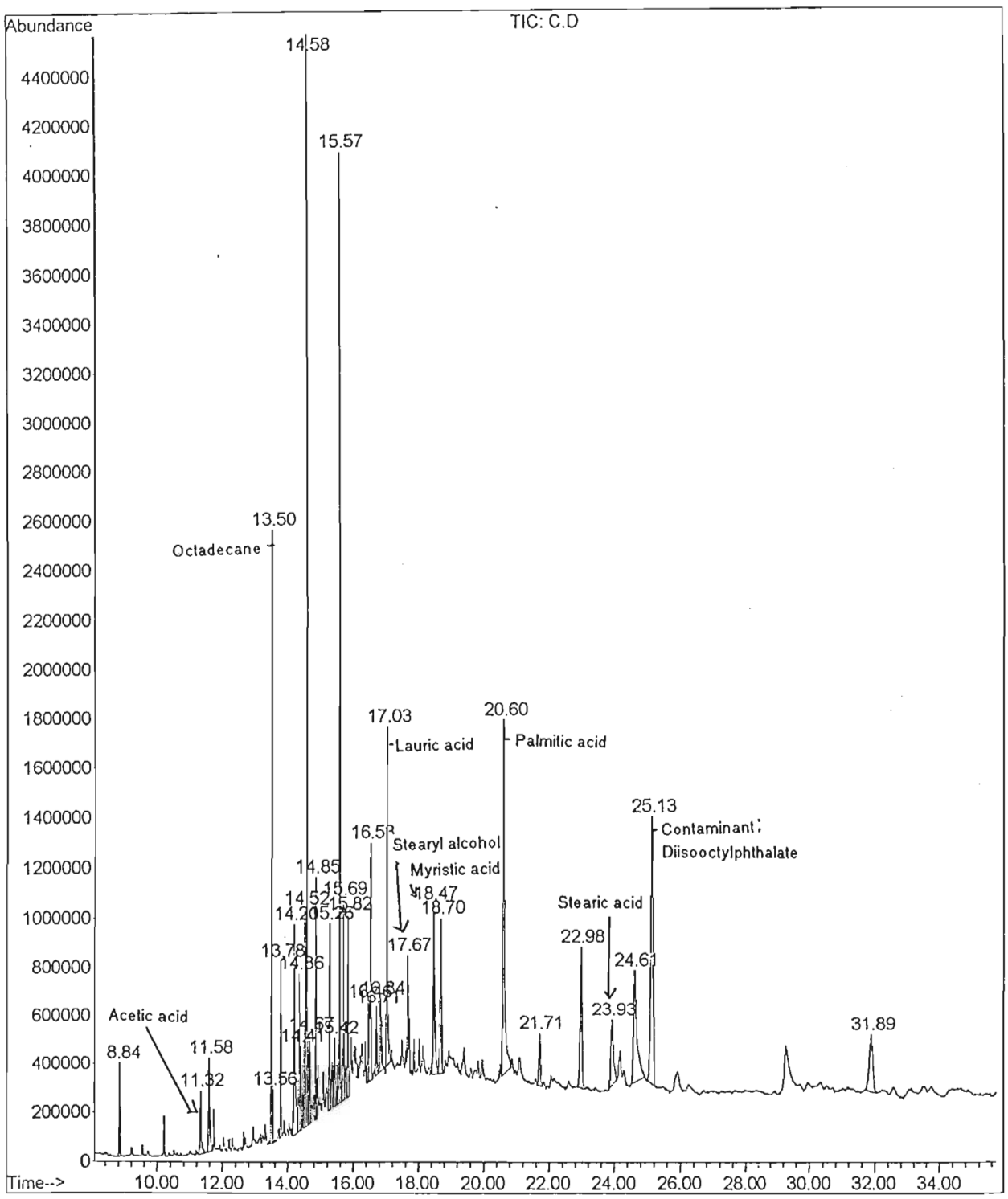


Fig. A.21: *H. depressa* GC-MS spectrum of isolated anti-bacterial Compound B(A)

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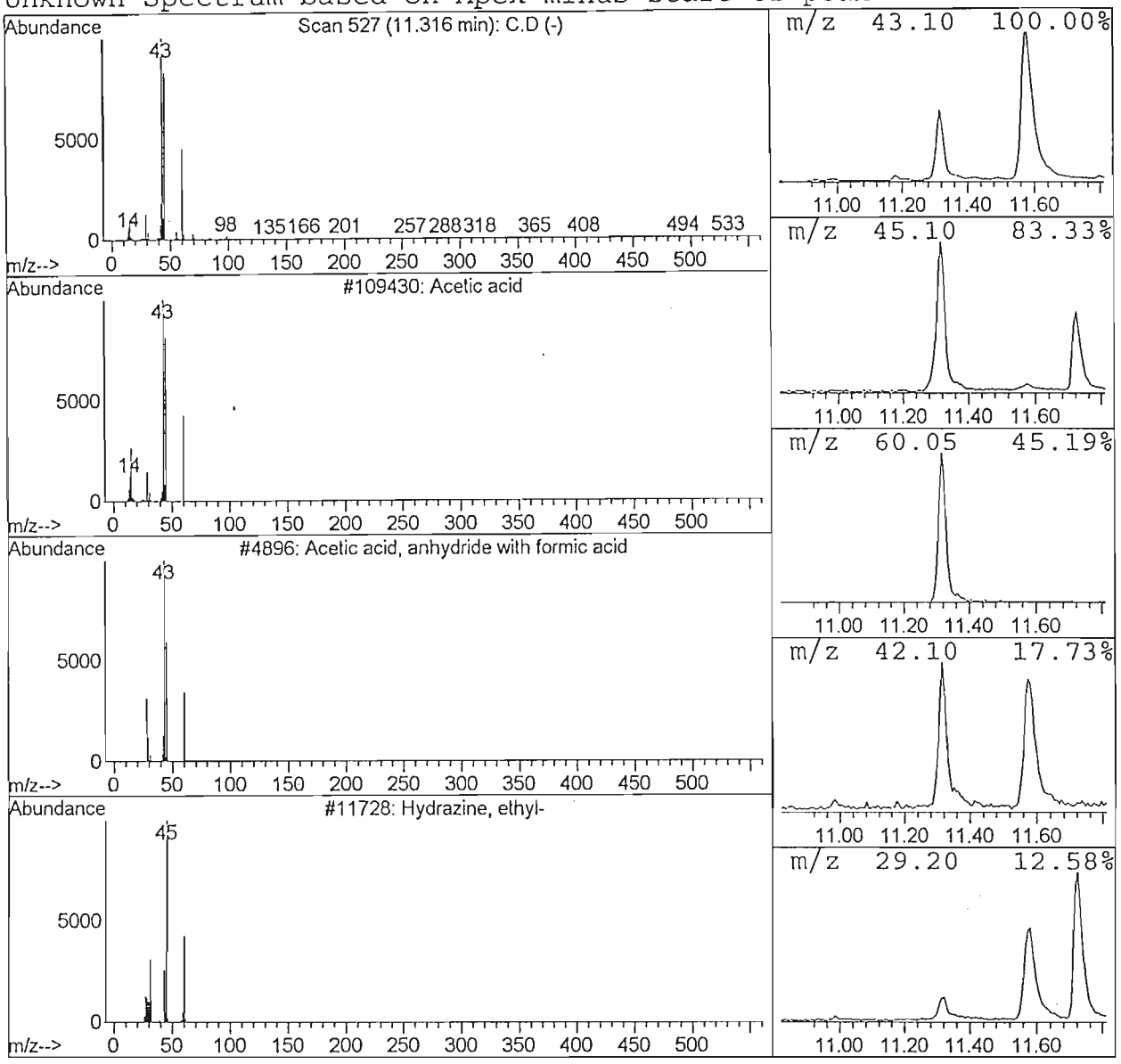
Unknown Spectrum based on Apex minus start of peak 219



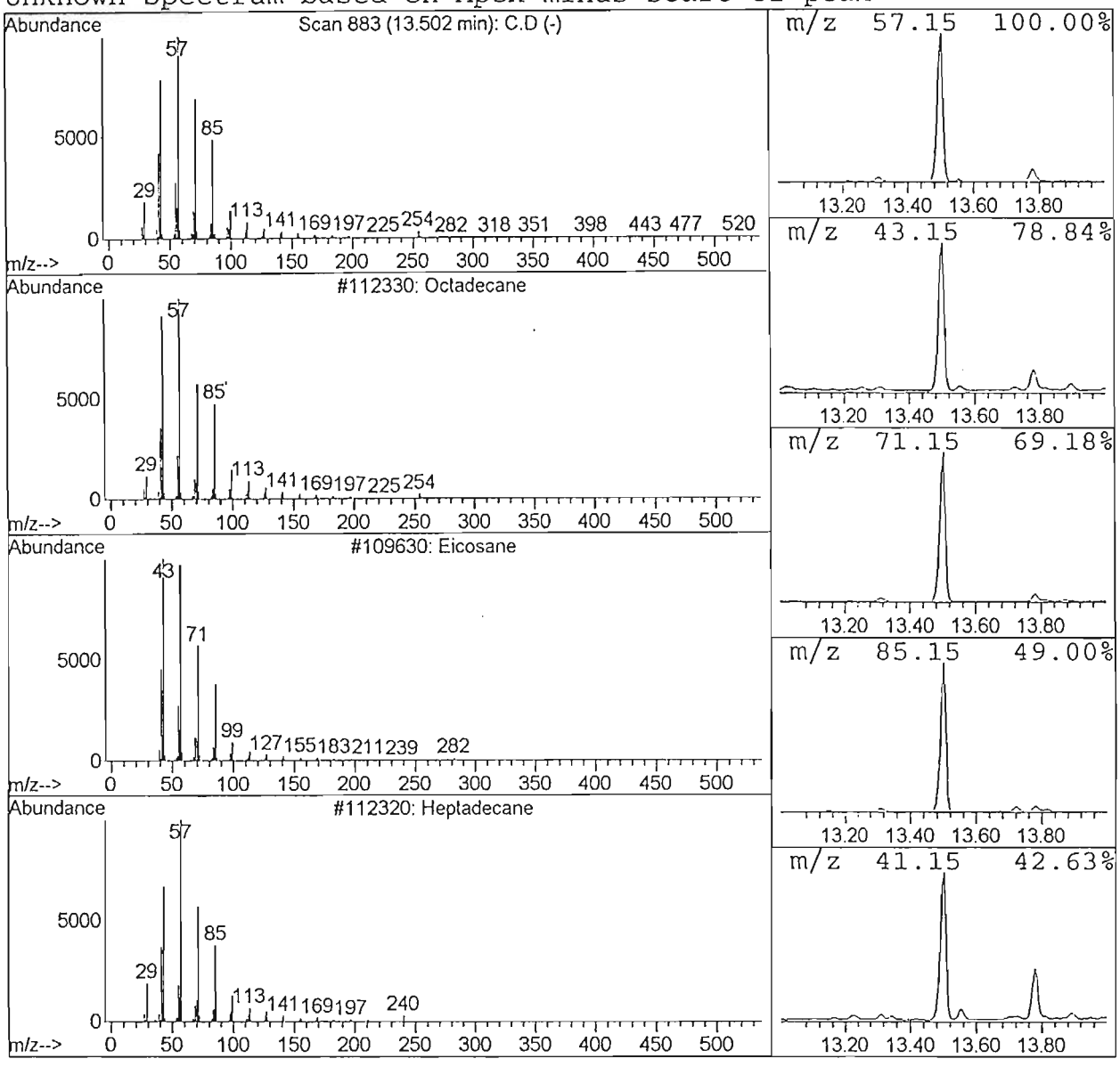
Peak Number: 2 at 11.32 min Area: 6234583 Area % 1.04

The 3 best hits from each library.	Ref#	CAS#	Qual
C:\Database\HPPest.L	No hits were retrieved.		
C:\Database\PMW_TOX2.L			
1 Acetic acid ...	1548	000064-19-7	78
2 Isopropanol ...	1546	000067-63-0	2
3 Ethylene glycol monomethylether ...	3779	000109-86-4	1
C:\Database\Nist98.l			
1 Acetic acid	109430	000064-19-7	90
2 Acetic acid, anhydride with form...	4896	002258-42-6	64
3 Hydrazine, ethyl-	11728	000624-80-6	38

Fig A. 22: GC -MS library search of *H. depressa* Compound B(A) at a retention time of 11.316 min corresponding with Acetic (ethanoic) acid spectrum

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex minus start of peak

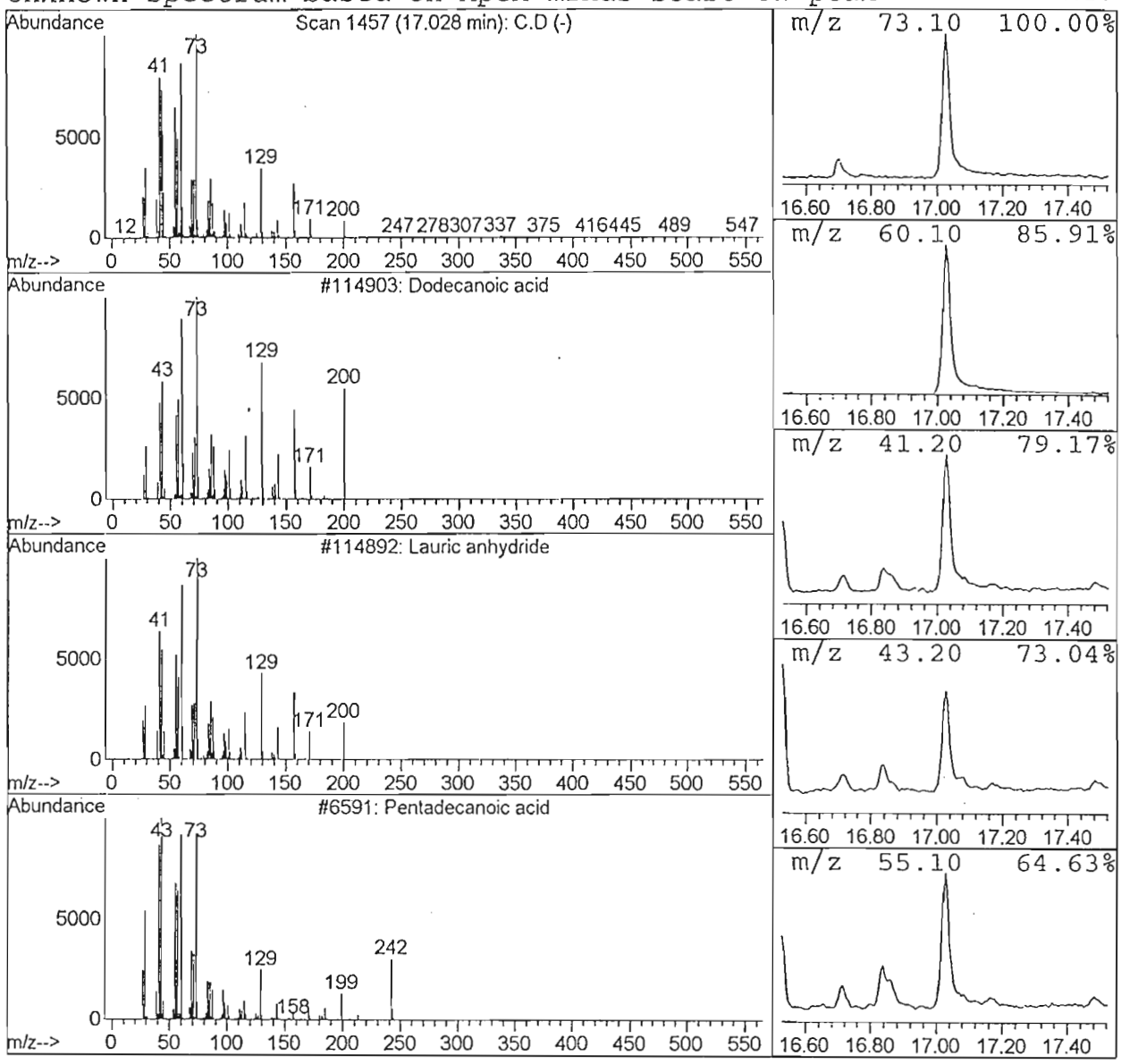


Peak Number: 4 at 13.50 min Area: 29763168 Area % 4.95

The 3 best hits from each library.		Ref#	CAS#	Qual
C:\Database\HPPest.L	No hits were retrieved.			
C:\Database\PMW_TOX2.L				
1 Octacosane	...	3797	000630-02-4	81
2 Eicosane	...	2352	000112-95-8	80
3 Hexadecane	...	2353	000544-76-3	74
C:\Database\Nist98.l				
1 Octadecane		112330	000593-45-3	99
2 Eicosane		109630	000112-95-8	97
3 Heptadecane		112320	000629-78-7	97

Fig. A. 23: GC -MS library search of *H. depressa* Compound B(A) at a retention time of 13.502 min corresponding with Octadecane spectrum

Unknown Spectrum based on Apex minus start of peak

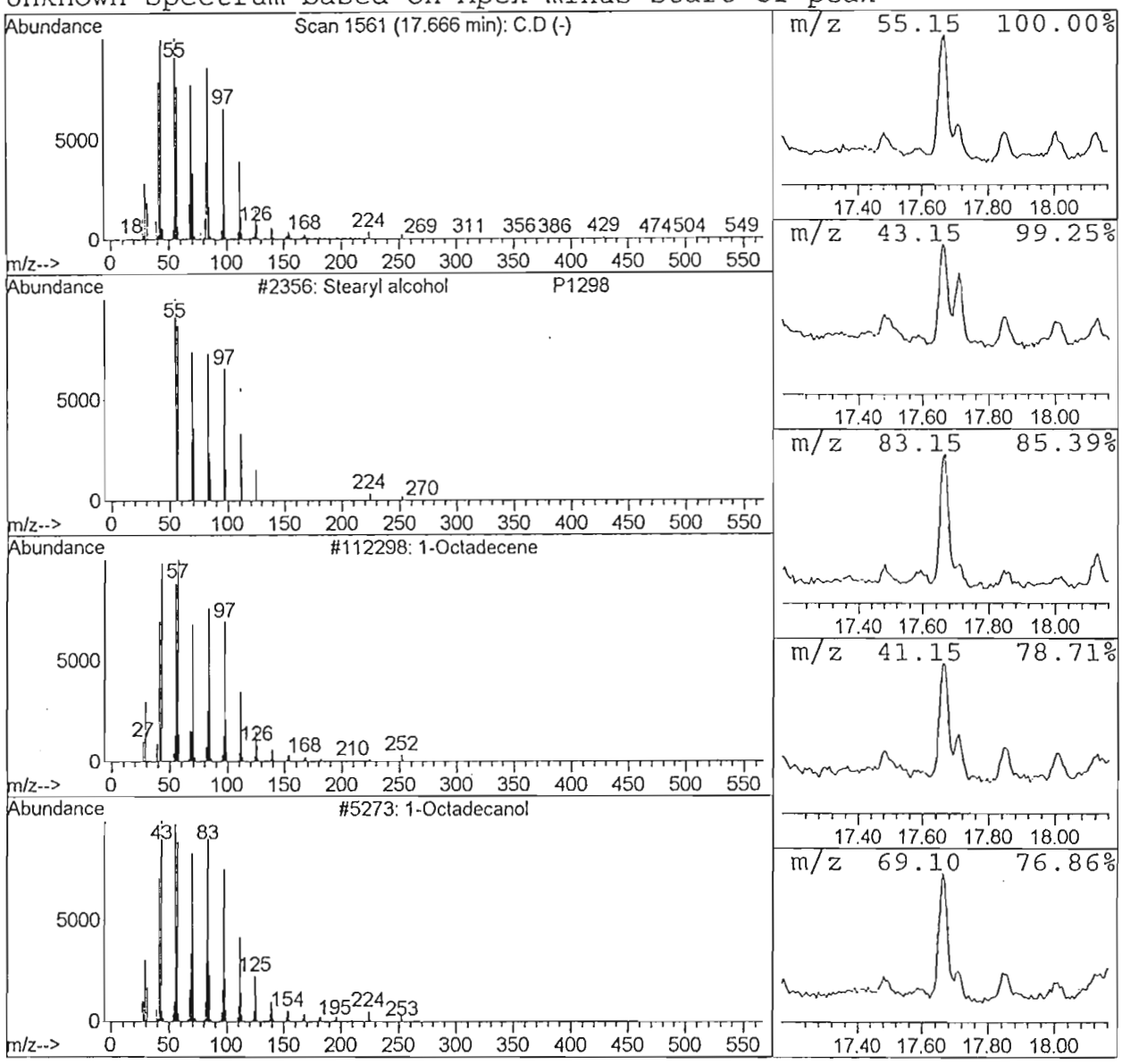


Peak Number: 23 at 17.03 min Area: 25124282 Area % 4.18

The 3 best hits from each library.		Ref#	CAS#	Qual
C:\Database\HPPest.L	No hits were retrieved.			
C:\Database\PMW_TOX2.L				
1 Myristic acid	...	1140	000544-63-8	74
2 Acetic acid	...	1548	000064-19-7	5
3 Cyclamate-M AC	...	1229	001124-53-4	1
C:\Database\Nist98.1				
1 Dodecanoic acid		114903	000143-07-7	94
2 Lauric anhydride		114892	000645-66-9	94
3 Pentadecanoic acid		6591	001002-84-2	81

Fig. A.24: GC -MS library search of *H. depressa* Compound B(A) at a retention time of 17.028 min corresponding with Lauric (Dodecanoic) acid spectrum

Unknown Spectrum based on Apex minus start of peak



Peak Number: 24 at 17.67 min Area: 6228682 Area % 1.04

The 3 best hits from each library.		Ref#	CAS#	Qual
C:\Database\HPPest.L				
1 CYCLOATE	...	112	001134-23-2	1
C:\Database\PMW_TOX2.L				
1 Stearyl alcohol	...	2356	000112-92-5	95
2 Cyclohexadecane	...	2355	000295-65-8	94
3 Cyclotetradecane	...	2354	000295-17-0	76
C:\Database\Nist98.l				
1 1-Octadecene		112298	000112-88-9	95
2 1-Octadecanol		5273	000112-92-5	95
3 9-Nonadecene		13042	031035-07-1	92

Fig. A.25: GC -MS library search of *H. depressa* Compound B(A) at a retention time of 17.666 min corresponding with Stearyl alcohol spectrum

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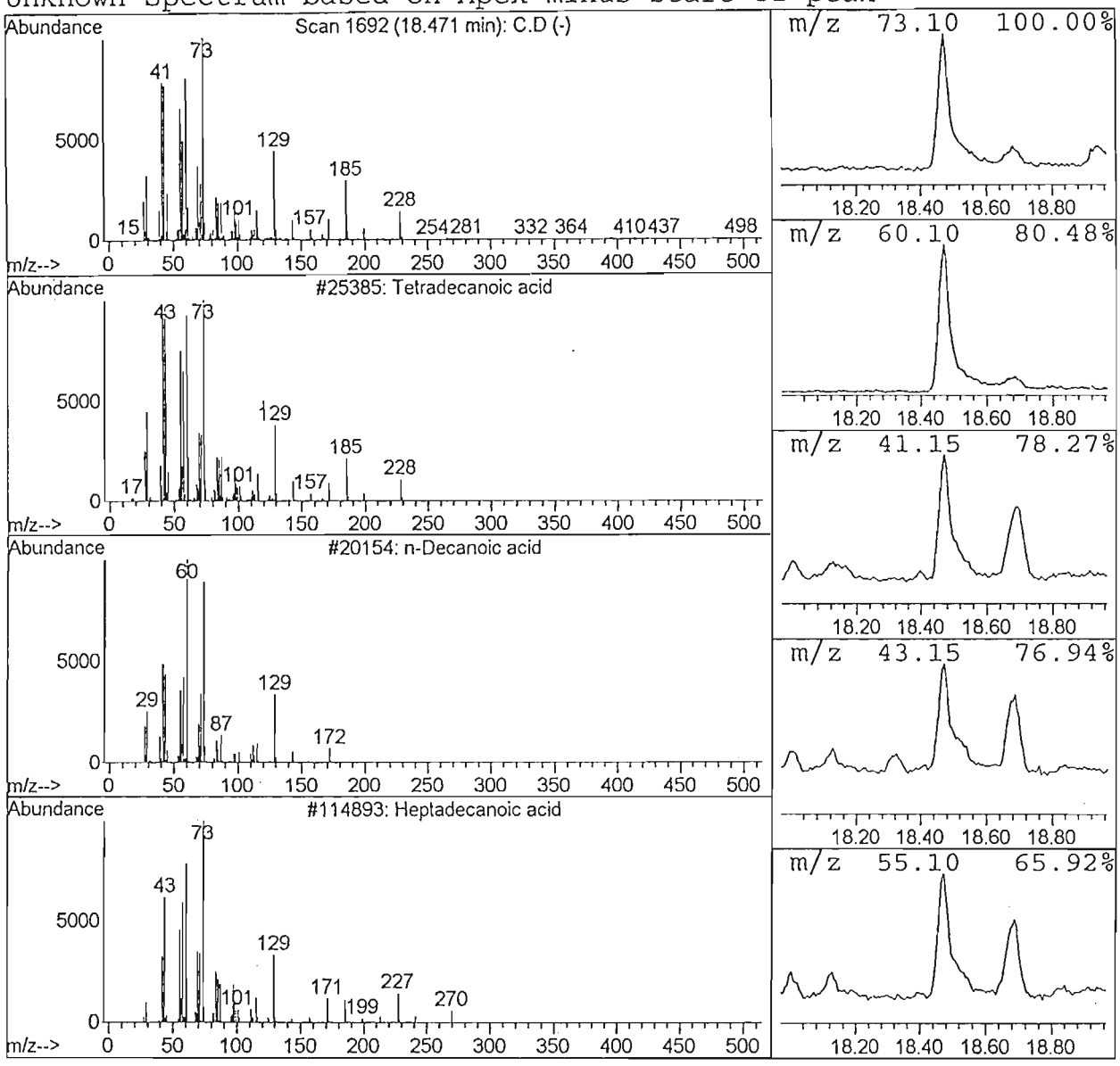
Unknown Spectrum based on Apex minus start of peak



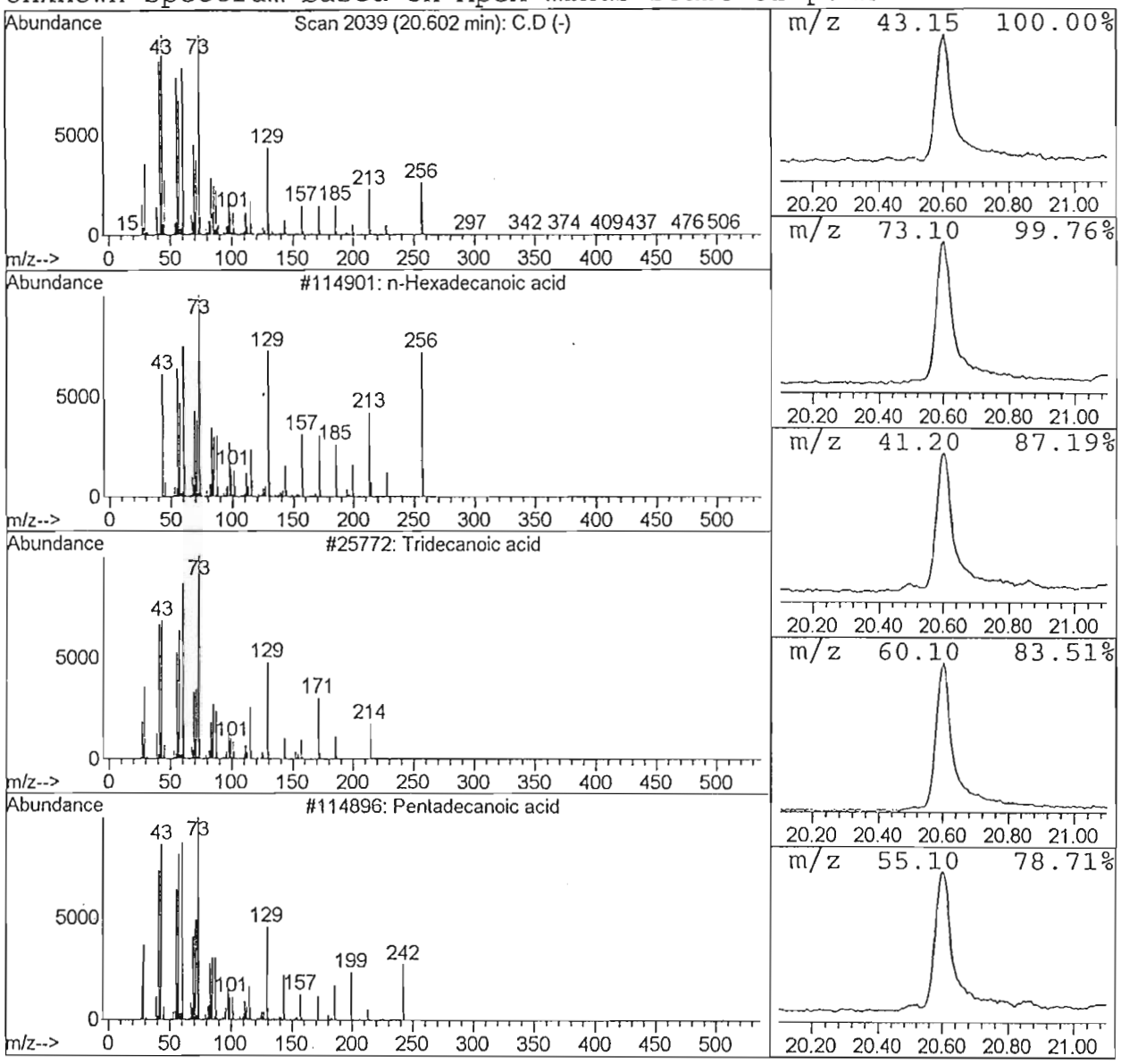
Peak Number: 25 at 18.47 min Area: 21137518 Area % 3.51

The 3 best hits from each library.		Ref#	CAS#	Qual
C:\Database\HPPest.L	No hits were retrieved.			
C:\Database\PMW_TOX2.L				
1 Myristic acid	...	1140	000544-63-8	95
2 Acetic acid	...	1548	000064-19-7	4
3 Cyclamate-M AC	...	1229	001124-53-4	1
C:\Database\Nist98.l				
1 Tetradecanoic acid		25385	000544-63-8	99
2 n-Decanoic acid		20154	000334-48-5	68
3 Heptadecanoic acid		114893	000506-12-7	62

Fig. A.26: GC -MS library search of *H. depressa* Compound B(A) at a retention time of 18.471 min corresponding with Myristic (Tetradecanoic) acid spectrum

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex minus start of peak



Peak Number: 27 at 20.60 min Area: 52918266 Area % 8.80

The 3 best hits from each library.		Ref#	CAS#	Qual
C:\Database\HPPest.L	No hits were retrieved.			
C:\Database\PMW_TOX2.L				
1 Palmitic acid	...	822	000057-10-3	97
2 Myristic acid	...	1140	000544-63-8	58
3 Diethylene glycol dipivalate	...	1904	000000-00-0	9
C:\Database\Nist98.l				
1 n-Hexadecanoic acid		114901	000057-10-3	99
2 Tridecanoic acid		25772	000638-53-9	95
3 Pentadecanoic acid		114896	001002-84-2	83

Fig. A.27: GC -MS library search of *H. depressa* Compound B(A) at a retention time of 20.602 min corresponding with Palmitic (Hexadecanoic) acid spectrum

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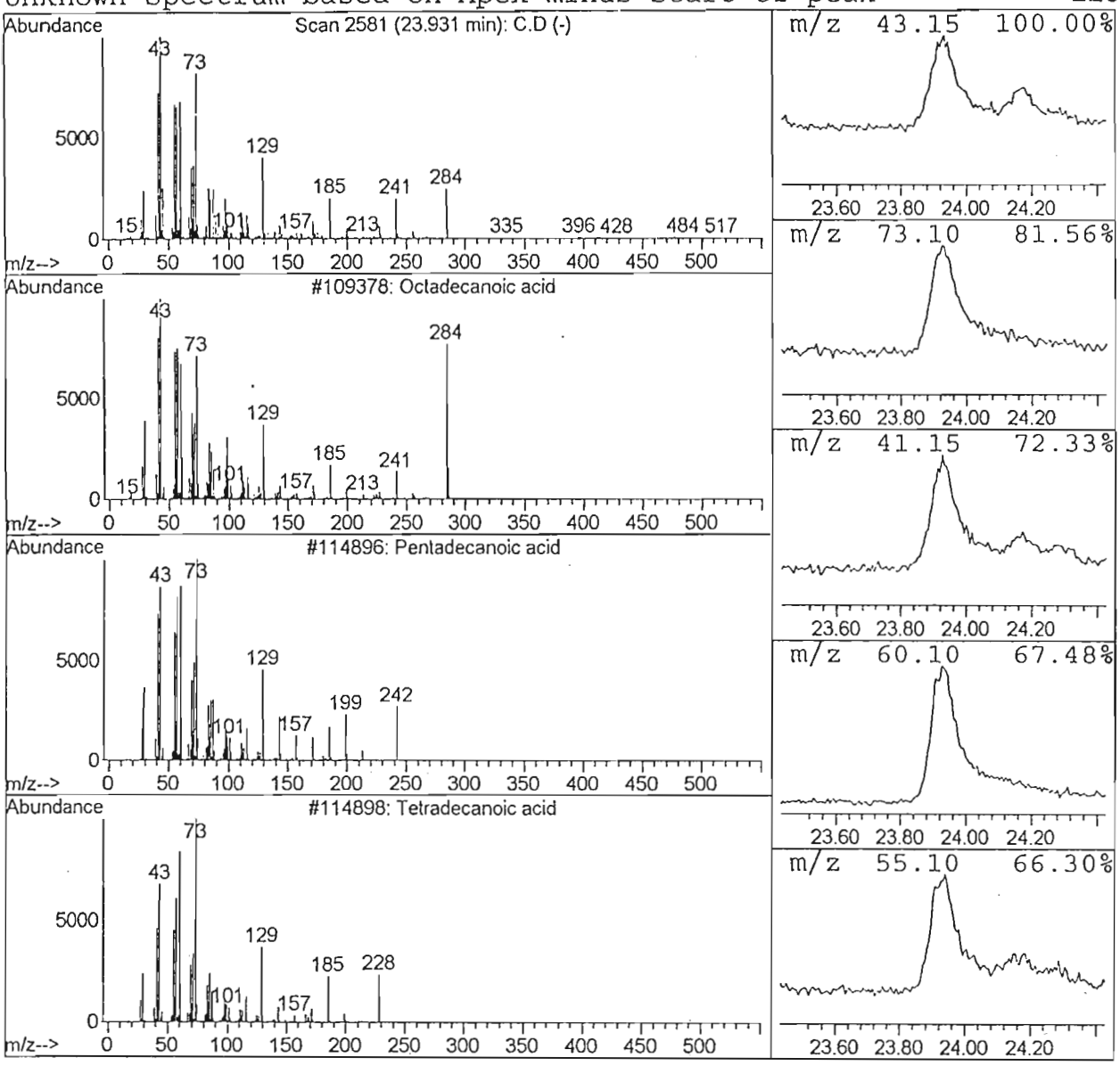
Unknown Spectrum based on Apex minus start of peak 225



Peak Number: 30 at 23.93 min Area: 15291215 Area % 2.54

The 3 best hits from each library.		Ref#	CAS#	Qual
C:\Database\HPPest.L	No hits were retrieved.			
C:\Database\PMW_TOX2.L				
1 Stearic acid	...	969	000057-11-4	86
2 Myristic acid	...	1140	000544-63-8	43
3 Diethylene glycol dipivalate	...	1904	000000-00-0	9
C:\Database\Nist98.1				
1 Octadecanoic acid		109378	000057-11-4	94
2 Pentadecanoic acid		114896	001002-84-2	86
3 Tetradecanoic acid		114898	000544-63-8	62

Fig. A. 28: GC -MS library search of *H. depressa* Compound B(A) at a retention time of 23.931 min corresponding with Stearic (Octadecanoic) spectrum

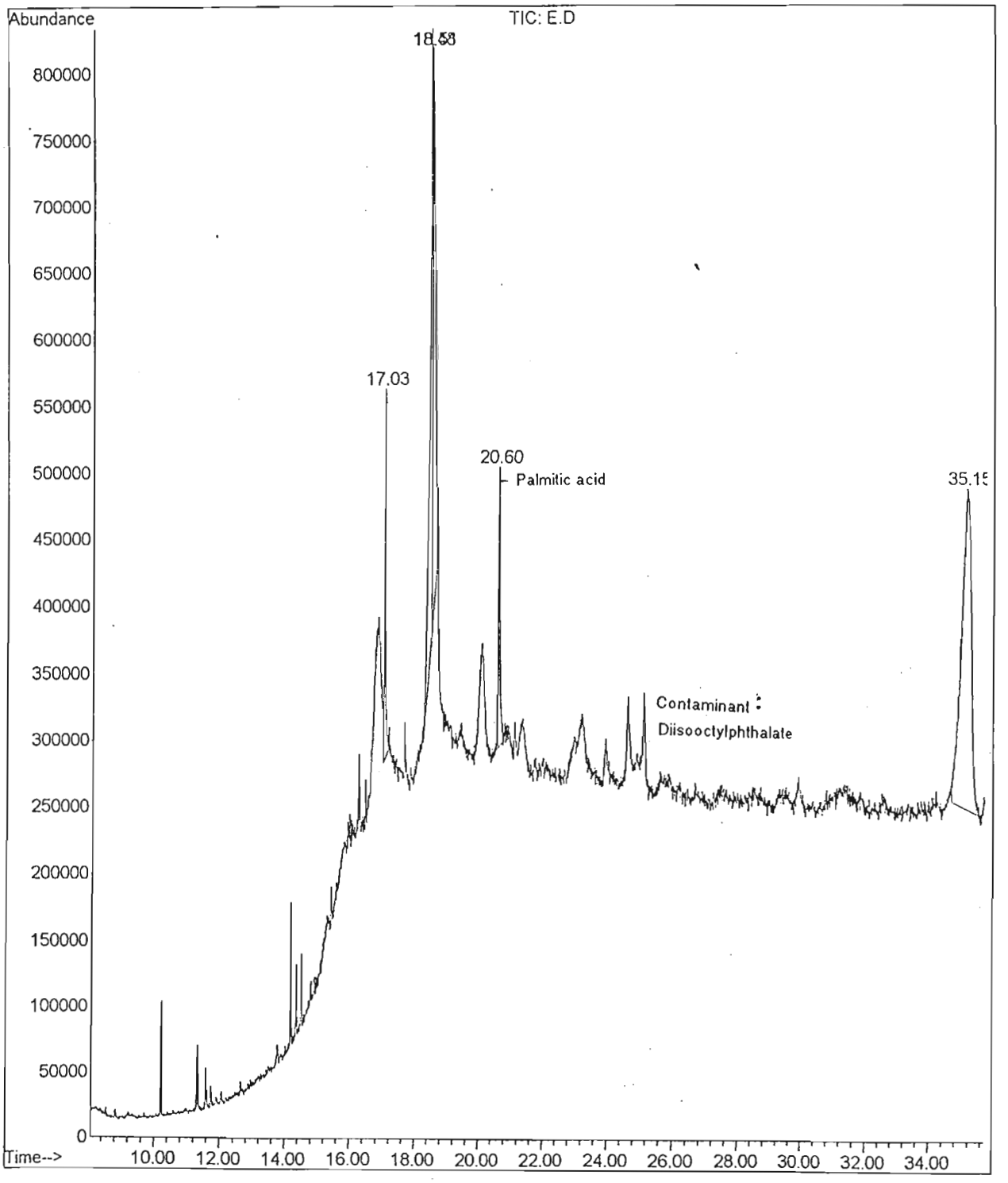
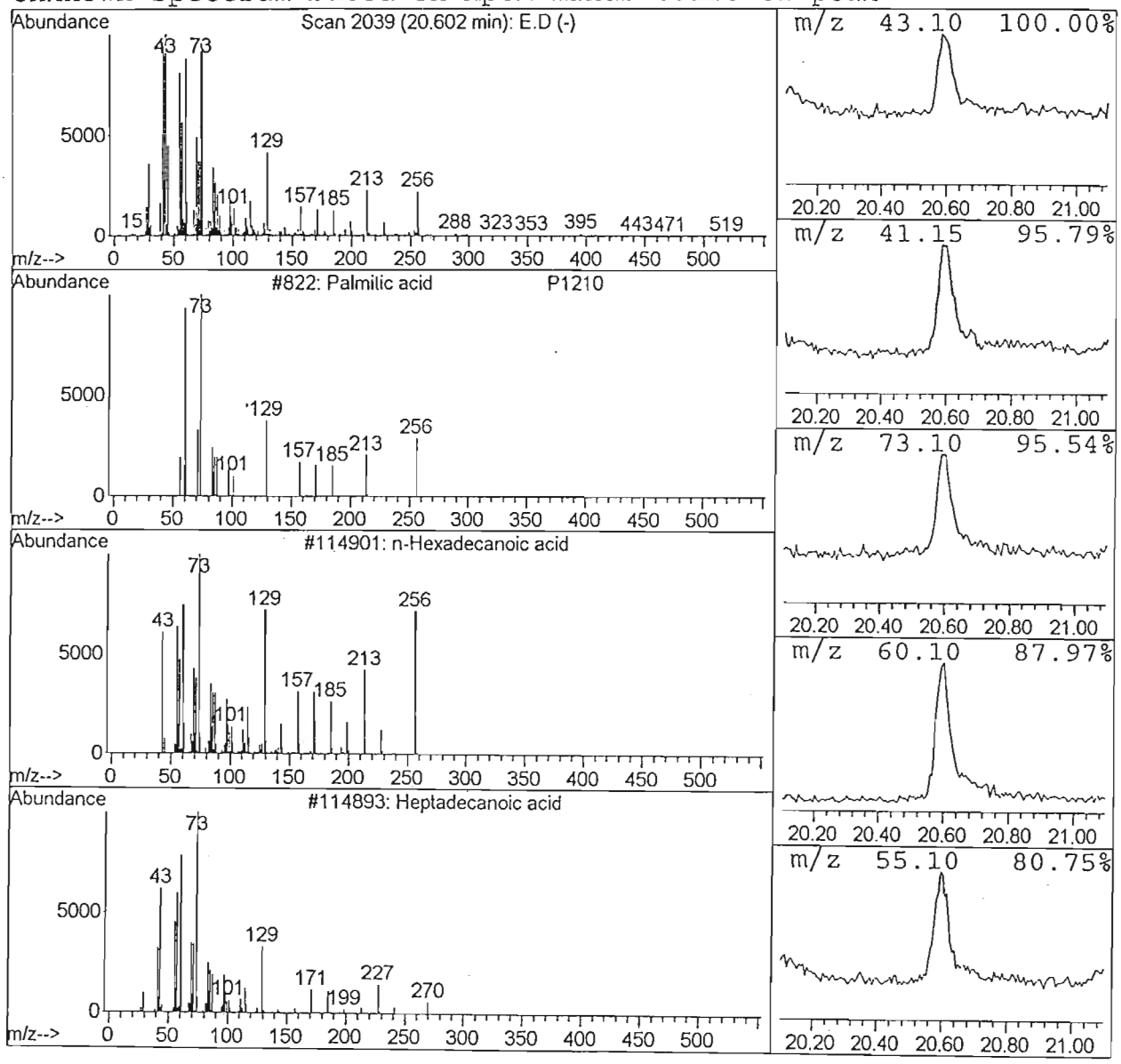


Fig. A.29: *H. depressa* GC-MS spectrum of isolated anti-bacterial Compound B(E)

Library Search Report - Chemstation Integrator

Unknown Spectrum based on Apex minus start of peak 227



Peak Number: 4 at 20.60 min Area: 7302086 Area % 6.61

The 3 best hits from each library.		Ref#	CAS#	Qual
C:\Database\HPPest.L	No hits were retrieved.			
C:\Database\PMW_TOX2.L				
1 Palmitic acid	...	822	000057-10-3	93
2 Myristic acid	...	1140	000544-63-8	64
3 Acetic acid	...	1548	000064-19-7	7
C:\Database\Nist98.l				
1 n-Hexadecanoic acid		114901	000057-10-3	90
2 Heptadecanoic acid		114893	000506-12-7	70
3 Lauric anhydride		114892	000645-66-9	64

Fig. A.30: GC -MS library search of *H. depressa* Compound B(E) at a retention time of 20.602 min corresponding with Palmitic (Hexadecanoic) acid spectrum

APPENDIX B

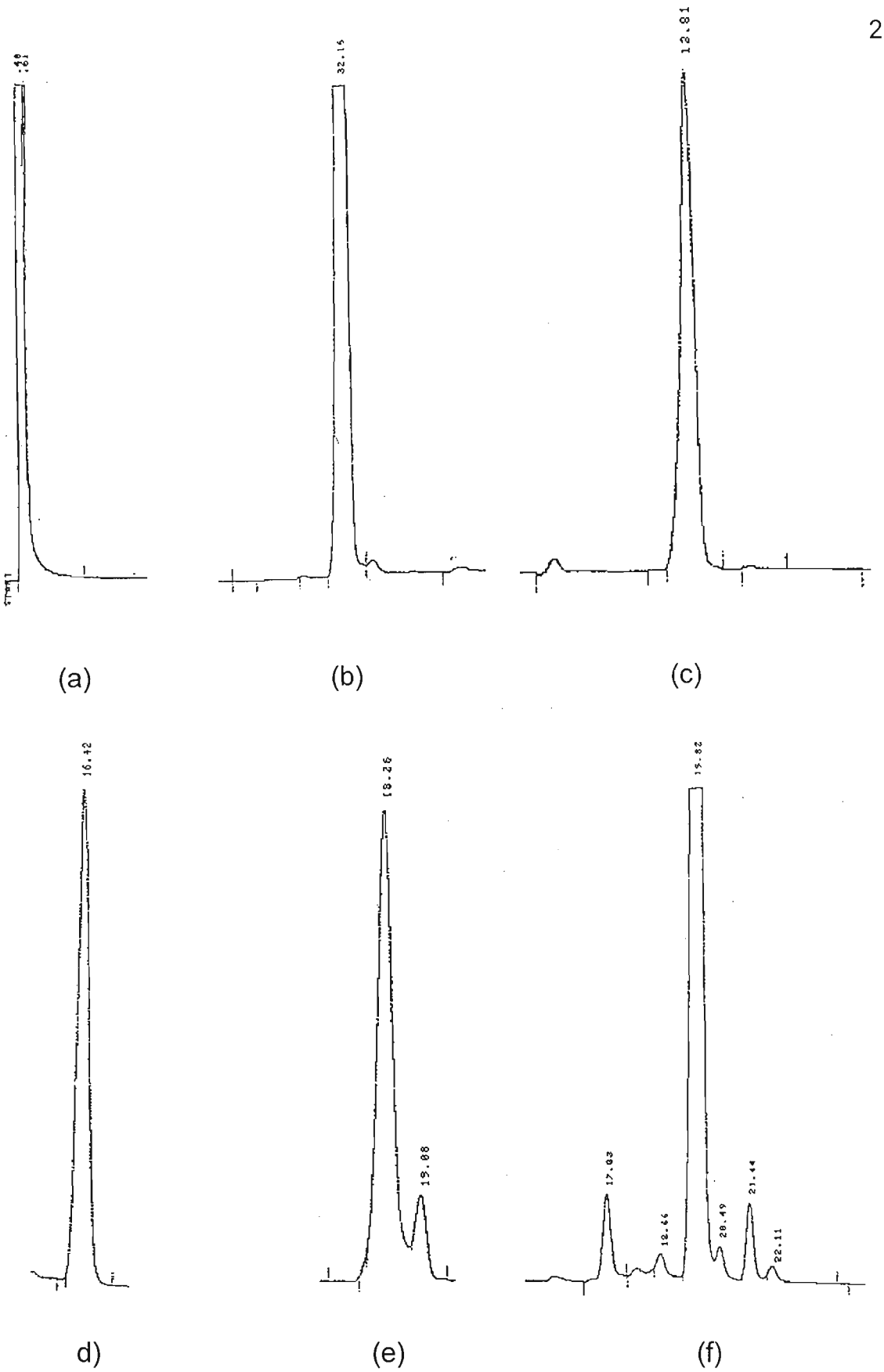


Fig B.1: Representative chromatograms for sugar standards. Peak (a) = solvent (dichloromethane), (b) = sucrose, (c) = L(+) hamnose, (d) = D(-)fructose,(e) = D(+)glucose, (f) = D-glucuronic acid

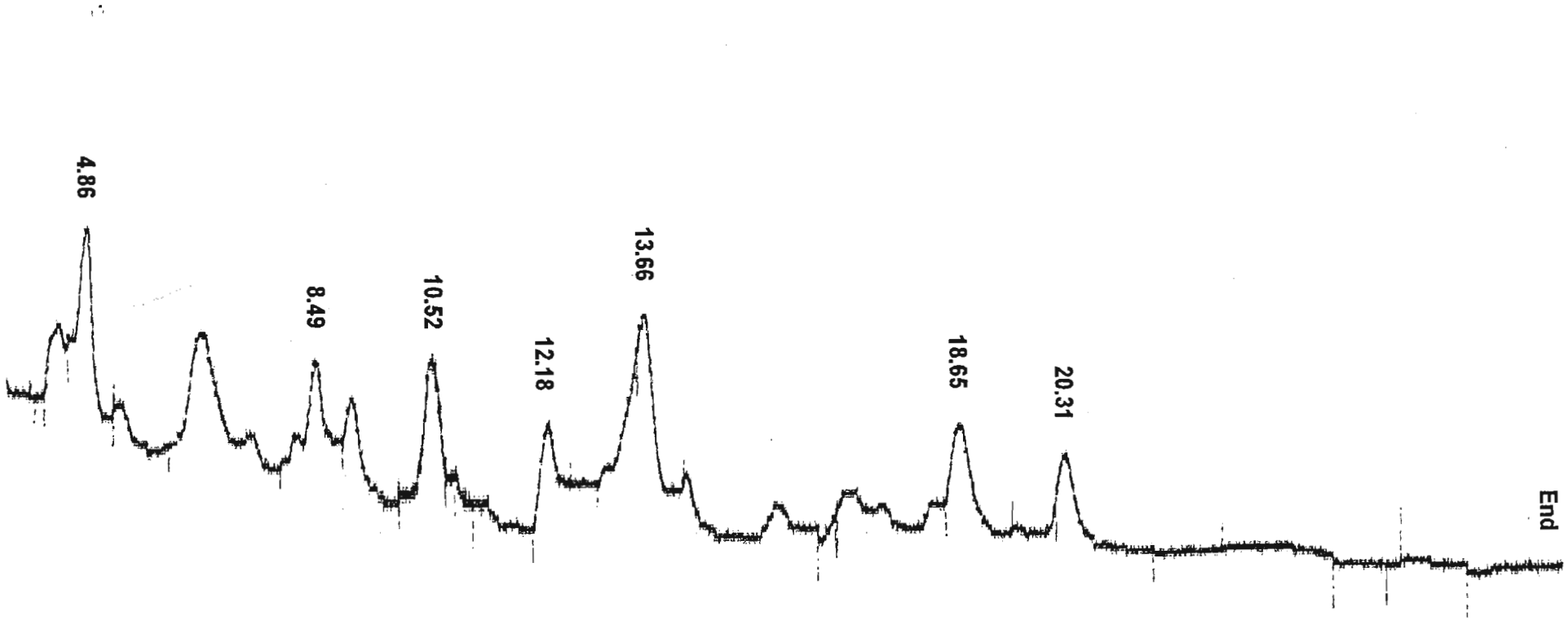


Fig B.2: Representative chromatogram for sugar detection with Sil-A of *H. depressa* aqueous extract.