THE ISOLATION AND CHARACTERISATION OF ANTIBACTERIAL COMPOUNDS FROM *COMBRETUM ERYTHROPHYLLUM* [BURCH.] SOND.

BY

NATALY DOMINICA MARTINI

BPharm, MSc (University of Pretoria)

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Promoter: Dr J.N. Eloff

Co-promoter: Prof. J.R. Snyman

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GLOSSARY OF ABBREVIATIONS

2A:3MDC	Acetone:methylene dichloride (2:3)
Acetone-d_6	Deuterated acetone
ATCC	American Type Culture Collection
BEA	Benzene:ethanol:ammonia (9:1:1%)
BuOH	Butanol
CCl_4	Carbon tetrachloride
C_5D_5N	Deuterated pyridine
CDC	Centre for Disease Control
$CDCl_3$	Deuterated chloroform
CEF	Chloroform:ethyl acetate:formic acid (5:4:1)
C_6H_{14}	Hexane
$CHCl_3$	Chloroform
CL	Chemiluminescence
COSY	^{1}H-^{1}H-COrrelation SpectroscopY
COSY-LR	Long Range COSY
CSIR	Centre for Scientific and Industrial Research
CT	Carbon tetrachloride
DCM	Dichloromethane
DMSO	Dimethyl sulphoxide
DMSO-d_6	Deuterated DMSO
DNA	Deoxyribonucleic acid
EMW	Ethyl acetate:methanol:water (45:5:4.4)
ESBLs	Extended Spectrum β-lactamases
EtOAc	Ethyl acetate
EtOH	Ethanol
FLD	Fluorescence detector
GC-MS	Gas Chromatography-Mass Spectrometry
GMOs	Genetically Modified Organisms
H_2O	Water

HMBC	^{1}H-^{13}C-Heteronuclear Multiple Bond Connectivity
HMQC	^{1}H-^{13}C-Heteronuclear Multiple Quantum Correlation
HPLC	High Performance Liquid Chromatography
HREIMS	High Resolution Electron Impact Mass Spectrometry
INT	p-Iodonitrotetrazolium violet
LDH	Lactate dehydrogenase
LPC	Lysophosphatidylcholine
MCW	Methanol:chloroform:water (12:5:3)
MDC	Methylene dichloride
MHB	Mueller Hilton Broth
MIC	Minimum Inhibitory Concentration
MWD	Multiple Wavelength Detector
NCCLS	National Committee for Clinical Laboratory Standards
NMR	Nuclear Magnetic Resonance
PBS	Phosphate Buffered Saline
PTLC	Preparative Thin Layer Chromatography
RNA	Ribonucleic acid
RP	Reverse phase
SOZ	Serum opsonized zymosan
TLC	Thin Layer Chromatography
TMSI	N-Trimethyl silylimidazole
UV	Ultraviolet
WHO	World Health Organisation
W/M	Water/methanol

SUMMARY

Previous studies [Martini, 1998] on the leaves of *Combretum erythrophyllum* (Combretaceae) confirmed the antimicrobial activity but the compounds responsible for the activity could not be identified due to insufficient material. The main aim of this study was therefore to isolate and identify the antimicrobial compounds from *Combretum erythrophyllum* leaf material. Trees around the Pretoria region were tested for variation in activity and a small difference in bioactivity between plants was noted. Leaf extracts were also tested for free radical scavenging activity exhibiting good antioxidant activity and possible anti-inflammatory activity. Toxicity tests using human lymphocytes showed that compounds isolated were not toxic to these human cells.

For preliminary testing four standard organisms were used to compare the activity of antimicrobial components, namely *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli* and *Pseudomonas aeruginosa.* These are isolates recommended by the National Committee for Clinical Laboratory Standards. The pure compounds isolated were tested using a wider spectrum of bacteria and fungi.

Acetone as solvent extracted 14 antibacterial inhibitors in previous studies and was hence used for the crude extraction. The extracts were complex and group separation by solvent/solvent extraction yielded six fractions. The hexane and chloroform fractions were selected for further study. The hexane fraction contained mainly non-polar compounds and the chloroform fraction both non-polar and polar compounds. Both these fractions showed activity against *S. aureus*.

Methods used for compound isolation were mainly column chromatography, preparative TLC and HPLC using solvents with different polarities and selectivity. Column chromatography produced the best results, yielding a number of pure compounds. Although PTLC was not as effective, seven highly active compounds were isolated from the hexane fraction but were too impure and in insufficient quantities for structure elucidation.

NMR was the method used for identification of isolated compounds and confirmed by MS. The hexane fraction yielded primarily waxes and fatty acids. Although these compounds are known to exhibit good antimicrobial activity, they have not been used in medicinal research due to their poor pharmacokinetic properties and as a result further research with this fraction was abandoned. Previous work with the chloroform fraction produced triterpenoids but these were in insufficient quantity for identification. This study yielded seven antibacterial flavonoids from the same fraction, possibly due to different extraction techniques. Three of these compounds were flavones, i.e. apigenin, genkwanin and 5-hydroxy-7,4’-dimethoxyflavone and four flavonols were identified i.e. kaempferol, rhamnocitrin, rhamnazin and quercetin-5,3’-dimethylether. Although all these compounds are fairly common flavonoids they are all reported for the first time in *Combretum erythrophyllum*, and in some cases in the family Combretaceae.

Bioassays showed selective antibacterial activity between different microorganisms. In some cases MIC values ranged in the order of 25-100 μg/ml with the overall best activity against *Vibrio cholerae*. For some of these compounds this is the first report of antibacterial activity.

OPSOMMING

Vorige studies [Martini, 1998] op die blare van *Combretum erythrophyllum* (Combretaceae) het die antimikrobiese aktiwiteit bevestig. Die verbindings verantwoordelik vir die aktiwiteit kon egter nie geidentifiseer word nie as gevolg van te min materiaal. Die hoof doelwit van hierdie studie was daarom om die antimikrobiese verbindings in die blare van *Combretum erythrophyllum* te isoleer en identifiseer. Bome in die Pretoria gebied was ondersoek om die moontlikheid van variasie in aktiwiteit vas te stel. Daar is nie ‘n noemenswaardige verskil in bioaktiwiteit tussen verskillende plante nie. Blaar ekstrakte was ook getoets vir hul potensiaal om vry radikale op te neem. Hierdie vermoë is aanduidend van antioksidant en moontlike anti-inflammatoriese werking. Toksisiteits toetse op menslike limfosiete het gewys dat die geisoleerde verbindings nie toksies vir hierdie selle is nie.

Vier standaard organismes is in voorlopige toetse gebruik om die aktiwiteit van die antimikrobiese verbindings te vergelyk, naamlik *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli* en *Pseudomonas aeruginosa.* Hierdie isolate is deur die “National Committee for Clinical Laboratory Standards” aanbeveel. Die nuwe suiwer geisoleerde komponente is getoets teen ‘n wyer spektrum bakteriee en swamme.

Asetoon as ekstraeermiddel, het 14 fraksies met antimikrobe aktiwiteit opgelewer en was dus vir die basiese ekstraksies gebruik. Die ekstrakte was kompleks en groep skeiding met vloeistof/vloeistof-ekstraksie het ses fraksies gelewer. Die heksaan en chloroform-fraksies was gekies vir verdere studie. Die heksaan-fraksie het hoofsaaklik nie-polêre verbindings bevat en die chloroform-fraksie beide polêr en nie-polêre verbindings. Beide hierdie fraksies het aktiwiteit teen *S. aureus* getoon.

Die metodes gebruik om verbindings te isoleer was hoofsaaklik kolom chromatografie, preparatiewe dun-laag chromatografie en HDVC met oplosmiddels met verskillende polariteite en selektiwiteit. Kolom chromatografie het die beste resultate gelewer met heel party suiwer verbindings. Alhoewel PDLC nie so effektief was nie, is sewe hoogs aktiewe verbindings geskei deur die heksaan fraksie. Laasgenoemde was egter te onsuiwer en te min vir struktuur uitklaring.

KMR was gebruik vir identifikasie van die geisoleerde verbindings en is bevestig deur MS. Die heksaan fraksie het hoofsaaklik wasse en vetsure gelewer. Alhoewel hierdie verbindings goeie antimikrobe aktiwiteit het, is hulle nie verder vir moontlike medisinale gebruik nagevors nie as gevolg van hul swak farmakokinetiese eienskappe. Vorige werk met die chloroform-fraksie het triterpenoide gelewer maar die opbrengs was te min vir identifikasie. Deur van ander tegnieke gebruik te maak is daar in hierdie studie sewe flavonoide met antimikrobe-aktiwiteit geekstraeer. Drie van hierdie verbindings was flavone, nl. apigenin, genkwanin en 5-hydroksie-7,4’-dimetoksieflavoon en vier was flavonole nl. kaempferol, rhamnocitrin, rhamnazin en quercetin-5,3’-dimetieleter. Alhoewel hierdie verbindings algemene flavonoide is, is hulle almal vir die eerste keer in *Combretum erythrophyllum* geraporteer en in party gevalle in die familie Combretaceae.

Bioessaeering het selektiewe antibakteriese aktiwiteit tussen verskillende mikroorganismes getoon. In sommige gevalle is MIK waardes tussen 25-100 µg/ml gerapporteer met die beste aktiwiteit teen *Vibrio cholerae*. Sommige van hierdie verbindings was nog nooit voorheen beskryf as om oor antimikrobe aktiwiteit te beskik nie.

CONTENTS

“In all things there is a poison, and there is nothing without a poison. It depends only upon the dose whether a poison is a poison or not”.

Paracelsus (1490 – 1541)

CHAPTER 1

1. INTRODUCTION TO ANTIBIOTICS

1.1 DEVELOPMENT OF ANTIBIOTICS

Antibiotics have been called the single most important therapeutic discovery in the history of medicine. An interesting feature of their historic discovery is that they occurred within the lifetime of many of the population living today. It would appear, however, that antibiotics were used even before their discovery. Ancient writings report the application of cloths impregnated with natural substances and other forms of organic matter onto wounds to help them heal. Anthropologists have also unearthed traces of tetracycline in thousand-year old Nubian mummies. The Nubian nation survived from ancient times to the 14th century and some scientists suggest that this might be partially linked to the presence and use of antibiotics such as the tetracyclines [Levy, 1984].

Modern antimicrobial chemotherapy developed in three eras. The first, from 1600 to 1900, involved the use of Cinchona bark in the treatment of malaria. Quinine, the active ingredient, was isolated only later in the 1820s. The synthetic era, which marked the development of various dyes to treat bacterial infections, arrived around the beginning of the 1900s. The first was pyocyanase, a blue pigment produced by *Pseudomonas aeruginosa*, which reduced the growth of other bacteria *in vitro*. When used *in vivo*, it was found to be unstable and toxic. Similar problems occurred with Salvarsan, used in the treatment of syphilis, and Prontosil, active against streptococci *in vitro* [Edwards, 1980].

Possibly the most important event in the discovery of antibiotics occurred around 1940 and began the era of the antibiotics. Although Alexander Fleming discovered penicillin in 1929, it was only recognised for its potential and potency by Florey and his team in 1940. These so-called “miracle drugs” were so scarce in the beginning that penicillin was often re-extracted from the urine for subsequent dosage. Early in the antibiotic era, this valuable agent was freely available to the public in over-the-counter preparations including throat lozenges, nasal ointments and even cosmetic creams. By 1955 most countries restricted its use to prescription only but widespread usage had already occurred, which led to widespread resistance. An answer to this resistance came in the form of methicillin in the early 1960s and this semi-synthetic penicillin was not vulnerable to the bacterial enzymes that degraded penicillin. Other derivatives soon followed but this advance soon revealed the growing pattern of resistance to all available antibiotics [Levy, 1984].

Today the biggest killers in western cultures include cardiovascular disease, neoplasms, nervous system ailments, and not surprisingly, microbial infections. In indigenous people, whose lifestyles differ considerably from western society, other ailments such as diarrhoea, maternity complications and inflammation are regarded as more of a threat to survival [Cox, 1994]. The role that bacterial diseases play in these cultures should not be underestimated since studies performed among inhabitants of West Africa have shown the main endemic bacterial diseases include leprosy, tuberculosis, bacillary dysentery, enteric fevers, undulant fevers and occasionally cholera [Oliver-Bever, 1986].

It is only since the 1940s that we have seen the rapid development and production of antibiotics and the quest for new antibiotics continues but at a slower pace. New drug discoveries often come up with a derivative member of a family already described but more initiatives to find truly novel antibiotics are required and are the current goals for the new millennium [Levy, 1984].

1.2 ANTIBIOTIC RESISTANCE

Drug abuse usually conjures up images of secret meetings in dark allies, gangs and “spaced out” teenagers lying on the floors of clubs. The use of heroin, cocaine, marijuana and even the dependency of stressed-out executives and housewives on anti-anxiety drugs or antidepressants is considered to be substance abuse. Recently a new way of detecting erythropoeitin, used by athletes for performance enhancement, has been brought into action to disqualify those abusing it.

Most of us seem to agree that the world is relying far too much on drugs to either keep us awake or help us to sleep, suppress or stimulate the appetite, control depression and stress and generally make our lives “more bearable”. Few of us would, however, consider the overuse of antibiotics as abuse. Yet the abuse of these compounds could spell more devastating worldwide health consequences to both humans and animals than the accumulated abuse of all the world’s narcotics put together [King, 2000].

Almost since the beginning of the antibiotic era, bacterial resistance has been seen as the major obstacle to successful treatment. Hardly any group of antibiotics has been introduced to which some bacterium has not developed resistance. Resistance was often minimised as a problem simply because the problem was not known or recognised. At the end of the 1960s the Surgeon General of the United States stated that “we could close the book on infectious diseases.” At the time he uttered these words the emergence of resistance did not seem to affect therapeutic options and although *S. aureus* had become resistant to benzylpenicillin and showing resistance to methicillin, it remained sensitive to gentamicin and infections could therefore still be treated [Amyes, 2000].

At the start of a new century, things look very different. Already at least three bacterial species capable of causing life-threatening illness (*Enterococcus faecalis, Mycobacterium tuberculosis* and *Pseudomonas aeruginosa*) are already known to be resistant to every one of the over 100 antibiotics available except for vancomycin. Vancomycin is the antibiotic of last resort for treatment of resistant infections and within the past year scientists have found strains of *Streptococcus pneumoniae* and *Staphylococcus aureus* to be resistant to this antibiotic. With the advent of these reports and new cases emerging continuously, an ingrown toenail, sore throat or a dirty cut could spell death.

Why is this happening? Well firstly we tend to view all bacteria as "the enemy" to be quickly dealt the deathblow with antiseptics, disinfectants and antibiotics. In human medicine alone, the US Centre for Disease Control and Prevention estimates that approximately one-third of the 150 million prescriptions for antibiotics written each year were unneeded [http://www.agric.gov., 2000].

Although antibiotic discovery has been exponential since the 1940s, no new clinically useful drugs were discovered after 1961 and almost all the drugs that have been launched since the 1960s are modifications of the antibiotics we already have. The introduction of organ transplantation in the late 1960s prompted the massive increase in antibiotic use, which was accompanied by an increase of methicillin resistance in staphylococci and vancomycin resistance in enterococci [Amyes, 2000].

Recent reports have also shown a marked increase of antibiotic resistance of food-poisoning bacteria due to non-rational and excessive uses of antibiotics as therapeutic agents or as growth promoter in livestock. Another aspect of resistance lies in the use of antibiotic resistant genes as selection markers in genetically modified organisms (GMOs). The main safety factor concern is the release of the resistant genes to sensitive organisms when these GMOs are introduced into the environment [http://www.biosafety, 1999].

1.2.1 Battlegrounds

There are two main zones of combat today, the hospital and the community, which are neither discrete nor separate. Patients are being discharged from the hospital into the community and those with communal infections are being hospitalised. The ease of travel means the transport of resistant organisms into foreign lands, making it a global problem. As a result of this increasing and dangerous problem, the Centre of Disease Control (CDC), World Health Organisation (WHO) and local public health groups have initiated various surveillance programs. Some of these programs have been monitoring resistance for decades and many pharmaceutical companies have supported national and international surveillance programs. Despite all of these efforts it is evident that a more deliberate attention is required and *grass-roots* methods employed [Operation Resistance, 2000].

1.2.2 Battle lines

Enterobacteriaceae

One of the largest groups of bacteria is the Enterobacteriaceae, presently comprising over 120 species. These are grouped in genera of which the most infamous include *Klebsiella, Enterobacter* and *Serratia* (K.E.S.). Also included are *Escherichia, Proteus, Salmonella, Shigella* and *Yersinia*.

These members are found in many different locations and have been associated with virtually every type of infection covering abscesses, pneumonia, meningitis, septicaemia and other site-specific infections. Collectively these organisms are responsible for one in six nosocomial infections. Internationally these organisms have caused significant hospital outbreaks in France, Germany, Australia, UK, Greece, Tunisia and the USA [Operation Resistance, 2000].

The production of simple β-lactamases by the Enterobacteriaceae has been recognised for years and since the 1970s, resistance to the current β-lactams, trimethoprim-sulphamethoxazole (TMP/SMX) and often the aminoglycosides, was causing outbreaks of nosocomial infections. In the 1980's, broader spectrum agents were developed, which included the second- and third-generation cephalosporins and the aminopenicillins. Soon it became evident that these organisms evolved their own β-lactamases in response to environmental pressure and these enzymes are known as Extended Spectrum β-Lactamases (ESBLs). These enzymes soon inactivated agents such as cefoxitin, cefotetan and more recently, the carbapenems. These ESBLs destroy most second- and third generation cephalosporins but also have activity against potentiating agents such as clavulanic acid, tazobactam and sulbactam. This resistance may also accompany resistance to other classes of antibiotics. Table 1.1 shows the susceptibility to a range of commonly prescribed hospital antibiotics.

Table 1.1: Antimicrobial Susceptibility (%) of Selected Nosocomial Pathogens [Adapted from Operation Resistance, 2000]

Antimicrobial agent	*E. coli*	*K. pneumoniae*	*E. aerogenes*	*E. cloaceae*
Cefuroxime	95	91	77	59
Ceftazidime	96	92	81	77
Cefotaxime	98	89	77	67
Ceftriaxone	97	96	75	77
Pipercillin-tazobactam	95	87	74	77
Imipenem	97	98	95	97
Gentamicin	97	95	97	96
Ciprofloxacin	96	88	88	93

Examination of data from the NNIS showed a linear increase in resistance of *Enterobacter* species and *Klebsiella pneumoniae* to ceftazidime over the periods 1987-1989 to 1990-1991 respectively:

K. pneumoniae 1.5% - 3.6%

Enterobacter sp. 32.9% - 38.6%

This ability to cause new mutations and therefore a broader range of β-lactam inactivating enzymes provide these organisms with a protective shield from beneath which they can cause significant morbidity and mortality.

Respiratory Tract Infections

Approximately 63% of antibiotic prescriptions are written for respiratory tract infections (RTIs) [Operation Resistance, 2000] and these infections account for nearly half of all antibiotics used in intensive care units [Singh, 2000]. Of these infections, the one most feared is that caused by *Streptococcus pneumoniae*. Globally each year at least one million people die due to *S. pneumoniae* infections and its increasing resistance to antibiotics is frightening. Already in 1945 resistant mutants were created in the laboratory setting and since 1967 it has travelled the globe a remarkable number of times. During these travels, MICs to penicillin have increased as well as resistance to other antimicrobial drugs including the macrolides, TMP/SMX, tetracyclines and chloramphenicol. One survey in the USA shows that penicillin resistance is present in approximately 35% of all community-derived isolates of *S. pneumonia* (see Figure 1.1) [Operation Resistance, 2000], while another reported an increase of 45% [Gotfried, 2000]. Slightly lower levels of resistance to other antimicrobials are also demonstrated with the exception of the newer quinolones where very few resistant strains have been seen.

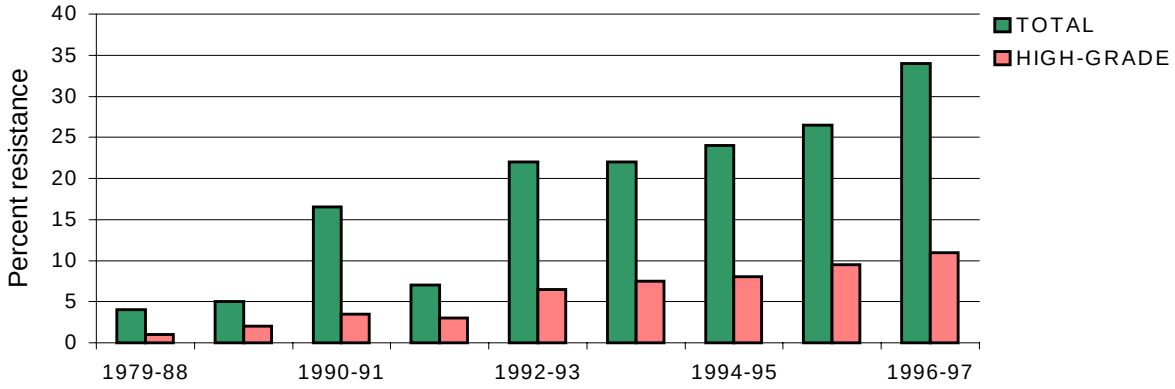


Figure 1.1: Percentage of the total and high-grade resistance of *S. pneumoniae* to penicillin [adapted from Operation Resistance, 2000]

Other respiratory pathogens include *Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus* and *Streptococcus pyogenes*. Since 1970 the incidence of β-lactamase producing *H. influenzae* strains have risen so that today over a third are resistant to amoxicillin. Only the quinolones are currently impervious to its resistance [Operation Resistance, 2000]. A surveillance study of patients with respiratory infections in Saudi Arabia showed a 13.2% resistance of *H. influenzae* to ampicillin, 7% to tetracycline, 5.4% to chloramphenicol, 3.9% to roxithromycin and 1.6% to amoxicillin/clavulanic acid [Abdel-Rahman, 1999].

Staphylococcus aureus is also a leading cause of nosocomial infections and is almost always resistant to β-lactams, macrolides and tetracyclines, leaving few alternatives [Operation Resistance, 2000].

According to the SENTRY Antimicrobial Surveillance Program (1998) the four most frequently isolated organisms isolated from the lower respiratory tract of patients hospitalised with pneumonia were *P. aeruginosa* (26.8%), *Staphylococcus aureus* (24.0%), *Klebsiella pneumoniae* (12.1%) and *Acinetobacter* spp. (10.5%). Of the more than 40 antimicrobial agents tested, amikacin (77.5% susceptible) was the most active drug tested against *P. aeruginosa*. Only meropenem (78.3%) and imipenem (81.3%) possessed susceptibility rates greater than 50% against the *Acinetobacter* spp. isolates. Resistance to oxicillin by *S. aureus* was nearly 50% and several clusters of multiple resistant organisms were observed. These results indicate the rapid rates of resistance among respiratory tract pathogens and the concern of multiple resistant strains in several medical centres [Lewis, 2000]. Figure 1.2 shows the percentage resistance of respiratory tract isolates versus non-respiratory tract isolates over a period of eight years. Respiratory tract isolates became notably more resistant to imipenem, ceftazidime, cefotaxime, ticarcillin/clavulanic acid and piperacillin than were non-respiratory isolates [Flournoy, 2000].

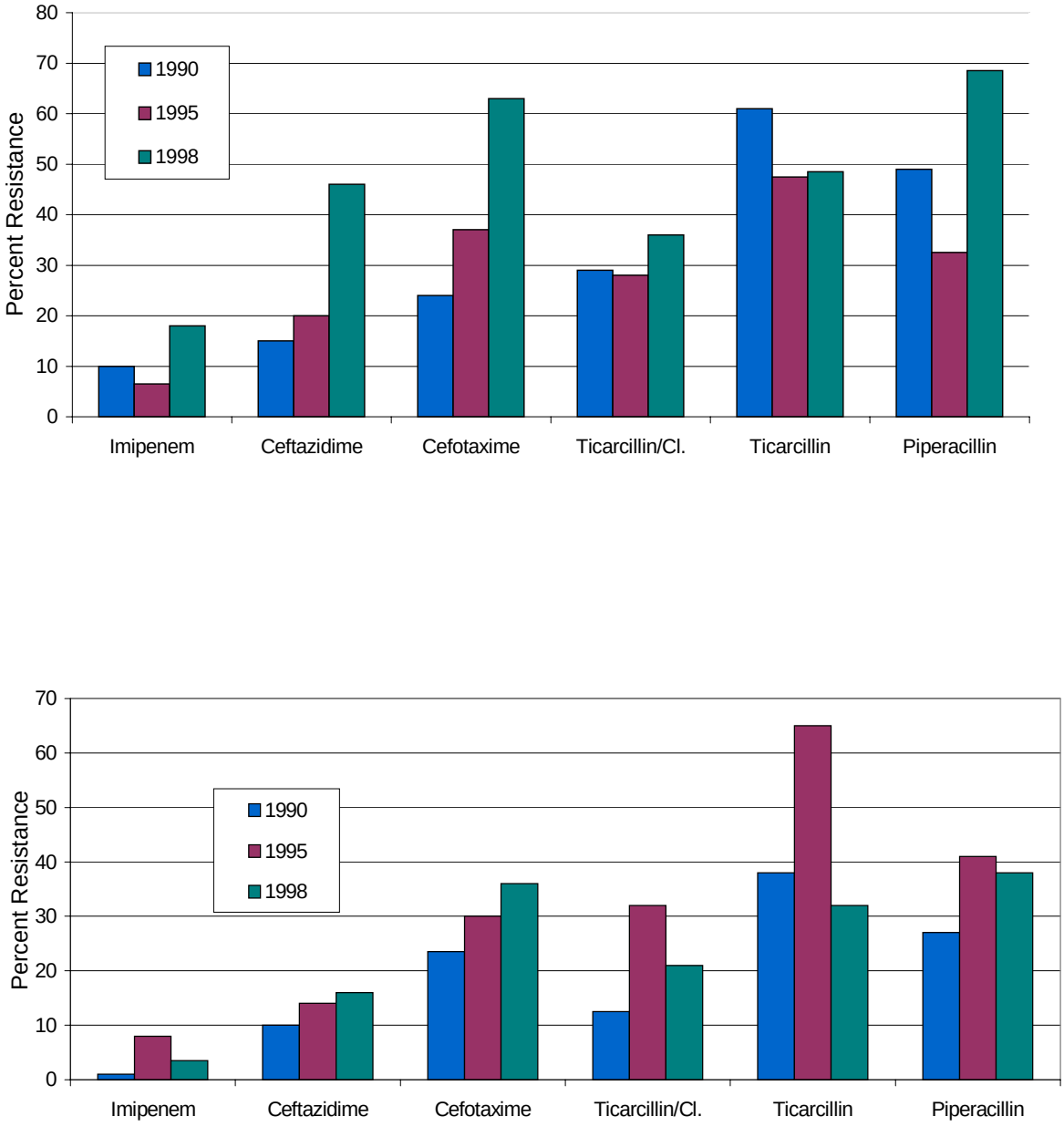


Figure 1.2: The increasing resistance of respiratory initial isolates (top graph) and non-respiratory isolates (bottom graph) between 1990 and 1998 to different antibiotics. (Ticarcillin/Cl. is the combination of Ticarcillin with clavulanate) [Adapted from Flournoy, 2000].

Urinary tract infections (UTIs)

The cost and treatment of this infection costs at least $1 billion annually in the USA. Table 1.2 shows the pathogens responsible for UTIs and susceptibility rates of various antimicrobials.

Table 1.2: Susceptibility rates of various antimicrobials in acute cystitis in percentage [Adapted from Operation Resistance, 2000]

Pathogen	Incidence	Ampicillin	TMP/SMX	Nitrofurantoin	Ciprofloxacin
E. coli	86	74	91	99	99.8
S. saprophyticus	4	0	92	100	?
Proteus spp	3	92	95	0	100
Klebsiella spp	3	2	91	46	100
Enterobacter spp	1.4	8	98	48	100
Citrobacter spp	0.8	12	97	82	100

As there has been only one novel class of antimicrobial agents in the last 25 years, it is essential that something be done to either prescribe the existing drugs more rationally, to try to minimise infection or to find novel antimicrobial agents [Operation Resistance, 2000].

1.3 QUANTITATIVE ASPECTS

Clinically, *in vitro* antimicrobial susceptibility tests are useful as a guide for determining antimicrobial chemotherapy whenever the susceptibility of a pathogen is unpredictable or when an infection has not responded to therapy that otherwise appears appropriate [NCCLS, 1991].

Minimum inhibitory concentration (MIC) is defined as the minimum concentration required to inhibit 50% of a bacterial population. MIC however, does not represent an absolute value and the “true” MIC is somewhere between the lowest

test concentration that inhibits the organism growth ("read" MIC) and the next lower test concentration. Even under the best of controlled conditions a dilution test may not yield the same end-point each time it is repeated but gives an indication of the concentration of an antimicrobial agent that must reach the site of infection to inhibit the infecting organism. Table 1.3 gives an indication of MIC values of a selected range of antibiotics against four reference strains.

Table 1.3: Acceptable quality control ranges of MICs (μg/ml) for reference strains (Adapted from NCCLS, 1991)

Antibiotic	***S. aureus*** **ATCC 29213**	***E. faecalis*** **ATCC 29212**	***E. coli*** **ATCC 25922**	***P. aeruginosa*** **ATCC 27853**
Amikacin	1-4	64-256	0.5-4	0.5-8
Azithromycin	0.28-1	-	-	-
Cefaclor	1-4	>32	1-4	-
Cefazolin	0.25-1	>16	1-4	-
Cefuroxime	0.5-2	-	2-8	-
Chloramphenicol	2-8	4-16	2-8	-
Clarithromycin	0.12-0.5	-	-	-
Erythromycin	0.12-0.5	1-4	-	-
Gentamicin	0.12-1	4-16	0.25-1	0.25-4
Lomefloxacin	0.25-2	2-8	0.03-0.12	1-4
Methicillin	0.5-2	>16	-	-
Ofloxacin	0.12-1	1-4	0.015-0.12	1-8
Penicillin G	0.25-1	1-4	-	-
Teicoplanin	0.25-1	0.06-0.25	-	-
Tetracycline	0.25-1	8-32	1-4	8-32
Vancomycin	0.5-2	1-4	-	-
Trimethoprim	1-4	≤1	0.5-2	>64

1.4 CONCLUSION

In a crisis such as this where we are faced with the desperately growing need for novel antimicrobial compounds in the fight against resistance, it seems logical to turn towards Mother Nature for answers. Secrets to her success have been discovered over many centuries within various sources and a survey conducted in 1967 calculated that over 58% of all antibiotics are produced by Actinomycetes, 18% by fungi, 12% by higher plants, 9% by bacteria and the remaining 3% by algae, litchens and animals [Edwards, 1980]. Although these antibiotics are now synthesised in laboratories, they were originally discovered inside living organisms. For this very reason it became the aim of this study to review natural resources, in particular *Combretum erythrophyllum* used by traditional healers in their treatment of infection, with the hope of isolating and identifying the compounds responsible for its activity. This is discussed in further detail in Chapters 2 and 3.

CHAPTER 2

2. PLANTS IN MEDICINE

2.1 APPROACHES TO PLANT-DERIVED DRUG DEVELOPMENT

2.1.1 Brief history

Fossils of plants date back as early as 3.2 billion years ago. These plants provided the foundation upon which animal life and later, human life were based on. They provide bodybuilding food and calories as well as vitamins essential for metabolic regulation. Plants also yield active principles employed as medicines [Shultes, 1992].

Finding healing powers in plants is an ancient idea. Hundreds, if not thousands, of indigenous plants have been used by people on all continents as poultices and infusions dating back to prehistory. There is evidence of Neanderthals, living 60 000 years ago in present-day Iraq, using hollyhock (*Alcea rosea* L.), which is still in ethnomedicinal use around the world today [Cowan, 1999]. The Bible offers descriptions of at least 30 healing plants of which frankincense (*Boswellia sacra* L.) and myrrh (*Commiphora myrrha* L.) were employed as mouthwashes due to their reported antiseptic properties.

The fall of ancient civilisations resulted in the destruction or loss of much of the documentation of plant pharmaceuticals but many cultures continued in the excavation of the older works as well as building upon them. Native Americans were reported to have used 1625 species of plants as food while 2564 found use as drugs, while the Europeans started turning towards botanicals when treatment in the 1800s became dangerous and ineffective [Cowan, 1999]. Today some 1500 species of medicinal and aromatic plants are widely used in Albania, Bulgaria, Croatia, France, Germany, Hungary, Spain, Turkey and the United Kingdom [Hoareau, 1999].

2.1.2 Secondary plant compounds

So why have plants been so widely used in the treatment of disease? And how would plant chemicals kill human and animal pathogens and influence immune systems, hormonal balances, organs and cells of the body?

Living organisms, whether they are plants, animals or microbes, are all made up of basically the same chemical components. Comparative biochemistry has shown that minor or major modifications of chemical processes have been evolved by different organisms to suit their particular requirements [Goodwin and Mercer, 1983]. Chemical compounds have been extensively used in plant systematics and two major categories of systematically useful chemical compounds can be recognised: secondary metabolites, which perform non-essential functions in the plant, and the information containing proteins, DNA and RNA.

Most secondary compounds function in defence against predators and pathogens, as allelopathic agents or attractants in pollination and seed dispersion. The major categories are discussed briefly below.

2.1.2.1 Terpenoids

Terpenoids are a large and structurally diverse group of secondary compounds that are important in numerous biotic interactions. They are widely distributed and many have primary physiological functions, either forming part of membrane-bound steroids, carotenoid pigments, side-chain of chlorophyll and the hormones giberellic acid and abcisic acid. Volatile monoterpenoids are the major components of essential oils and often function as floral odour glands [Goodwin and Mercer, 1983]. More information regarding these compounds is discussed in chapter 3.

2.1.2.2 Alkaloids

Cinchona alkaloids present in the bark of *Cinchona* spp. have quinine as their main constituent, known since 1630 for its antimalarial properties. All alkaloids contain nitrogen, which frequently forms part of a heterocyclic ring and make them basic in nature. Widespread distribution in all parts of plants has stimulated searches for a function of these compounds in the general metabolism of plants. These compounds offer protection against predators, act as growth regulators, maintain ionic balance, act as a nitrogen reserve and possibly serve as nitrogen excretion products [Goodwin and Mercer, 1983].

2.1.2.3 Flavonoids

These represent a widespread group of water-soluble phenolic derivatives, many of which are brightly coloured. At least nine classes of flavonoids are recognised and are of great interest in phytochemistry. They probably function in defence against herbivores and in regulation of auxin transport. Attraction of insects and birds also play an important role in seed dispersal and pollination [Goodwin and Mercer, 1983].

2.1.2.4 Others

Stilbenes and tannins are both synthesised via the same pathway resulting in isoflavones and other phenolic metabolites [Figure 8.1]. These compounds are used by plants as growth inhibitors and antimicrobial compounds [Goodwin and Mercer, 1983].

2.1.3 The birth of pharmacology

Natural products have been used to elucidate physiological processes and even define them, hence the naming of ‘nicotinic’ and ‘muscarinic’ receptors and even more recently ‘endorphins’ from ‘endogenous morphines’. Natural products are the basis of many standard drugs used in modern medicine and are so widely used that even some members of the medical profession are not aware of their plant origin. Some of the newer pharmacological tools such as colforsin (an adenyl cyclase stimulator), ginkgolide B (a specific platelet activating factor (PAF) antagonist) and phorbol esters that activate proteinkinase C, are at the forefront of biochemical research and are obtainable only from plant sources [Williamson, 1996].

Although medicinal plants may not always lead to the discovery of novel compounds which may be employed in the treatment or cure of disease, plants may give valuable insight into the pathology of diseased conditions or the disturbed human mind. Hallucinogenic plants have been thought to transport the mind to realms of ethereal wonder and some were even considered to be gods. It is only recently, the past twenty years, that modern westernised societies have realised the significance of these plants in shaping the history of primitive and even advanced cultures. Some of these plants contain chemicals capable of inducing visual, auditory, tactile, olfactory and gustatory hallucinations or causing artificial psychoses and the question is whether a thorough understanding of the chemical composition of these drugs may lead to discovery of new drugs for the treatment of psychiatric conditions. As a result of the complexity of the human brain and central nervous system, psychiatry has not developed as rapidly as other fields of medicine, mainly due to the lack of adequate tools, therefore these drugs may provide the necessary pharmacological tools for the discovery of more appropriate and effective drugs [Shultes, 1992].

Although the first chemical substance to be isolated from plants was benzoic acid in 1560, the search for useful drugs of known structure did not begin until 1804 when morphine was separated from *Papaver somniferum* L. (Opium). Since then many drugs from higher plants have been discovered but less than 100 of defined structure are in common use today. Less than half of these are accepted as useful drugs in industrialized countries [Farnsworth, 1984].

Table 2.1: Plant-derived drugs widely employed in Western medicine (Adapted from Farnsworth, 1984]

Acetyldigoxin	Ephedrine*	Pseudoephedrine*
Aescin	Hyoscyamine	Quinidine
Ajmalicine	Khellin	Quinine
Allantoin*	Lanatoside C	Rescinnamine
Atropine	Leurocristine	Reserpine
Bromelain	α-Lobeline	Scillarens A & B
Caffeine*	Morphine	Scopolamine
Codeine	Narcotine	Sennosides A & B
Colchicine	Ouabain	Sparteine
Danthron*	Papain	Strychnine
Deserpidine	Papaverine*	Tetrahydrocannabinol
Digitoxin	Physostigmine	Theobromine*
Digoxin	Picrotoxin	Theophylline*
L-Dopa*	Pilocarpine	Tubocurarine
Emetine	Protoveratrines A & B	Vincaleukoblastine
		Xanthotoxin

* Produced industrially by synthesis

Less than 10 of these well-established drugs are produced commercially by synthesis although laboratory synthesis has been described for most of them [Farnsworth, 1984].

In 1875 a folk healer told the English physician William Withering that the leaves of *Digitalis purpurea* L. were useful for treating dropsy, a swelling of the body caused by inadequate pumping action of the heart. In an effort to standardise the dosage, he began to administer water infusions of leaves and later ground leaf powder.

Since then more than 30 cardiac glycosides have been isolated from *D. purpurea*. Withering's success in translating folk knowledge into new pharmaceuticals is not unique. Of all plant-derived drugs listed in Samuelsson [1992], at least 50 were derived from ethnobotanical leads [Table 2.2). Farnsworth [1990] estimated that there are at least 88 ethnobotanically derived drugs [Cox, 1994].

Plants seem to have served as models in drug development for 3 reasons. Firstly at least 25% of all prescriptions contain active principles extracted from higher plants, which has persisted for at least the last 25 years [Farnsworth & Morris 1976, Farnsworth 1982b] and as many as two-thirds of people in developing countries rely on plants as sources of drugs [Farnsworth, 1984]. Secondly, biologically active substances derived from plants may have poor pharmacological or toxicological profiles for use in humans per se. They can, however serve as templates for synthetic modification and structure-function studies with anticipation of useful drugs in man will result. Thirdly, many secondary, highly active, plant constituents are found to be useful in studying biological systems and disease processes.

2.1.4 Current developments

The rationale for studying plants as traditional medicines is that 80% of 5200 million people live in less developed countries. The WHO estimates that 80% of these people rely almost exclusively on traditional medicine for their primary health care needs. Since medicinal plants are the 'backbone" of traditional medicine, this means that more than 3300 million people utilize medicinal plants on a regular basis. [Farnsworth, 1994].

It cannot be denied that higher plants have yielded many useful drugs to alleviate the medical problems facing the World's population. In 1985, Farnsworth identified 119 secondary metabolites isolated from higher plants that were being used globally as drugs [Table 2.2][Farnsworth, 1990]. About 75% of these drugs have the same or related uses as the plant from which they were discovered. These 119 useful

drugs are still obtained commercially for the most part by extraction from only about 90 species of plants. With more than 250 000 species of higher plants more useful drugs remain to be discovered.

There is a great demand and potential for medicinal plant research as shown by the growing market in medicinal herbs. They are high in value, low in shipping volume, popular with the public interested in natural products and strong competitors for synthetic drugs developed at high costs.

In 1980 the consumer paid about $ 8.0 billion for prescription drugs in which the active principles were derived from plants [Farnsworth & Morris, 1976, Farnsworth 1982b]. Dollar values from 1994 also provide strong support: $6.5 billion in Europe, $2.1 billion in Japan, $2.3 billion in the rest of Asia and $1.5 billion in North America. It is estimated that medicinal plants are therefore a $12 billion market and expected to increase for another 10 or 20 years [http://www.bizjournals]. In spite of this, not a single pharmaceutical manufacturer in the United States had a serious research program designed to discover new drugs from the plant kingdom at that stage [Farnsworth, 1984].

In developed countries, the cost of taking a drug from the discovery stage to the market place can exceed $50 million and span a period of several years. The industry is therefore reluctant to invest in the development of any drug when its investment cannot be recovered.
Failure of many programs to produce useful drugs after several years of intensive effort and millions of dollars, signals to many that plants are an uninteresting source of useful drugs [Farnsworth, 1984].

In spite of the widespread usage of plants and plant products, there is often no evidence to support their use. No clear rationale is proposed for most product use over the traditional beliefs and superstition and therefore a scientific explanation is warranted.

Table 2.2: Fifty drugs discovered by ethnobotanical leads [Farnsworth, 1990]

Drug	Medical use	Plant Source
Ajmaline	Heart arrhythmia	Rauvolfia spp.
Aspirin	Analgesic, anti-inflammatory	Filipendula ulmaria
Atropine	Pupil dilator	Atropa belladonna
Benzoin	Oral disinfectant	Styrax tonkinensis
Caffeine	Stimulant	Camellia sinensis
Camphor	Rheumatic pain	Cinnamomum camphora
Cascara	Purgative	Rhamnus purshiana
Cocaine	Ophthalmic anaesthetic	Erythoxylum coca
Codeine	Analgesic, antitussive	Papaver somniferum
Colchicine	Gout	Colchicium autumnale
Demecoline	Leukaemia, lymphomata	C. autumnale
Deserpidine	Antihypertensive	Rauvolfia canescens
Dicoumarol	Antithrombotic	Melilotus officinalis
Digoxin	Atrial fibrillation	Digitalis purpurea
Digitoxin	Atrial fibrillation	D. purpurea
Emetine	Amoebic dysentery	Psychotria ipecacuanha
Ephedrine	Bronchodilator	Ephedra sinica
Eugenol	Toothache	Syzygium aromaticum
Gallotanins	Haemorrhoid suppository	Hamamelis virginia
Hyoscamine	Anticholinergic	Hyoscyamus niger
Ipecac	Emetic	Psychotria ipecacuanha
Ipratropium	Bronchodilator	H. niger
Morphine	Analgesic	Papaver somniferum
Noscapine	Antitussive	P. somniferum
Papain	Attenuator of mucous	Carica papaya
Papaverine	Antispasmodic	Papaver somniferum
Physostigmine	Glaucoma	Physostigma venenosum
Picrotoxin	Barbiturate antidote	Anamirta cocculus
Pilocarpine	Glaucoma	Pilocarpus jaborandi
Podophyllotoxin	Condyloma acuminatum	Podophyllum peltatum
Proscillaridin	Cardiac malfunction	Drimia maritima
Protoveratrine	Antihypertensive	Veratrum album
Pseudoephedrine	Rhinitis	E. sinica
Psoralen	Vitiligo	Psoralea corylifolia
Quinine	Malaria prophylaxis	Cinchona pubescens
Quinidine	Cardiac arrhythmia	C. pubescens
Rescinnamine	Antihypertensive	R. serpentia
Reserpine	Antihypertensive	R. serpentia
Sennosides A,B	Laxative	Cassia angustifolia
Scopolamine	Motion sickness	Datura stramonium
Sigma sterol	Steroidal precursor	Physostigma venenosum
Strophanthin	Congestive heart failure	Strophanthus gratus
Tubocurarine	Muscle relaxant	Chondrodendron tomentosum
Teniposide	Bladder neoplasms	Podophyllum peltatum
Tetrahydrocannibinol	Antiemetic	Cannabis sativa
Theophylline	Diuretic, antiasthmatic	Camellia sinensis
Toxiferine	Relaxant in surgery	Strychnos guianensis
Vinblastine	Hodgkin’s disease	Catharanthus roseus
Vincristine	Paediatric leukaemia	C. roseus
Xanthotoxin	Vitiligo	Ammi majus

Table 2.3: Number of records of worldwide medicinal plant uses according to NAPRALERT

Induce menstruation	4110	Epilepsy	299
Induce abortion	2630	Genitourinary problems	298
Reduce inflammation	1879	Kills fish	294
Prevent post-partum bleeding	1547	Insecticide	289
Bacterial diseases	1521	Vermifuge	265
Induce diuresis	1327	High blood pressure	264
Reduce fever	1299	Induce vomiting	260
Impotence	1275	Induce perspiration	255
Pain relief	1255	Snake and spider bites	250
Contraception	1249	Cardiotonic	224
Laxative / cathartic	1032	Flatulence	214
Diarrhoea	922	Produces hallucinations	213
Antihelmitinic	867	Tuberculosis	178
Antispasmodics	856	Prevents vomiting	146
General tonics	749	Treponema infections	145
Jaundice / liver disease	733	Haemorrhoids	145
Stimulate lactation	629	Fungal infections	142
Suppress cough	621	Emollient	118
Dysmenorrhoea	620	Narcotic	115
Sedative	606	Stomach ulcers	110
Accelerates wound healing	576	Increases bile flow	107
Malaria	565	Schistosomiasis	101
Cancer	552	Flu symptoms	98
Digestive aids	540	Tapeworm	91
Diabetes	514	Stimulates hair growth	84
Expectorant	473	Bitters (appetite stimulant)	80
Fertility promotion	432	Dissolves kidney stones	79
Food products	414	Anticoagulant	78
Asthma	410	Haematinics	71
Astringent	380	Burn wounds	70
Haemostatic	323	Caustic	67
Central nervous system stimulant	321	Prevents miscarriage	63
Viral infections	315	Insect repellent	62

2.2 PLANTS AS A SOURCE OF ANTIBIOTICS

Plants have developed an arsenal of weapons to survive attacks by microbial invasion. These include both physical barriers as well as chemical ones, i.e. the presence or accumulation of antimicrobial metabolites. These are either preformed in the plant (prohibitins) or induced after infection, the so-called phytoalexins. Since phytoalexins can also be induced by abiotic factors such as UV irradiation, they have been defined as ‘antibiotics formed in plants via a metabolic sequence induced either biotically or in response to chemical or environmental factors’.

When an infection or damage to a plant takes place, a number of processes are activated and some of the compounds produced become activated immediately whereas phytoalexins take two to three days to produce. Sometimes it is difficult to determine whether the compounds are phytoalexins or prohibitins and moreover, the same compound may be a preformed antimicrobial substance in one species and a phytoalexin in another [Grayer and Harborne, 1994]. The chemical class in which these substances can be found varies greatly as can be seen in Table 2.4, which makes the isolation thereof even more intriguing.

Since the advent of antibiotics in the 1950s, the use of plant derivatives as antimicrobials has been virtually non-existent but that pace is rapidly on the increase as we begin to realise the need for new and effective treatments. The worldwide spending on finding new anti-infective agents is expected to increase 60% from 1993 and plant sources are especially being investigated. A summary of useful antimicrobial phytochemicals is given in Table 2.4 [Cowan, 1999].

Table 2.4: Plants containing antimicrobial activity [Adapted from Cowan, 1999]

Common name	Scientific name	Compound	Class	Activity[d]	R.T[b]
Alfalfa	Medicago sativa	?		Gram-positive	2.3
Allspice	Pimenta dioica	Eugenol	Essential oil	General	2.5
Aloe	Aloe barbadensis, Aloe vera	Latex	Complex mixture	*Corynebacterium*, *Salmonella*, *Streptococcus*, *S. aureus*	2.7
Apple	Malus sylvestris	Phloretin	Flavonoid derivative	General	3.0
Ashwagandha	Withania somniferum	Withafarin A	Lactone	Bacteria, fungi	0.0
Aveloz	Euphorbia tirucalli	?		*S. aureus*	0.0
Bael tree	Aegle marmelos	Essential oil	Terpenoid	Fungi	
Balsam pear	Mormordica charantia	?		General	1.0
Barberry	Berberis vulgaris	Beriberine	Alkaloid	Bacteria, protozoa	2.0
Basil	Ocimum basilicum	Essential oils	Terpenoids	Salmonella, bacteria	2.5
Bay	Laurus nobilis	Essential oils	Terpenoids	Bacteria, fungi	0.7
Betel pepper	Piper betel	Catechols, eugenol	Essential oils	General	1.0
Black pepper	Piper nigrum	Alkaloid	Alkaloid	Fungi, Lactobacillus, Micrococcus, *E. coli*, *E. faecalis*	1.0
Blueberry	Vaccinium spp.	Fructose	Monosaccharide	*E. coli*	
Brazilian pepper	Schinus terebinthifolius	Terebinthone	Terpenoids	General	1.0
Buchu	Barosma setulina	Essential oil	Terpenoid	General	2.0
Burdock	Articum lappa		Polyacetylene, tannins, terpenoids	Bacteria, fungi, viruses	2.3
Buttercup	Ranunculus bulbosus	Protoanemonin	Lactone	General	2.0
Caraway	Carum carvi		Coumarins	Bacteria, fungi, viruses	
Cascara sagrada	Rhamnus purshiana	Tannins	Polyphenols Anthraquinone	Bacteria, fungi, viruses	1.0
Cashew	Anacardium pulsatilla	Salicylic acids	Polyphenols	*P. acnes*, bacteria, fungi	
Castor bean	Ricinus communis	?		General	0.0
Ceylon cinnamon	Cinnamomum verum	Essential oils, others	Terpenoids, tannins	General	2.0 2.3
Chamomile	Matricaria chamomilla	Anthemic acid	Phenolic acid	*M. tuberculosis*, *S. typhimurium*, *S. aureus*, helminths	
Chapparal	Larrea tridentata	Nordihydroguaiarti acid	Coumarins Lignan	Viruses Skin bacteria	2.0
Chili peppers	Capsicum annuum	Capsaicin	Terpenoid	Bacteria	2.0
Clove	Syzygium aromaticum	Eugenol	Terpenoid	General	1.7
Coca	Erythroxylum coca	Cocaine	Alkaloid	Gram-negative and positive cocci	0.5
Cockle	Agrostemma githago	?		General	1.0
Coltsfoot	Tussilago farfarva	?		General	2.0
Coriander, cilantro	Coriandrum sativum	?		Bacteria, fungi	
Cranberry	Vaccinium spp.	Fructose Other	Monosaccharide	Bacteria	
Dandelion	Taraxacum officinale	?		*C. albicans*, *S. cerevisiae*	2.7
Dill	Anethum graveolens	Essential oil	Terpenoid	Bacteria	3.0
Echinacea	Echinaceae angustifolia	?		General	
Eucalyptus	Eucalyptus globulus	Tannin -	Polyphenol Terpenoid	Bacteria, viruses	1.5
Fava bean	Vicia faba	Fabatin	Thionin	Bacteria	
Gamboge	Garcinia hanburyi		Resin	General	0.5
Garlic	Allium sativum	Allicin, ajoene	Sulfoxide Sulphated terpenoids	General	
Ginseng	Panax notoginseng		Saponins	*E. coli*, *Sporothrix schenckii*, *Staphylococcus*, *Trichophyton*	2.7
Glory lily	Gloriosa superba	Colchicine	Alkaloid	General	0.0
Goldenseal	Hydrastis candensis	Berberine, hydrastine	Alkaloids	Bacteria, *Giardia duodenale*, trypanosomes Plasmodia	2.0
Gotu kola	Centella asiatica	Asiatocoside	Terpenoid	*M. leprae*	1.7
Grapefruit peel	Citrus paradisa		Terpenoid	Fungi	
Green tea	Camellia sinensis	Catechin	Flavonoid	General, *Shigella*, *Vibrio*, *S. mutans*, viruses	2.0
Harmel, rue	Peganum harmala	?		Bacteria, fungi	1.0
Hemp	Cannabis sativa	β-Resercyclic acid	Organic acid	Bacteria and viruses	1.0
Henna	Lawsonia inermis	Gallic acid	Phenolic	*S. aureus*	1.5
Hops	Humulus lupulus	Lupulone, humulone	Phenolic acids (Hemi)terpenoids	General	2.3
Horseradish	Armoracia rusticana	-	Terpenoids	General	
Hyssop	Hyssopus officinalis	-	Terpenoids	Viruses	
(Japanese) herb	Rabdosia trichocarpa	Trichorabdal A	Terpene	*Helicobacter pylori*	
Lantana	Lantana camara	?		General	1.0
-	Lawsonia	Lawsone	Quinone	*M. tuberculosis*	-
Lavender-cotton	Santolina chamaecyparissus	?		Gram positive bacteria, Candida	1.0

Legume	Millettia thonningii	Alpinumisoflavone	Flavone	Schistosoma	
Lemon balm	Melissa officinalis	Tannins	Polyphenols	Viruses	
Lemon verbena	Aloysia triphylla	Essential oil	Terpenoid	Ascaris	1.5
			?	E.*coli*, *M. tuberculosis*, *S. aureus*	
Licorice	Glycyrrhiza glabra	Glabrol	Phenolic alcohol	*S. aureus*, *M. tuberculosis*	2.0
Lucky nut (yellow)	Thevetia peruviana	?		Plasmodium	0.0
Mace, nutmeg	Myristica fragrans	?		General	1.5
Marigold	Calendula officinalis	?		Bacteria	2.7
Mesquite	Prosopis juliflora	?		General	1.5
Mountain tobacco	Arnica montana	Helanins	Lactones	General	2.0
Oak	Quercus rubra	Tannins	Polyphenols		
		Quercetin (available commercially)	Flavonoid		
Olive oil	Olea europaea	Hexanal	Aldehyde	General	
Onion	Allium cepa	Allicin	Sulfoxide	Bacteria, Candida	
Orange peel	Citrus sinensis	?	Terpenoid	Fungi	
Oregon grape	Mahonia aquifolia	Berberine	Alkaloid	Plasmodium, Trypanosomes, general	2.0
Pao d'arco	Tabebuia	Sesquiterpenes	Terpenoids	Fungi	1.0
Papaya	Carica papaya	Latex	Mix of terpenoids, organic acids, alkaloids	General	3.0
Pasque-flower	Anemone pulsatilla	Anemonins	Lactone	Bacteria	0.5
Peppermint	Mentha piperita	Menthol	Terpenoid	General	
Periwinkle	Vinca minor	Reserpine	Alkaloid	General	1.5
Peyote	Lophophora williamsii	Mescaline	Alkaloid	General	1.5
Poinsettia	Euphorbia pulcherrima	?		General	0.0
Poppy	Papaver somniferum	Opium	Alkaloids and others	General	0.5
Potato	Solanum tuberosum	?		Bacteria, fungi	2.0
Prostrate knotweed	Polygonum aviculare	?		General	2.0
Purple prairie clover	Petalostemum	Petalostemumol	Flavonol	Bacteria, fungi	
Quinine	Cinchona sp.	Quinine	Alkaloid	*Plasmodium* spp.	2.0
Rauvolfia	Rauvolfia serpentina	Reserpine	Alkaloid	General	1.0
Rosemary	Rosmarinus officinalis	Essential oil	Terpenoid	General	2.3
Sainfoin	Onobrychis viciifolia	Tannins	Polyphenols	Ruminal bacteria	
Sassafras	Sassafras albidum	?		Helminths	2.0
Savory	Satureja montana	Carvacrol	Terpenoid	General	2.0
Senna	Cassia angustifolia	Rhein	Anthraquinone	*S. aureus*	2.0
Smooth hyrangea, seven barks	Hydrangea arborescens	?		General	2.3
Snakeplant	Rivea corymbosa	?		General	1.0
St. John's wort	Hypericum perforatum	Hypericin, others	Anthraquinone	General	1.7
Sweet flag,calamus	Acorus calamus	?		Enteric bacteria	0.7
Tansy	Tanacetum vulgare	Esssential oils	Terpenoid	Helminths, bacteria	2.0
Tarragon	Artemisia dracunculus	Caffeic acids, tannins	Terpenoid Polyphenols	Viruses, helminths	2.5
Thyme	Thymus vulgaris	Caffeic acid	Terpenoid	Viruses, bacteria, fungi	2.5
		Thymol	Phenolic alcohol		
		Tannins	Polyphenols		
		-	Flavones		
Tree bard	Podocarpus nagi	Totarol	Flavonol	*P. acnes*, other Gram-positive Bacteria	
		Nagilactone	Lactone	Fungi	
Tua-Tua	Jatropha gossyphiifolia	?		General	0.0
Tumeric	Curcuma longa	Curcumin	Terpenoids	Bacteria, protozoa	
		Turmeric oil			
Valerian	Valeriana officinalis	Essential oil	Terpenoid	General	2.7
Willow	Salix alba	Salicin	Phenolic glucoside		
		Tannins	Polyphenols		
		Essential oil	Terpenoid		
Wintergreen	Gaultheria procumbens	Tannins	Polyphenols	General	1.0
Woodruff	Galium odoratum	-	Coumarin	General, viruses	3.0
Yarrow	Achillea millefolium	?		Viruses, helminths	2.3
Yellow dock	Rumex crispus	?		*E. coli*, *Salmonella*, *Staphylococcus*	1.0

[b] Relative toxicity: 0, very safe; 3, very toxic

[d] "General" denotes activity against multiple types of microorganisms (e.g. bacteria, fungi and protozoa) and "bacteria" denotes activity against Gram-positive and Gram-negative bacteria.

Literally thousands of phytochemicals with inhibitory effects on microorganisms have been found to be active *in vitro*. One may argue that these compounds have not been tested *in vivo* and therefore activity cannot be claimed but one must take into consideration that many, if not all, of these plants have been used for centuries by various cultures in the treatment of disease. Another argument could possibly be that at very high concentrations, any compound is likely to inhibit the growth of microorganisms. Firstly if this is the case, the high concentrations required would no doubt have serious side-effects on the patient unfortunate enough to contract an illness and secondly, these compounds are compared with those of standard antibiotics already available on the market. This means that the concentrations used must compare favourably to those that have already passed the test.

Asiaticoside, an antimicrobial compound isolated from *Centella asiatica* (used traditionally in skin diseases and leprosy), has been studied in normal as well as delayed-type wound healing. The results indicated significant wound healing in both models [Shukla, 1999]. Another compound, cryptolepine, isolated from *Cryptolepis sanguinolenta* and active against *Campylobacter* species, has been used traditionally in Guinea Bissau in the treatment of hepatitis and in Ghana for the treatment of urinary and upper respiratory tract infections and malaria [Paulo, 1994]. The debate continues…

Preliminary screening tests performed by Noristan scientists on a number of plants species resulted in the finding that *Combretum erythrophyllum* had antimicrobial activity but due to changes in company policy no further work was performed. A comprehensive study into this family and specie was therefore initiated to determine whether there was activity and whether these compounds could be isolated. This is discussed in further detail in chapter 3.

CHAPTER 3

3. SELECTION OF PLANTS

3.1 FAMILY COMBRETACEAE

3.1.1 Taxonomy

The Combretaceae family belongs to the order Myrtales, which is sub-divided into two sub-families of which only the Combretoideae is of interest. Of the two tribes comprising this sub-family, only the Combreteae, which is further divided into three subtribes, is of relevance in Africa. The five genera from the three sub-tribes are *Combretum, Terminalia, Pteleopsis, Quisqualis* and *Lumnitzera* [Arnold and De Wet, 1993]. Reports in the literature indicate that traditional healers have confined themselves almost exclusively to the use of species form the genus *Combretum* and to a lesser extent *Terminalia* in the treatment of a wide range of illnesses [Rogers and Verotta, 1996].

3.1.2 Use in Traditional Medicine

The first published scientific study carried out was on the West-African drug "Kinkeliba", isolated from *C. micranthum* leaves and used in the treatment of biliary fever, colic and vomiting [Paris, 1942]. This plant also has cholagog and diuretic properties and shows antimicrobial properties against both Gram positive and negative organisms [Rogers and Verotta, 1996].

Some medicinal uses of the Combretaceae are listed in Table 3.1.

Table 3.1: Traditional medicinal uses of the Combretaceae [Adapted from Rogers and Verotta, 1996]

COMBRETUM SPECIES	***TRADITIONAL USE***
C. apiculatum	Snake and scorpion bite, bloody diarrhoea, leprosy, abdominal disorders, conjunctivitis and weak body
C. erythrophyllum	Fattening tonic for dogs, to reduce the size of the vaginal orifice
C. fragrans	Chest coughs, syphilis, aphrodisiac
C. glutinosum	Hepatic disease, antihypertensive, diuretic, bronchial disease
C. hereroense	Bilharzia, headache, infertility in women
C. imberbe	Coughs, colds, diarrhoea
C. microphyllum	Lunacy, lucky charm
C. molle	Hookworm, stomach ache, snakebite, leprosy, fever, dysentery, chest complaints, anthelmintic, headache, diarrhoea, convulsions, dressing for wounds, stop bleeding after childbirth and to fatten babies
C. platypetalum	Swelling caused by mumps, pneumonia, abdominal pains, diarrhoea, antiemetic, dysmenorrhoea, infertility in women, earache, epistaxis, haemoptysis
C. zeyheri	Toothache, cough, scorpion bite, bloody diarrhoea, arrest menstrual flow, eye lotion, embrocation, abdominal disorders

From data provided by Cunningham [1990] it was calculated that 20.2 tons of Combretaceae (Table 3.2) was used annually in the herbal trade in Zululand [Eloff, 1998]. This is a rough indication of its considerable popularity in traditional medicinal usage.

Table 3.2 : Total amount of medicinal plant material in tons yearly used in Kwazulu-Natal [Adapted from Eloff, 1998]

FAMILY	TOTAL	FAMILY	TOTAL
Liliaceae	131.7	Combretaceae	20.2
Myrsinaceae	84.4	Rutaceae	20.2
Anacardiaceae	56.4	Sapotaceae	20.2
Sapindaceae	49.2	Rubiaceae	17.2
Compositae	47.9	Halorrhagidaceae	17.0
Amarillidaceae	47.7	Dioscoreaceae	16.3
Umbelliferae	39.9	Canellaceae	15.8
Passifloraceae	36.9	Crassulaceae	14.2
Meliaceae	31.4	Convovulceae	12.5
Melianthaceae	29.8	Vitaceae	12.2
Lauraceae	23.1	Cycadales	11.7
Leguminosae	21.2	Ranunculaceae	11.4

3.1.3 Metabolites isolated from Combretaceae

Recently a number of clinical trials initiated with Deerghayu, a *Terminalia arjuna* based polyherbal formulation, have shown encouraging results in heart patients. Arjuna exhibits good anti-oxidant properties, reduces cardiac size and downregulates beta-receptors, therefore proving beneficial in coronary artery disease. It has also shown good antiviral and antimicrobial properties. Researchers contribute this activity to various active constituents including tannins such as gallic acid, ellagic acid and oligomeric proanthocyanidins, triterpenoid saponins, flavonoids (luteolin, arjunone, arjunolone), phytosterols and certain minerals [Narendran, 2001].

More substantial chemical work has, however, been done on the genus *Combretum*. In 1973, Letcher and co-workers isolated a number of substituted phenanthrenes, dihydrophenanthrenes and bibenzyls from various *Combretum* spp. [Letcher and Nhamo, 1973].

A group from the University of Durban-Westville isolated and characterised a number of novel compounds, [Pegel and Rogers 1985, Rogers and Thevan 1986, Osborne and Pegel 1985] namely triterpenes and saponins like mollic acid, jessic acid and their derivatives. The sodium salts of mollic acid glycoside isolated from *C. molle* were found to be toxic to *Biomphalaria glabrata* snails at a concentration of 12 ppm [Rogers, 1989].

More recently a series of stilbenes and dihydrostilbenes (combretastatins) with potent cytotoxic activity and acidic triterpenoids and their glycosides with molluscicidal, antifungal and anti-inflammatory activity have been isolated from *Combretum* species. Combretastatin is the first of a series of unique stilbenes highly active against the murine P-388 lymphocytic leukaemia cell line and was originally isolated from *C. caffrum* [Rogers and Verotta, 1996]. These bioactive compounds were later also found in *C. kraussi* and *C. erythrophyllum* species and clinical trials began in November 1998 [Schwikkard, 2000].

Novel pentacyclic triterpenes were isolated from *Combretum nigricans* and identified as combregenin and its glycoside, combre-glucoside [Jossang, 1996]. This species is used in folk medicine for the treatment of gastrointestinal diseases and also as a fish poison. Two triterpenes, arjunolic acid and mollic acid, and two flavonoids, 3-*O*-methylquercentin and 3-*O*-α-L-rhamnopyranosylquercetin, have been isolated and identified from *Combretum leprosum* [Facundo, 1993] and two phenanthrenes (4,7-dihydroxy-2,3,6-trimethoxyphenanthrene, 2,7-dihydroxy-3,4,6-trimethoxydihydrophenanthrene) and a dihydrostilbene (4,4'-dihydroxy-3,5-dimethoxydihydrostilbene) have been isolated from the heartwood of *C. apiculatum* [Grayer and Harborne, 1994]. These compounds showed total inhibition of *Penicillium expansum* when 20 μg was spotted on a TLC plate.

Although many compounds have been isolated and chemically identified, few plant species have been subjected to bioassays in order to determine pharmacological activity. Some of those tested however, have shown remarkable activity. *Combretum micranthum* for example, traditionally used in the treatment of malaria, has shown strong antimalarial activity against both a chloroquine-sensitive and -resistant strain of *Plasmodium falciparum* [Benoit, 1996]. Leaves of *C. paniculatum* have shown promising results in the inhibition of HIV-1 and HIV-2 replication [Asresk, 2001].

Alexander *et al*. [1992] investigated the antimicrobial activity of 12 *Combretum* spp. and found several active components in some species. They did not follow up the antimicrobial components in *C. erythrophyllum*, but found up to five different inhibitors in extracts of other *Combretum* spp. using different test organisms. Preliminary bioassays of extracts were found to be active against *S. aureus*. Breytenbach and Malan [1989] isolated three antimicrobial components from *C. zeyheri* and proposed structures for two of them. All had activity against *S. aureus*.

A number of Combretaceae species tested for antifungal activity showed promising results. Of the seven species tested, *C. nigricans* was strongly active on dermatophytes [Baba-Moussa, 1998].

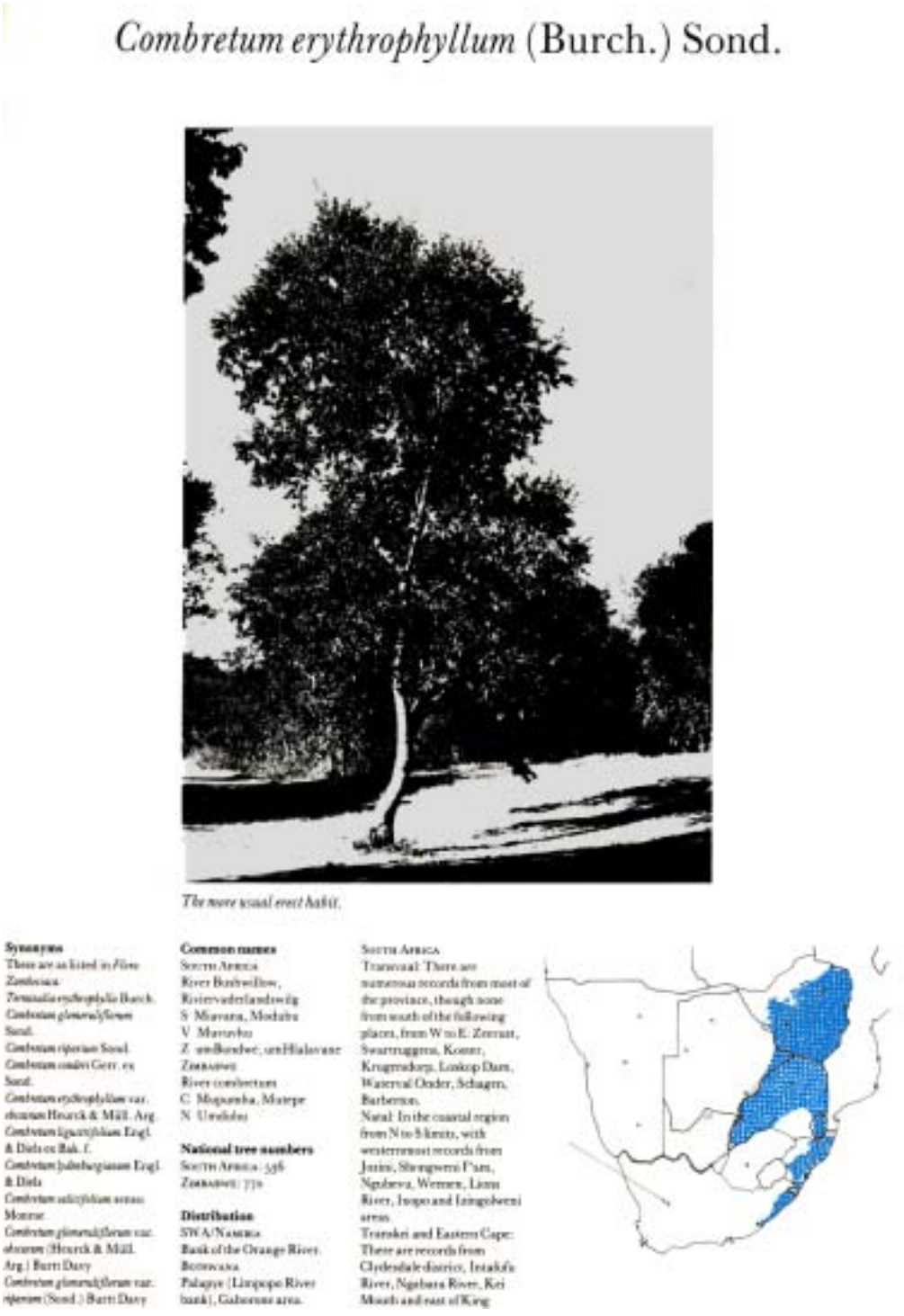
3.2 PRESENT STUDY

3.2.1 Background on *Combretum erythrophyllum* (Burch.) Sond.

Combretum erythrophyllum (see figures 3.1 and 3.2) was nominated as the tree of the year in 1995. It is commonly known as the river bushwillow and is adapted to a great variety of climatic conditions. It occurs in almost the entire eastern part of South Africa and as can be deduced from its name, the river bushwillow prefers riverbanks as its natural habitat leaning over the water in true willow fashion. In the west it occurs mainly along the Orange River. The growth form is mostly multi-stemmed and bushy with branches growing out horizontally, sometimes lying on the ground and with a large number of upright branches sprouting up from these. The tree flowers in winter to late spring, just after the young leaves have appeared [http://www.sa-embassy]. In autumn the leaves sometimes turn red, which explains the scientific name. The gum is found to be slightly antibacterial and can be applied to sores in a powdered form [pamphlet, National Botanical Gardens, Pretoria].

In Zulu medicine, unspecified parts are used in pregnancy to facilitate labour, leaves are used for coughs and abdominal pain and the bark for infertility and during pregnancy. Small doses of root have been administered to dogs as a fattening tonic. In Zimbabwe the roots are used as a cure and prophylactic against venereal disease and inserted vaginally as an aphrodisiac and to reduce the size of the vaginal orifice. This practice is not recommended as many women have died after vaginal insertion of powdered roots, with symptoms including abdominal pain, severe vomiting and confusion. Roots have a purgative effect and the fruit can produce persistent hiccoughs. The poisonous principle in the fruit has not yet been identified. The gum is used for tanning and has a slight positive test for antibiotic activity. Extracts from leaves have significant antimicrobial activity against *Branhamella catarrhalis, Mycobacterium phlei* and *Serratia marecescens* [as quoted by Hutchings et al, 1996].

Figure 3.1: *Combretum erythrophyllum* (Adapted from Carr]



Combretum erythrophyllum (Burch.) Sond.

The more usual erect habit.

Synonyms
These are as listed in *Flora Zambesiaca*
Terminalia erythrophylla Burch.
Combretum glomeruliflorum Sond.
Combretum riparium Sond.
Combretum randii Gerr. ex Sand.
Combretum erythrophyllum var. *obovatum* Heurck & Müll. Arg.
Combretum ligustrifolium Engl. & Diels ex Bak. f.
Combretum paludosum Engl. & Diels
Combretum salicifolium sensu Monroe
Combretum glomeruliflorum var. *obovatum* (Heurck & Müll. Arg.) Burtt Davy
Combretum glomeruliflorum var. *riparium* (Sond.) Burtt Davy

Common names
South Africa
River Bushwillow,
Rivierwaterlandswilg
S Muvaru, Modubu
V Murovhu
Z umBondwe, umHlalovane
Zimbabwe
River combretum
C Mupamba, Mutepe
N Umholoti

National tree numbers
South Africa: 536
Zimbabwe: 770

Distribution
SWA/Namibia
Bank of the Orange River.
Botswana
Palapye (Limpopo River bank), Gaborone area.
South Africa
Transvaal: There are numerous records from most of the province, though none from south of the following places, from W to E: Zeerust, Swartruggens, Koster, Krugersdorp, Loskop Dam, Waterval Onder, Schagen, Barberton.
Natal: In the coastal region from N to S limits, with westernmost records from Jozini, Shongweni Farm, Ngubevu, Wemmer, Lions River, Inspo and Izingolweni areas.
Transkei and Eastern Cape: There are records from Clydesdale district, Intaka's River, Ngabara River, Kei Mouth and east of King

Figure 3.2: Common names and distribution of *C. erythrophyllum* [Adapted from Carr]

Seven novel triterpenoid acids and lactones were isolated in the search for compounds responsible for the toxicity of this plant [Rogers, 1998]. The representative compound was given the name erythrophyllic acid [Lawton and Rogers, 1991]. Attempts to saturate one of the triterpenoids by hydrogenation, using Adams catalyst, resulted in two unusual hydrogenation products, 3-oxo-9,19-cycloart-11-en-21-oic acid and 23-hydroxycycloart-11-en-21-oic acid. The water-soluble sodium salt fractions were found to be active against *Citrobacter, Enterobacter* and *Salmonella* species [Mocktar, 1997].

3.2.2 Background to Triterpenoids

Since triterpenoids have shown to exhibit antibacterial activity [Mocktar, 1997] and Rogers [1998], as mentioned above, isolated seven novel triterpenoids from *Combretum erythrophyllum*, it is necessary to include a more detailed background to these secondary metabolites and their functional role in plants. This is also a continuation of a previous study, which isolated five triterpenoids [Martini, 1998].

Terpenoids are also called terpenes and can occur as monoterpenes, diterpenes, triterpenes and tetraterpenes (C_{10}, C_{20}, C_{30} and C_{40} respectively) as well as hemiterpenes (C_5) and sesquiterpenes (C_{15}). When they contain additional elements, usually oxygen, they are termed terpenoids. They differ from fatty acids in that they contain extensive branching and are cyclized. Examples of common terpenoids are menthol and camphor, as well as artemisin, a compound currently used in the treatment of cerebral malaria [Cowan, 1999].

Triterpenoids are non-steroidal secondary metabolites. The physiological function of these compounds is generally believed to be a chemical defence against pathogens and herbivores. It is therefore expected that they should act against certain pathogens causing human and animal diseases [Mahato, 1997]. Although medicinal uses of this class of compounds are rather limited, possibly due to their hydrophobic nature, recent work in this regard indicates their great potential as drugs. Moreover, despite the remarkable diversity already known to exist, new variants continue to emerge [Mahato, 1992].

Due to the wide occurrence and structural diversity of triterpenoids, evaluation of their biological activity has always attracted attention and although application of these compounds is very limited, extensive exploratory activities have been underway during recent years. A number of triterpenoids have been found to have antitumour and anticancer activity. Possibly the best-known one is ursolic acid, which was found to inhibit tumour promotion in mouse skin [Mahato, 1997]. *In vitro* and *in vivo* studies of carbenoxelone have also demonstrated intrinsic mineralocorticoid activity in rats. This compound increased the overall mean of prostaglandin E_2 concentrations in gastric juice as well as decreasing the gastric juice. Glycyrrhetinic acid was studied in hyperlipemia and caused significant changes in lipid metabolism. It has been found to have hypolipemic and antiartherosclerotic activity greater than that of the established antiartherosclerotic polysponin. Other activities include anti-inflammatory activity, analgesic and antipyretic properties. Some have been found to prevent liver injury and lower urinary tract infections by increasing the mucopolysaccharide layer in the bladder. Antitussive and expectorant activities, antiviral and antimicrobial effects have also been well documented [Mahato, 1992].

Terpenoids are found to be active against bacteria, fungi, viruses and protozoa. Their mechanism of action is not fully understood but it is speculated that these lipophilic compounds cause disruption of membranes because by increasing the hydrophilicity of kaurene diterpenoids, antimicrobial activity was drastically reduced.

Capsaicin, a constituent of chilli peppers, is bactericidal to *Helicobacter pylori*, although possibly detrimental to the gastric mucosa. Another terpenoid called petalostemumol, isolated from the prairie clover (*Dalea* sp.) shows excellent activity against *Bacillus subtilis* and *S. aureus* as well as *Candida albicans*. Some other examples are cited in Table 2.4 [Cowan, 1999].

Currently the terpenoids isolated from *Combretum* species include jessic acid and methyl jessate from *C. elaegnoides*; imberbic acid from *C. imberbe* [Mahato, 1992]; combregenin, combre-glucoside, arjungenin and arjunglucoside from *C. nigricans* [Jossang, 1996]and arjunolic and mollic acid from *C. leprosum* [Facundo, 1993]. So far the tritepenoids isolated from *C. erythrophyllum* seem to belong to almost exclusively two distinct groups, namely 30-carboxy-1α-hydroxycycloartanes and 29-carboxy-1α-hydroxyoleanes [Rogers 1998] [Figure 3.1].

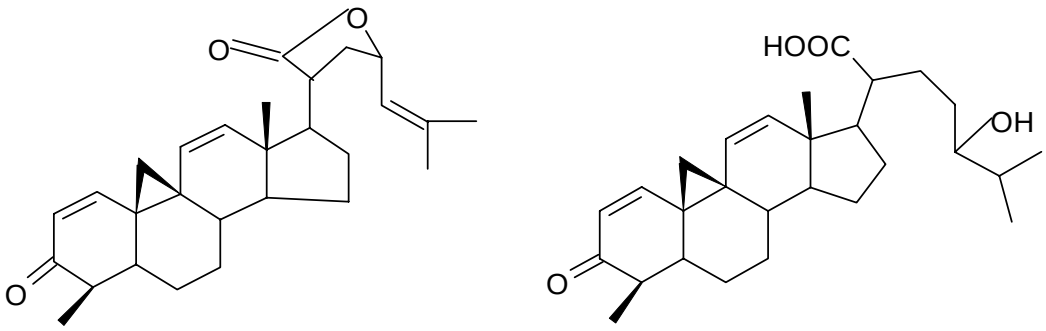


Figure 3.3: Cycloartane dienone lactones from *C. erythrophyllum*

3.3 PREVIOUS ISOLATION AND IDENTIFICATION WORK

This study is a continuation of work done previously [Martini, 1998]. Previous work involved the extraction of crude material with acetone and subsequently solvent/solvent extraction using various solvents (discussed further in chapter 4). The carbon tetrachloride and chloroform fractions were further fractionated by column chromatography (CC) and preparative TLC (PTLC). This method afforded five compounds but unfortunately due to the minute quantities extracted, structural elucidation was not possible. It was, however, established by means of NMR that these five compounds were triterpenoids and appeared to be novel structures. This was not validated however, but inspired continuation of the study.

3.4 AIM OF STUDY

The primary objective of this study is to isolate sufficient quantities of bioactive compounds, to elucidate their structures and determine biological activity.

Secondary objectives include toxicity screening of the pure compounds using an LDH method as well as determining antioxidant and anti-inflammatory activity using lymphocytes isolated from healthy volunteers. It has been suggested that the phytochemistry of a plant varies with the environment and the plant's maturity. It was decided to investigate this variability by determining antimicrobial activity of plant material sourced from various regions around Pretoria and variations in activity between young leaves versus older and mature material.

CHAPTER 4

4. MATERIALS AND METHODS

4.1 PLANT MATERIAL

Fresh or dried plant material can be used as a source for secondary plant components. Most scientists have opted to use dry material for several reasons: (i) traditional healers frequently use dried plant material, (ii) the time delay between collecting plant material and processing it makes it difficult to work with fresh material and (iii) there are fewer problems associated with the large scale extraction of dried plant material. Freshly harvested and dried materials are more commonly used since old dried material may undergo some quantitative losses and qualitative changes. In the present study dried material was used.

Combretum erythrophyllum [Burch] Sond. leaves were collected from a tree in the Pretoria National Botanic Gardens. The tree was identified by the plant label and the identity was confirmed by Mrs C.L. Bredenkamp, the curator of Combretaceae at the National Botanical Institute. A voucher specimen was deposited in the National Herbarium (C.L. Bredenkamp 1542 PRE). With later studies leaves were collected from various sites around Pretoria and voucher specimens were deposited in the National Herbarium.

4.2 PREPARATION OF LEAF MATERIAL AND EXTRACTION

Farnsworth [1994] states that the biggest problem in drug development from plants is choosing the appropriate extracting solvent.
Soxhlet extraction of dried plant material is widely used employing solvents of increasing polarity, e.g. ether, petroleum ether, chloroform, ethyl acetate and ethanol. This method only works for compounds that can withstand the high temperatures of the boiling solvent but cannot be used for thermolabile compounds. This problem can be overcome by boiling at reduced pressure but this can be difficult and was therefore not used in this study.

The choice of solvent also depends on what is planned with the extract. If the extract is to be used for antimicrobial screening, the effect of the solvent on subsequent bioassay is important. From previously published papers where authors screened plants for antimicrobial properties, it can be noted that the authors used a variety of extractants. These have varied from 80% ethanol [Vlietinck, 1995], methanol [Taylor, 1995], petroleum ether, chloroform, ethanol, methanol and water [Salie, 1996]. In previous studies it was found, however, that acetone extracted a greater number of inhibitors (14) than those extracted with methanol (9), methylene dichloride (11), ethanol (8) and water (1), and was therefore used in this study to extract the crude material [Eloff, 1998].

The leaves were carefully examined and old, insect-damaged; fungus-infected leaves, twigs and flowers were removed. Healthy leaves were spread out and dried in the laboratory at room temperature for 5-8 days or until they broke easily by hand. Once completely dry, leaf material was ground to a fine powder using a Jankel and Künkel Model A10 mill. Larger quantities were sent to Phytotron at the Department of Plant Production and Soil Science, where a Wiley mill was used to grind material to a fine powder of c. 1.0 mm diameter. Material was stored in a closed container at room temperature until required.

Extraction was initially performed on a Labotec Model 20.2 shaking apparatus with a 10 ml: 1 g solvent to dry weight ratio. With larger quantities of material, a ratio of 5 ml: 1 g was used and shaken continuously for an hour each time. This procedure was repeated three times and all extracts were decanted and combined.

The extracts were filtered before drying using Whatman filter paper no. 2 on a Büchner funnel and the solvent removed by vacuum distillation in a Büchi rotary evaporator at 60ºC, care being taken to decrease the temperature to 40°C for the final drying. For quantitative determination, the extracts were placed in pre-weighed flasks before drying. Technical grade acetone [Merck] was used as solvent.

4.3 ANALYSIS BY TLC

Thin layer chromatography [usually 5 µl of a 100 mg extract/ml solution] was on Merck TLC F_{254} or Macherey-Nagel Alugram Sil G $_{254}$ or Polygram Sil G UV_{254} plates using chloroform:ethyl acetate:formic acid [5:4:1], acetone:methylene dichloride [2:3] and benzene:ethanol [9:1] as eluents. Streaking of polar components was minimised by the addition of 1% ammonium chloride to the benzene:ethanol solution. In some cases DC Alufolien RP18 F_{254} [Merck] plates were used, eluting with varying ratios of water: methanol. Samples were applied quickly and run without delay to minimise the possibility of oxidative or photo-oxidative change. Separated components were visualised under visible and ultraviolet light [254 and 360 nm, Camac Universal UV lamp TL-600]. Plates were also sprayed with 5% anisaldehyde in 5% sulphuric acid in ethanol and 0.36% vanillin in 3.6% sulphuric acid in methanol and heated for 2-5 minutes at 100°C to allow for development of colour changes [Carr and Rogers, 1986].

4.4 BIOASSAY AND TEST ORGANISMS

The biological assays employed were chosen because of their simplicity, reproducibility, and sensitivity and relatively low cost while being rapid and simple at the same time.

4.4.1 Antibacterial activity

Minimum inhibitory concentration [MIC] was determined by serial dilution of extracts beyond the level where no inhibition of growth of test organisms was observed [Eloff, 1998]. This was performed in microplates by filling all wells, with 50 µl sterile Mueller Hilton broth. In row A, 50 µl [100%] of the extract was placed with a micropipette. From row A, 50 µl was transferred to row B after taking up and releasing three times to ensure adequate mixing. The process was repeated until all the rows were completed and the additional 50 µl from row H was discarded. Two wells were used as a sterility and growth control respectively with the sterility control containing only Oxoid® Mueller Hilton broth (MHB), whilst the growth control containing both MHB as well as test organism.

After adding 50 µl of the bacterial suspension to each row (except for the sterility control), the microplate was sealed and incubated at 37°C at 100% relative humidity overnight. The following morning 50 µl of a 0.2 mg/ml solution of INT (p-iodo-nitrotetrazolium violet) was added to each row and the plate was returned to the incubator for at least half an hour to ensure adequate colour development. p-INT is a dehydrogenase activity detecting reagent, which is converted into corresponding intensely coloured formazan by metabolically active micro-organisms [Navarro, 1998].

Inhibition of growth was indicated by a clear solution or a definite decrease in colour reaction. This value was taken as the minimum inhibitory concentration [MIC] of the extract. Extracts used for MIC determination were either dissolved in acetone or solubilized in DMSO (100 to 200 µl/mg) and made up as a stock solution (200 µg/ml) with distilled water. Well A had typically a final concentration of 100 µg/ml. Positive controls for test organisms were usually made up to a concentration of 1000 µg/ml and are listed in section 4.4.3.

4.4.2 Bioautography

Chromatograms were dried overnight to remove solvents from the plate. In the case of TLC plates run in CEF, plates were dried for at least 40 hours to ensure adequate removal of formic acid. The plates were then sprayed with a suspension of actively growing *Staphylococcus aureus* bacterial cells and incubated overnight at 37°C in a chamber at 100% relative humidity. The following morning, the plates were sprayed with a 2 mg/ml solution of p-iodonitrotetrazolium violet [Sigma]. Inhibition of growth was indicated by clear zones on the chromatogram [Begue and Kline, 1972]. This method was chosen for its simplicity, low cost, accuracy and fast results.

4.4.3 Test organisms used

The following test organisms were commonly used to test minimum inhibitory concentration of the extracts: *Staphylococcus aureus* (Gram-positive) [American Type Culture Collection number 29213] *Pseudomonas aeruginosa* (Gram-negative) [ATCC 27853], *Escherischia coli* (Gram-negative) [ATCC 25922] and *Enterococcus faecalis* (Gram-positive) [ATCC 21212]. *S. aureus* is considered to be one of the most important pathogenic bacteria and is used as primary bioassay organism for this reason.

All these organisms are important nosocomial pathogens widely used in screening tests (Table 4.1) and are reference isolates recommended by the National Committee for Clinical Laboratory Standards, USA [NCCLS, 1990].

Table 4.1: Test organisms widely used in screening tests

Vlietinck [1995]	**Fourie et al. [1992]**	**Wallhäuser [1966]**
Microsporium canis		*Microsporium gypseum*
Trichophyton mentagrophytes		
Staphylococcus aureus	*Staphylococcus aureus*	*Staphylococcus aureus*
Pseudomonas aeruginosa	*Pseudomonas aeruginosa*	
Candida albicans	*Candida albicans*	*Candida albicans*
Escherischia coli	*Escherischia coli*	
	Streptococcus pyogenes	
	Aspergillus niger	
		Bacillus subtilis
		Micrococcus flavus
		Saccaromyces cerivisiae
		Sarcina lutea

Previous studies showed inhibition of these four test organisms to different degrees. The two Gram-positive organisms were more sensitive than the Gram-negative with *S. aureus* being the most sensitive (100%) followed by *E. faecalis* (36%), *E. coli* (11%) and *P. aeruginosa* (3%). It was therefore decided to include a larger spectrum of organisms (including a fungus) in order to obtain a wider knowledge of both the spectrum as well as the type of inhibition (bactericidal or bacteriostatic) that these compounds exhibit. For this reason *Klebsiella pneumoniae, Salmonella typhimurium, Streptococcus faecalis, Shigella sonei, Micrococcus luteus, Aspergillus niger* and *A.. fumigatus, Bacillus subtilis* and *Vibrio cholerae* were also included and were kindly supplied by Mrs. A. Lombard of the Department of Microbiology and Plant Pathology, University of Pretoria.

Incubation times and positive controls used in MIC determinations for these organisms are listed below (Table 4.2).

The cultures were grown at 37ºC and regularly subcultured (1% inoculum) in Mueller Hilton broth. Every c. 3 months, new cultures of *S. aureus, P. aeruginosa, E. faecalis* and *E. coli* were obtained from Dr Flavia Huygens, Department Medical Microbiology, University of Pretoria.

Table 4.2: Incubation periods and positive controls for test organisms used in the bioassays

ORGANISM	POSITIVE CONTROL	INCUBATION PERIOD
Aspergillus	Amphotericin B	24 hours
Bacillus subtilis	Gentamicin	24 hours
Enterococcus faecalis	Gentamicin	4 hours
Escherischia coli	Gentamicin	4 hours
Klebsiella pneumoniae	Gentamicin	4-6 hours
Micrococcus luteus	Ampicillin	24 hours
Pseudomonas aeruginosa	Gentamicin	4 hours
Salmonella typhimurium	Ampicillin	4 hours
Shigella sonei	Ampicillin	4 hours
Staphylococcus aureus	Ampicillin	4 hours
Streptococcus faecalis	Ampicillin	4 hours
Vibrio cholerae	Chloramphenicol	12-24 hours

MHB is recommended by the NCCLS for broth dilution susceptibility testing and was therefore chosen for the bioassays.

4.5 GROUP SEPARATION OF EXTRACTS

4.5.1 Solvent / solvent extraction

The purpose of this procedure is to simplify extracts by fractionating the chemical compounds into broad groups based on their solubilities. The solvent/solvent group separation procedure used by the USA National Cancer Institute as described by Suffness and Douros [1979] was applied with slight variation. After extracting the dry leaf material, the acetone extract was taken to dryness in a Büchi RE-120 rotary evaporator under reduced pressure, rotating at c. 100 rpm and the water bath temperatures initially at 60°C but not exceeding 40°C when the extract was nearly dry. This extract was dissolved in 1:1 mixture of chloroform and water and the two phases were separated in a separatory funnel. The water fraction was mixed with an equal volume of n-butanol in a separatory funnel to yield the water [W] and butanol [B] fractions. The chloroform fraction was taken to dryness in a rotary evaporator under reduced pressure as described above and extracted with an equal volume of hexane and 10% water/methanol mixture. This yielded the hexane [H] fraction and the 10% water/methanol mixture was diluted to 20% water/ methanol by the addition of water. This was then mixed with carbon tetrachloride in the separatory funnel to yield the carbon tetrachloride [CT] fraction. The 20% water/methanol was further diluted to yield a mixture of 35% water/ methanol and mixed with chloroform to yield the chloroform [CL] and 35% water in methanol [WM] fractions. In all cases, equal volumes of solvents were used and the process repeated until the extracting solution was light in colour. In some cases centrifugation had to be applied to separate the solvent phases.

For quantitative determination, extractants were placed in pre-weighed flasks and the solvents removed under vacuum in the rotary evaporator. The extracts were later redissolved in acetone or acetone/water for further analysis. The process is schematically represented in Figure 4.1.

From previous studies with *S. aureus* as test organism, the chloroform soluble fraction contained by far the largest quantity of inhibiting components (100%), followed by the fractions soluble in water (23%), 35% methanol in water (18%), carbon tetrachloride (2%) and hexane (traces) [Martini, 1998]. Due to the relatively low complexity of the hexane fraction (one inhibitory compound) and the great diversity of inhibitory compounds present in the chloroform fraction, these two fractions were evaluated further. Bioassay-guided fractionation was used throughout the study.

4.5.2 Analysis and bioassay of fractions

The hexane, chloroform, carbon tetrachloride, butanol and 35% water/methanol fractions were dissolved in 100% acetone. The water fraction was dissolved in 30% acetone in water. Thin layer chromatography [5 µl of 100 mg/ml extract] was on Merck TLC F254 plates with acetone:methylene dichloride (2:3), chloroform:ethyl acetate:formic acid (5:4:1) and benzene:ethanol:ammonium chloride (9:1:1%). In some cases ethylacetate:methanol:water (40:5.4:4) and other variations thereof were used for comparison. Separated components were visualised under visible and ultraviolet light [254 and 360 nm, Camac Universal UV lamp TL-600]. Plates were then sprayed with 0.36% vanillin, 3.6% sulphuric acid in methanol and heated at 100°C for 2-5 minutes [Carr and Rogers, 1986]. Minimum inhibitory concentrations were determined by means of serial dilution as described in paragraph 4.4.1.

SOLVENT/SOLVENT EXTRACTION

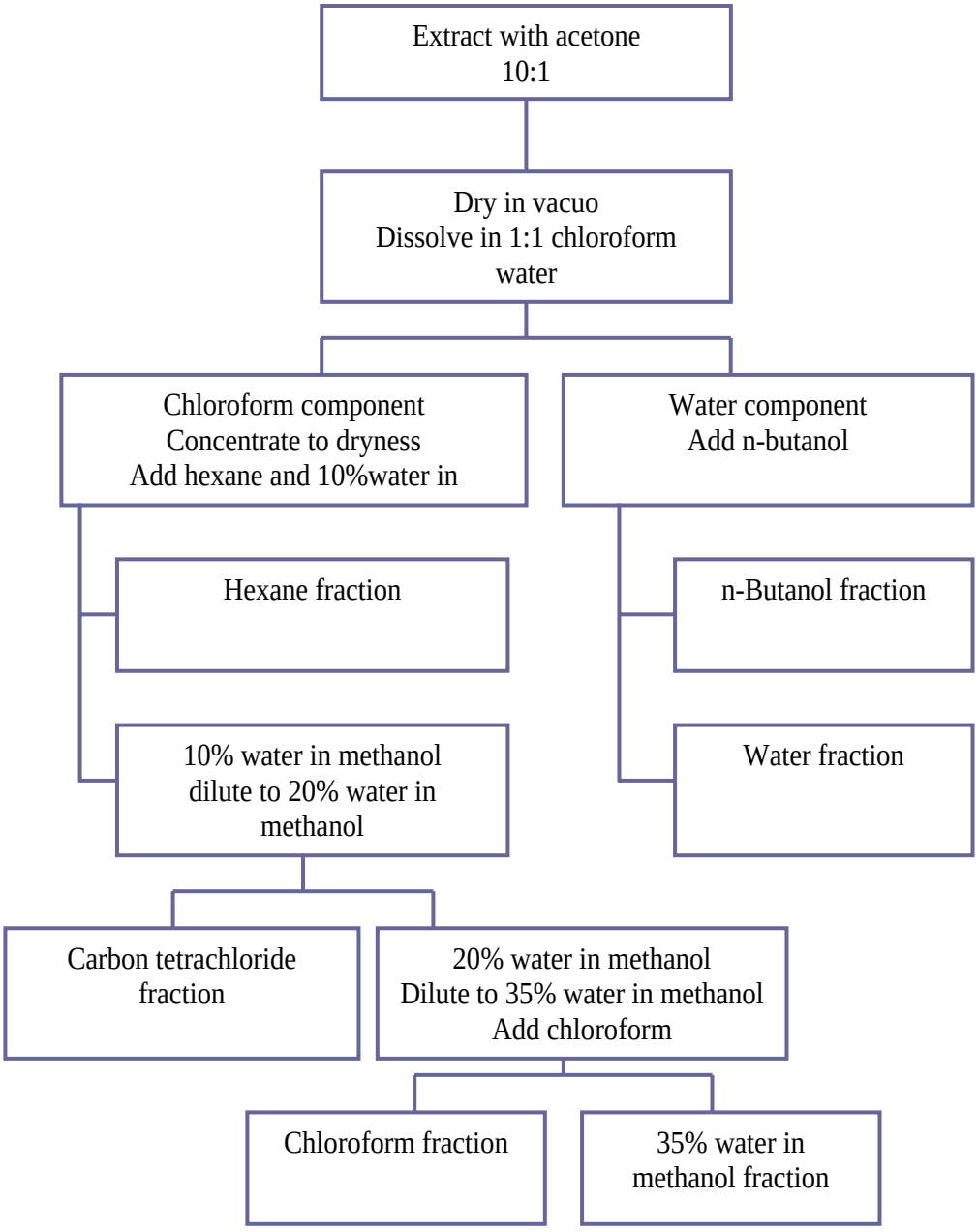


Figure 4.1: The procedure used for solvent/solvent fractionation

4.6 ISOLATION OF BIOACTIVE COMPONENTS

4.6.1 Column chromatography

Column chromatography (CC) is the most widely used technique to isolate the components of complex mixtures (preparative chromatography). CC can also be used to determine the quantity of different compounds present in the mixture (analytical chromatography). Various stationary phases were used to separate compounds either according to polarity (normal and reverse phase silica gel, Sephadex) or size of compounds (Toyopearl - size-exclusion chromatography).

After solvent-solvent group fractionation (paragraph 4.5.1), the fractions were dried in the Büchi rotary evaporator to determine the mass extracted in each solvent. Initially the hexane fraction was chosen for column chromatography due to its relatively low complexity as seen with TLC and bioautography. Later the chloroform fraction was also employed in column chromatography.

In order to select the best mobile phase for eluting the hexane fraction, 5 µl of a 100 mg/ml solution was spotted on TLC and ran with combinations of solvents. In this way the solvent system that exhibited the most favourable separation of compounds was chosen.

In all cases a filter (Whatman filter no. 2) was cut to size and inserted at both the bottom and top of the stationary phase to prevent disruption during elution of the compounds.

Schematic presentations of the methods employed in separating and analysing the various fractions are depicted in Figures 4.2 and 4.3 at the end of the chapter.

4.6.1.1 Silica gel

Silica gel (Kieselgel 60, 0.015-0.04 mm) was suspended in required solvent and left for c. 2 hours to swell after which it was poured into the column. The fraction obtained during solvent/solvent extraction was suspended in the minimum amount of particular solvent in which it would dissolve and filtered to remove impurities and any large particles which could cause diffusion problems whilst developing the column. This fraction was applied to the top of the column using a pipette with great care as not to disturb the top of the column. After application, the solvent flask was raised to facilitate solvent flow into the column and was run either using gravitational force or with pressure by connecting a Labotec pump to the column to facilitate elution. In the case where BEA was used as eluent, the column was developed in a fume hood to limit toxic benzene vapours in the atmosphere.

A concentration gradient of eluents was used. With the hexane fraction a 500 ml mixture of hexane:methylene dichloride (1:1) was used, gradually introducing a mixture of acetone:methylene dichloride (1:1). 100% acetone was introduced next and finally methanol was added to elute any components that could not be moved with the other solvents. The column was left to run overnight, connected to a fraction collector, at a flow rate of 0.6 ml/min.

The chloroform fraction was run initially with 40% hexane in dichloromethane (DCM), gradually increasing the DCM concentration to 100%. Methanol was introduced initially at 5% and increasing to a final concentration of 80% methanol in DCM.

4.6.1.2 Sephadex

Sephadex LH-20 [Fluka®] was employed in separating the chloroform fraction. It was poured into the column in the dry state and the solvent added thereafter. A gradient was used starting with 100% hexane, 1:1 hexane to chloroform and then gradually increasing the chloroform to 100%. Methanol was added in small increments starting with 5% in chloroform and increasing to 100%.

4.6.1.3 Reverse phase

Bondesil C_{18} (40μm preparative grade) silica was used as reverse phase stationary phase. Reverse phase elutes the most polar compounds first and was thus employed in separating polar compounds not eluted with normal phase silica.

The stationary phase was mixed with 10% water in methanol and poured into a column. After a filter (Whatman filter paper no. 2) was cut to size and inserted at the top of the column, glass beads were thrown on top of the column to hold down the stationary phase. A small polypropylene pre-column was inserted on top of the main column containing the same stationary phase as that of the main column. The sample was applied to this pre-column and eluted with solvents to remove impurities and large particles thus preventing adsorption of compounds on the main column. Once fully eluted, the column was connected to the Labotec pump and solvent was pumped through to wash the column.

4.6.1.4 Toyopearl®

Toyopearl HW-40F has a particle size of 45 μm and is supplied as a suspension in 20% ethanol. Toyopearl is a semirigid, macroporous, spherical resin synthesized from a hydrophilic vinyl copolymer exclusively composed of C, H and O atoms and is an excellent resin for the separation of biomolecules [Toyopearl information brochure].

A long burette was filled with Toyopearl and allowed to settle. Acetone (100%) was pushed through the burette using medium pressure (Labotec pump was connected to the burette) to replace the ethanol. The sample was dried using nitrogen gas to c. 0.5 ml and applied to the top of the column by means of a Pasteur pipette. The flow rate was adjusted to 100 drops/minute and samples were collected by means of fraction collector.

4.6.2 Analysis and concentration of fractions

As soon as column chromatography was completed, test-tubes were placed under a stream of air to facilitate concentration of the fractions for TLC analysis and bioassay. After c. 40% of the volume of the eluent had evaporated, every second fraction was analysed by TLC using c. 0.1% of each fraction. In some cases test-tubes were not pre-weighed and the fractions were not dried, therefore it was not possible to determine the exact concentration applied to the TLC plates. Fractions were analysed using BEA (9:1:1%), CEF (5:4:1) and 2A:3MDC as described in paragraph 4.3. EMW (45:5:4.4) was also attempted but with poor results. Separated components were sprayed with vanillin reagent.

4.6.3 Combination of fractions

From TLC results, fractions were combined according to their separation profile. Combined fractions were placed under an air current to facilitate drying. Once dry, the components were weighed to calculate the total mass extracted, dissolved in the minimum amount of acetone possible and transferred to a glass vial with a screw top to prevent evaporation.

4.6.4 Preparative TLC

Fractions were dissolved in the smallest amount of solvent (usually acetone) in which it would dissolve (c. 1 ml) and applied in a band across the preparative TLC plate (Silica gel 60 F_{254}) starting and ending at least 1 cm from either side. The plate was developed repetitively (at least three times) in the mobile phase and the bands visualised under ultraviolet light (254 and 360 nm). A small section on the side of the plate was sprayed with vanillin and heated with a heating gun, whilst protecting the rest of the plate with foil. Components were easily visualised and marked using a soft 2B pencil. After spraying the plate with water to facilitate easier removal of components, the bands were scraped off the glass plate. The components were collected into separate beakers and crushed to a fine powder using a glass rod. The adsorbent powder was eluted with c. 5 ml acetone, depending on the quantity and pigment recovered by filtration using a sintered glass funnel. The process was repeated at least twice or until the powder regained its original white colour. This was followed by 1% acetic acid in methanol for a final rinse to remove any polar components not removed with acetone. Each component was collected into a separate vial and concentrated under a stream of cold air.

4.7 GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

A splitless mode was chosen with helium as carrier gas. Initial temperature and pressure of the front inlet were 250 °C and 115.5 kPa respectively. Injection volume of the sample was 1.0 microliters and run period c. 48 minutes at a flow rate of 3.4 ml/min. No prior knowledge of compound structure was known and therefore a steroid method, recommended by Dr. T. Laurens, was chosen as it was thought to be the most effective in identifying plant-derived compounds. The column was a capillary column (Model no. J&W 122-1031) of 30.0 m in length, 250 μm diameter i.d. and 0.10 μm thickness.

Due to the minute quantities required for GC-MS analysis and the difficulty in weighing such small amounts, quantitative analysis was not possible. A small quantity of the fraction precipitate was taken, dissolved in the smallest quantity of chloroform into which would go, vortexed and injected onto the capillary column for detection. Later, 100μl of N-trimethylsilylimidazole (TMSI) was used to dissolve the dry precipitate. This was left overnight in an oven at 100°C and injected the following morning onto the GC-MS column. Polar compounds have a tendency to adsorb onto the support surface causing distorted peaks, which are broadened and often exhibit a tail. These support materials can be deactivated by silanization with TMSI, which has a strong affinity for polar organic molecules and tends to retain them by adsorption.

The supernatant, which was already dissolved in acetone, was also injected onto GC-MS to compare peaks found in the precipitate. The MS was programmed to incorporate the solvent after a delay of 3 minutes.

4.8 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Analysis was performed on a Hewlett Packard 1050 HPLC with a Luna 5u C18 (250 mm x 4.60 mm i.d.) Phenomenex (U.K.) column. Fractions were diluted five to ten times before injection onto the column and eluted with 10% water: 90% methanol. Temperature was kept off during the run and average run time was 25 minutes. Parameters were set as follows: flow rate: between 1.0 and 2.0 ml/min with an average of 1.5 ml/min; minimum and maximum pressure limits at 20 and 400 bar respectively. The multiple wavelength detector (MWD) was set to measure signals at wavelengths of 204 nm and 254 nm. Fluorescence detection (FLD) was set at Em: 345 nm and Ex: 254 nm. Solvents used were Riedel-de-Häen HPLC grade [Merck].

4.9 NUCLEAR MAGNETIC RESONANCE (NMR)

After column chromatography, precipitation of some fractions began to take place. These precipitates were collected, cleaned using various solvent systems starting with non-polar solvents e.g. hexane and then introducing ethanol, methanol, ethyl acetate, chloroform and acetone. The samples were passed through a Pasteur pipette plugged with cotton wool to facilitate the removal of impurities. The clean samples were weighed and dissolved in maximum 2 ml deuterated solvents used for NMR [Merck]. In the studies, DMSO-d_6 was used as the solvent of choice, although other solvents were also attempted, because of its good ability to dissolve a wide range of compounds. The samples were then pipetted into NMR tubes [Wilmad, economy] with the aid of a Pasteur pipette and sent to Mr Eric Palmer of the Chemistry department, University of Pretoria. ^{1}H NMR was run at either 300 or 400 MHz and ^{13}C at 75 MHz using the solvent signal as reference. Structures were elucidated by Dr D. Katerere (Department of Pharmacology).

4.10 MASS SPECTROMETRY (MS)

High Resolution Electron Impact Mass Spectroscopy (HREIMS) was performed on samples sent for analysis using a MASPEC II system [II32/A002]. Dr P. Boshoff from the Cape Technikon and Dr D. Katerere of the University of Pretoria, performed the analysis.

4.11 VARIATION IN BIOLOGICAL ACTIVITY

Previous studies compared the antibacterial activity of the leaves of seven different *C. erythrophyllum* trees situated in the University of Pretoria grounds. Results showed no difference in antibacterial activity but the TLC plates did show slight variations. Comparisons of trees growing in different areas of Pretoria as well as those present in other provinces were therefore carried out to determine whether other factors such as climactic and soil conditions would cause any differences in biological activity. Conditions of age and state of development among many other things can affect the bioactivity of plant material and the levels of secondary metabolites can differ with these parameters.

Plant material from each specimen was dried, ground to a fine powder (as described in section 4.1 and 4.2) and 500 mg was extracted with 5 ml acetone by shaking for 5 minutes on a shaking apparatus. The extracts were centrifuged for five minutes at 3500 rpm, the process repeated three times and the extracts decanted and combined. The extracts were transferred to pre-weighed glass vials and evaporated to dryness under a stream of air. Extracts were dissolved in acetone to produce a 100 mg extract/ml solution and 5 µl applied to Merck TLC F254 plates. These extracts were used in bioautography and MICs calculated according to the procedure described in sections 4.4.1 and 4.4.2.

4.12 TOXICITY ASSAY

Toxicity of fractions was determined by the lactate dehydrogenase assay, which requires the use of human neutrophils at a concentration of 1 x 10^7 cells/ml. This assay was chosen for its simplicity and ease of determination as no specialised equipment is required. It is also a rather fast test, which allows for several determinations in a short span of time.

4.12.1 Cell separation

Heparinized blood was taken from healthy volunteers and diluted in a ratio of 1:2 with phosphate-buffered saline (PBS). The diluted blood was layered onto Ficoll (Histopaque 1077, Sigma Diagnostics) in a ratio of 2:1 and centrifuged for 30 minutes at 2100 rpm (1000xg). The serum was discarded up to 2 mm from the top layer and the mononuclear cells were aspirated and removed into a clean 50 ml tube. The suspension was centrifuged for 5 minutes at 1200 rpm (400xg) after adding 40 ml PBS and the resulting pellet (lymphocytes and monocytes) washed again with PBS.
Lysis buffer (0.83% (w/v) NH_4Cl) was added to the remaining cells to induce hypotonic lysis of the contaminating erythrocytes and suspended cells were incubated on ice for 5-6 minutes before centrifuging for 5 minutes at 1000 rpm. This procedure was repeated and the remaining pellet resuspended in 1-5 ml PBS after which cells were counted and adjusted to a concentration of 10^7/ml with PBS. All cells were kept in an ice bath prior to use.

4.12.2 Lactate dehydrogenase assay (LDH)

Neutrophils (10^7 cells/ml) were incubated with Hanks' balanced salt solution (HBSS) for 5 minutes at 37°C in a ratio of 1:4 i.e. 20 µl cells + 80 µl HBSS. As a positive control, 20µl lysophosphatidylcholine (LPC) was added to one ependorff tube and the volume adjusted to 200µl with HBSS. Two other controls were selected, one with distilled water and the other with the same ratio of DMSO to water in which the compounds were brought to solution and 100 µl of each added to their respective tubes. In the other ependorffs 100 µl of the test compounds were added into their respective tubes to make up a final concentration of 100 µg/ml. The tubes were incubated for a further 10 minutes at 37°C after which they were centrifuged at 1000 rpm for 5 minutes. The supernatant (100 µl) of each was pipetted and transferred to a microplate into which 100 µl PBS was added. Into each well 20 µl of NADH (1.3 mg/ml) was added and just before reading 20 µl pyruvate (1.1 mg/ml) was added to initiate the reaction. The microplate was read at 340 nm at time 0, 1, 5 and 10 minutes and measurements were done in triplicate.

4.13 ANTIOXIDANT / ANTI-INFLAMMATORY ACTIVITY

4.13.1 Serum opsonization of Zymosan

Zymosan A (Sigma Chemical Co.) was mixed with distilled water (2.5 mg/ml), boiled for 5 minutes and centrifuged at 900 rpm for 5 minutes until sedimented. The zymosan was washed with 10 ml PBS and resuspended before spinning off again at 900 rpm for 5 minutes. Freshly pooled serum (10 ml) from a healthy volunteer was added to the zymosan and incubated for 60 minutes at 37°C.

After centrifugation (900 rpm, 5 minutes), the supernatant was discarded and the pellet washed twice with PBS before finally resuspending in saline solution at a concentration of 2.5 mg/ml and used as the serum opsonized zymosan (SOZ).

4.13.2 Preparation of test compounds and chemiluminescence assay

Isolated test compounds were dissolved in DMSO and distilled water to yield a concentration of 200 μg/ml. The final concentration for each stimulant in solution with lymphocytes was: SOZ, 279 μg/ml and compounds, 50 μg/ml.

In each cuvette 100 μl of the cell suspension (2 x 10^6 cells/ml), was incubated with 100 μl luminol (5-amino-2,3-dihydro-1,4-phthlazindione; Sigma; final concentration 10^{-4} M) and the test compounds (100 μl) for 30 minutes at 37°C. The cuvettes were inserted in the chemiluminometer and the stimulant (SOZ) added just before recording. A sample that was not stimulated with SOZ but contained cells, luminol and RPMI to adjust the volume was included as the blank control. Two other controls, one containing RPMI instead of test compound and the other DMSO and water in the same concentration used to suspend compounds, were also included in the assay. Measurements were done in triplicate and each sample measured for 30 minutes using a luminescence assay. All buffers and reagents used from the same batch to limit variations in results. The peak response (peak CL) and the time for peak CL (peak time, minutes) were read from the recorder (BioORbit Oy, 1991).

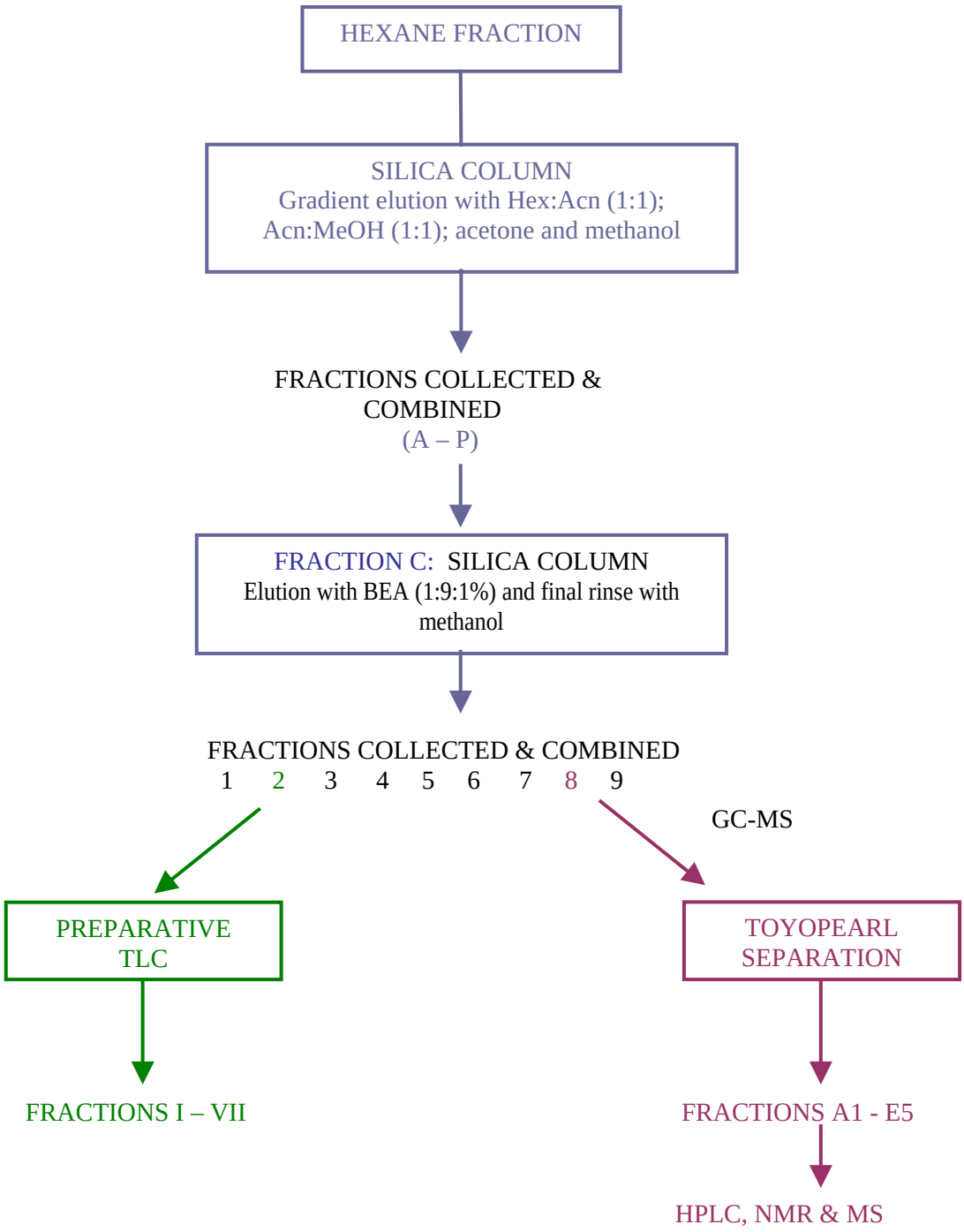


Figure 4.2: Schematic presentation of the compound isolation procedure followed with the hexane fraction of *C. erythrophyllum*, following solvent-solvent fractionation

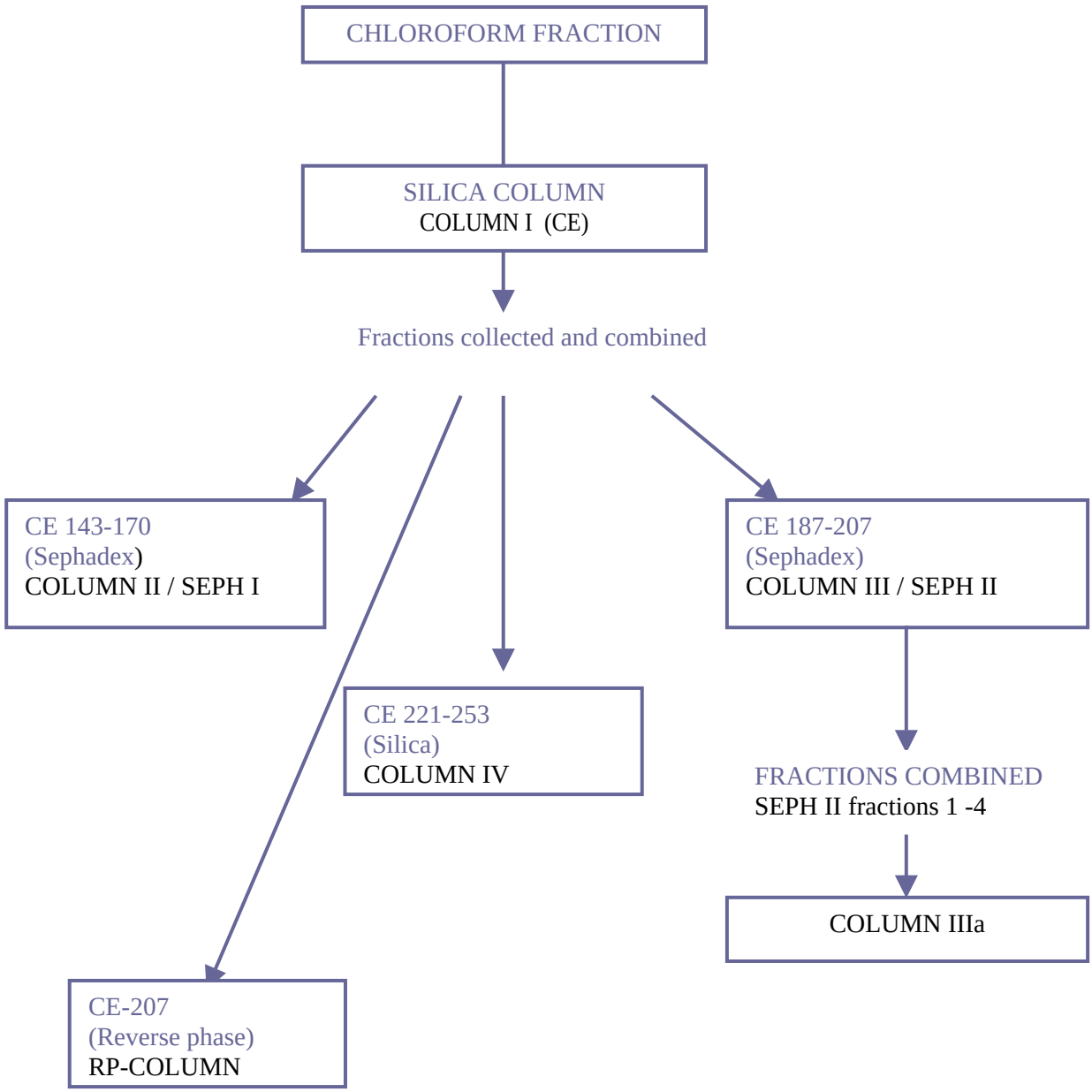


Figure 4.3: Schematic presentation of the compound isolation procedure followed using the chloroform fraction of *C. erythrophyllum*, following solvent-solvent fractionation

CHAPTER 5

5. RESULTS AND DISCUSSION

5.1 EXTRACTION

Although the total quantity extracted, after three successive extractions, was not as great as with some other solvents (Table 5.1), previous extraction studies performed on *C. erythrophyllum* using different solvent systems showed acetone to extract the greatest number of inhibitory components (Table 5.2).

Table 5.1: Percentage extracted from leaf material using various solvent systems

Solvent	1st	2nd	3rd	TOTAL
Acetone	9.2	2.4	1	12.6
Methanol	22.6	7.8	3.2	33.6
Ethanol	10.4	3.2	1.4	15
MCW†	24	9.2	4	37.2
MDC‡	8.8	2.4	1.4	12.6
Water	15	7.8	2.8	25.6

† Methanol/chloroform/water (12:5:3)

‡ Methylene dichloride

Table 5.2: The number of components visible on TLC chromatograms and the number of *S. aureus* growth inhibitors from different extracts of *C. erythrophyllum* leaf material

	C. erythrophyllum	
Solvent	**Components**	**Inhibitors**
Acetone	9	14
Methanol	9	9
Ethanol	8	8
MCW	4	13
MDC	8	11
Water	3	1

5.2 SOLVENT / SOLVENT EXTRACTION

The percentage extracted by each solvent was calculated by drying off the solvent from pre-weighed glass flasks. The highest percentage of extract was present in the hexane and carbon tetrachloride fractions (Table 5.3).

Table 5.3: Amount [as %] extracted from *Combretum erythrophyllum* by each solvent in the solvent/solvent extraction process.

	% of dry weight
Carbon tetrachloride (CCl_4)	37.3
Hexane (C_6H_{12})	24.3
Chloroform ($CHCl_3$)	21.8
Water (H_20)	5.1
35% water/methanol (35%W/M)	3.4
Butanol (BuOH)	1.5

There was some loss of original extract (c. 7%) due to incomplete phase separation. Antimicrobial components resided in all of the above fractions (Fig. 5.3) with the greatest inhibition of *S. aureus* exerted by 35% water in methanol and the least with hexane (Table 5.4). The number of components were visualised with TLC using three different mobile phases (see fig 5.1 and 5.2).

Table 5.4: The minimum inhibitory concentration in mg/ml of different fractions obtained by solvent/solvent extraction of *C. erythrophyllum* leaves.

	H_20	35%W/M	BuOH	$CHCl_3$	CCl_4	C_6H_{14}
S. aureus	1.56	<0.39	1.56	0.78	0.78	6.25
E. coli	3.13	3.13	6.25	25	>50	>50
E. faecalis	0.78	6.25	3.13	12.5	25	>50
P. aeruginosa	1.56	1.56	12.5	12.5	>50	>50

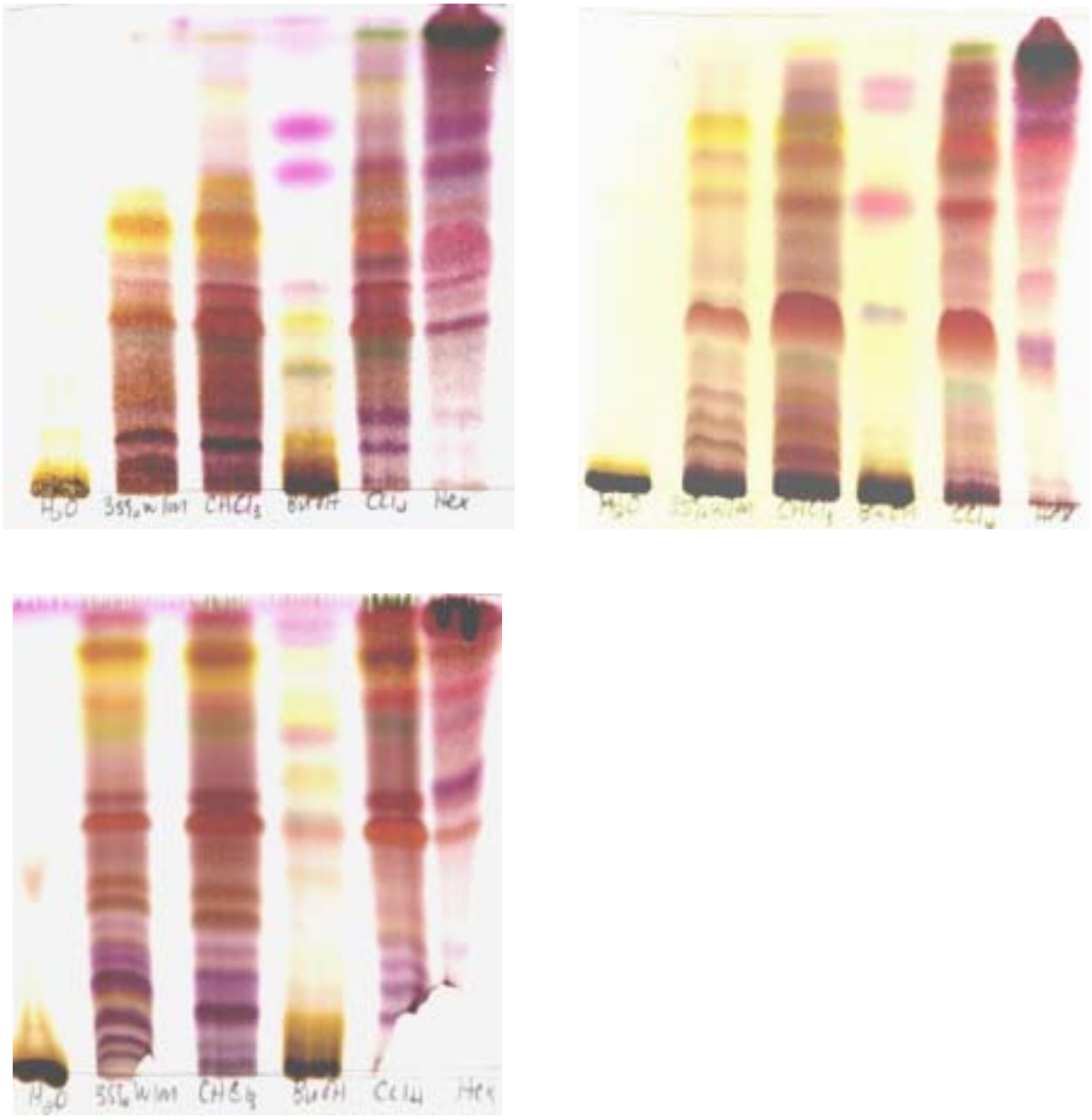


Figure 5.1: Solvent/solvent fractions depicting from left to right: water, 35% water/methanol, chloroform, butanol, carbon tetrachloride and hexane. Clockwise from the top are the fractions run in BEA, 2 acetone:3 MDC and CEF

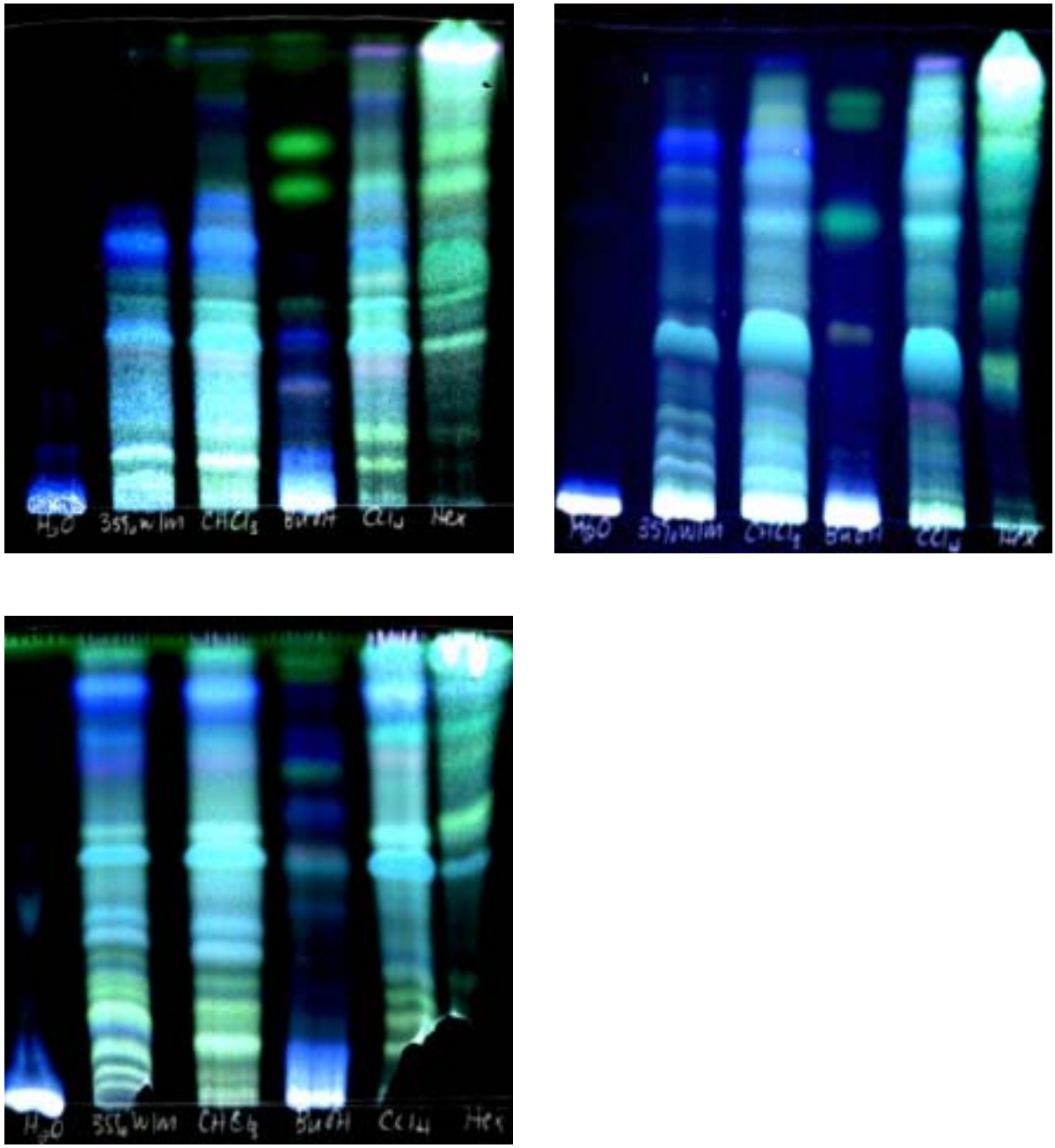


Figure 5.2: Negative image of solvent/solvent extraction depicting from left to right: water, 35% water in methanol, chloroform, butanol, carbon tetrachloride and hexane. Clockwise from the top are the mobile phases BEA, 2A:3MDC and CEF

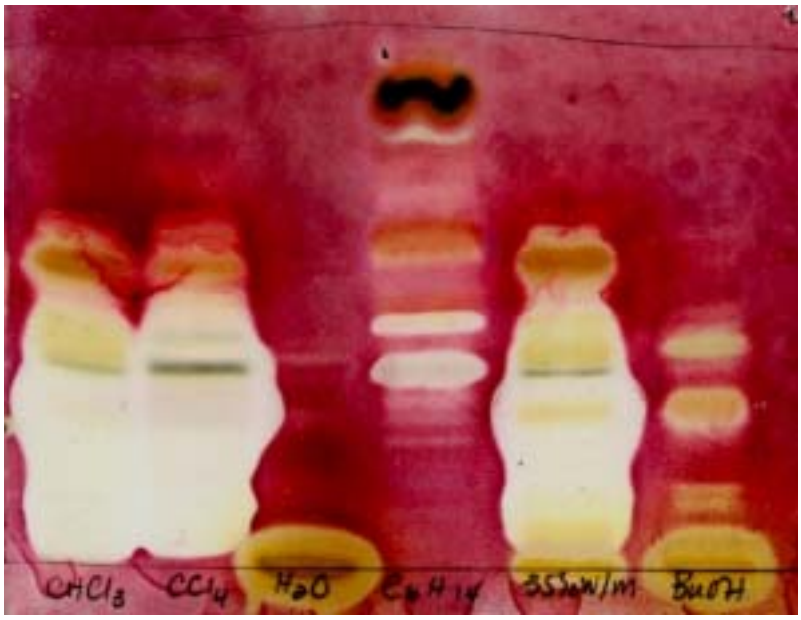


Figure 5.3: Bioautography of the solvent/solvent fractions using BEA as mobile phase. From left to right are chloroform, carbon tetrachloride, water, hexane, 35% water in methanol and butanol.

5.3 COLUMN CHROMATOGRAPHY

5.3.1 Hexane fraction

After examining the solvent/solvent extraction, hexane was chosen for column separation as a result of its relatively low complexity as seen with TLC (Fig. 5.1) in comparison to the chloroform and carbon tetrachloride fractions. The inhibitory compounds present in hexane exhibited a good separation with BEA (Fig. 5.3) and appeared easier to separate from the mixture than the other fractions. The more polar fractions i.e. the water and 35% water/methanol fractions were not even considered for analysis due to the difficulty in separating and analysing these fractions.

After attempting various combinations of solvent systems (fig 5.4) with the hexane fraction on TLC, a combination of hexane:methylene dichloride (1:1) and acetone:methylene dichloride (1:1) produced a good separation of the non-polar and polar fractions respectively. Methanol was employed in the final stages of separation to remove components not moved by the other mobile phases.

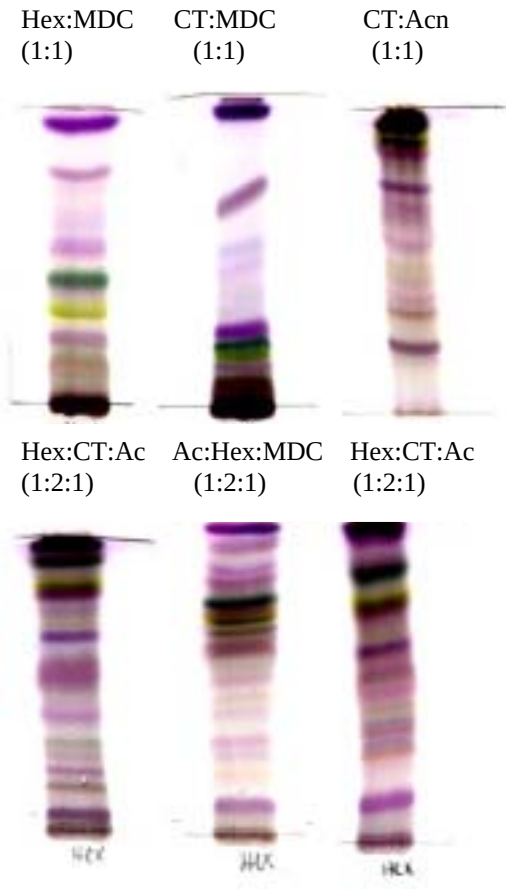


Figure 5.4: TLC plates showing the separation obtained by various mobile phases using the hexane fraction. Hexane: methylene dichloride (1:1), shown top left, separated the non-polar fractions and acetone: methylene dichloride (1:1) (not shown) separated the polar fractions the best.

5.3.1.1 TLC analysis and combination of fractions

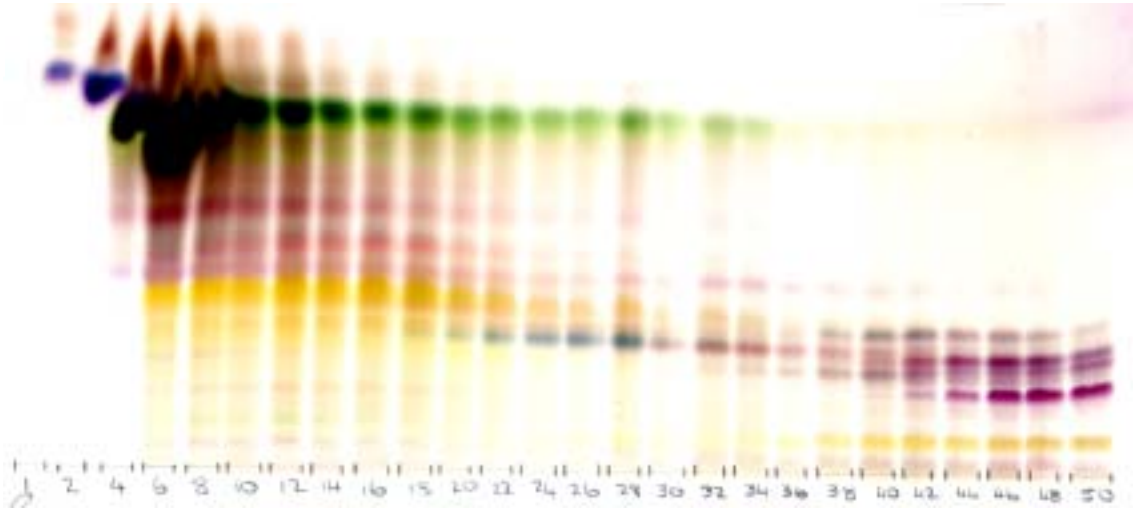
BEA was used for TLC analysis for the first 50 non-polar fractions and CEF for the remaining polar fractions (Figure 5.5), which enabled us to combine fractions with similar TLC profiles. Combination of fractions resulted in 16 fractions (A-P), which were then further analysed by TLC and bioautography (Figure 5.6 and 5.7 respectively) and the complexity and mass determined (Table 5.5).
Some of the samples did not dry completely, possibly due to oil content, and therefore mass was not accurately determined for those fractions.

Table 5.5: Mass and complexity of fractions A-P after combining the hexane fractions obtained after column chromatography

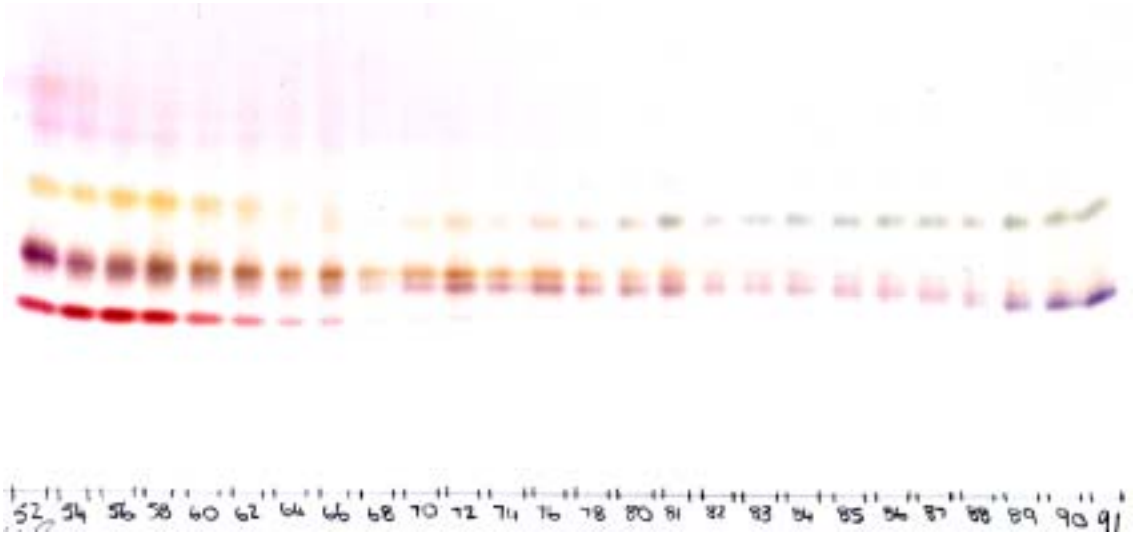
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
Combined	1-3	4-8	9 19	20 29	30 39	40 50	51 68	69 88	89 108	109 117	118 121	122 127	128 138	139 153	154 167	168 180
Complex	2	3	3	4	4	5	5	5	5	5	5	5	3	3	2	2
Mass (mg)	460	1166	5375		708	492	455	366	250	160	78	225	96	92	220	202

A white precipitate had formed in some of the fractions and consequently fraction K was separated into the precipitate and supernatant. The precipitate was dissolved in chloroform and applied to a preparative TLC plate with the hope of obtaining a pure sample. The mobile phase in which it was run was BEA and eight fractions were collected. The powdered silica was eluted with chloroform and acetone, fractions weighed and reapplied to TLC. Masses ranged between 3 and 17 mg and the experiment was subsequently scrapped due to insufficient material.

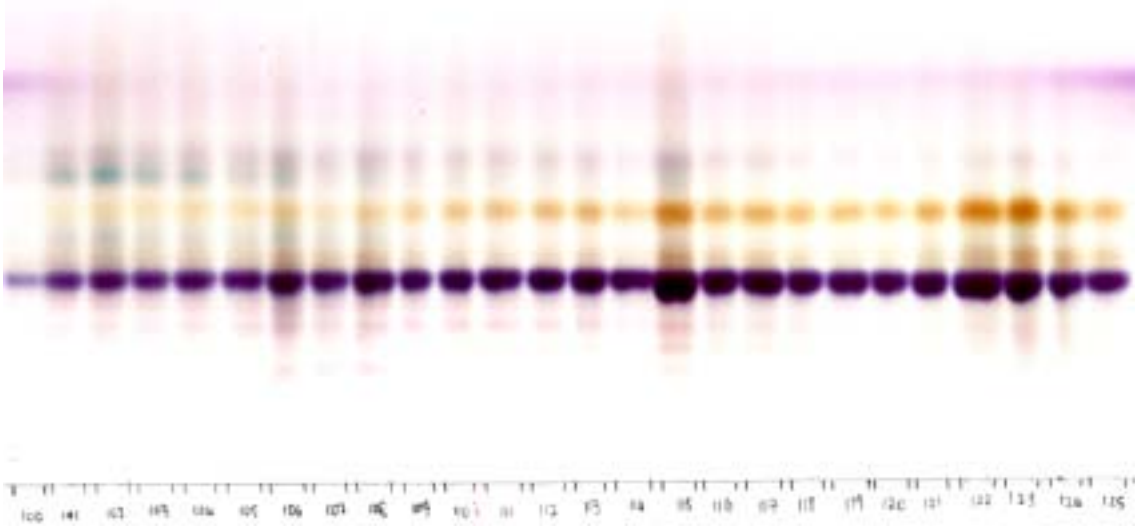
A



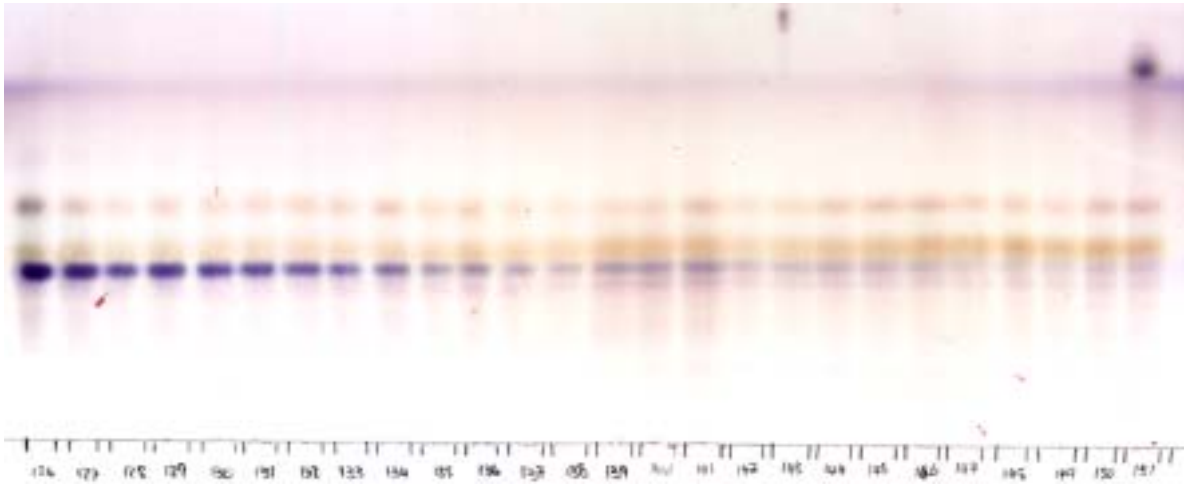
B



C



D



E

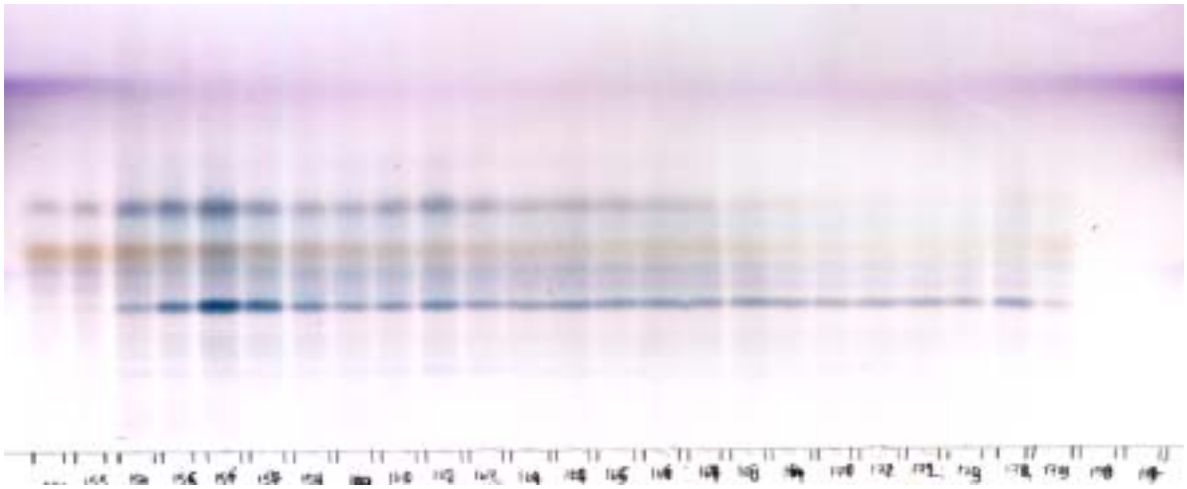


Figure 5.5: TLC of hexane fractions collected after passing through a silica gel column. A depicts the first 50 samples eluted with BEA, while B (52-91), C (100-125), D (126-151) and E (152-178) are eluted with CEF. All plates were sprayed with vanillin.

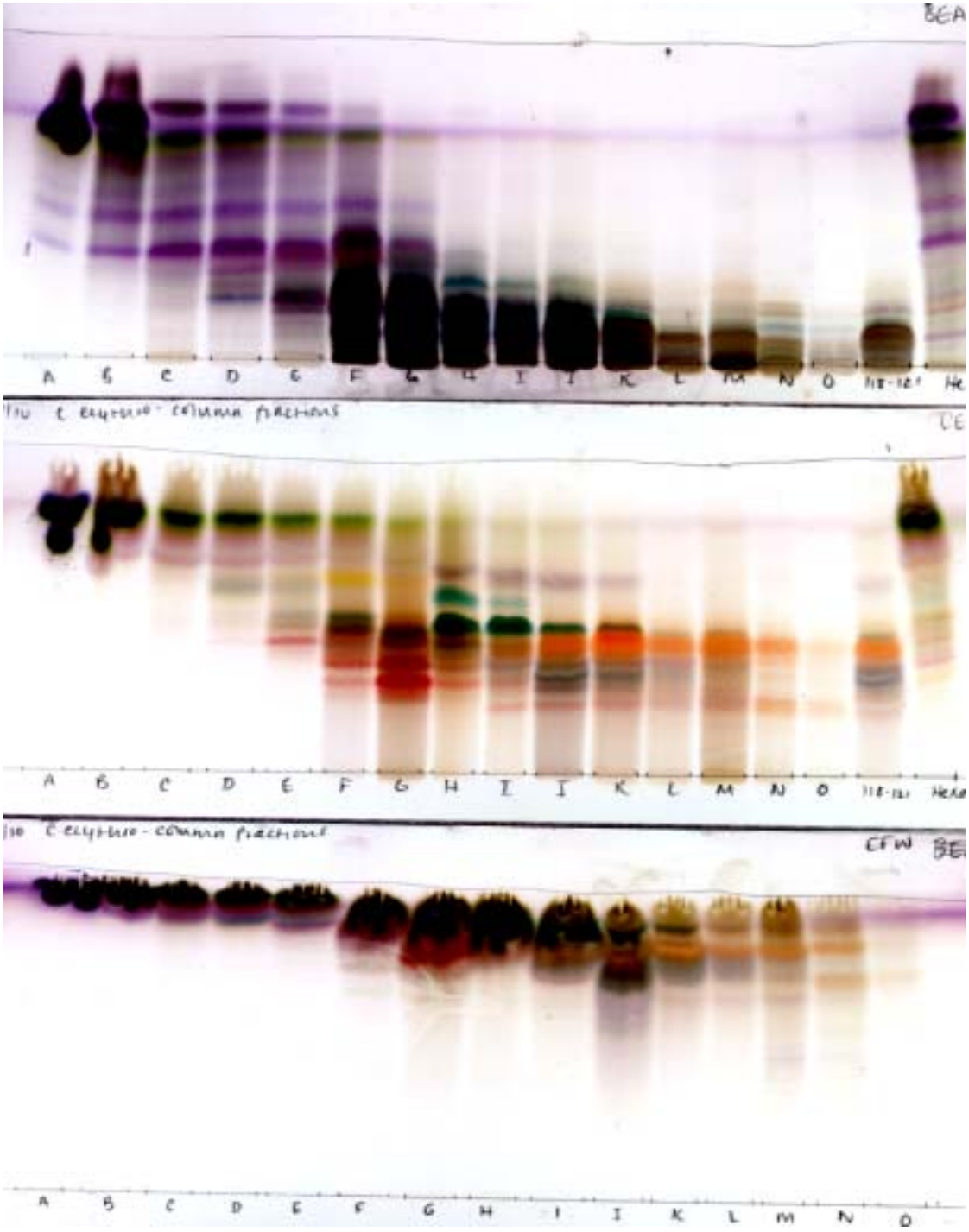


Figure 5.6: TLC of the fractions, which were combined according to the TLC results depicted in Figure 5.5. From the top are the fractions eluted with BEA, CEF and EMW and sprayed with vanillin.

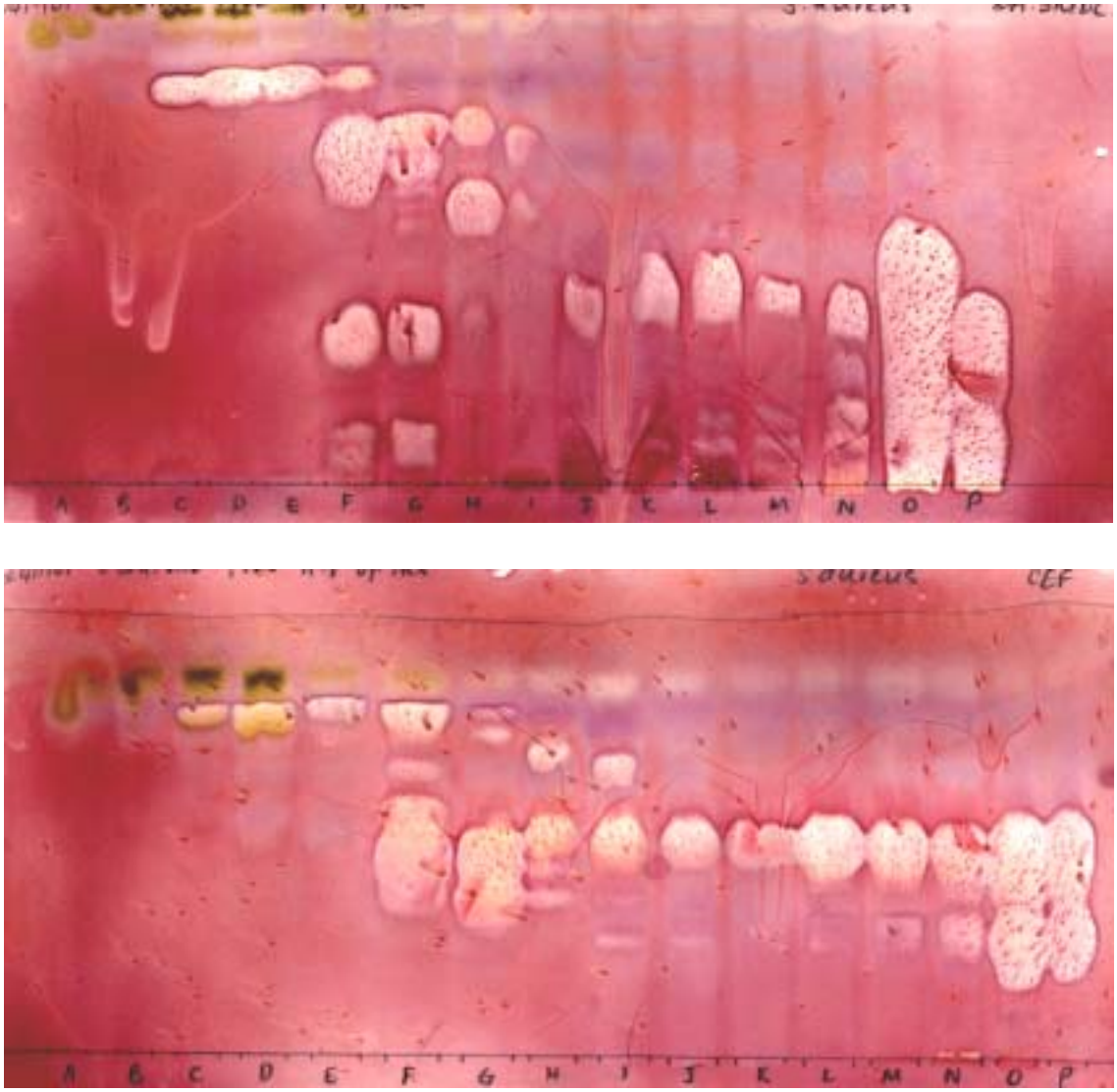


Figure 5.7: Bioautography of fractions A-P using *S. aureus* as test organism and two different mobile phases, i.e. 2A:3MDC (top) and CEF (bottom).

5.3.1.2 Separation of fraction C

Although fractions A and B exhibited few compounds, it was decided to use fraction C for further analysis due to the large quantity extracted i.e. c. 5.375 g and favourable separation with BEA (Fig 5.6). This solvent system was subsequently employed as mobile phase in the column. Once again, methanol was used in the final stages of separation to remove any components not eluted with the BEA solution.

In total 80 fractions were collected of which the first 30 were analysed with BEA and the following 50 fractions with both BEA and CEF. From these results (Figure 5.8) combination yielded nine fractions (1-9) (Figure 5.9).

Bioautography with *S. aureus* produced 1 clear inhibition zone (Rf =0.55) for fraction 2 of C (2C) with CEF (Fig 5.10). Initially the results were not very clear due to problems experienced with the bioautography procedure and were performed at a later stage with 2A:3MDC, CEF and BEA with results shown in Table 5.6.

Table 5.6: Data of fractions 1 – 9 obtained by column chromatography of *C. erythrophyllum* hexane fraction C

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Combined	3	4	5-6	7-14	15-22	23-25	26-29	30-50	51-80
Complexity	1	2	3	3	3	3	3	2	2
Activity	1	2	2	2	2	3	4	4	2
Mass (mg)	3	46	146	158.3	78.4	11	37.5	128.2	47.4
MIC (mg/ml)	0.047	0.18	9.13	>79.2	9.8	0.085	<0.075	<0.25	1.48
Total Activity	63.8	255.6	16	<2	8	129.4	>500	>512.8	32

Complexity of extracts was defined as the number of components that could be visualised on the TLC plate after spraying the plate with vanillin. They were given a value between 1 and 5, with less than three components = 1; between three and five = 2; six to ten = 3; 10-14 = 4 and greater than 15 = 5.

Activity was defined as the number of inhibitory zones present after spraying the plate with a *S. aureus* suspension. Values assigned were between one and five with less than one zone = 1; between one and two = 2; two to three = 3, three to four = 4 and greater than or equal to five = 5.

The total activity is defined as the mass (in mg) divided by the MIC value. A high value denotes a low MIC value with a relatively good quantity of compound to work with. For this reason fraction 2C, with a total activity of 255.6, was prepared for preparative TLC as the mass (46 mg) was too little for good separation via column chromatography. The other three components that showed good MIC values, i.e. 6C, 7C and 8C, were analysed by using varying ratios of EMW in an attempt to separate the non-polar components from each other. Fraction 6C was not used in any further analysis due to the small quantity present (11 mg).

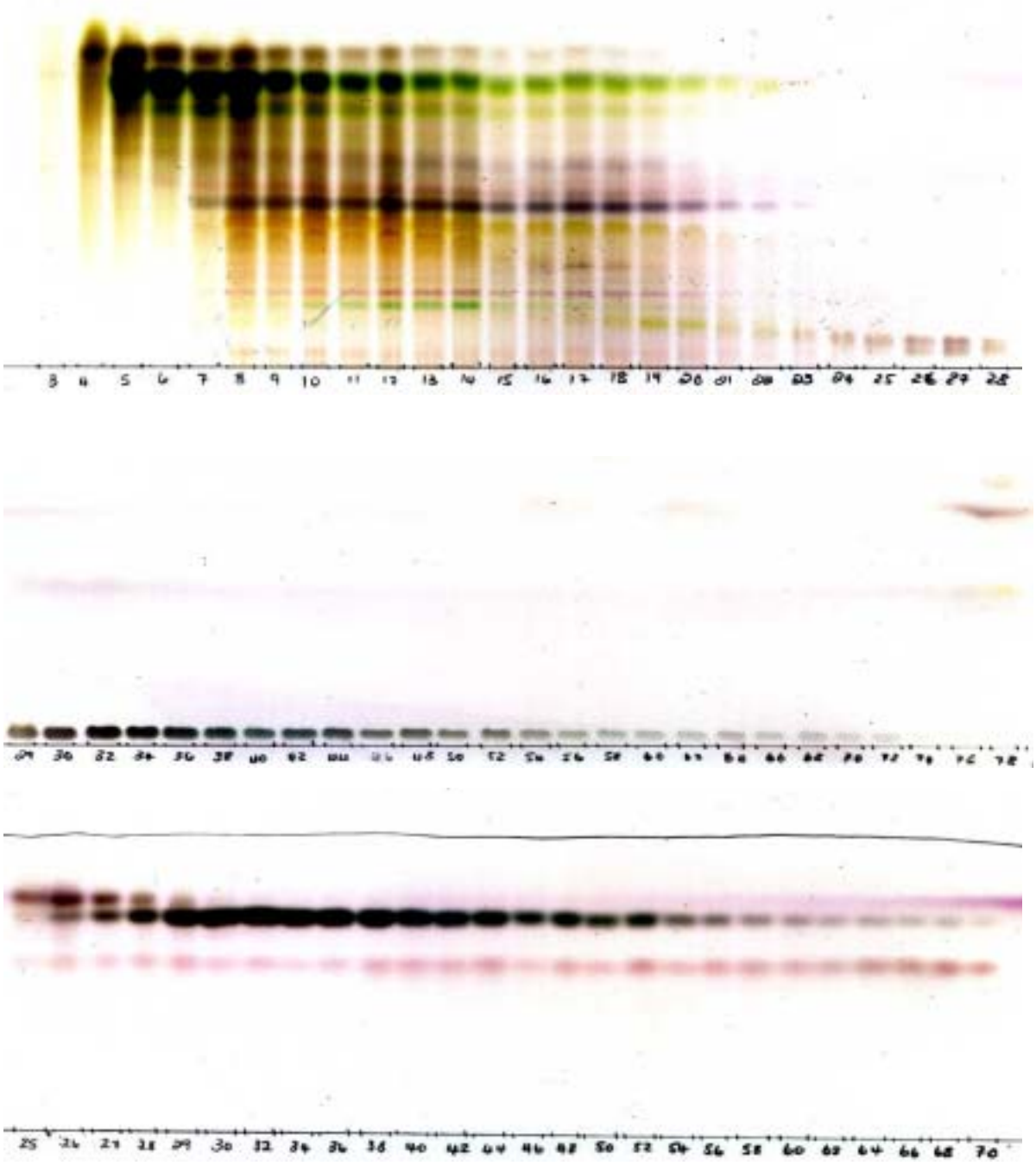


Figure 5.8: TLC of fraction collected from column chromatography of sample C. First 28 samples were eluted using BEA (top) and the rest with both BEA and CEF (middle and bottom) and sprayed with vanillin.

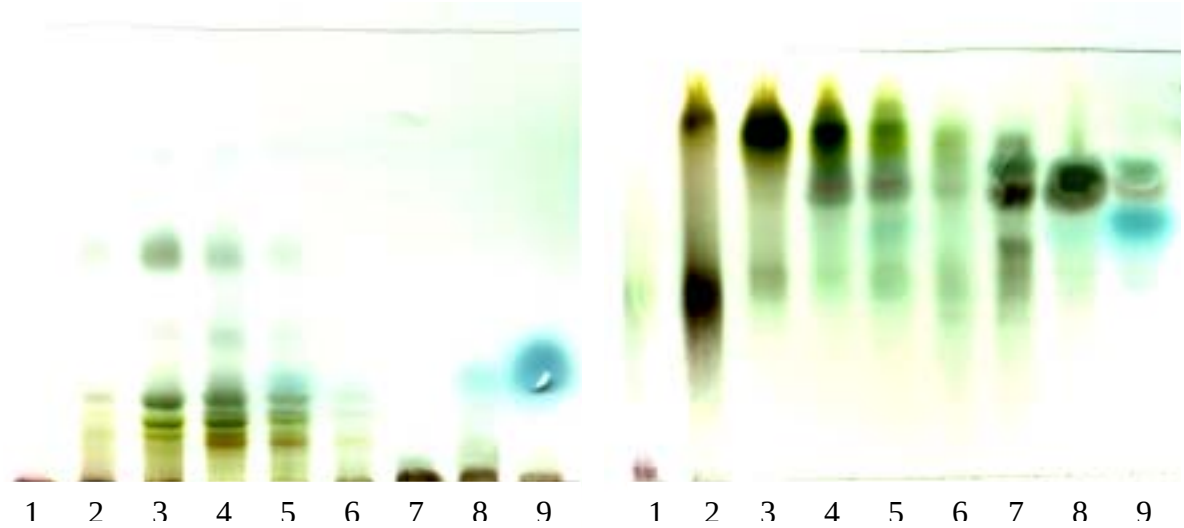


Figure 5.9: Combination of the fractions shown in figure 5.8 into 9 fractions. The TLCs show the fractions eluted with BEA (left) and CEF (right) and sprayed with vanillin.

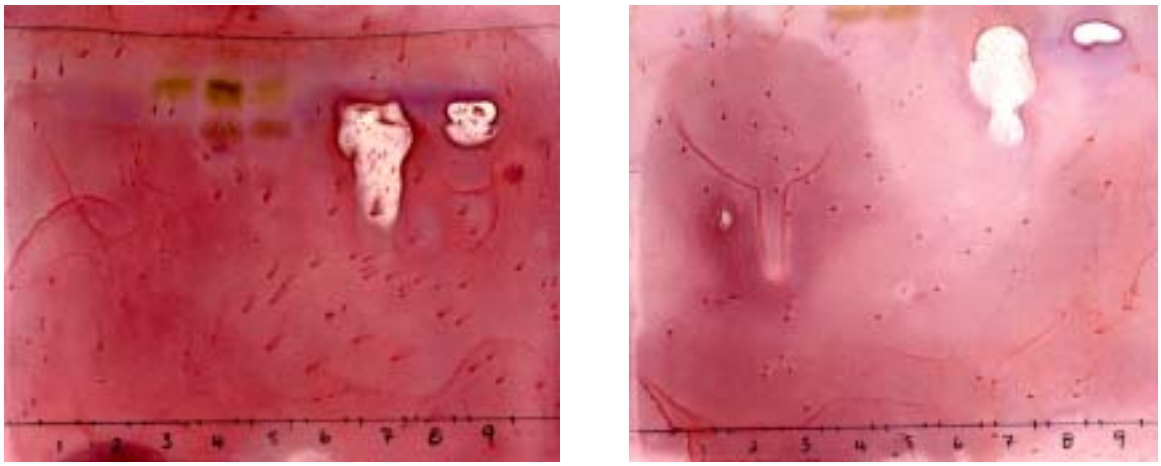


Figure 5.10: Bioautography showing inhibitory compounds of fractions 1-9 eluted with CEF (left) and 2A:3MDC (right). Although depicted on the plate, fractions 2 and 8 were not included as they had already been used for further analysis.

5.3.1.3 Preparative TLC of fraction 2C

Fraction 2C was dissolved in as little acetone as possible and applied to a preparative TLC plate. CEF was used as eluent as it showed the most favourable separation profile (Fig 5.9). The seven bands (I-VII) were marked off using UV and visible light as a visual aid (Fig 5.11) and then scraped off and extracted first with acetone (Fig 5.12); chloroform and then 1% acetic acid in methanol to remove polar components. Products were dried under a stream of cold air to avoid heat decomposition and stored in the fridge. Only the acetone fractions were subjected to the bioassays as the quantities extracted with chloroform and 1% acetic acid/methanol were insignificant.

The results are tabulated in Table 5.7 showing MIC's calculated at 30 minutes and 24 hours after INT addition. The change in values shows a bacteriostatic rather than bactericidal activity. Fraction VII had accidentally been dissolved in an unknown solvent which was difficult to remove therefore results were not obtained. Complexity and activity are as defined in paragraph 5.3.1. Total activity was calculated using MIC values obatained at 30 minutes.

Table 5.7: Preparative TLC results obtained for hexane fraction 2C

	I	**II**	**III**	**IV**	**V**	**VI**	**VII**
Complexity	2	2	3	4	4	1	1
Activity	4	2	2	1	3	4	5
Mass (mg)	11	7	15	21	21	6	?
MIC (mg/ml) 30 min	0.086	0.11	0.12	1.31	0.66	0.09	?
MIC (mg/ml) 24 hours	1.38	0.88	1.88	1.31	0.66	0.19	?
Total activity	127.9	63.6	125	16	31.8	66.7	?

Although these results show good MIC values after 30 minutes, the quantities extracted were insufficient for further cleaning and analysis of the samples. Even those which showed an activity value of 4 (fractions I and VI), had between three and five other compounds present on TLC, possibly more. Since the mass extracted was between 11 mg and 6 mg respectively for these two compounds, further cleaning would have resulted in even less material and therefore insufficient for structural elucidation.

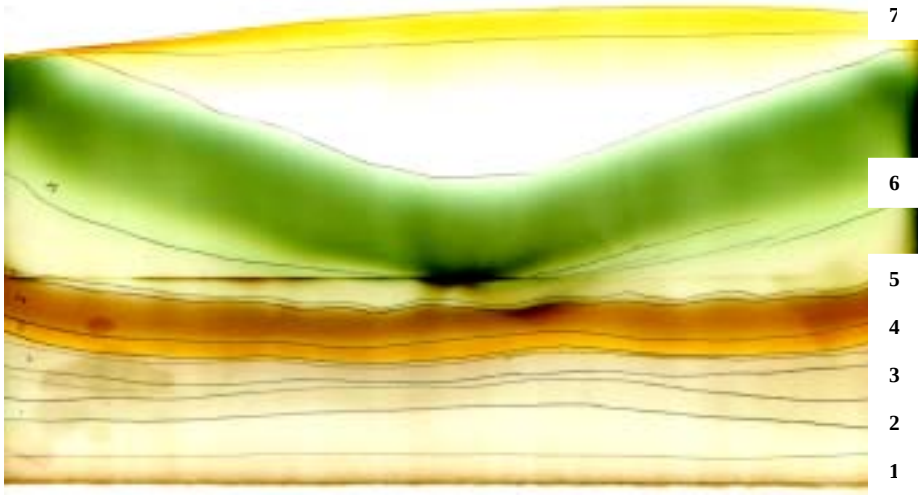


Figure 5.11: Preparative TLC of fraction 2C eluted with CEF. Compounds marked off were seen under both UV and visible light.

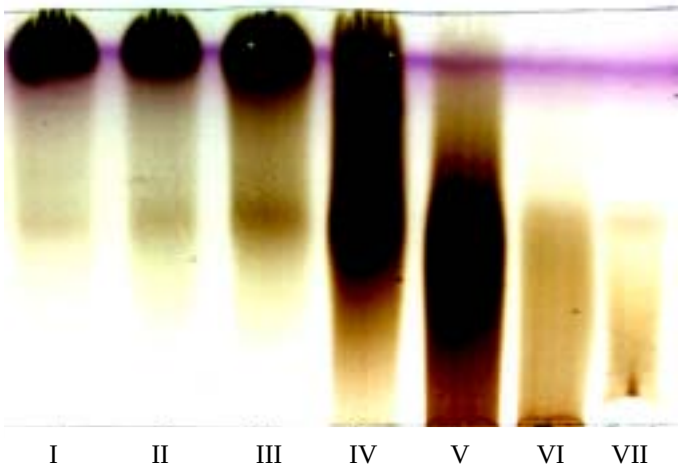


Figure 5.12: TLC of fractions I – VII after PTLC, eluted with CEF and sprayed with vanillin

5.3.1.4 Analysis of fraction 8C by GC-MS

When subjected to a cold air current, crystals had begun forming in the supernatant of fraction 8C. Although this fraction was initially soluble in acetone, the precipitate dissolved in chloroform and when applied to TLC at least two bands were visible after spraying with vanillin. The mixture of both supernatant and precipitate were slowly heated in a water bath and then gradually cooled in the refrigerator with the hope of forming large crystals. The two fractions were separated from each other by centrifugation in a larger tube containing ice to prevent re-dissolution of the precipitate. Both were sent for GC-MS analysis to determine types of compounds present. Initially it was thought that the active compounds could be present in the precipitate and therefore after initial analysis of supernatant and precipitate, TMSI was added to the precipitate. Since dissolution in the TMSI was poor, the mixture was left overnight at 100°C and injected the following morning onto the GC column.

Some compounds that were initially visible had disappeared on addition of the TMSI, which could possibly mean that unbinding of certain compounds had taken place and was therefore not possible to determine original structure composition. The results seen with the precipitate indicated that it contained primarily wax-like structures (Appendix A), which tend to precipitate out of solution under reduced temperatures.

Since only a small quantity of both the supernatant and precipitate fractions were taken and dried off to calculate mass, the accuracy of the results is not very reliable. MIC values were calculated in µg/ml and were 0.02 for *S. aureus*, 0.133 for *E. coli*, 0.625 for *P. aeruginosa* and 0.078 µg/ml for *E. faecalis* respectively.

5.3.1.5 Toyopearl separation

A long burette was filled with Toyopearl to the top and allowed to settle. The 20% ethanol in which the suspension is marketed, was replaced with acetone and approximately 420 mg of sample 8C was applied to the top of the column. Acetone was used as the mobile phase and the samples collected were dried under a stream of cold air. According to the TLC results the fractions were combined to form five major fractions (A1-E5). These five fractions were applied to UV absorbant TLC plates to determine Rf values of compounds (Table 5.9), their mass calculated and solutions reapplied to TLC to determine standard of purity.

Table 5.8: Rf x 100 values of fractions A1 – E5 eluted with 2A:3MDC and CEF. Absorption of UV, either at 254 or 360 nm, is denoted in brackets.

	A1	B2	C3	D4	E5
2A:3MDC	0(254)	90(360) 77 (254) 0 (254)	90(360) 4(360) 0(254)	80(254) 6(360) 0(254)	0(254)
CEF		71(254)	71(360) 60(360) 0(360)	67(254) 58(254) 0(254)	

Results of the MIC determination and bioautography are shown in Table 5.9 and Fig 5.13 respectively.

Table 5.9: MIC values in mg/ml (total activity) of fractions A1-E5 of Toyopearl samples.

Sample	*S. aureus*	*P. aeruginosa*	*E.coli*	*E. faecalis*
A1	1.25 (4)	>1.25 (<4)	>1.25 (<4)	0.156 (32)
B2	0.55 (129)	8.9 (8)	8.9 (8)	0.28 (254)
C3	0.69 (32)	5.5 (4)	5.5 (4)	0.17 (129)
D4	0.53 (64)	2.13 (16)	2.13 (16)	0.27 (126)
E5	1.25 (8)	2.5 (4)	>2.5 (<4)	0.31 (32)

Fraction B2 was chosen for further analysis since it exhibited the greatest total activity against both Gram-positive organisms.

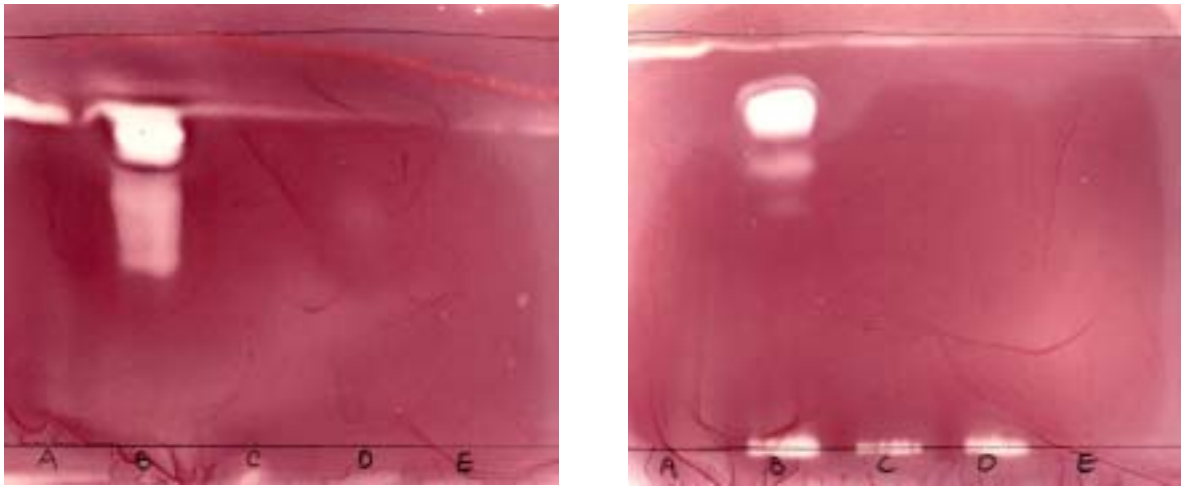


Figure 5.13: Bioautography of samples A1-E5 using *S. aureus* as test organism and eluting samples with CEF (left) and 2A:3MDC (right)

5.3.1.6 HPLC and preparative HPLC of fraction B2

In order to prepare the correct solvent system for fraction B2, it was initially run on reverse phase TLC plates with varying ratios of methanol:water. The best separation was obtained by using 10% water in methanol and this system was subsequently used for HPLC analysis. The column used was a C_{18} reverse phase column but since the compound seemed to absorb at wavelengths similar to that of solvents used, it was not possible to distinguish the solvents from the compounds (Appendix B).

Since the apparatus available was not good enough for structural analysis, fraction B2 was sent to the CSIR for preparative HPLC analysis. Since the only column available to them was also a reverse phase, separation of components was not possible. They had subsequently sent the sample for mass spectroscopy and NMR analysis (Appendix C) but due to the small quantity available to them (30 mg) no conclusive results were reached.

5.3.1.7 Preparative TLC of fraction C3

The bioautography results show an inhibitory compound at an Rf value of 0.44, run with a solvent system of 15% methanol in chloroform. This sample was dried off and spotted on a preparative TLC (PTLC) plate and run with the same mobile phase. Only one compound was strongly visible under UV absorbance and when collected, by washing the silica with acetone, an oily emulsion had formed. This emulsion had initially dissolved in acetone but once dried off to determine mass, it had to be solubilised in deuterated dimethylsulphoxide (DMSO-d_6).

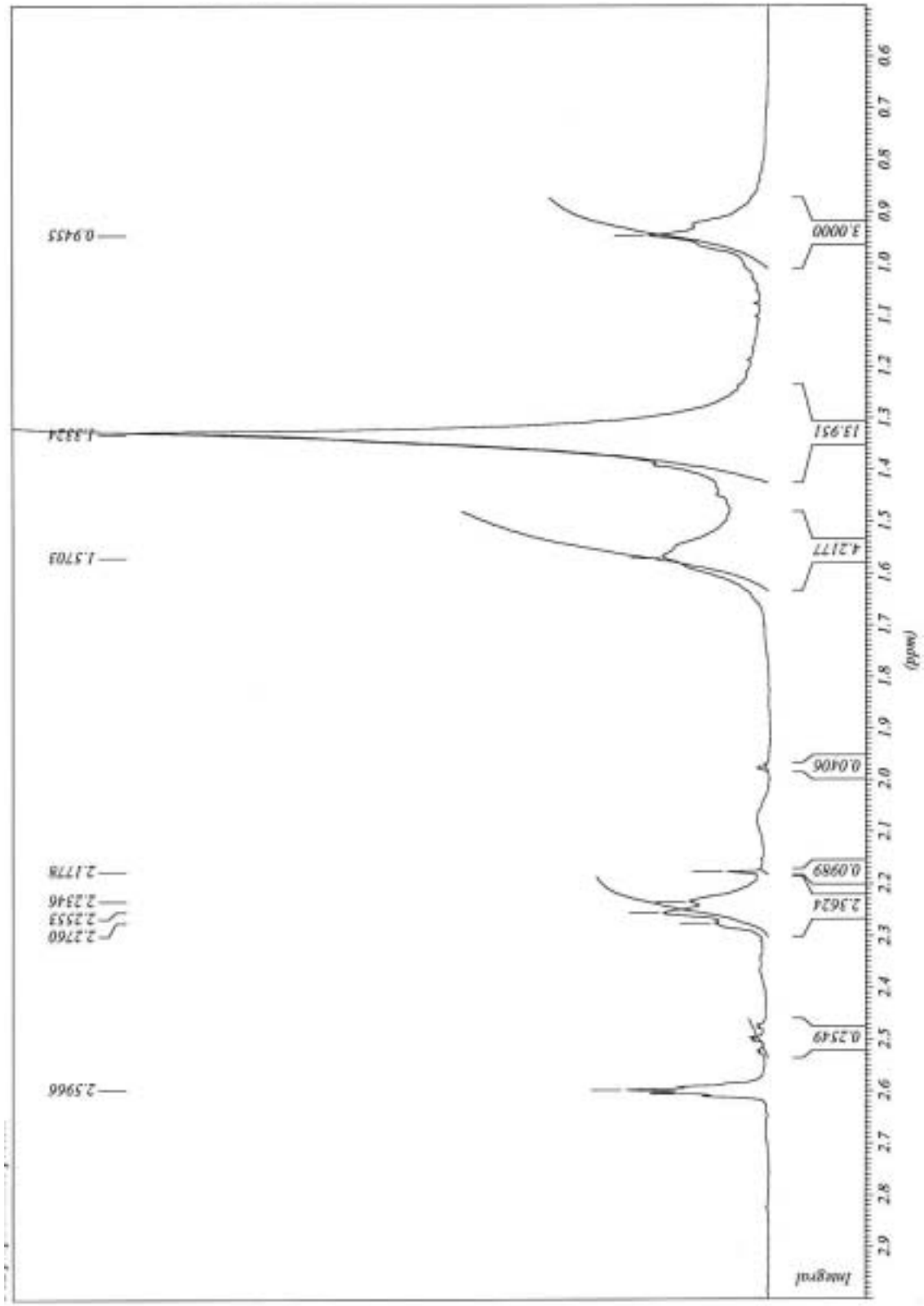
5.3.1.8 NMR of fraction B2 and C3

These two fractions were both sent for NMR analysis for structure elucidation. The ^{1}H-NMR spectrum of sample B2 (dissolved in deuterated chloroform and measured at 400 MHz) was not clear and peaks could not be identified (Appendix C). Due to the small quantity of sample (30 mg), it could not be cleaned for further analysis and was subsequently discarded.

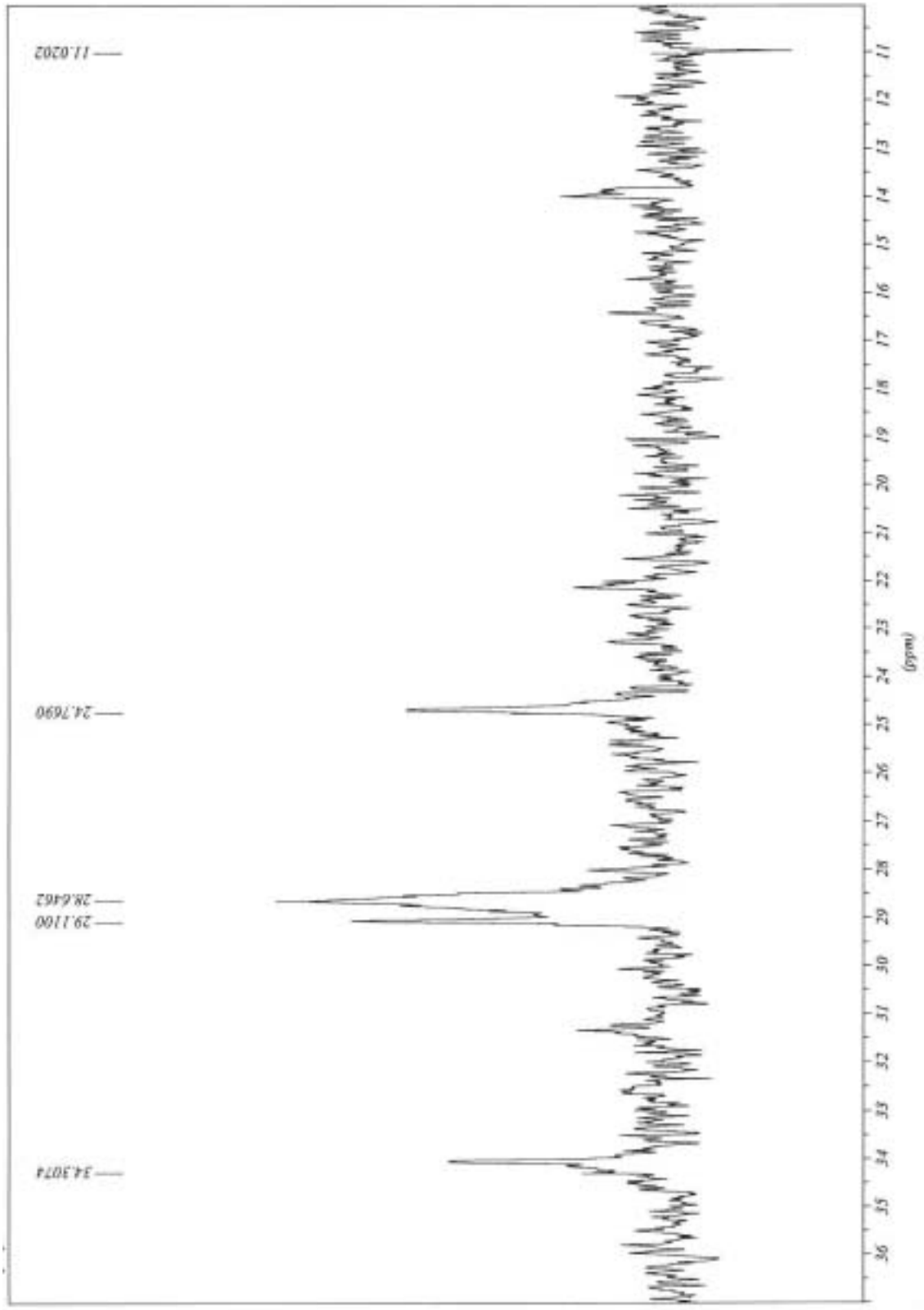
Fraction C3 was dissolved in deuterated DMSO and ran at 300 MHz but once again the ^{1}H NMR exhibited many peaks of which none could identify the compound. All that could be deduced from this spectrum was that this was an alkane (Spectrum 5.1). ^{13}C NMR was very noisy and could not help in the identification of this compound. Peaks were seen at δ_C 11.2, 24.8, 28.6, 29.1 and 34.3 implying the presence of methyls, methylenes and methines (Spectrum 5.2).

Confirmation of the class of compound was done by HREIMS analysis (Spectrum 5.3). The M^+ peak was seen at *m/z* 690 (100%), which gives the molecular formula of $C_{47}H_{62}O_4$. Since there are four oxygens instead of two, as would be seen with a fatty acid (-COOH), it was concluded that this compound was a long chain ester. Other fragments include *m/z* 646 $[M\text{-}C_3H_8]^+$ (8%); *m/z* 602 $[M\text{-}C_6H_{16}]^+$ (11%); *m/z* 575 $[M\text{-}C_8H_{18}]^+$ (44%) and *m/z* 558 $[M\text{-}C_9H_{24}]^+$.

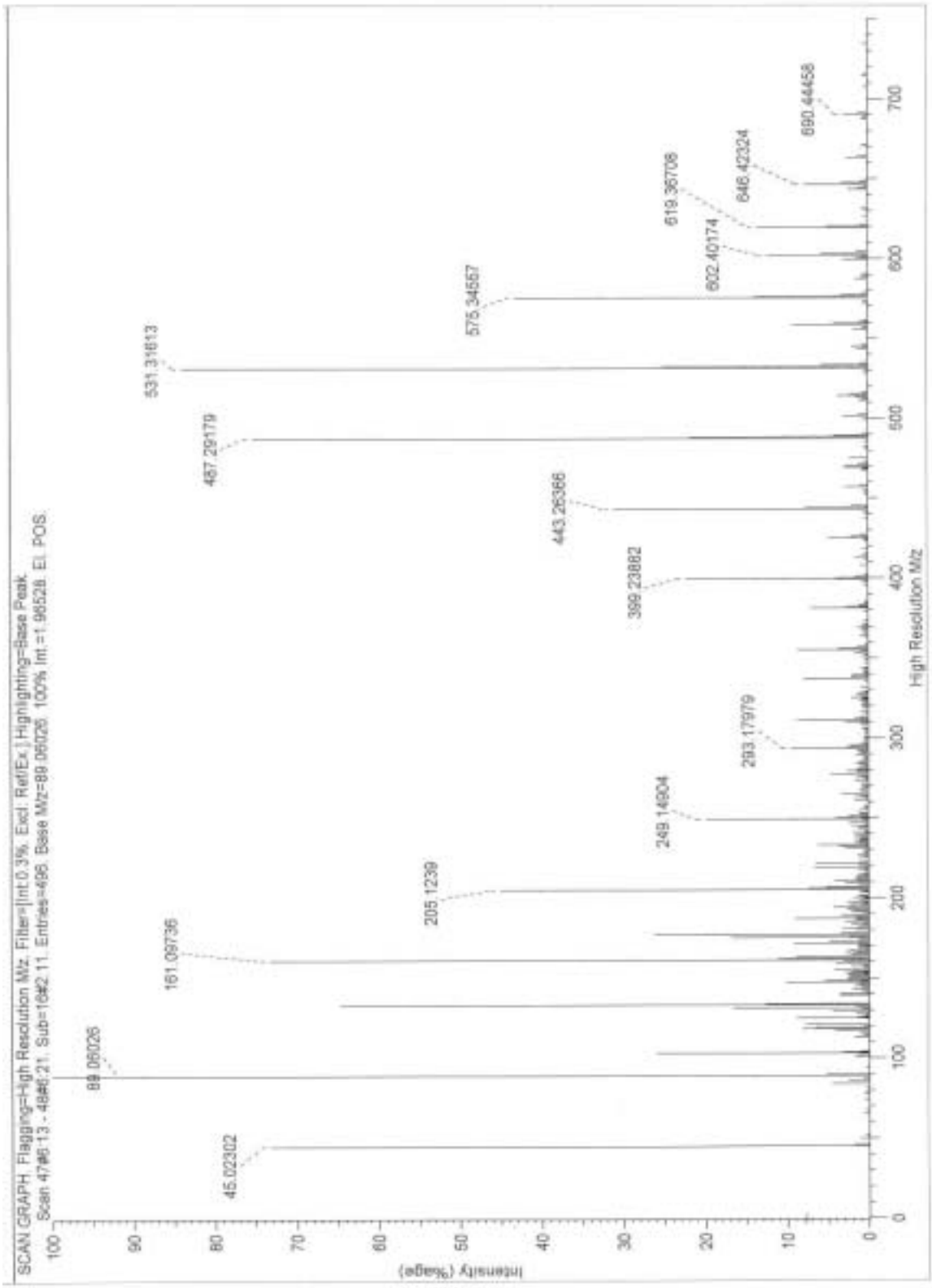
Other fractions that were collected from the hexane column chromatography, were sent for NMR analysis. They were all suggestive of long chain aliphatic compounds. No follow up work was done due to lack of time. Selected spectra are shown in Appendix C.



Spectrum 5.1: ^{1}H NMR (300MHz, DMSO-d_6) of C3



Spectrum 5.2: ^{13}C NMR (75MHz, DMSO-d_6) of C3



Spectrum 5.3: HREIMS of C3

5.3.2 Chloroform fraction

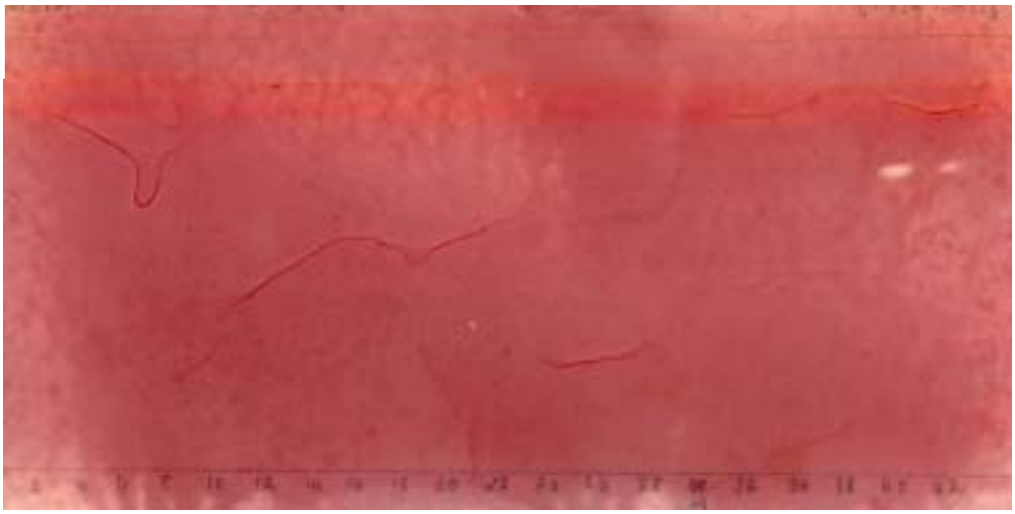
5.3.2.1 Column I (CE)

Since chloroform extracted both polar and non-polar compounds from solution, exhibited a good TLC profile of compounds (Figure 5.1 and 5.2), and extracted a number of inhibitory compounds, it was decided that this would possibly be the ideal fraction to work with. This fraction (17.651 g) was packed onto a silica gel column and eluted with a mixture of solvents starting at low polarity.

The first few samples collected were eluted with 40% hexane in dichloromethane (DCM) followed by 10% hexane/DCM, 100% DCM and then gradually methanol was added until a final concentration of 80% methanol/DCM.

In total 324 fractions were collected and every second fraction was spotted on TLC and bioautography performed (Figure 5.14a and b). In some of the samples crystals had begun to form and were consequently cleaned using various solvents and sent for NMR analysis. Other samples were combined according to preliminary TLCs and reapplied to TLC when more concentrated (Fig. 5.15). Mobile phases used were mainly different ratios of methanol:chloroform, depending on the samples. Bioautography was again performed on the combined fractions (Fig. 5.16)

A



B

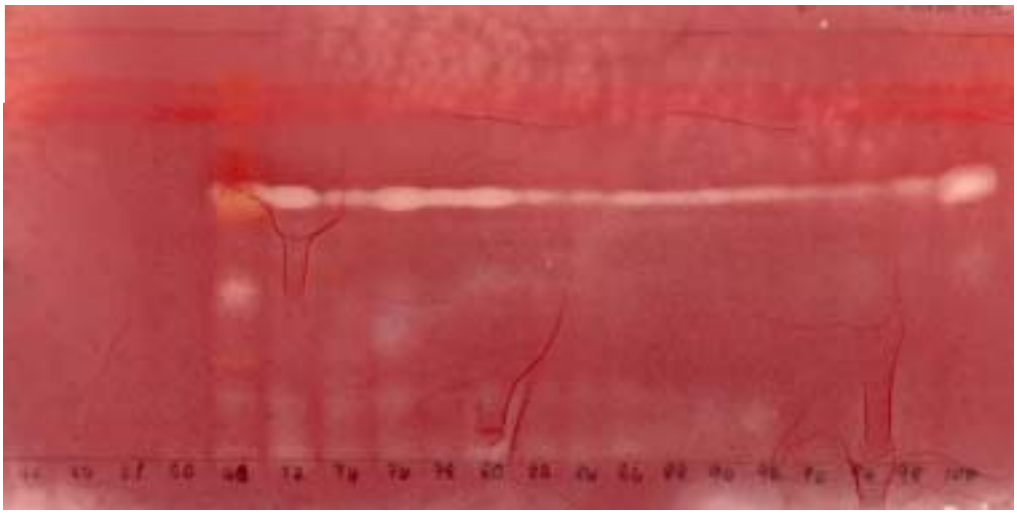
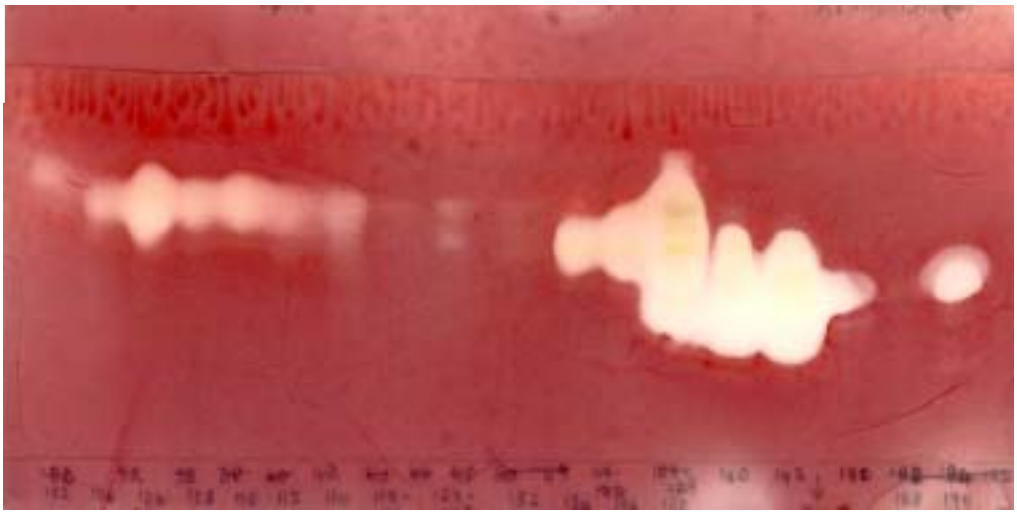


Figure 5.14a: Bioautography of fractions A (2-42) and B (44-100) of Column I eluted with 1% methanol/chloroform and sprayed with *S. aureus*

C



D

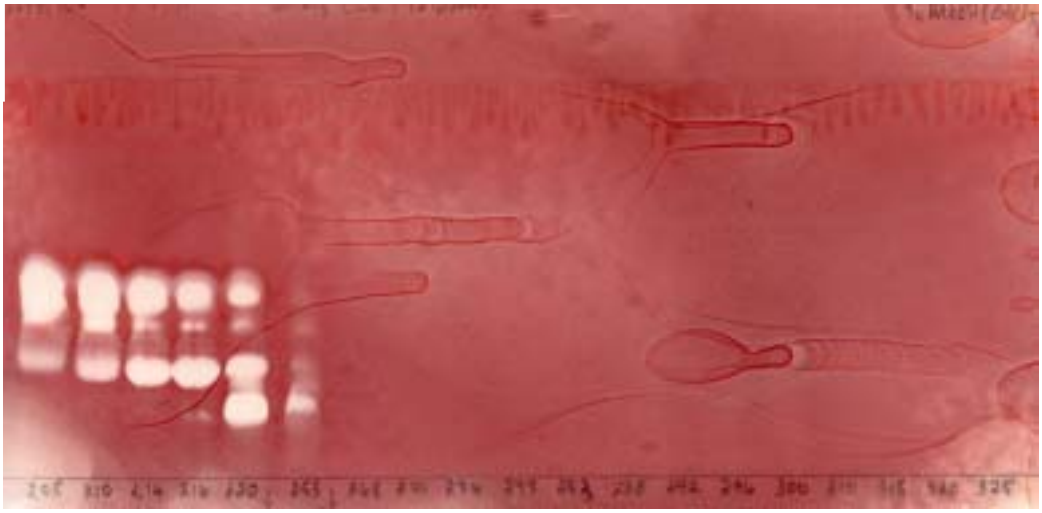


Figure 5.14b: Bioautography of fractions C (102-190) and D (205-325). And eluted with 10% methanol/chloroform and 15% methanol/chloroform respectively.

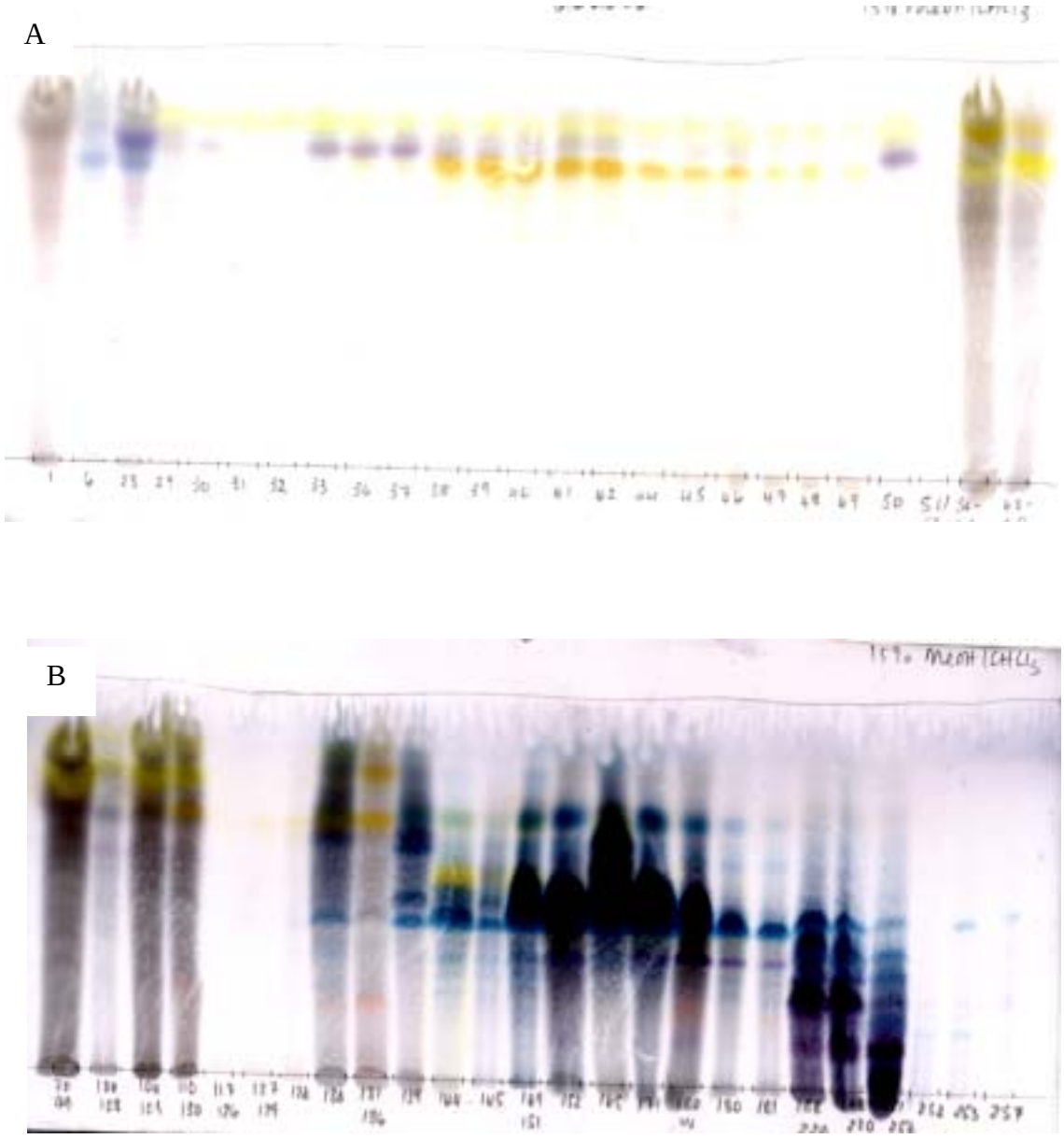


Figure 5.15: TLC of Column I of chloroform fraction. A are the fractions 1-69 and B are fractions 70-259 both eluted with 15% methanol in chloroform.

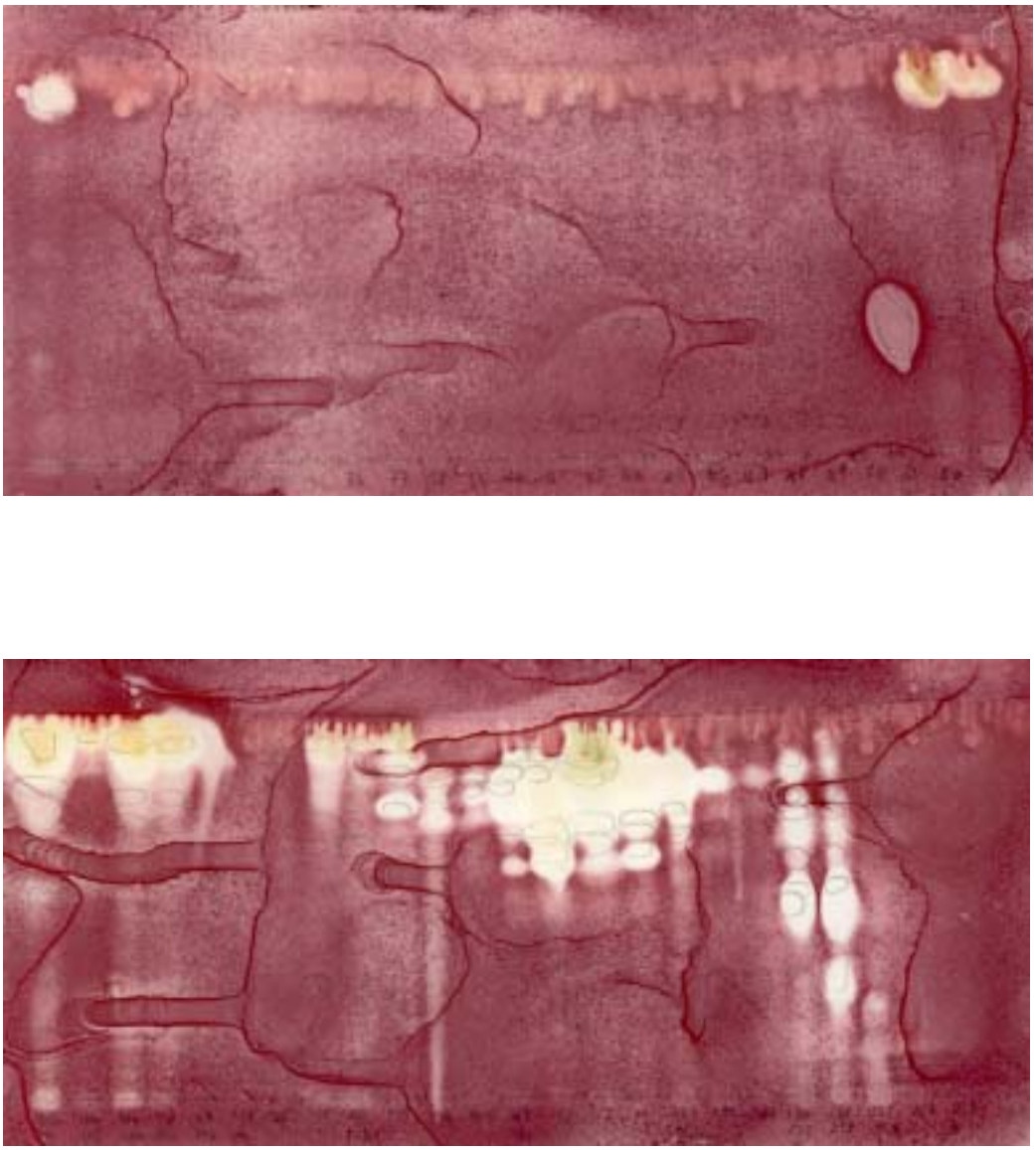


Figure 5.16: Bioautography of the combined fractions of the chloroform column fractionation (fig. 5.15) using 15% methanol/chloroform as eluant.

5.3.2.2 Column II / Sephadex I

Fractions CE 143-170 of column I were combined and eluted on a column packed with Sephadex-LH20. Elution was initiated with 100% chloroform after which 50% methanol was introduced and finally 100% methanol until no further compounds were eluted. Approximately 100 fractions were collected, which were analysed using TLC and similar fractions combined (Fig 5.17). Bioautography was performed at various stages i.e. before and again after combination in order to determine which fractions to combine (Fig. 5.18 and 5.19). Unfortunately fractions 1-9 were accidentally lost although they had shown significant activity.

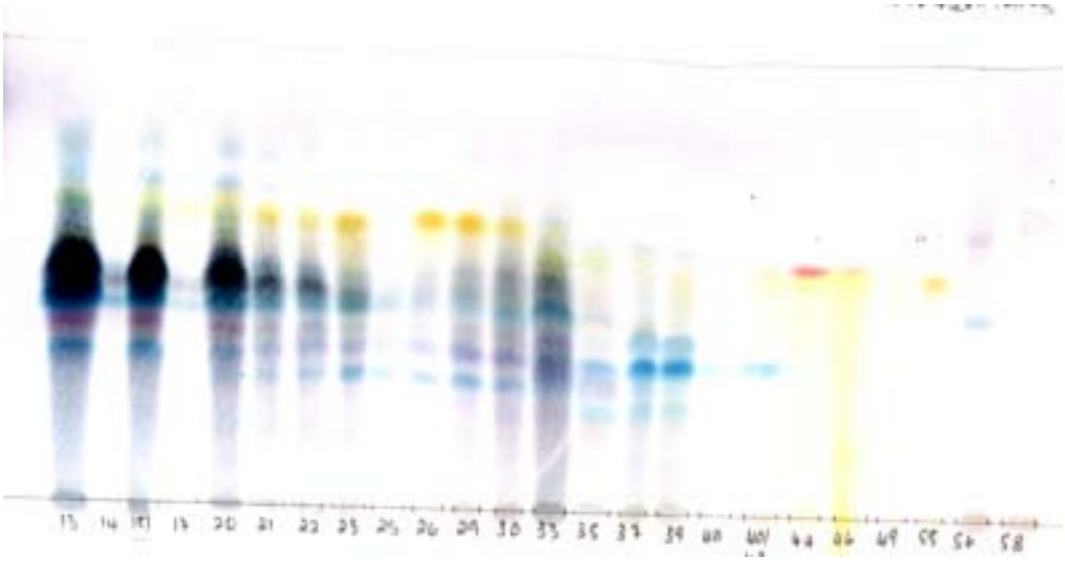


Figure 5.17: TLC of combined fractions of column II using 15% methanol in chloroform as eluant.

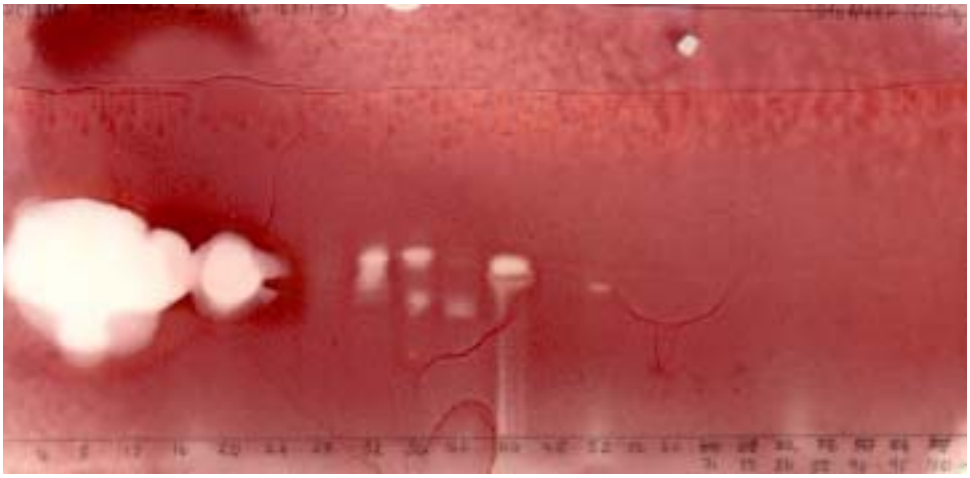


Figure 5.18: Bioautography of random fractions from Column II before combination. Elution was performed using 10% methanol in chloroform.

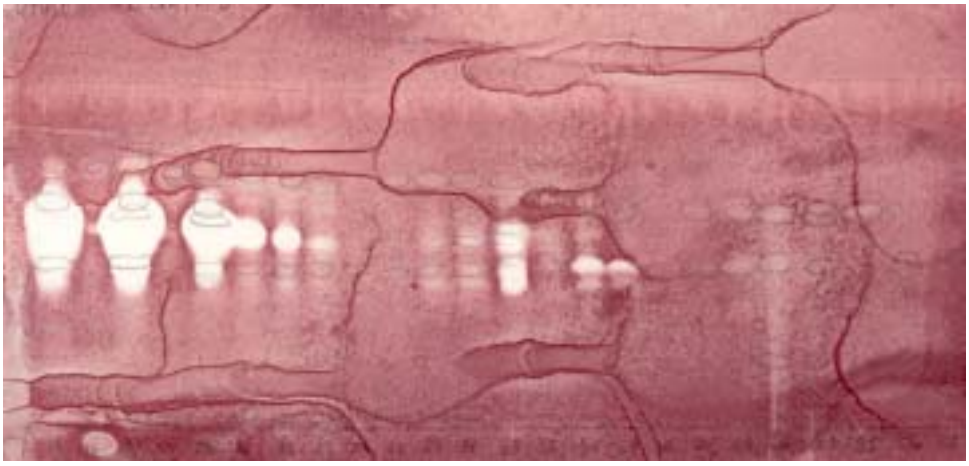


Figure 5.19: Bioautography of combined fractions from Column II coinciding with those of Fig. 5.17. Elution was performed using 15% methanol in chloroform.

5.3.2.3 Column III / Sephadex II

Fractions CE 187-207 of column I were combined and eluted on Sephadex-LH20 using the same eluting solvents as with Column II (Fig 5.20). Bioautography was performed on the combined (Fig. 5.21) fractions as well as prior to combination.

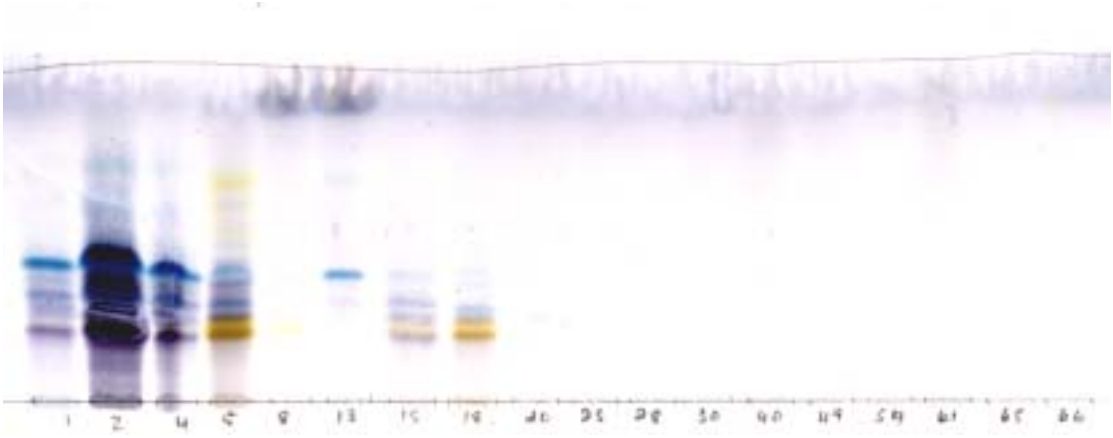


Figure 5.20: TLC of combined Column III fractions eluting with 15% methanol in chloroform.

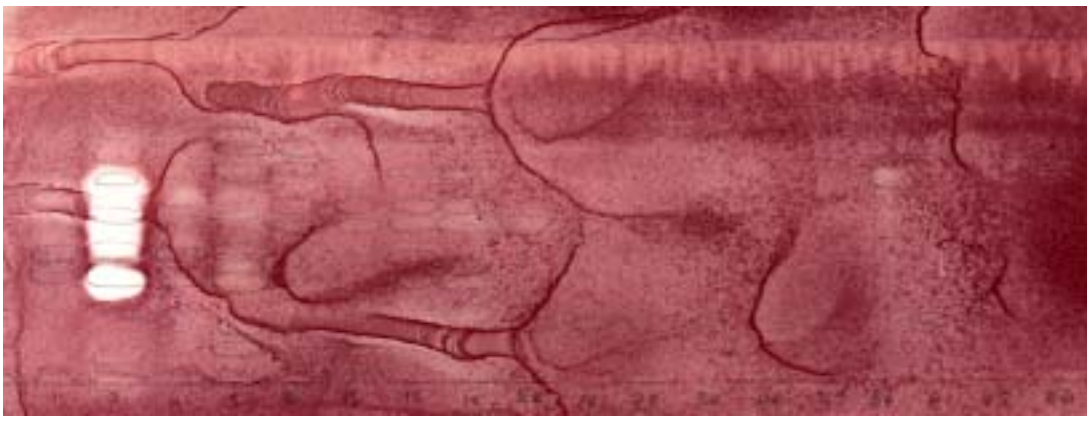


Figure 5.21: Bioautography of the same fractions applied to TLC in Figure 5.21 and eluted with 15% methanol in chloroform.

5.3.2.4 Column IIIa

Since activity appeared greatest in fractions 1-4 of Column III, they were combined and fractionated on a silica gel column. Approximately 400 fractions were collected and every fifth fraction spotted on TLC. From these results fractions were combined and reapplied on TLC (Fig. 5.22) followed by bioautography (Fig. 5.23).

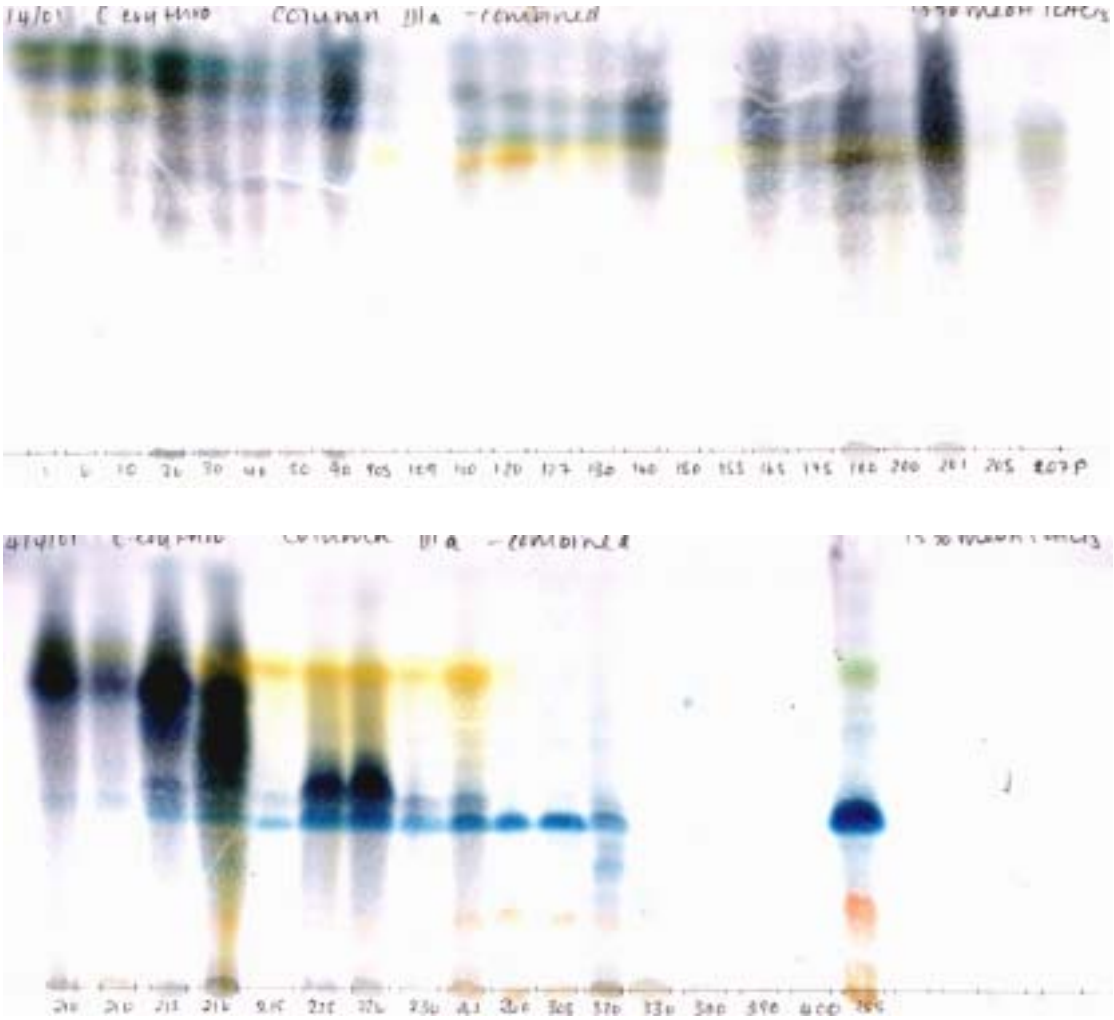


Figure 5.22: TLC of combined fractions of Column IIIa eluted with 15% methanol in chloroform.

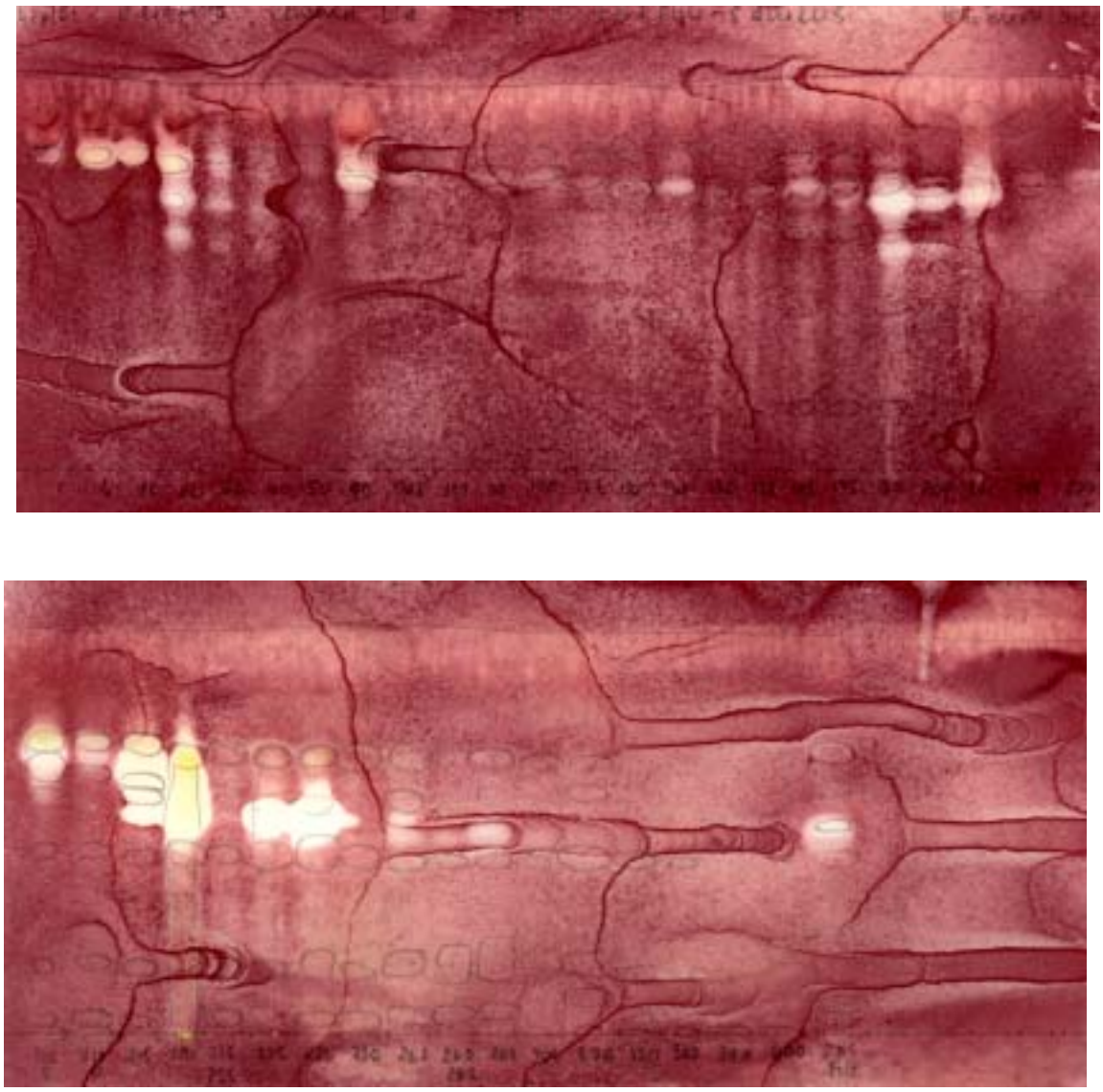


Figure 5.23: Bioautography of combined fractions of Column IIIa corresponding to those shown in Figure 5.22. Elution was with 15% methanol in chloroform.

5.3.2.5 Column IV

Fractions CE 221-253 (1.19 g) were combined and eluted on silica gel with the solvent system as described for Column I. Approximately 100 samples were collected and combined (Fig. 5.24) and bioautography performed (Fig. 5.25).

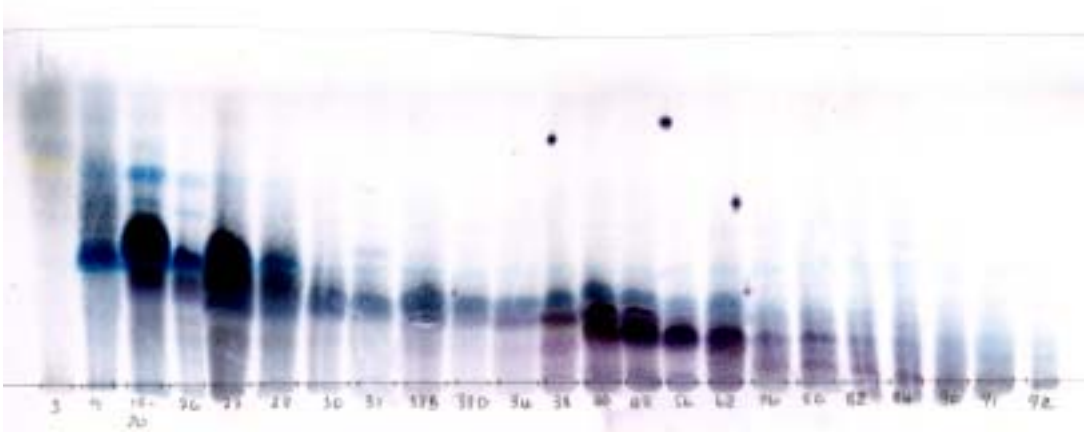


Figure 5.24: TLC of combined fractions of Column IV eluted with 15% methanol in chloroform.

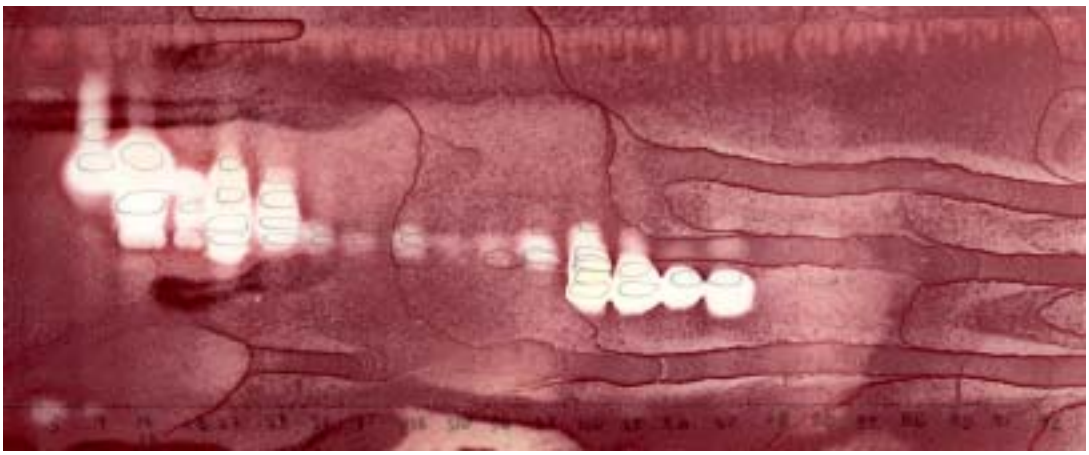


Figure 5.25: Bioautography of combined Column IV fractions corresponding to those shown in Figure 5.24.

5.3.2.6 RP-207S

Fraction CE 207 was chosen at random to determine whether reverse phase silica would elute compounds easier and faster. Since this fraction had formed a precipitate, the supernatant was separated by decantation and applied to the Bondesil C18 silica column. Elution was performed using 10% water in methanol as it shown the best separation on prior TLC analysis. A pre-column was connected prior to elution to prevent contaminants entering the column and approximately 120 fractions collected. These fractions were analysed on both normal and reverse phase TLC and certain fractions combined (Figure 5.26). Bioautography showed minimal inhibition and only those fractions that appeared to contain inhibitory compounds are shown in Figure 5.27.

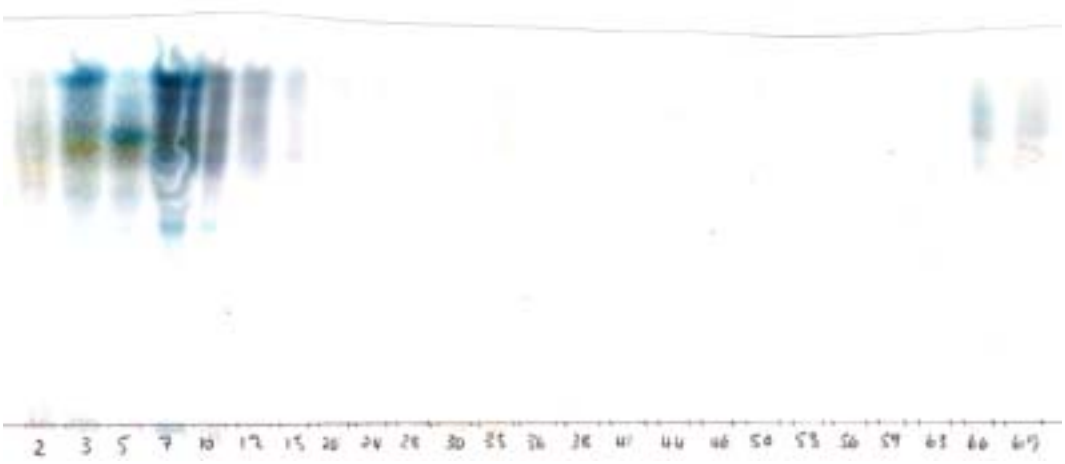


Figure 5.26: TLC of the first few fractions of the RP207S column. Compounds were eluted using 15% methanol in chloroform on normal phase TLC plates.

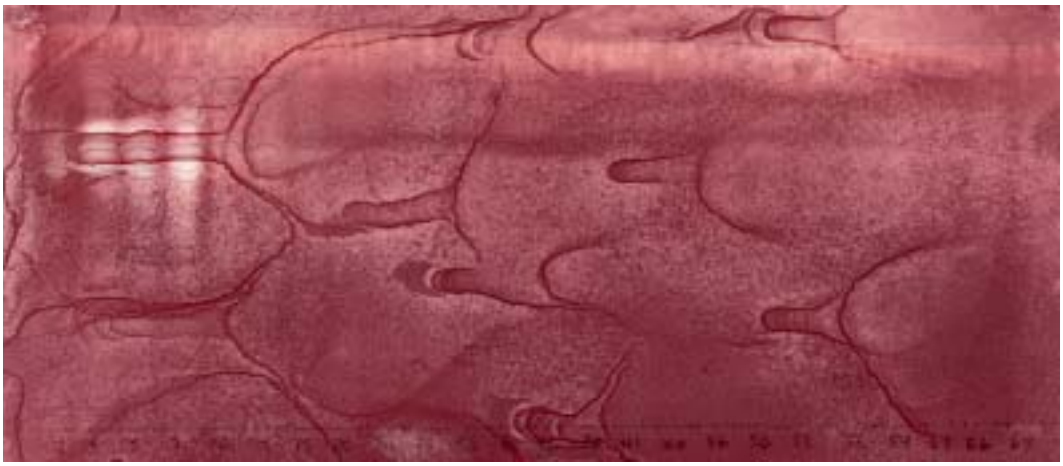


Figure 5.27: Bioautography corresponding to the samples show in Figure 5.26 elutes with 15% methanol in chloroform on normal phase TLC plates.

5.3.2.7 Column 221

Since fraction CE 221 had sufficient material to work with and exhibited activity, it was attempted to analyse this fraction by passing it through a Sephadex-LH20 column. This column did not separate compounds to any great extent (Figure 5.28) and bioautography results did not show particularly good results (Fig. 5.29)

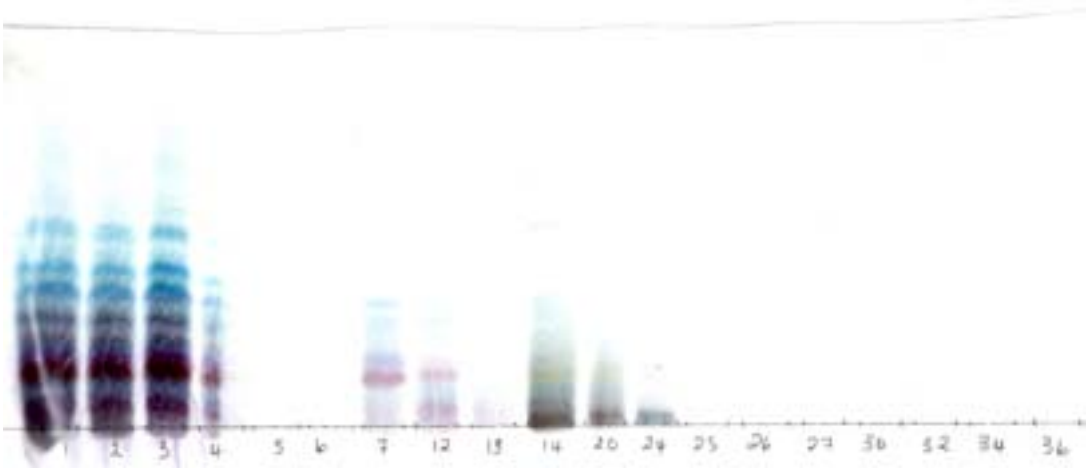


Figure 5.28: TLC of fractions collected from Column 221 and eluted with 15% methanol in chloroform.

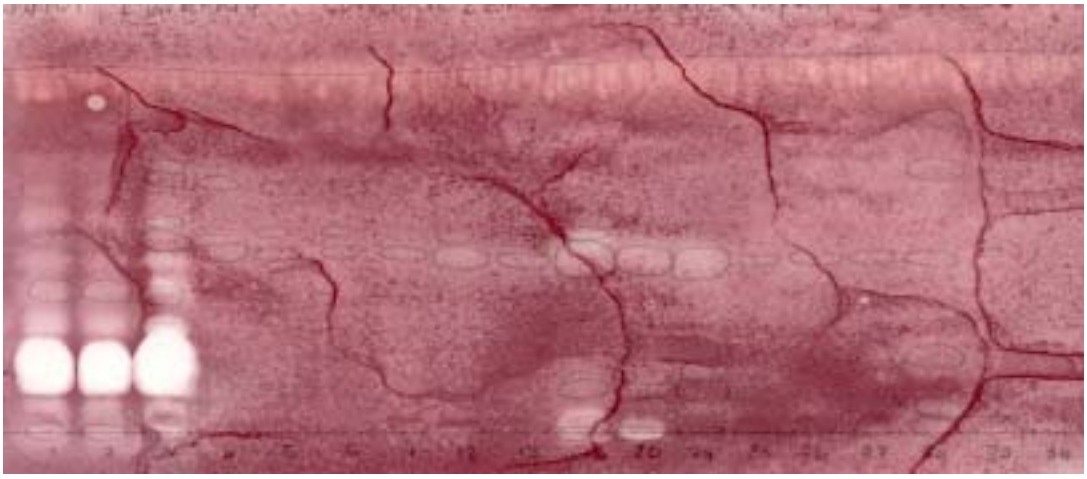


Figure 5.29: Bioautography results corresponding to fractions shown in Figure 5.28 eluted with 15% methanol in chloroform.

5.4 VARIATION IN BIOLOGICAL ACTIVITY

Comparison of the seven trees around the Pretoria area did not show significant differences in biological activity. Bioautography (Fig. 5.30) results showed strong anti-*Staphylococcus aureus* activity with Tree number 4, with zones of inhibition at different Rf values to the others. MIC values, however did not differ significantly from one another (Table 5.10).

Table 5.10: MIC values (mg/ml) and total activity of the leaves from seven *C. erythrophyllum* trees around Pretoria

	1	2	3	4	5	6	7
MIC	0.34	0.31	0.23	0.40	0.40	0.22	0.25
Total activity	129	129	130	128	126	127	128

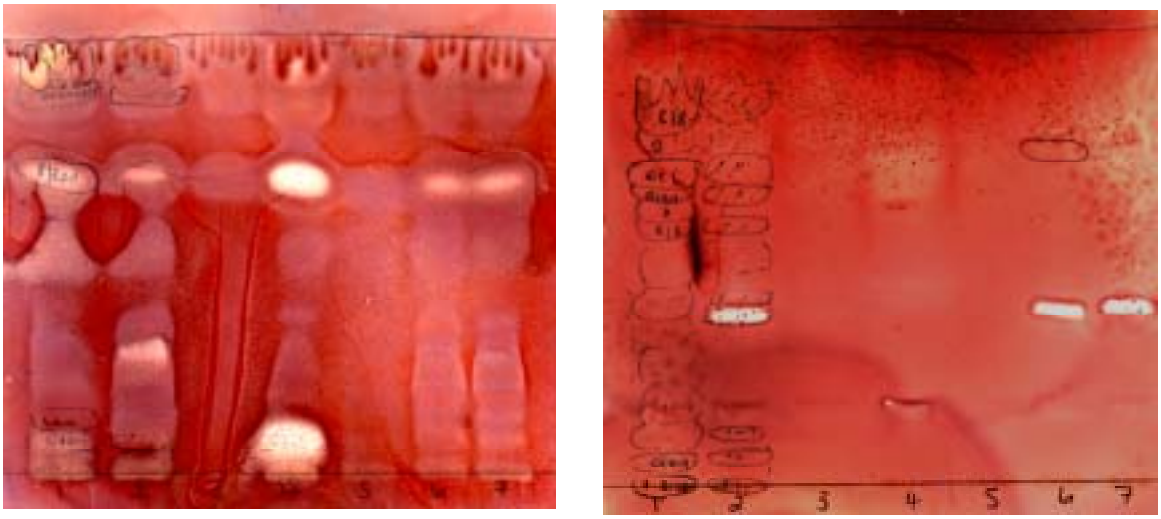


Figure 5.30: Bioautography of *C. erythrophyllum* leaves collected from 7 different plants around Pretoria, eluted with 2A:3MDC (left) and CEF (right)

CHAPTER 6

During the isolation procedure described in Chapter 5, a number of compounds had crystallised out of solution. After cleaning using various solvents these compounds were sent for spectroscopic analysis. Although some had not shown prior antibacterial activity, it was agreed to send them for analysis with the hope of discovering novel compounds.

6. STRUCTURE ELUCIDATION

The spectroscopic methods described in Chapter 4 (sections 4.9 and 4.10) were used to elucidate the structures of isolated compounds in this study. Seven compounds were isolated and identified as flavonoids, three of these being flavones and the remaining four flavonols.

The characterisation of flavonoids provides an ideal application for NMR spectra with the typical skeleton represented in Figure 6.1. The patterns of the aromatic protons reveal the nature of substitution of the various aromatic systems. The chemical shifts of the protons of rings A and B are independent of one another but are affected by ring C. Peaks arising from A occur upfield and are easily recognised, therefore examination of an unfamiliar spectrum usually starts with recognition of these peaks. Remaining peaks in the aromatic region reveal the pattern of oxygenation of ring B and confirm the nature of ring C. Comparison of the individual compounds often reveals the nature of the oxygen-borne groups [Batterham, 1963].

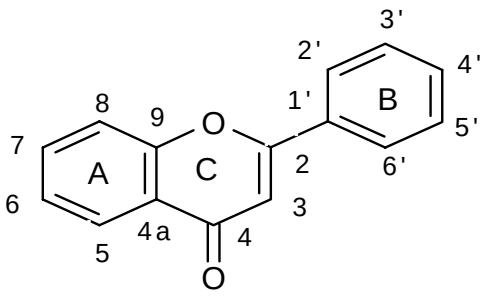
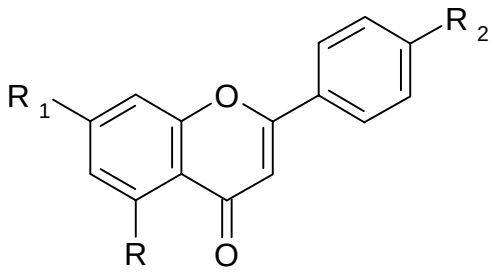


Figure 6.1: Skeleton structure of the flavonoids showing the numbering system

6.1 FLAVONES

Three flavones were isolated from *C. erythrophyllum* leaf extracts and identified as 5,7,4'-trihydroxyflavone (apigenin) (coded as CE144), 5,4'-dihydroxy-7-methoxy-flavone (genkwanin) (IIIa150) and 5-hydroxy-7,4'-dimethoxyflavone (CE36) (Fig. 6.2). These are reported in this family for the first time.



Compound	R	R_1	R_2
CE144	OH	OH	OH
IIIa150	OH	OCH_3	OH
CE36	OH	OCH_3	OCH_3

Figure 6.2: Flavones isolated from *C. erythrophyllum* leaf extracts

6.1.1 General characterization of flavones

Flavones are dehydrogenated in positions 2 and 3 (Fig. 6.2). Both the C-2 and C-3 resonances are olefinic, resonating between 157.4 – 165.8 and 102.3 – 113.7 ppm respectively. The presence of a 2,3-olefinic bond results in the quaternary behaviour of the C-2 resonance and methine behaviour of C-3 resonance. In unsubstituted flavones at C-2' and C-6' the chemical shift of the C-3 is usually between 102.0 and 108.6 ppm but when a hydroxyl or methoxyl group is attached to C-2' and/or C-6', a downfield shift occurs to between 110 and 115.5 ppm [Agrawal, 1989].

The 2,3-olefinic bond also leads to the upfield shift of the carbonyl group (C-4) that appears between 175.2-183.4 ppm, possibly due to conjugation. The presence of a hydroxyl at C-5 position causes a downfield shift of the C-4 resonance resulting in the appearance of C-4 resonance 182.5±0.7 ppm. This is as a result of intra-molecular hydrogen bond interactions between the ketone group (C-4) and C-5-attached hydroxyl. Another characteristic feature in 5-hydroxyflavones is the appearance of C-3 at 1.5±0.8 ppm upfield relative to the unsubstituted flavones at C-5 [Agrawal, 1989].

The carbon at 4a can also yield information about the substitution in ring A but is frequently obscured by larger peaks. With methoxyl substitution at C-5 and C-7, C-4a is shifted upfield from its usual position at 123 ppm by 10 and 6 ppm respectively. In simple benzene derivatives, carbons *ortho* to methoxyl are shielded by ca. 15 ppm and *para* carbons are shielded by ca. 9 ppm. Meta carbons are only slightly affected and therefore a change in the position of the methoxyl group around rings B and A will result in a sequential shielding of carbons which are in an *ortho* position to methoxyl. Methoxyl substitution in ring A at any given carbon will cause a large shift of the *ortho* carbon if the two carbons are joined by bonds of predominantly double-bond character, eg. C-5 and C-6. If, however, the bond is of single bond character, e.g. C-6 and C-7, methoxyl substitution seems to result in a small shift [Kingbury, 1974].

The splitting patterns for C-5 to C-8 are also informative as these carbons are split into either doublets or double doublets depending on whether or not the position *meta* to the carbon in question is substituted by methoxyl [Kingsbury, 1974].

The C-6 and C-8 resonances in flavones appear at between 92 - 100 ppm with C-6 downfield relative to C-8 resonance. This is important in establishing the site of C-alkylation at these sites [Agrawal, 1989].

The ^{1}H NMR spectrum shows that the presence of the double bond in ring C of flavones and flavonols causes a marked downfield shift of the 6,8 protons, producing a two-doublet pattern. In flavones, the presence of the C-ring double bond causes a shift of the 2',6'-protons and the spectrum shows the two complex multiplets, one centered at δ_H 8.0 (2',6') and the other at δ_H 7.6 (3',4',5').
With the introduction of a 4'-hydroxyl group the B ring protons appear effectively as a four-peak pattern. The hydroxyl group causes relative shielding on the adjacent 3',5'-protons and their peaks move substantially upfield. Introduction of the 2,3-double bond of flavones causes these protons to resonate at a much lower field (δ_H 8.0), while in flavonols the 3-hydroxy group causes a further slight downfield shift. In ring C the olefinic protons give rise to peaks in the general aromatic region. The singlets are easily identifiable and occur near δ = 6.8 [Batterham, 1963].

Table 6.1: ^{1}H NMR spectral data of isolated flavones from *C. erythrophyllum* leaf extracts

Position	CE144[a]	IIIa150[b]	CE36[b]
3	6.85 (s)	6.94 (s)	6.98 (s)
6	6.29 (d,2)	6.64 (d,2)	6.65 (d,2)
8	6.57 (d,2)	6.73 (d,2)	6.73 (d,2)
2'/6'	8.01 (d, 8.8)	7.97 (d, 8.8)	7.99 (m)
3'/5'	7.02 (d, 8.8)	7.28 (d, 8.8)	7.15 (m)

[a] Data obtained in DMSO-d_6 at 300MHz

[b] Data obtained in C_5D_5N at 400MHz

Coupling constant *J* in Hz is shown in parentheses

Table 6.2: ^{13}C NMR spectral data of isolated flavones from *C. erythrophyllum* leaf extracts

Position	CE144	IIIa150	CE36
2	164.5	164.4	-
3	103.2	103.4	104.8
4	182.1	182.3	-
4a	103.2	105.0	-
5	161.5	157.6	-
6	99.2	98.3	98.4
7	164.5	165.5	-
8	94.3	93.0	93.0
9	157.7	161.6	-
1'	121.5	121.4	-
2'/6'	128.8	128.9	128.4
3'/5'	116.3	116.3	114.9
4'	161.8	161.6	-

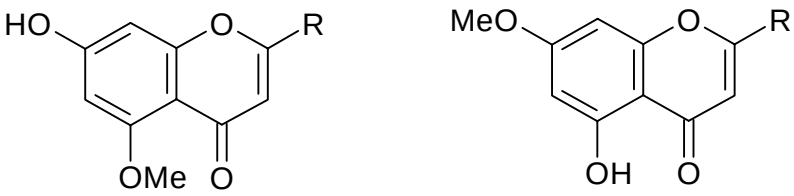
All data obtained in DMSO-d_6 except CE36 in $CDCl_3$

6.1.1.1 Characterisation of IIIa150

Compound IIIa150 was isolated as a yellow powder from column IIIa of the chloroform fraction. The Rf values were determined using three solvent systems and were calculated as follows: 0.34 (CEF), 0.70 (BEA) and 0.93 (2A:3MDC).

The ^{1}H NMR spectrum showed signals between 3.8 and 7.9 ppm indicating the presence of a methoxyl group δ_H 3.8, *meta*-coupling protons at δ_H 6.64 and δ_H 6.73 and aromatic protons at δ_H 6.94, 7.97 and 7.28. The *meta*-coupled protons (s, J = 2 Hz) are typical of the H-6 and H-8 in the A-ring of flavonoids. The singlet at δ_H 6.94 is the proton at H-3 while the signals at δ_H 7.97 and δ_H 7.28 (d, J = 8.8 Hz) are due to the *ortho*-coupling of protons 2'/6' and 3'/5' respectively [Spectrum 6.1]. ^{13}C NMR corresponded to the values obtained in Agrawal (1989) [Spectrum 6.2].

Two possible structures were suggested : 8-hydroxy-6-methoxyflavone (I) and 6-hydroxy-8-methoxyflavone (II). Agrawal [1989] suggests that C-4 resonates at a position that is dependant on the electron movements at C-5. Therefore in 5-hydroxylated flavones, C-4 is deshielded at δ_C 182.5±0.7, which correlates with ^{13}C NMR data (Table 6.2) and therefore Compound IIIa150 is likely to be II.



I II

Figure 6.3: Two possible substitution patterns in IIIa150

A Long Range COrrelation SpectroscopY (COSY-LR) experiment was also performed to provide evidence for the positioning of the methoxyl group on the A-ring of the flavone. Cross-peaks were visible between the methoxyl group and H-6 and H-8 confirming the positioning of the methoxyl group at C-7 [Spectrum 6.3].

Confirmation of structure was done by HREIMS (Spectrum 6.4), which showed the base peak and molecular ion M^+ to be at *m/z* 284 corresponding to $C_{16}H_{12}O_5$. Other prominent peaks were seen at *m/z* 255 $[M\text{-}CO]^+$ (26%), *m/z* 241 $[M\text{-}C_2H_3O]^+$ (10%), *m/z* 166 $[M\text{-}C_8H_6O]^+$ (8%) and *m/z* 118 $[M\text{-}C_8H_6O_4]^+$ (5.5%). The fragmentation is typical of the flavones and is illustrated in Fig. 6.4.

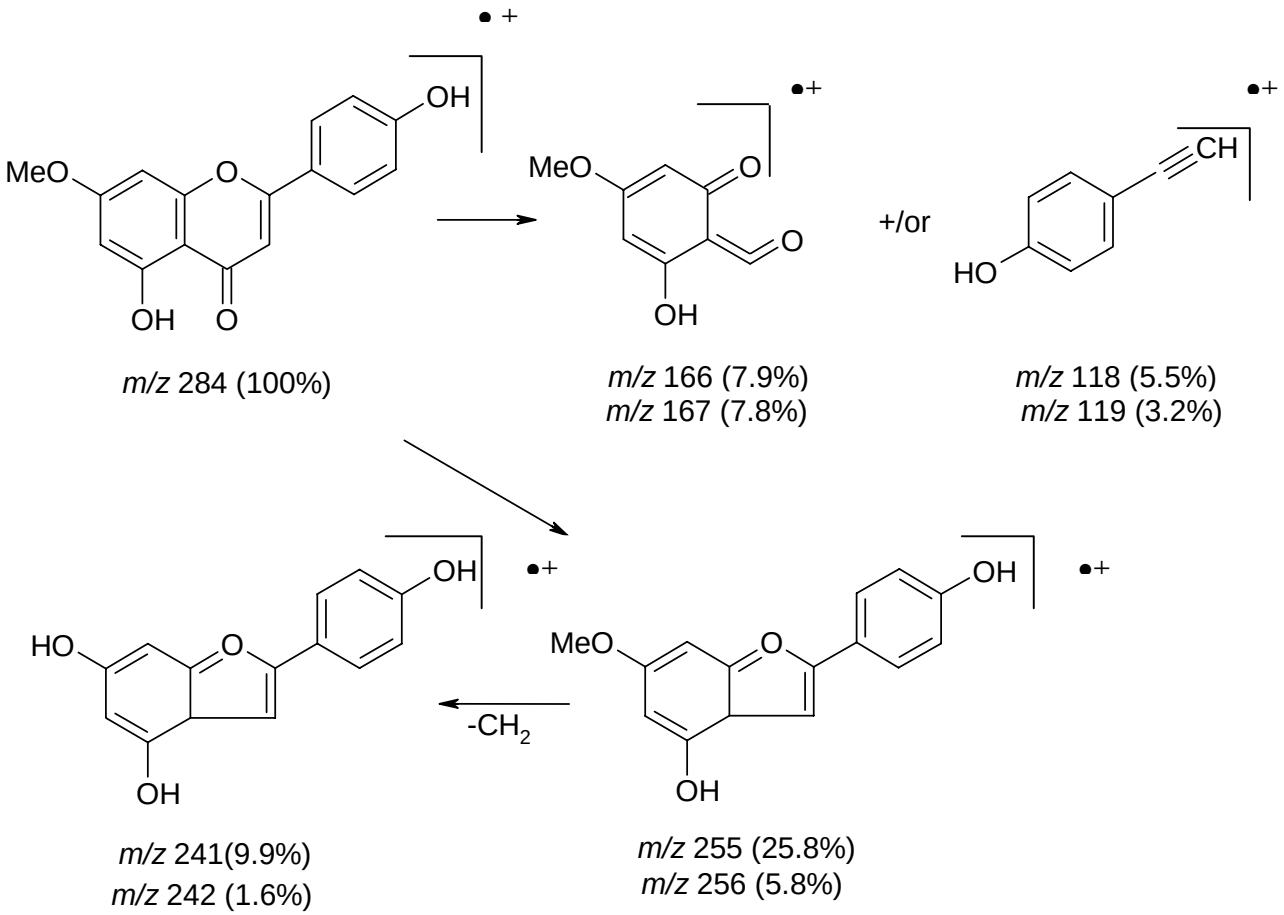


Figure 6.4: Suggested fragmentation pattern of IIIa150, typical of the flavones

IIIa150 was identified as genkwanin (5,4'-dihydroxy-7-methoxyflavone), which has previously been isolated from *Artemisia, Eupatorium, Alnus, Betula, Ostrya, Beyeria Rosmarinus, Salvia, Populus, Daphne, Larrea, Cheilanthes, Notholaena* species and *Pityrogramma tartarea* to name a few [Wollenweber, 1980]. This appears to be the first report of its isolation in Combretaceae.

6.1.1.2 Characterisation of CE144

CE144 was crystallised from fraction 144 and 145 of the first silica gel column using the chloroform fraction.

^{13}C NMR data revealed identical values for the C-atoms in the B and C-rings compared to that of IIIa150 [Spectrum 6.6]. Since hydroxylation in positions C-5 and C-7 corresponds to δ_C 99.2 and 94.4 of C-6 and C-8 respectively [Agrawal, 1989], it was concluded that this compound differed from IIIa150 only in the C-7-attached group. In comparison to hydroxy-substitution, methoxyl substitution at C-7 causes a downfield shift of the carbons in the *ortho*-position (C-6 and C-8) and an upfield shift of the C-4a carbon which lies in the *para*-position. ^{1}H NMR [Spectrum 6.5] was not compared to that of IIIa150 as different solvents were used but appeared similar to IIIa150 except for absence of a methoxyl group.

HREIMS gave the molecular ion M^+ at *m/z* 270, which corresponds to $C_{15}H_{10}O_5$ as the molecular formula. Other prominent fragments were *m/z* 242 $[M\text{-}CO]^+$, *m/z* 152 $[M\text{-}C_8H_6O]^+$ (13%), *m/z* 138 $[M\text{-}C_8H_4O_2]^+$ (4%) and *m/z* 119 $[M\text{-}C_7H_3O_4]^+$ (5%) [Spectrum 6.7]. The fragmentation pattern is typical of flavones as illustrated earlier (Figure 6.4).

By comparison of the ^{13}C NMR data with that of the literature, CE144 was identified as 5,7,4'-trihydroxyflavone, commonly known as apigenin [Agrawal, 1989].

Apigenin is a very commonly occuring flavone and has been isolated in many plant species including *Barleria christata, Chrysanthemium cinereriaefolium, Alnus* spp., *Betula* spp., *Garcinia multiflora, Coleus amboinicus,Salvia glutinosa, Acacia ixiophylla, Crudia amazonia, Prosopis* spp., *Elaegia utilis, Populus* spp., *Antirhinum* spp., *Larrea* spp., *Cheilanthes* spp. and *Notholaena* spp [Wollenweber, 1980]. In *Combretum* spp. it has been recently isolated in *C. apiculatum* [Katerere, unpublished 2001].

6.1.1.3 Characterisation of CE36

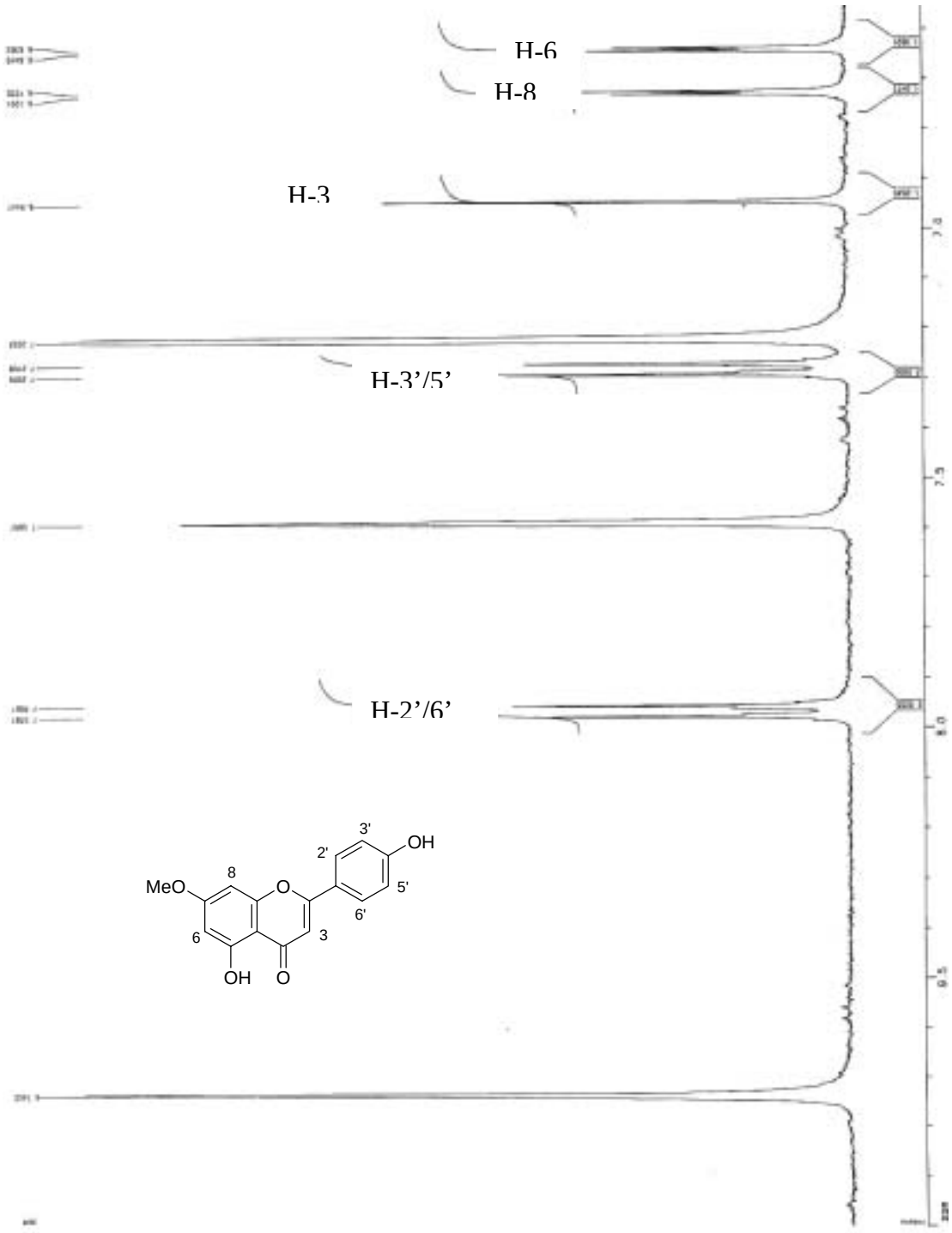
CE36 was one of the first compounds to come off column I of the chloroform fraction. It formed yellow needle-like crystals in solution and exhibited the following Rf values in the three solvent systems: 0.70 (CEF), 0.78 (BEA) and 0.95 (2A:3MDC).

^{1}H NMR revealed resonances at δ_H 3.78 (s, 3H) and δ_H 3.81 (s, 3H), representing 2 methoxyl groups. H-6 (δ_H 6.65) and H-8 (δ_H 6.73) were superimposable on spectra obtained for IIIa150, signifying a possible methoxyl-substitution at H-7 [Spectrum 6.8]. ^{13}C NMR verified the C-7 methoxyl substitution since C-6 and C-8 were identical to spectra obtained with IIIa150 (i.e. δ_C 98.3 and δ_C 93.0 respectively) [Spectrum 6.9]. These values also suggested the hydroxyl group to be at position C-5. Values obtained for C-3' and C-5' were further upfield (δ_C 114.9) than that obtained with a hydroxyl group (δ_C 116.3) and it was therefore suggested that the methoxyl groups were at positions C-7 and C-4'.

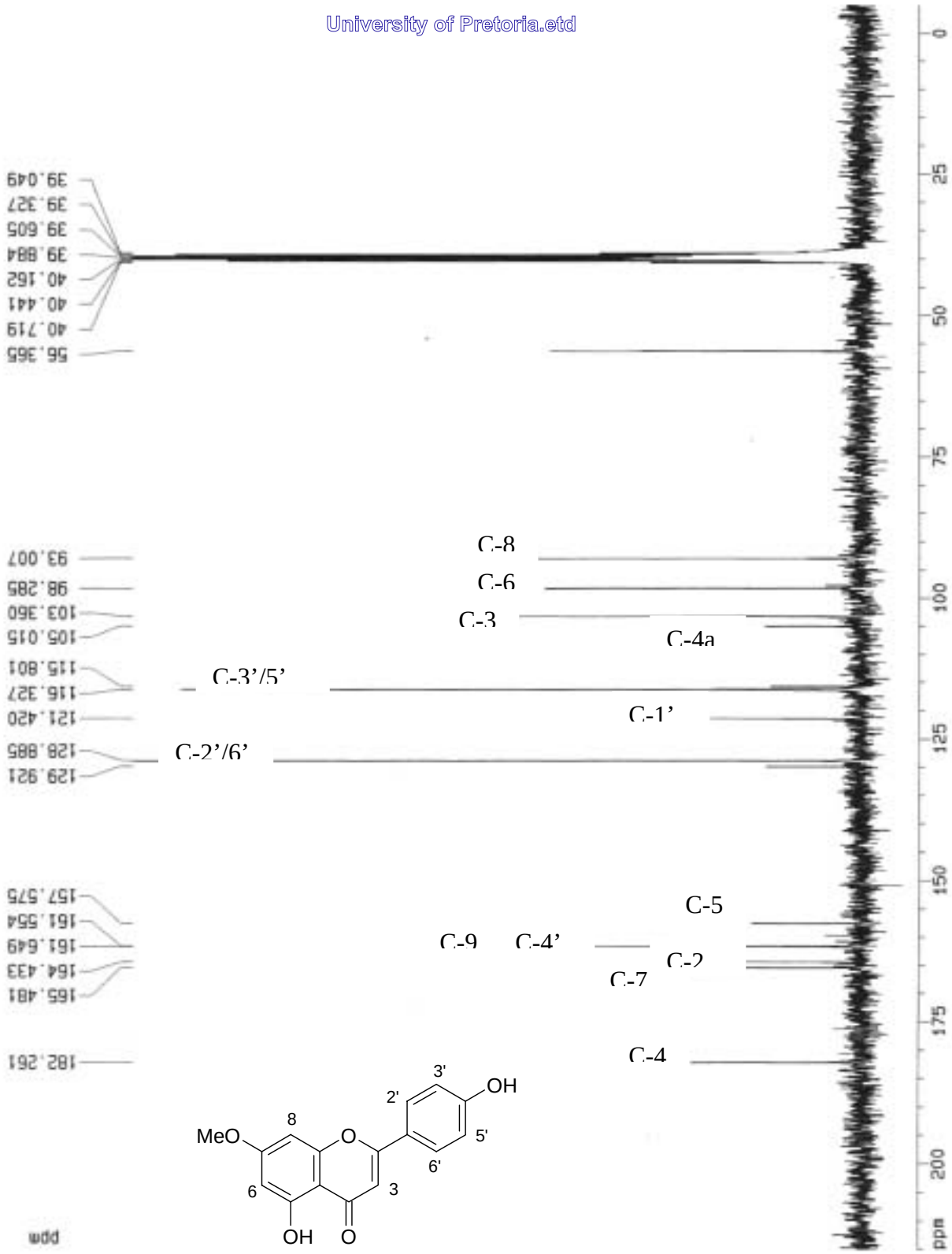
COSY-LR was performed to verify the structure [Spectrum 6.10]. Cross-peaks were visible between H-6 (δ_H 6.65) and H8 (δ_H 6.73) and the methoxyl group confirming the presence of the methoxyl group on C-7. Another cross-peak was also seen between the protons attached to C-3' and C-5' and the methoxyl group (C-5), which further confirms the positioning of the methoxyl group on the B-ring at position C-4'.

HREIMS data gave a molecular formula of $C_{17}H_{14}O_5$ (*m/z* 298) with other prominent peaks at *m/z* 297 $[M-H]^+$ (29%), *m/z* 281 $[M-OH]^+$ (23%), *m/z* 256 $[M-C_2H_2)\}^+$ (17%), *m/z* 219 $[M-C_2H_7)_3]^+$(33%), *m/z* 167 $[M-C_9H_7)]^+$ (10%) and *m/z* 193 $[M-C_7H_50]^+$ (34%) [Spectrum 6.11]. The fragmentation is typical of flavones (Figure 6.4). CE36 was identified as 5-hydroxy-7,4'-dimethoxyflavone.

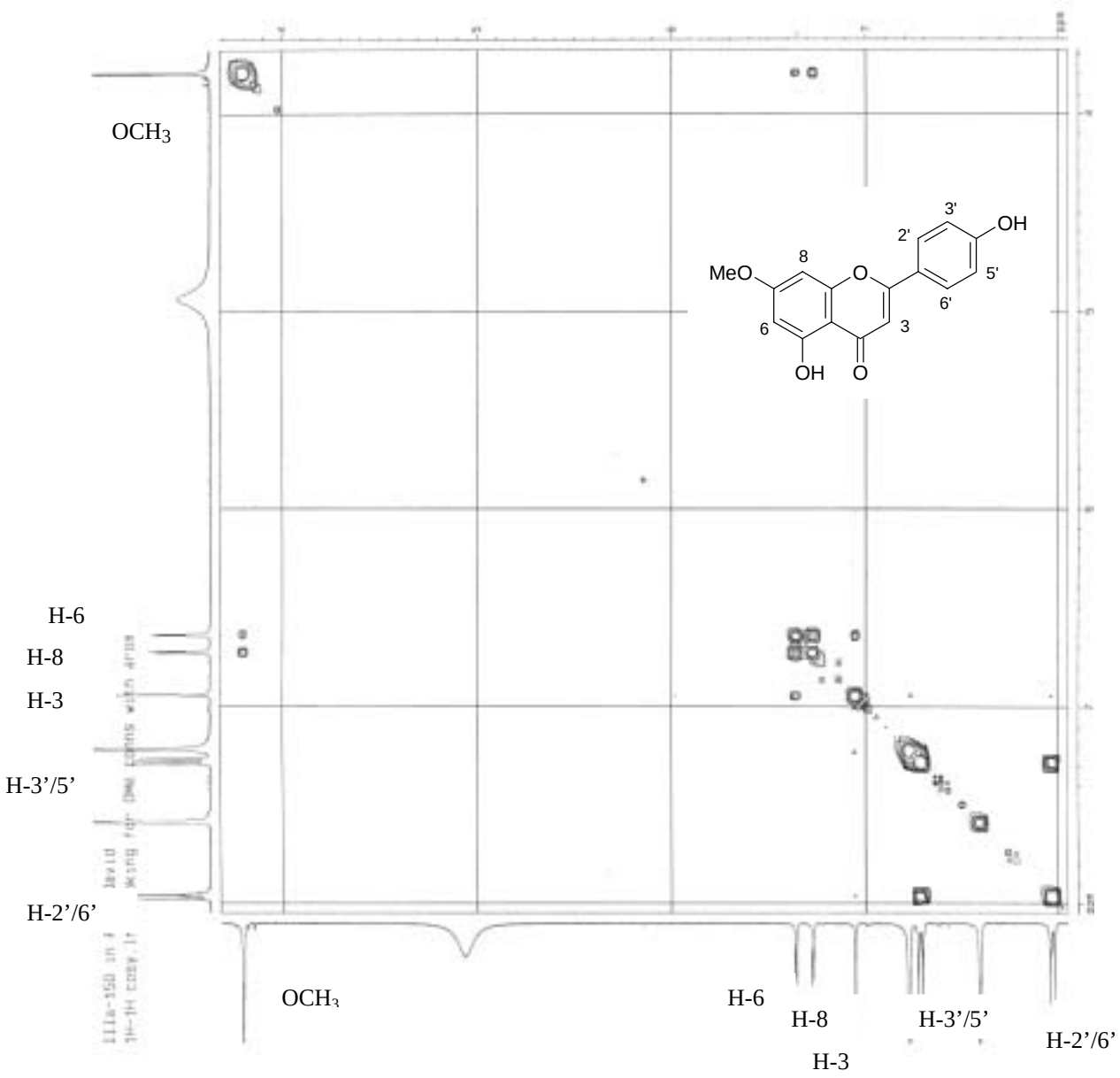
This compound has been found in *Biota orientalis* [Yang, 1995] and in the leaves of *Rosmarinus officinalis* [Brieskorn, 1967]. No known reports in Combretaceae have been documented. This is the first report of its isolation from *C. erythrophyllum* and family Combretaceae.



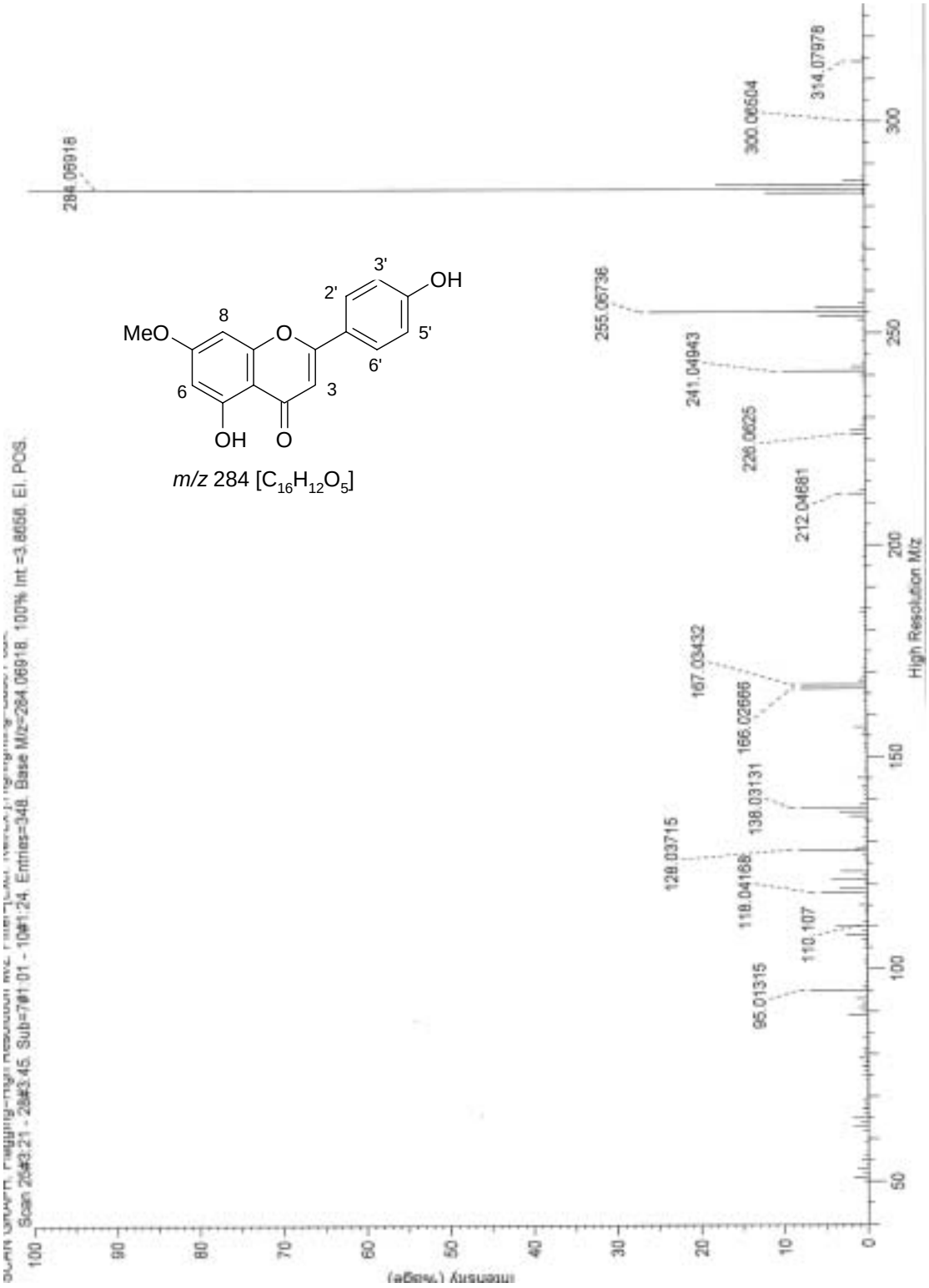
Spectrum 6.1: ^{1}H NMR (400 MHz, C_5D_5N) of IIIa150



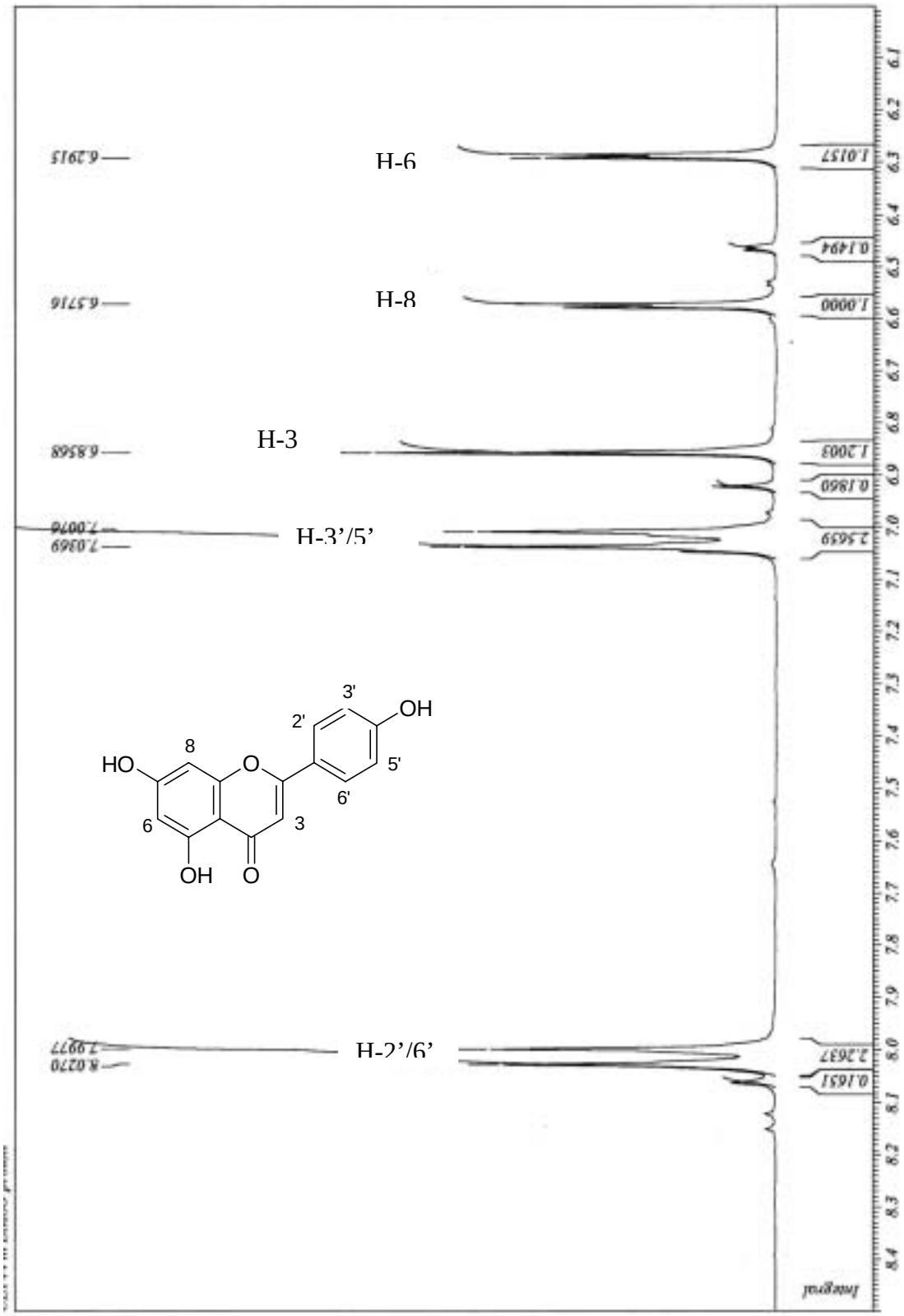
Spectrum 6.2: ^{13}C NMR (75 MHz, DMSO-d_6) of IIIa150



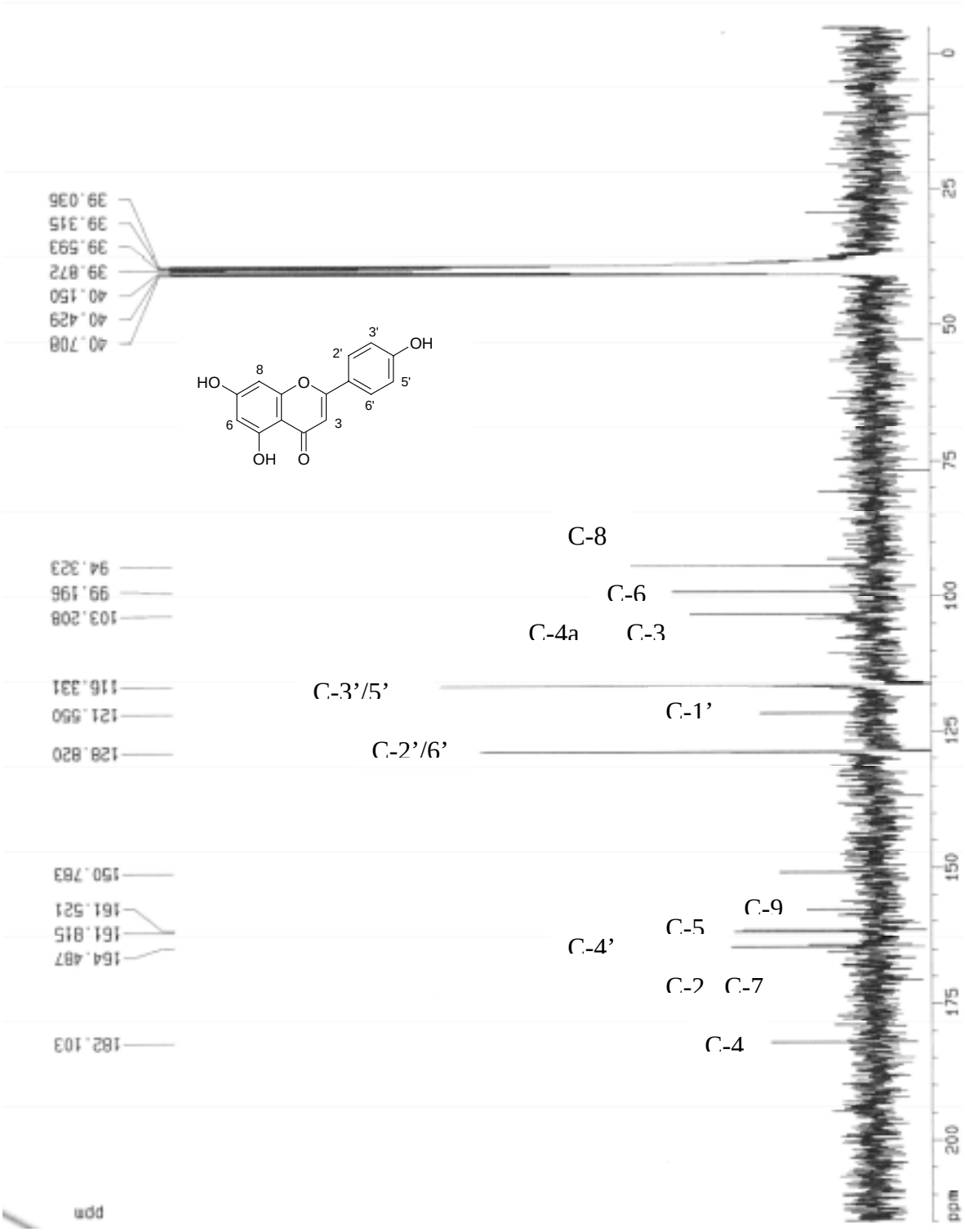
Spectrum 6.3: ^{1}H-^{1}H COSY-LR (400 MHz, C_5D_5N) of IIIa150



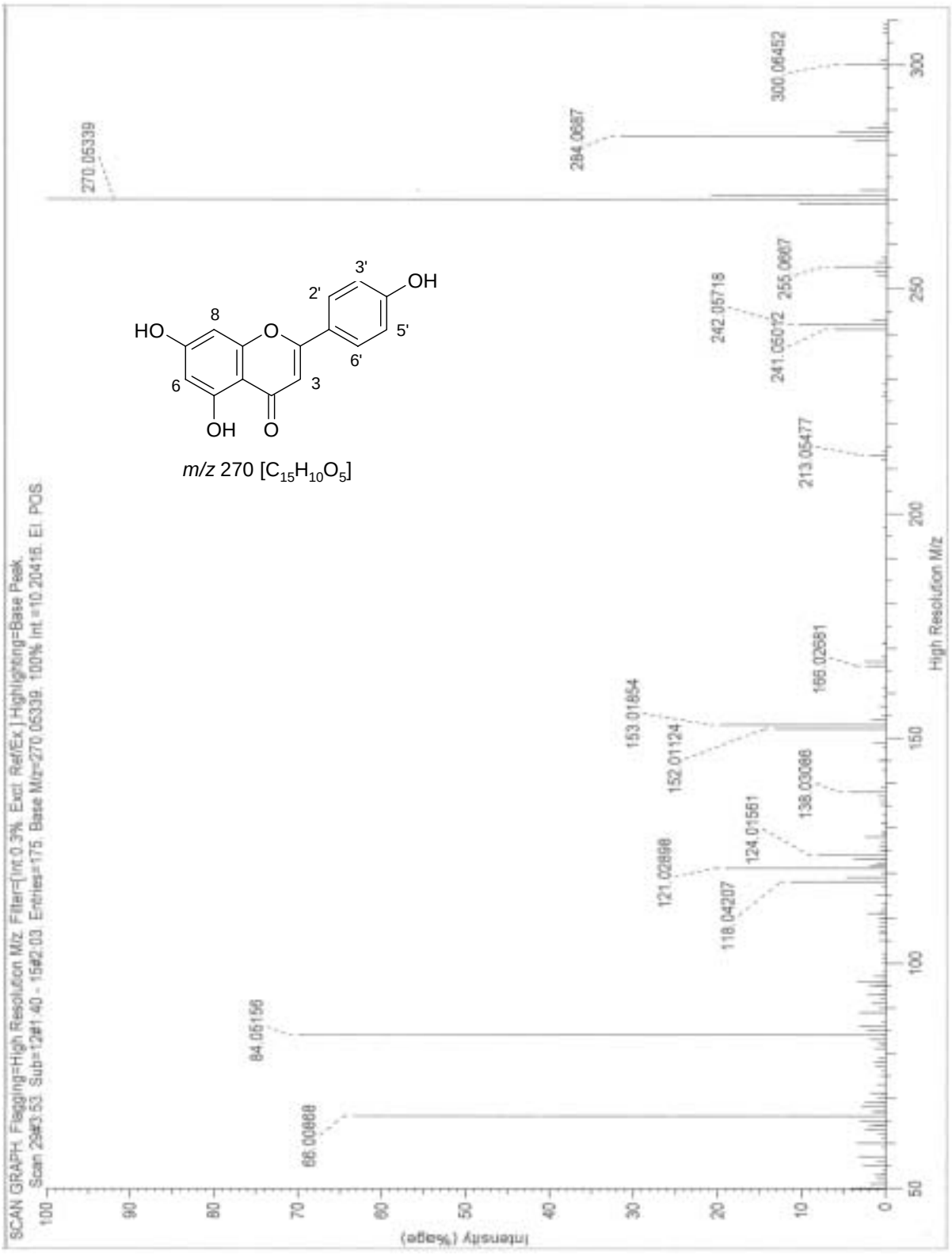
Spectrum 6.4: HREIMS of IIIa150



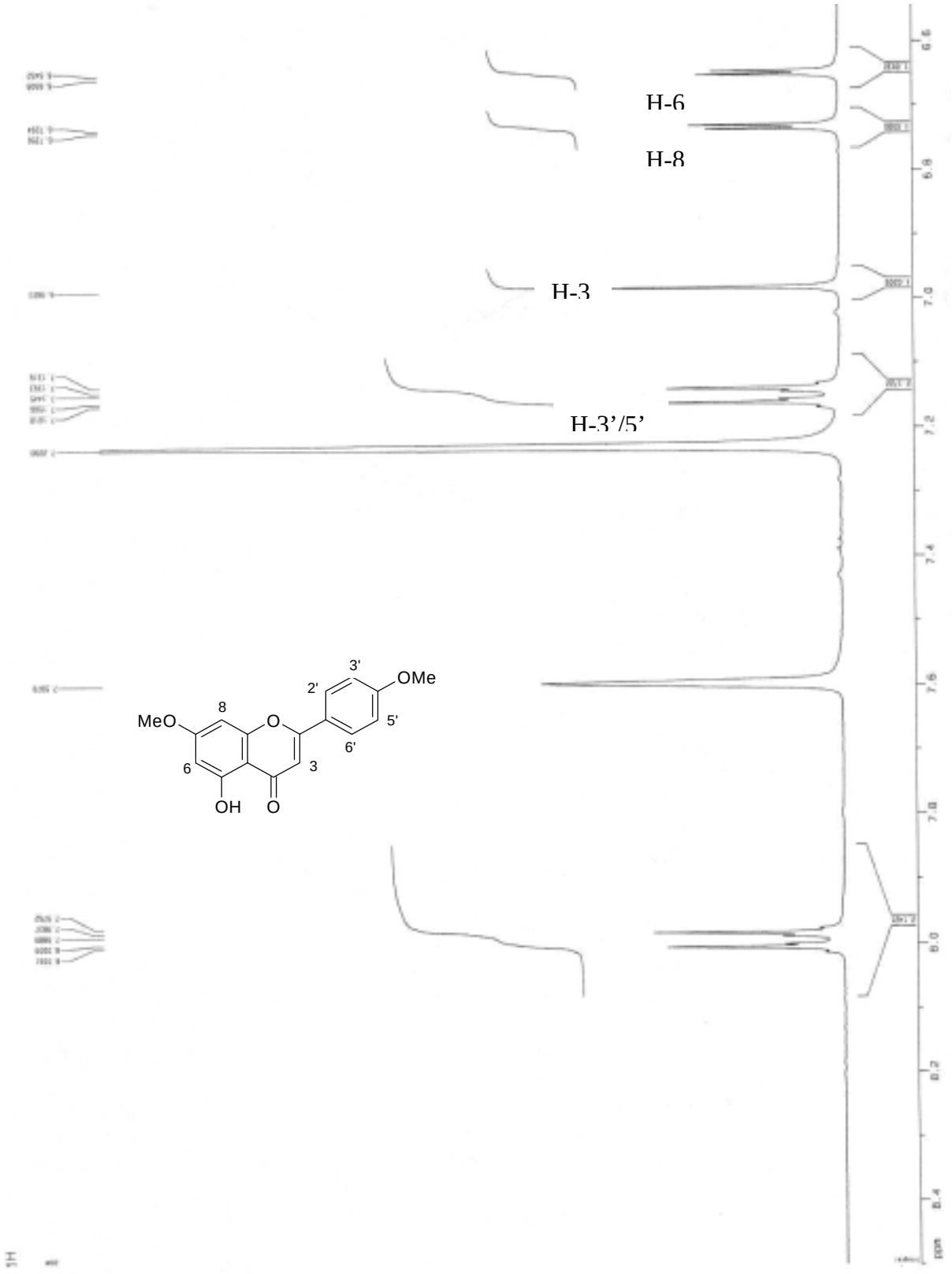
Spectrum 6.5: ^{1}H NMR (300 MHz, DMSO-d_6) of CE144



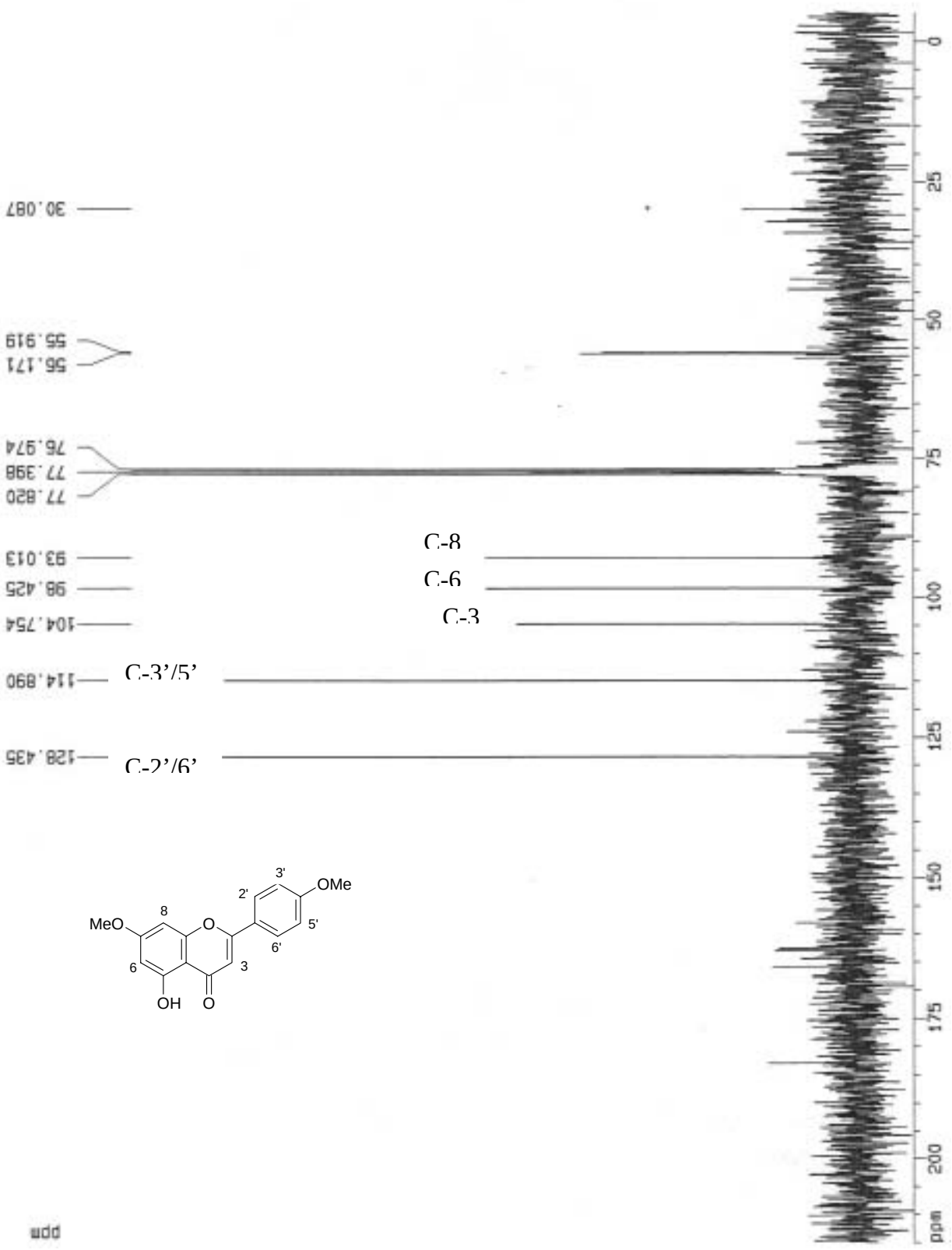
Spectrum 6.6: ^{13}C NMR (75 MHz, DMSO-d_6) of CE144



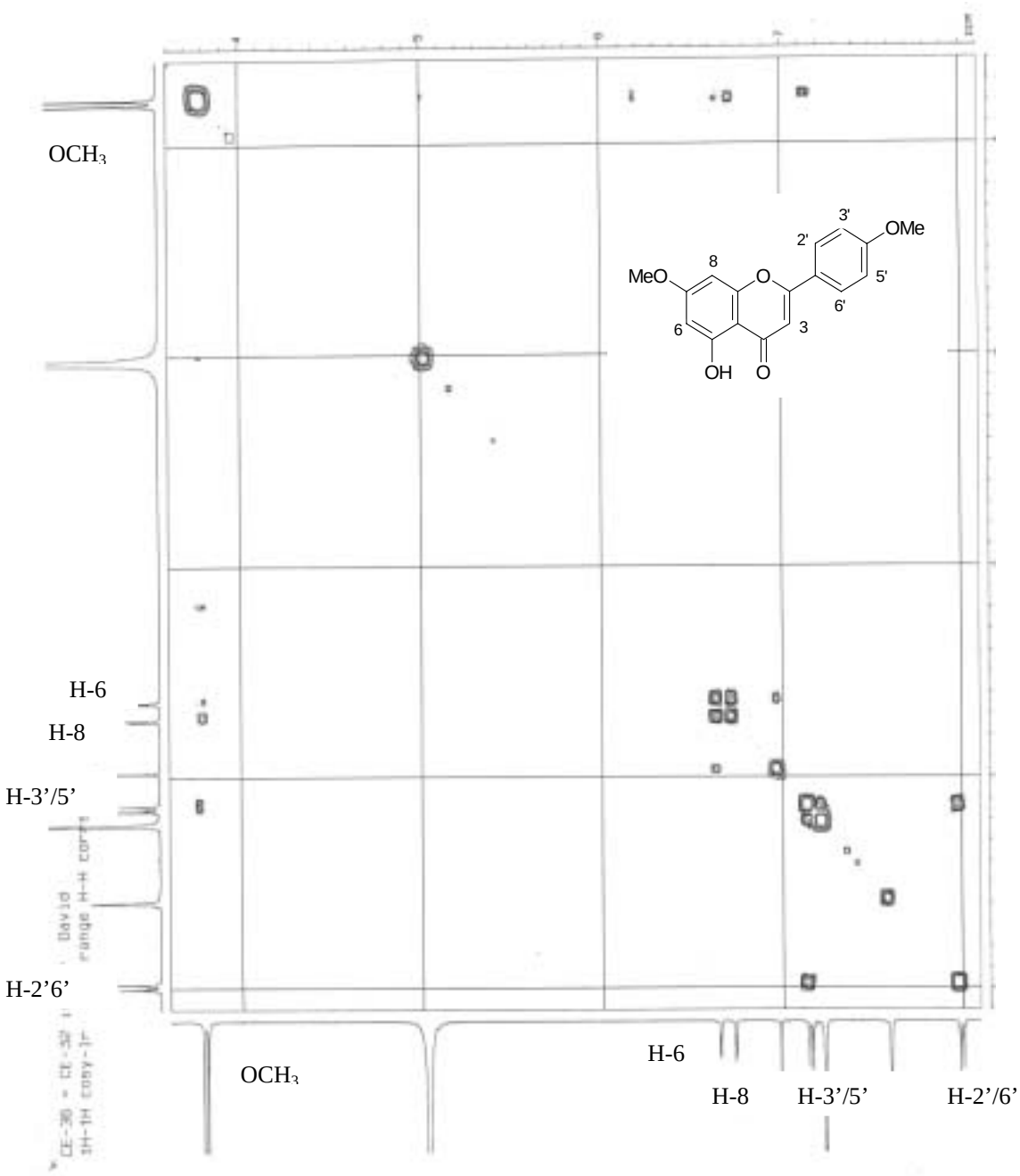
Spectrum 6.7: HREIMS of CE144



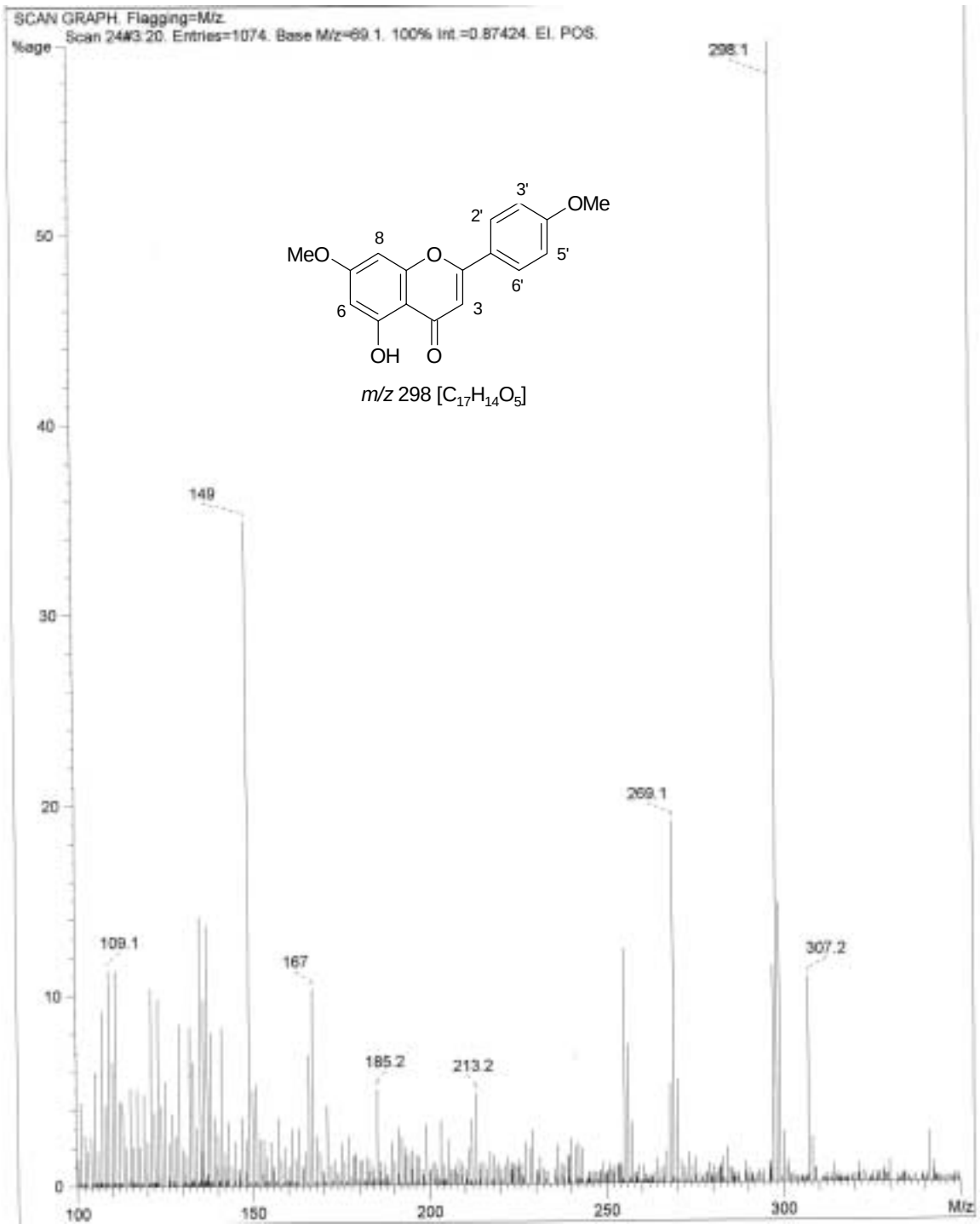
Spectrum 6.8: ^{1}H NMR (400 MHz, C_5D_5N) of CE36



Spectrum 6.9: ^{13}C NMR (75 MHz, $CDCl_3$) of CE36



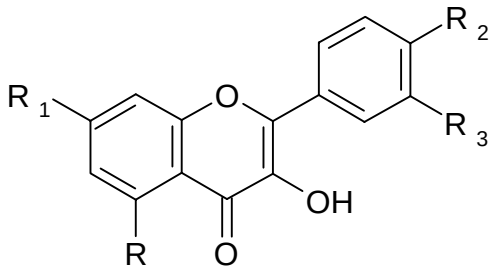
Spectrum 6.10: ^{1}H-^{1}H COSY LR (400MHz, C_5D_5N) of CE36



Spectrum 6.11: HREIMS of CE36

6.2 FLAVONOLS

Four flavonols were isolated from the *C. erythrophyllum* leaf extracts and identified as 5,6,4'-trihydroxyflavonol (kaempferol) (coded Seph 51), 5,4'-dihydroxy-7-methoxyflavonol (rhamnocitrin) (IIIa90), 5,4'-dihydroxy-7,5'-dimethoxyflavonol (rhamnazin) (CE51) and 7,4'-dihydroxy-5,5'-dimethoxyflavonol (quercetin-5,3'-dimethylether) (CE46). These are reported in Combretaceae for the first time.



Compound	R	R_1	R_2	R_3
IIIa90	OH	OCH_3	OH	H
Seph51	OH	OH	OH	H
CE46	OCH_3	OH	OH	OCH_3
CE51	OH	OCH_3	OH	OCH_3

Figure 6.5: Flavonols isolated from *C. erythrophyllum* leaf extracts

6.2.1 General characterisation of flavonols

Flavonols are 3-hydroxy derivatives of flavones. The chemical shifts of C-2 and C-3 are dramatically affected by the introduction of the 3-OH substituent on the C-ring of the flavone skeleton. The C-3 shifts downfield by ca. 32 ppm and C-2 and C-4 move upfield by ca. 17 and 5 ppm respectively. C-2 usually resonates at 5.0 – 13.0 ppm lower than C-3 resonance. In this case C-2 and C-3 are oxygenated quaternary carbons resonating around δ_C 133.5 and 151.2 respectively. Generally the resonance appearing at 140.0 – 151.2 ppm corresponds to C-2 and 133.5 – 140.0 ppm to C-3 [Agrawal, 1989].

In 5,7-dihydroxylated flavonols, C-6 resonates at about 5 ppm lower field relative to C-8 [Agrawal, 1989]. The presence of the double bond in ring C of the flavonols, as in the flavones, causes a marked shift of H-6 and H-8 in ^{1}H NMR, producing a two-doublet pattern. Introduction of a 3-hydroxy group causes a further downfield shift of the 2'/6'-protons and 3'/5'-protons than the flavones [Batterman, 1963].

Table 6.6: ^{1}H NMR (300MHz) and ^{13}C NMR (75MHz) spectral data of isolated flavonols

C	^{13}C	^{1}H	C	^{13}C	^{1}H
CE46[a]			**Seph 51[b]**		
6	98.8	6.63 (d,2)	6	99.6	6.31 (d,2)
8	93.2	6.80 (d,2)	8	94.9	6.57 (d,2)
2’		8.27(dd,2,8.4)	2’	130.7	8.19 (m,8.8)
3’	116.5	7.43 (d,8.4)	3’	116.8	7.06(m, 8.8)
5’	116.5	-	5’	116.8	7.06(m,8.8)
6’		8.33 (d,2)	6’	130.7	8.19(m,8.8)
CE51[a]			**IIIa90[a]**		
6	NOT	6.63 (d,2)	6	98.8	6.63 (d,2)
8	DONE	6.80 (d,2)	8	93.1	6.73 (d,2)
2’		8.27 (dd	2’	130.8	8.57 (m)
3’		7.43 (d,8.4)	3’	116.8	7.37 (m)
5’		-	5’	116.8	7.37 (m)
6’		8.32 (d,2)	6’	130.8	8.57 (m)

[a] Data obtained in C_5D_5N

[b] Data obtained in Acetone-d_6

Coupling constant *J* is shown in parentheses

6.2.1.1 Characterisation of CE51

Analysis of the ^{1}H-NMR spectra of CE51 showed the presence of two methoxyl groups δ_H 3.78 and δ_H 3.91[Spectrum 6.12]. The resonances of two *meta*-coupled doublets at δ_H 6.63 and δ_H 6.80 (1H, d, *J*=2 Hz), are typical of the 6- and 8-protons of the A-ring. The doublet at δ_H 7.43 1H, d, *J*=8.4) was attributed to H-3' which is *ortho*-coupled to H-2' at δ_H 8.27 (1H, dd, *J*=8.4). It is also further split by *meta*-coupling to the proton at δ_H 8.32.

To confirm the positioning of the methoxyl groups, a COSY-LR was done and this showed cross-peaks between H-6 and H-8 with a methoxyl group (δH 3.78), placing the methoxyl at position H-7. Another cross-peak was seen between H-6' and the methoxyl group (δH 3.9) placing it at position H-5'. From these results it was concluded that the methoxyl groups were situated at H-7 and H-5' and the hydroxyl groups at H-5 and H-4' [Spectrum 6.14].

This suggested structure was confirmed by HREIMS which exhibited a molecular peak at *m/z* 330 $[M]^+$ (100%) and fragmentation peaks at *m/z* 329 $[M-H]^+$ (20%), *m/z* 298 $[M-O_2]^+$ (11.6%), *m/z* 287 $[M-C_2H_30]^+$ (9%); *m/z* 167 $[M-C_9H_7O_3]^+$ (12.5%) and *m/z* 149 $[M-C_9H_9O_4]^+$(39.8%) [Spectrum 6.15]. This fragmentation pattern is closely related to that of flavones, illustrated in Figure 6.6.

CE51 was identified as 5,4'-dihydroxy-7,5'-dimethoxyflavonol, commonly called rhamnazin and is found in *Artemisia pygmaea, Alnus* spp., *Betula* spp., *Aesculus* 4 spp., *Polygonum hydropiper, Rhamnus* spp., *Populus* spp., *Larrea* spp., *Cheilanthes* spp and *Notholaena* spp. to name a few [Wollenweber, 1980]. It has not previously been reported in Combretaceae.

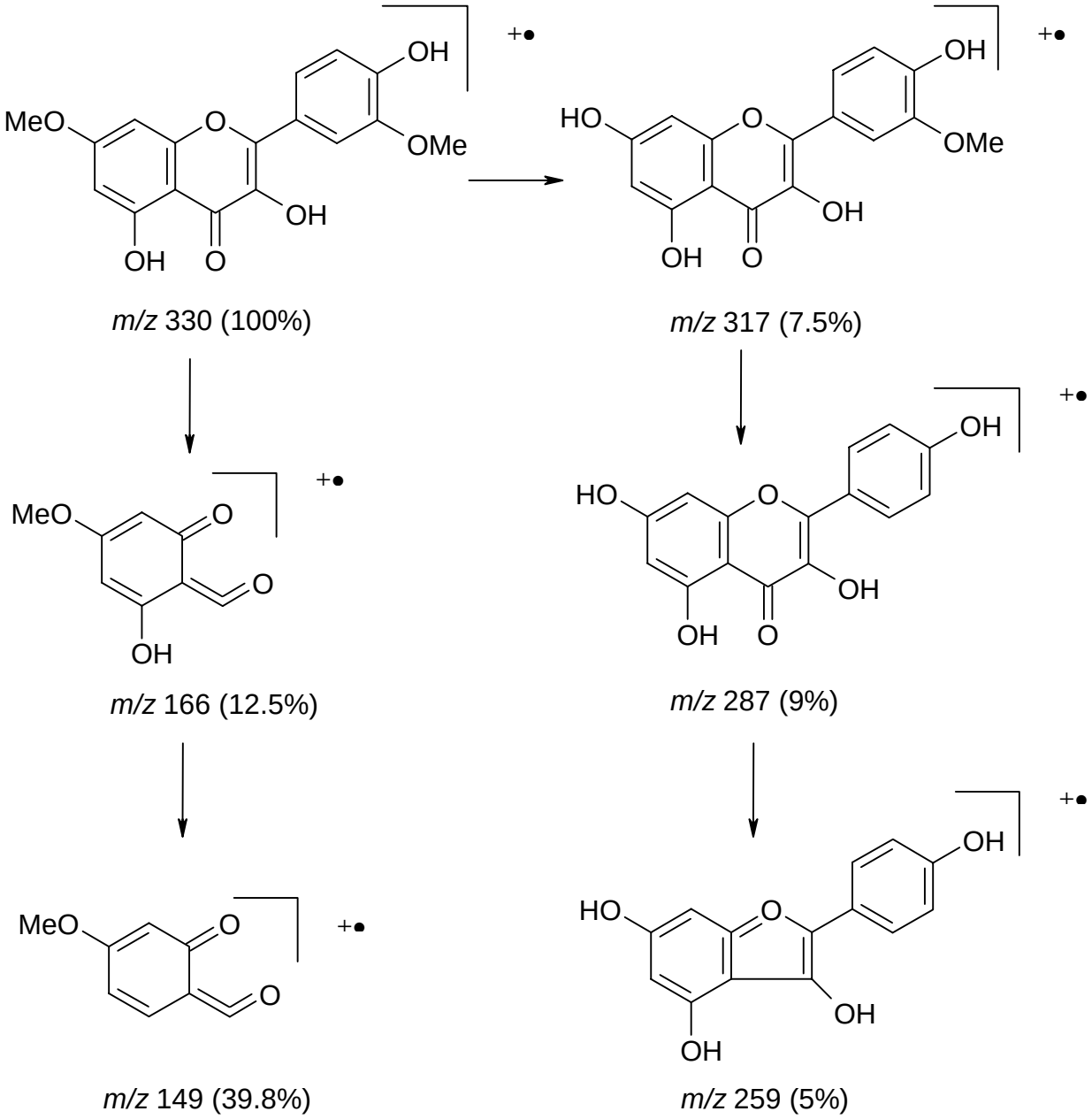


Figure 6.6: Suggested fragmentation pattern for CE51, which is typical of the flavonols

6.2.1.2 Characterisation of CE46

This compound was isolated as a yellow amorphous powder from Column I and exhibited Rf values of 0.38 with CEF, 0.74 with BEA and 0.93 with 2A:3MDC.
The ^{1}H-NMR spectra of CE46 matched that of CE51 showing the presence of two methoxyl groups δ_H 3.78 and δ_H 3.91 and the two *meta*-coupled protons at δ_H 6.63 and δ_H 6.80 (1H, d, *J*=2 Hz) characteristic of H-6 and H-8. The doublet at δ_H 7.43 (*J*=8.4) was attributed to H-3' while the double-doublets at δH 8.27 and doublet δH 8.32 were attributed to the *meta*-coupled H-2' and H-6' [Spectrum 6.8].

Initially it was thought that the A-ring methoxyl group was at C-5 and therefore a COSY-LR was done [Spectrum 6.14]. This showed cross-peaks between H-6 and H-8 with stronger absorption at H-8 placing the methoxyl at position H-7. Another cross-peak was seen between H-6' and the methoxyl group (δH 3.9) placing it at position H-5'. From these results it was concluded that the methoxyl groups were situated at H-7 and H-5' and the hydroxyl groups at H-5 and H-4' making this compound possibly identical to CE51. HMBQ data would be able to deliver more accurate results but was unfortunately not available in time for inclusion.

6.2.1.3 Characterisation of IIIa90

IIIa90 was isolated from column IIIa as a yellow powder and exhibited the following Rf values in the three solvent systems: 0.28 (CEF), 0.75 (BEA) and 0.94 (2A:3MDC).

^{1}H NMR spectra showed the presence of one methoxyl group at δ_H 3.81. Two protons with *meta*-coupling in ring A appeared at δ_H 6.63 and δ_H 6.73 (d, J = 2) and also two double doublets at δ_H 7.37 and δ_H 8.57 representing H-3'/5' and H-2'/6' respectively. ^{13}C NMR [Spectrum 6.17] of C-2'/6' and C-3'/5' (δ_C 130.8 and δ_C 116.8 respectively) indicated the presence of a hydroxyl group at C-4' [Spectrum 6.16].

Confirmation of structure was by COSY-LR [Spectrum 6.18] and HREIMS [Spectrum 6.19]. COSY-LR showed cross-peaks between H-6 and H-8 and the methoxyl group, confirming the position of the methoxyl at H-7.

HREIMS gave the molecular formula of $C_{16}H_{12}O_6$ *m/z* 300 $[M]^+$. Other fragmentation peaks were as follows *m/z* 299 $[M-H]^+$ (14.6%); *m/z* 284 $[M-OH]^+$ (16.6%); *m/z* 271 $[M-CHO]^+$ (9.2%); *m/z* 257 $[M-C_2H_30]^+$ (13.6%); *m/z* 167 $[M-C_9H_7O_3]^+$ (11.2%); *m/z* 149 $[M-C_9H_9O_4]^+$ (20%) and *m/z* 121 $[M-C_9H_7O_4]^+$ (37.7%).

This compound was identified as 5,4-dihydroxy-7-methoxyflavonol, commonly known as rhamnocitrin and has been found in *Alnus* spp., *Betula* spp., *Ostrya* spp., *Aesculus* spp., *Rhamnus* spp., *Populus* spp., *Alpinia japonica, Alpinia kumatake, Larrea* spp., *Cheilanthes* spp., *Notholeana* spp. and *Pityrogramma tartarea* [Wollenweber, 1980]. This appears to be the first report of its isolation in Combretaceae.

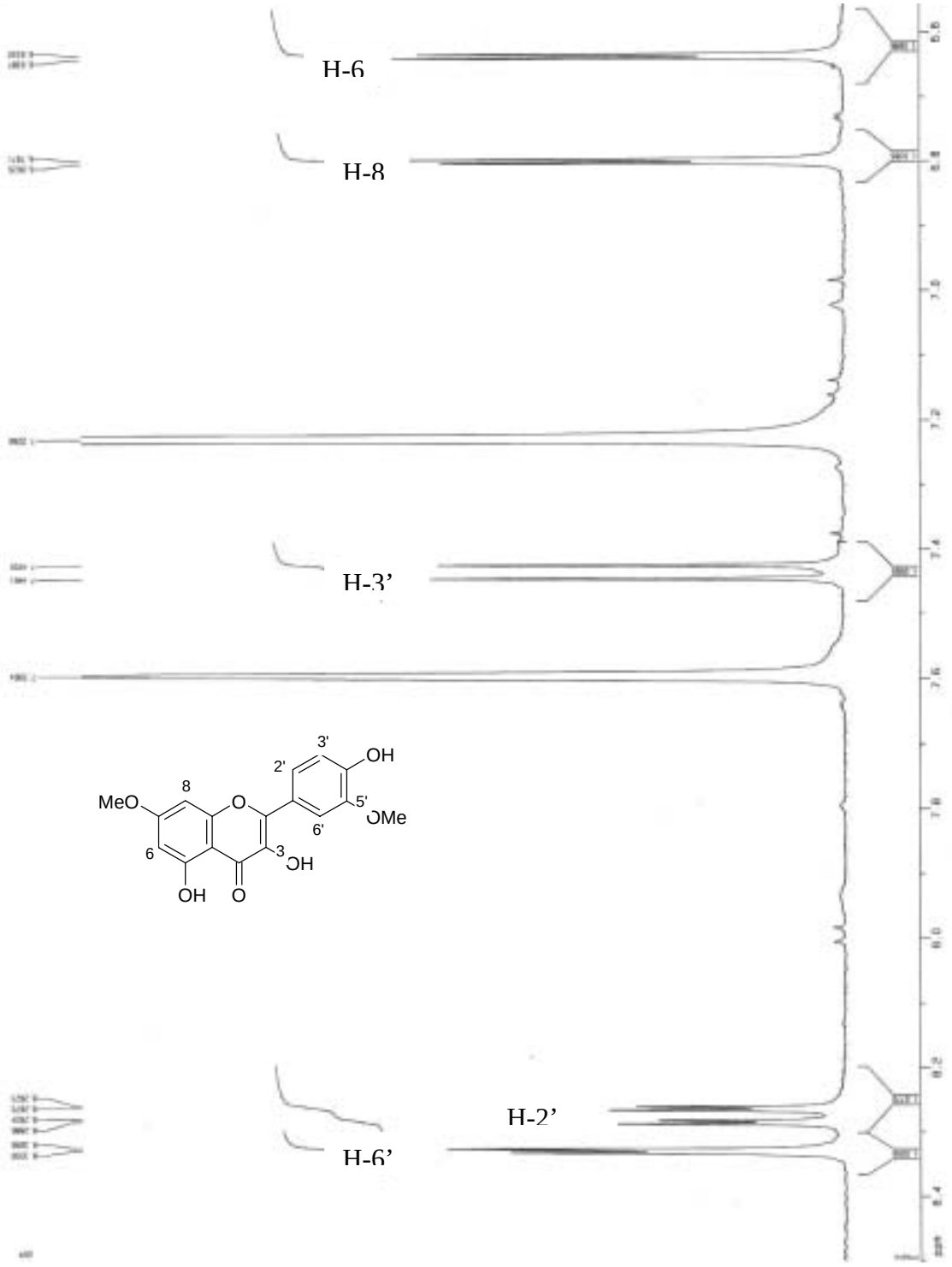
6.2.1.4 Characterisation of Seph 51

This compound was isolated from Column II and afforded a yellow powder. ^1H NMR [Spectrum 6.20] exhibited doublets at δ_H 6.31 (1H, *J* = 2) and δ_H 6.57 (1H, *J* =2), which corresponded to *meta*-coupled protons H-6 and H-8 respectively. These protons are attached to C-6 (δ_C 99.6) and C-8 (δ_C 94.9) carbons with ^{13}C NMR [Spectrum 6.21].

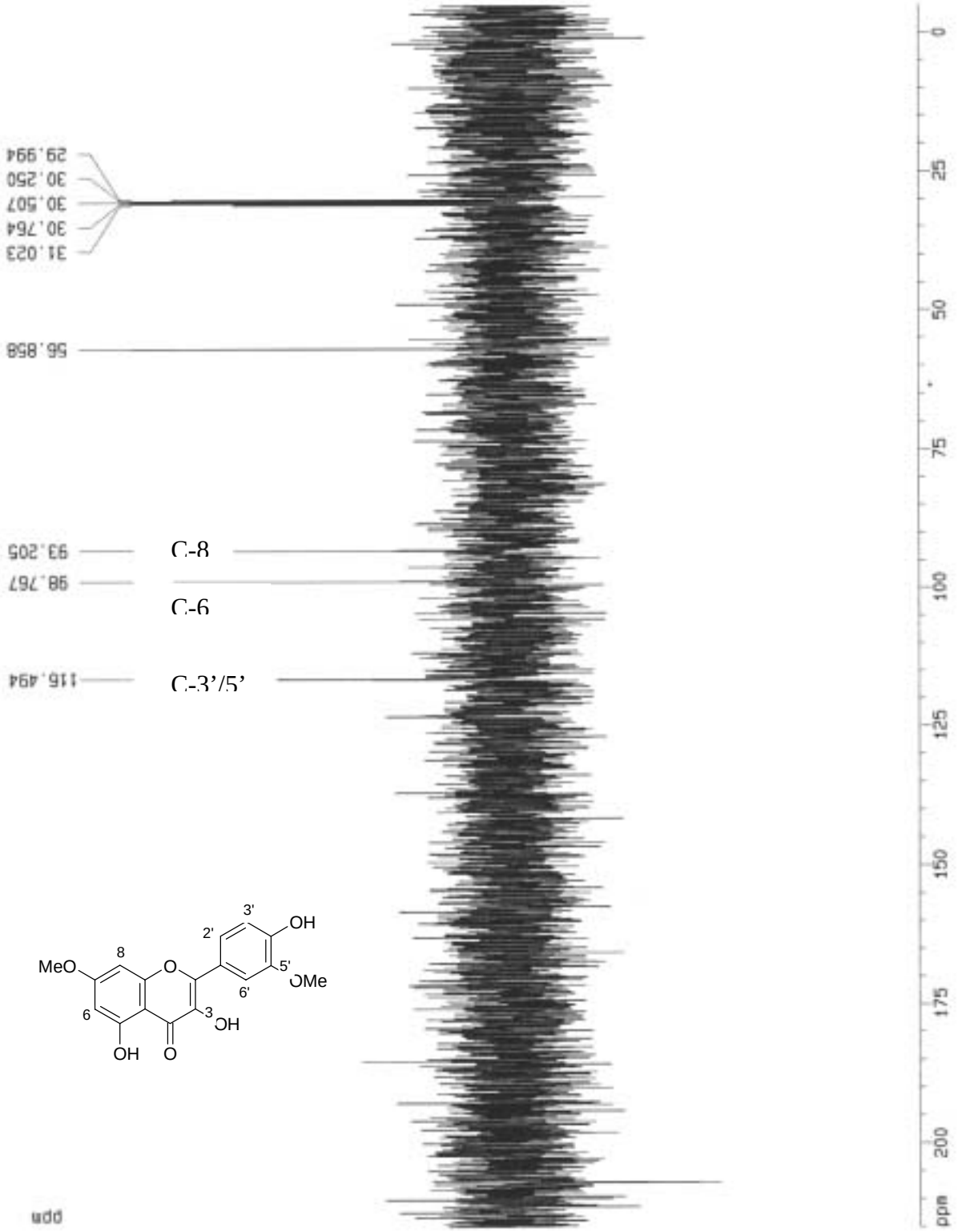
^{13}C NMR of the C-ring carbons was superimposable on the IIIa90 carbon-13 spectrum with resonances at δ_C 130.7 (C-2'/6') and δ_C 116.8 (C-3'/5'), suggesting therefore that a hydroxyl group was present on C-4'. C-6 and C-8 resonated at δ_C 99.6 and δ_C 94.9 respectively suggesting a hydroxyl group on position C-7 [Spectrum 6.17].

This structure was confirmed by HREIMS, which gave a molecular ion peak at *m/z* 286 and corresponds to $C_{15}H_{10}O_6$. Other fragments were seen at *m/z* 258 $[M\text{-}CO]^+$ (10%), *m/z* 153 $[M\text{-}C_8H_6O_2]^+$ (6.4%), *m/z* 134 $[M\text{-}C_7H_4O_4]^+$ (3%), *m/z* 229 $[M\text{-}C_2O_2H]$+ (7.8%) [Spectrum 6.22].

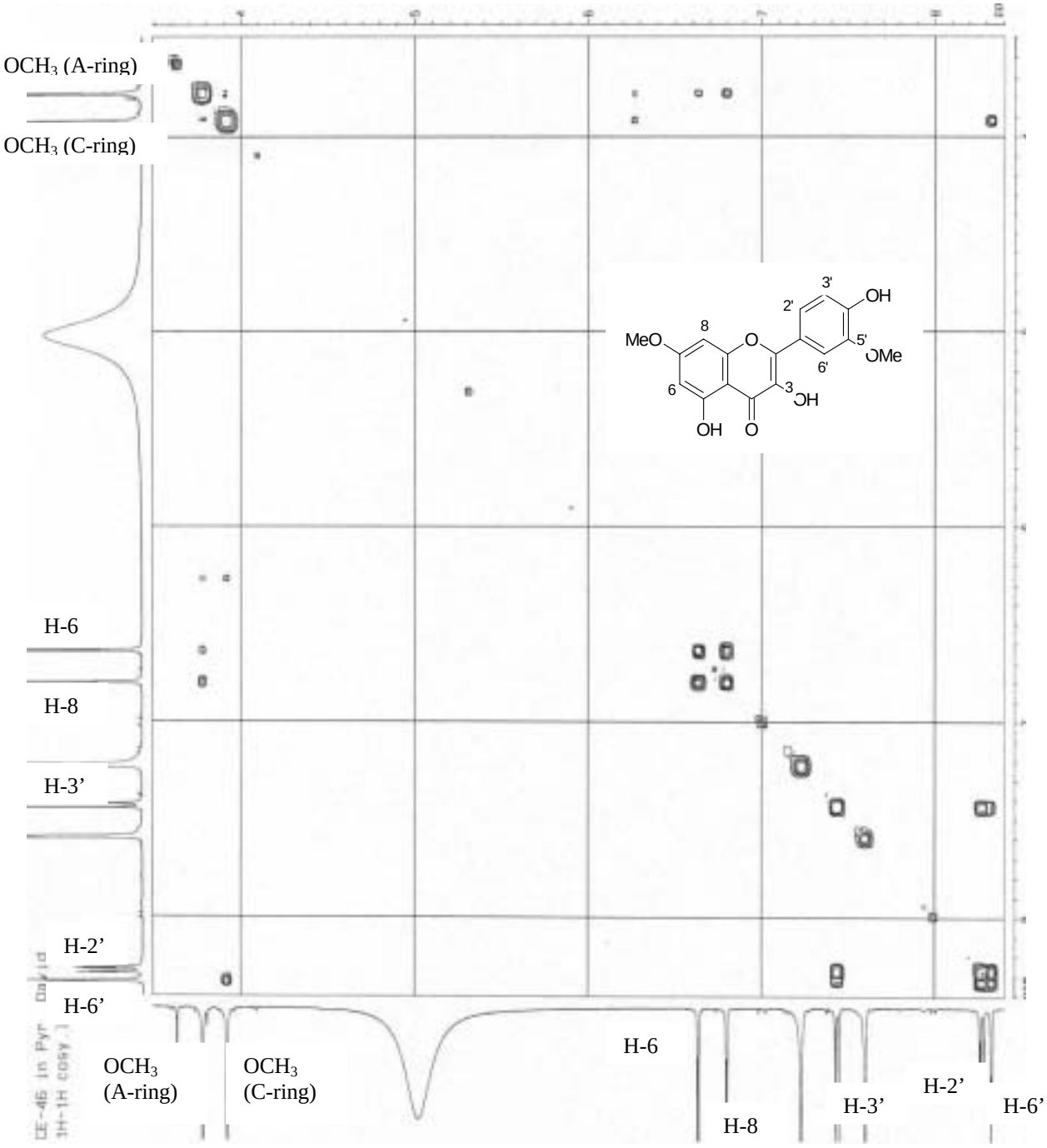
This compound was characterised as 5,6,4'-trihydroxyflavonol. It is also known as kaempferol. This is a very common compound and is found in many species including *Betula* spp., *Alluaudia ascendens, Acrotema uniflora, Aesculus* spp., *Prunus* spp., *Populus* spp., *Pterospermum acerifolium, Alpinia officinarum, Larrea* spp., *Cheilanthes* spp., *Notholaena* spp. and *Pityrogramma* spp [Wollenweber, 1980]. As far as it can be ascertained, this appears to be the first report of its isolation in Combretaceae.



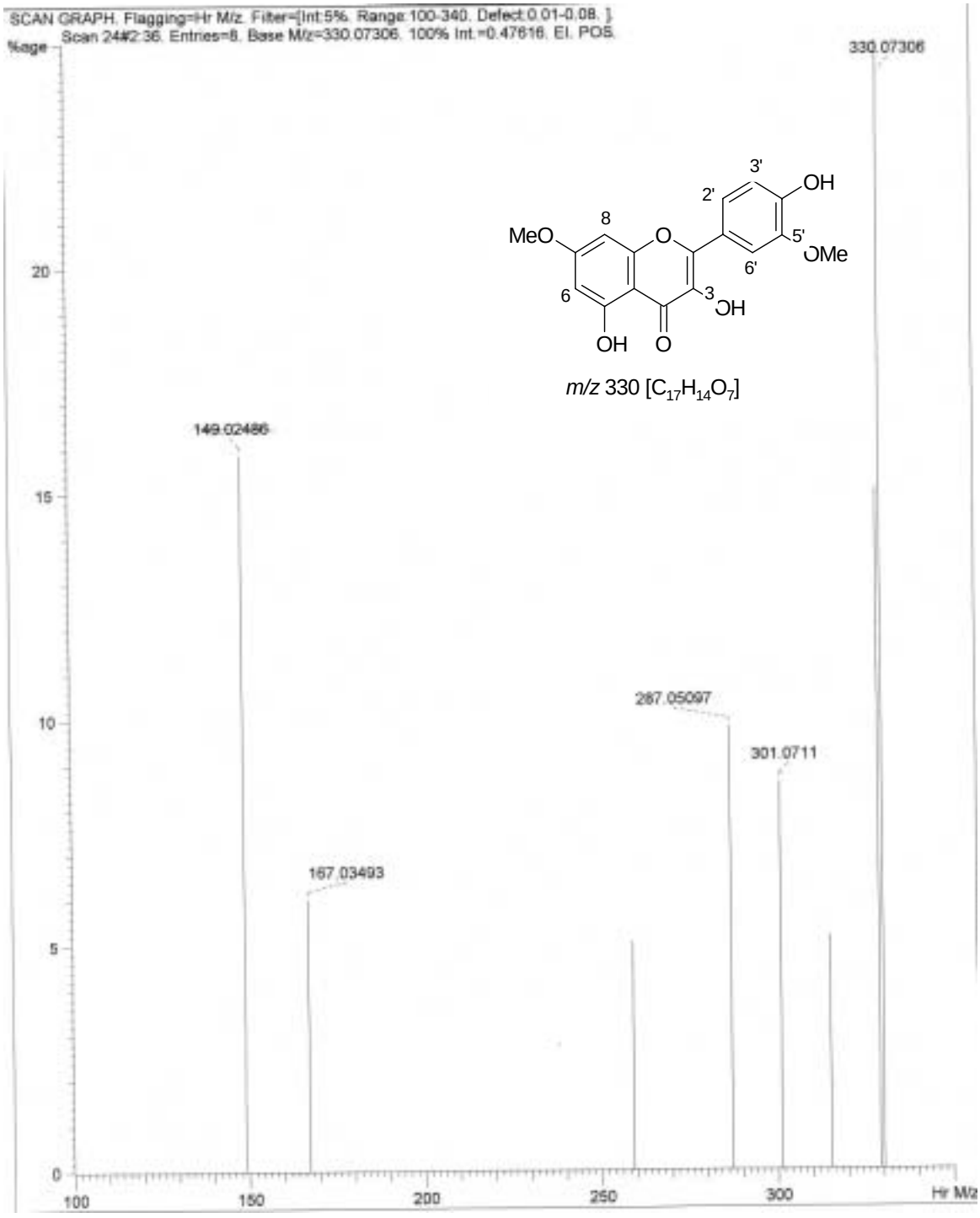
Spectrum 6.12: ^{1}H NMR (400MHz, C_5D_5N) of CE46 / CE51



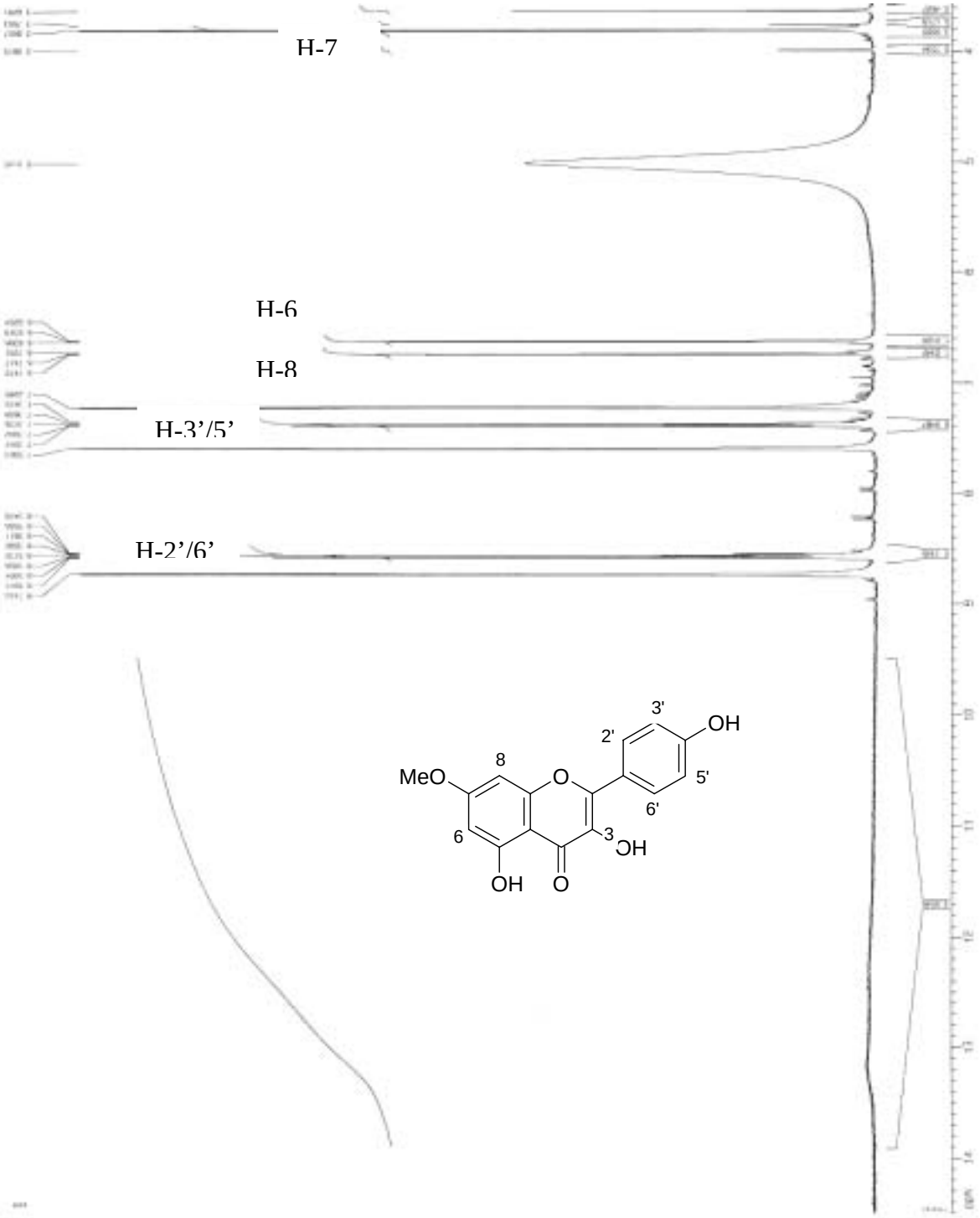
Spectrum 6.13: ^{13}C NMR (75 MHz, Acetone-d_6) of CE46



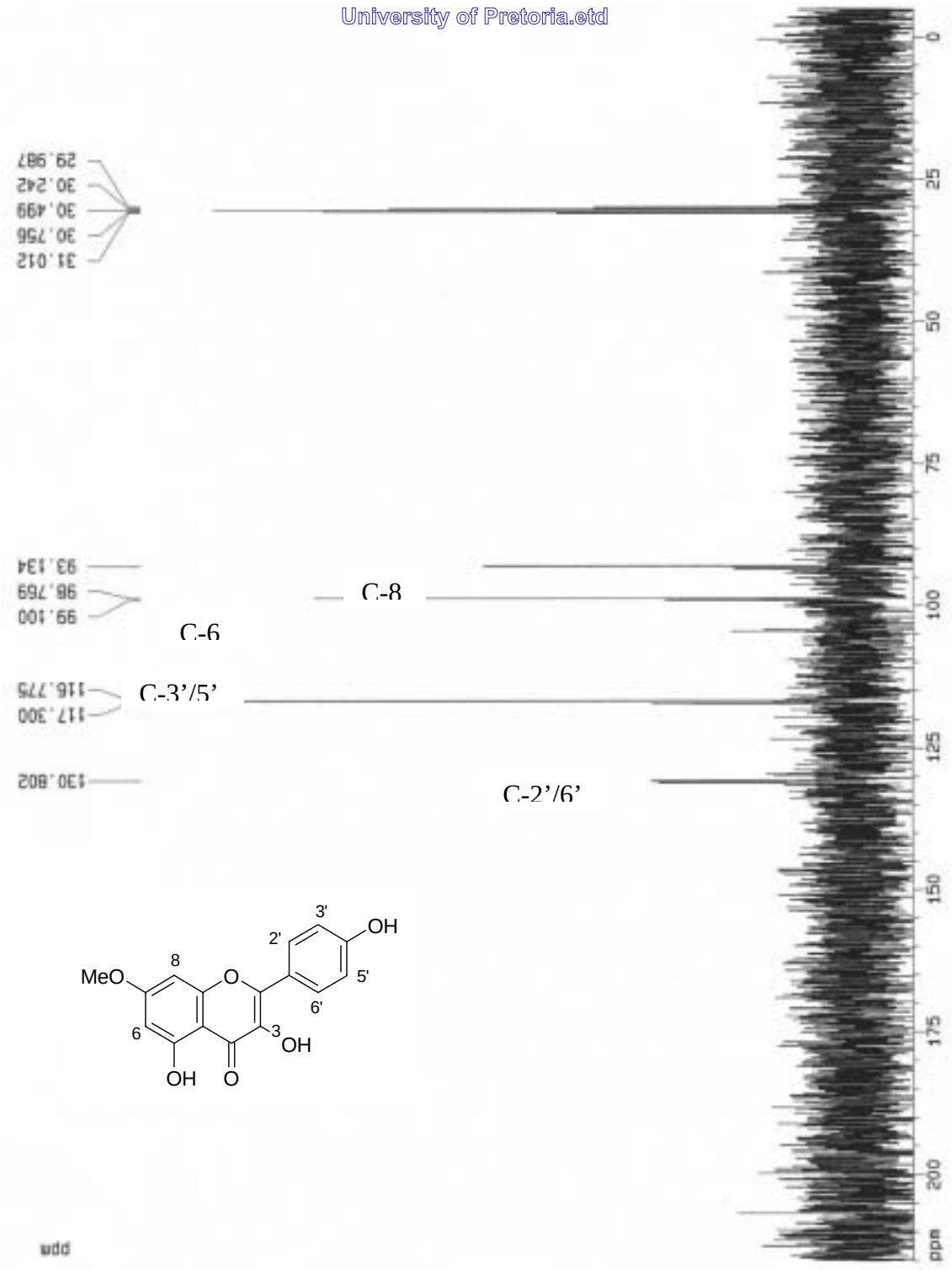
Spectrum 6.14: ^{1}H-^{1}H COSY LR (400MHz, C_5D_5N) of CE46



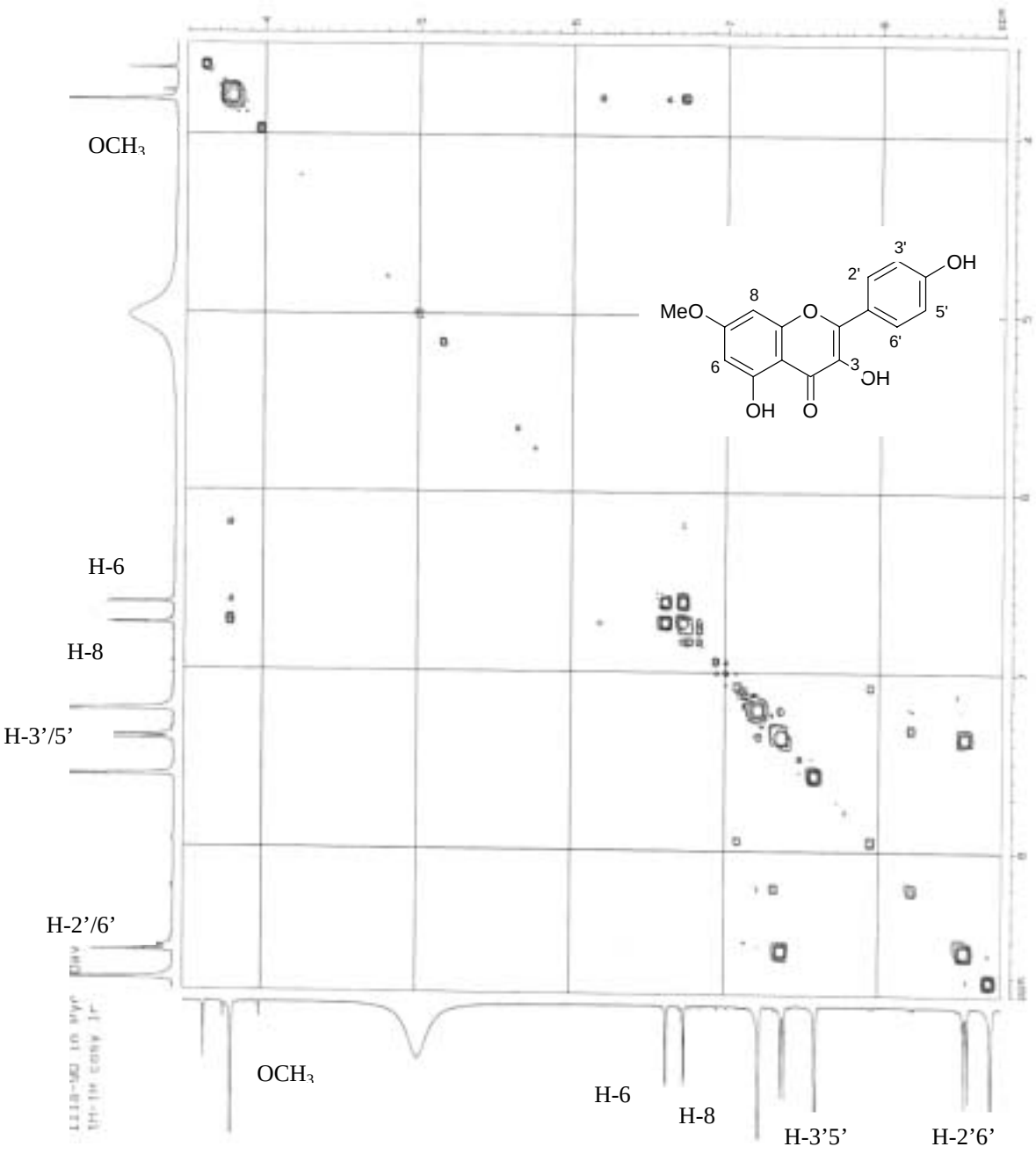
Spectrum 6.15: HREIMS of CE46



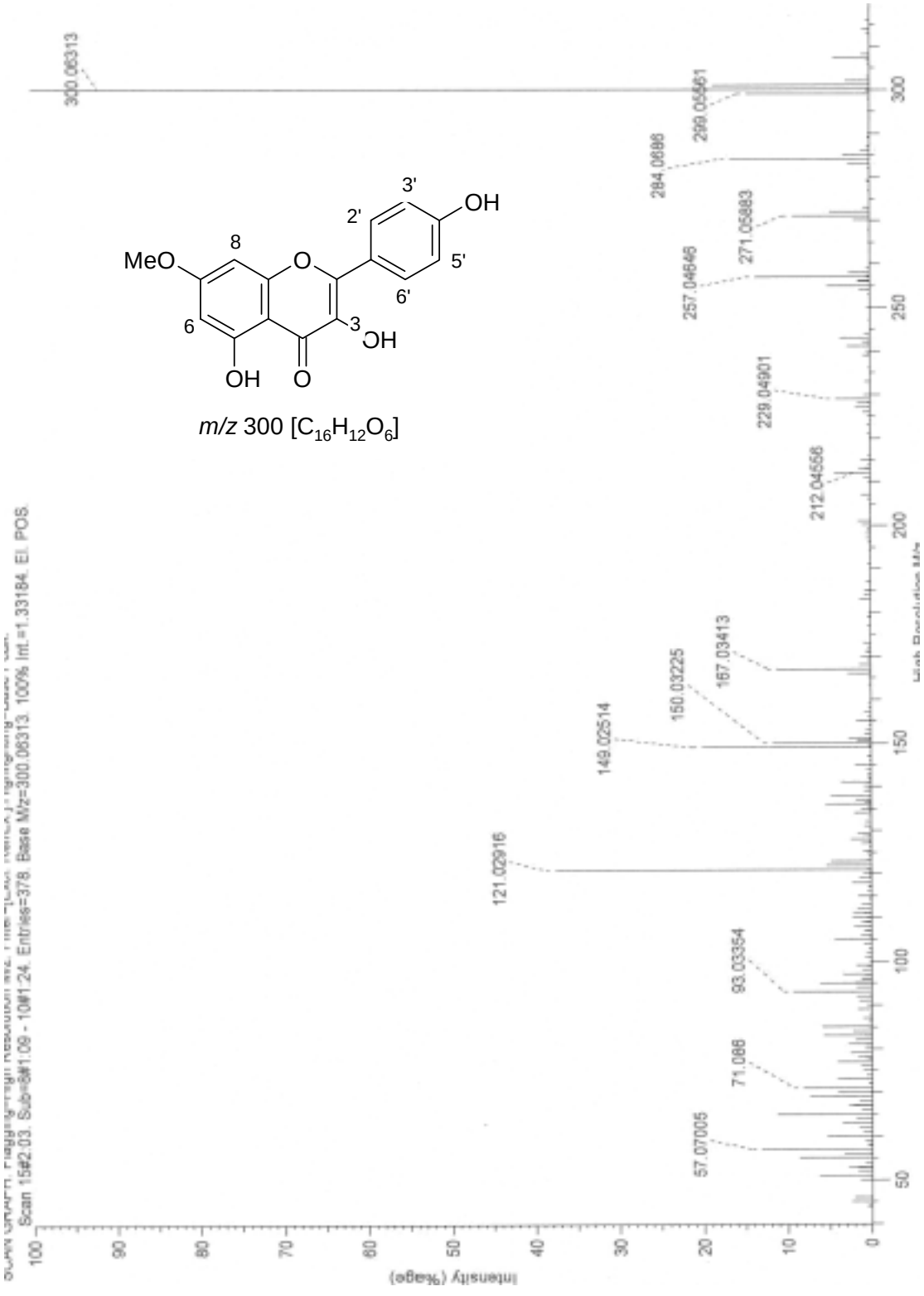
Spectrum 6.16: ^{1}H NMR (400MHz, C_5D_5N) of IIIa90



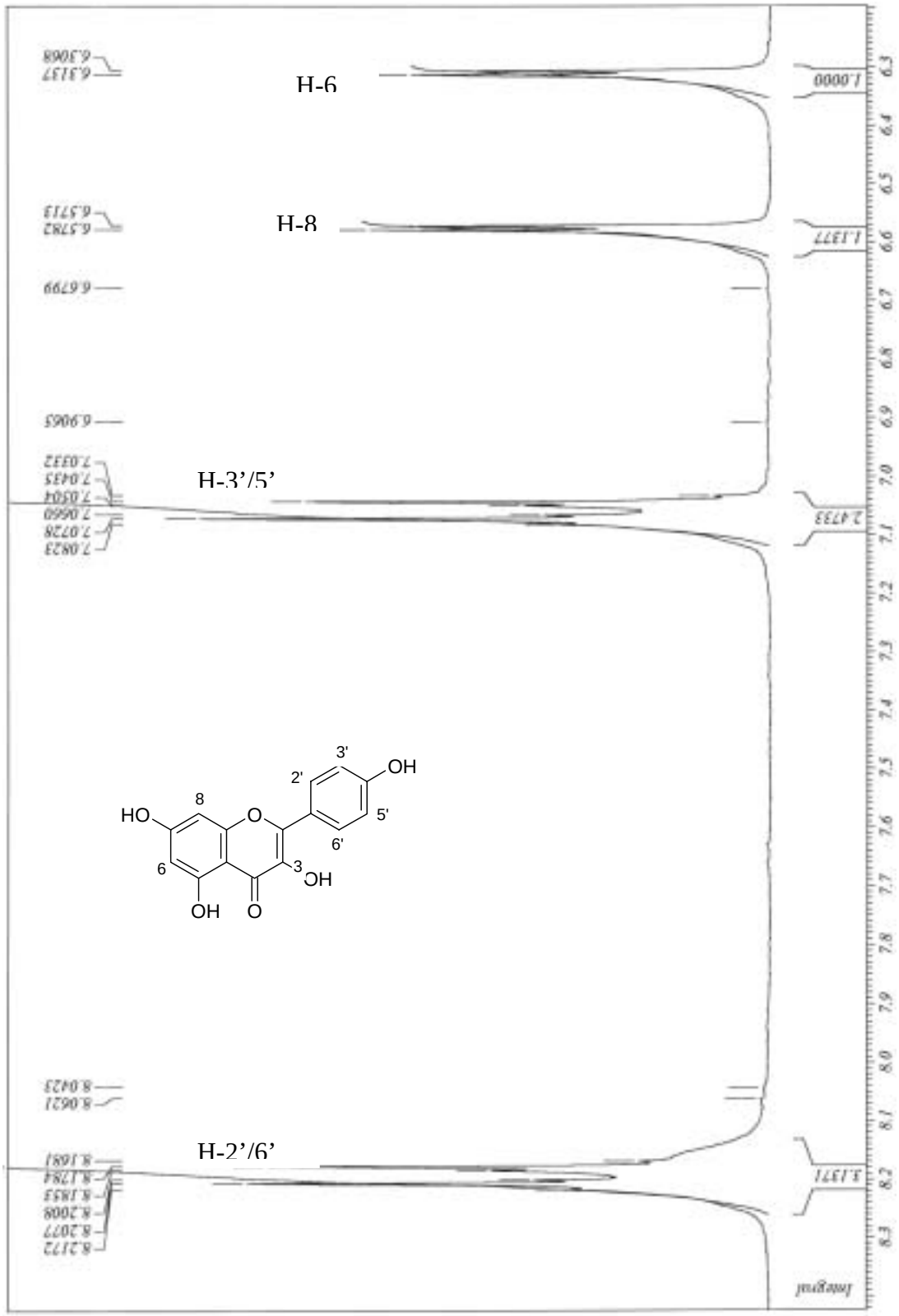
Spectrum 6.17: ^{13}C NMR (75MHz, Acetone-d_6) of IIIa90



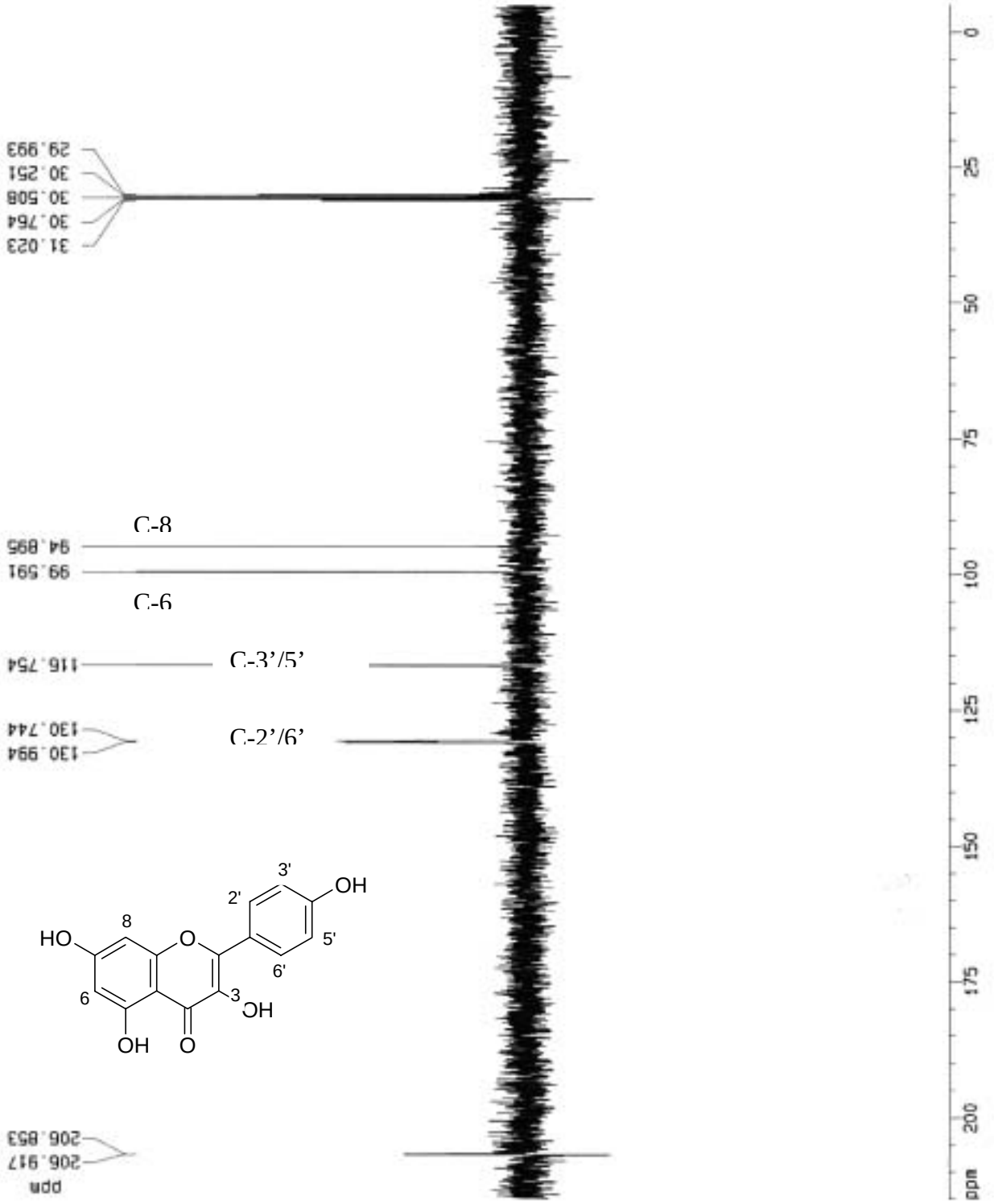
Spectrum 6.18: ^{1}H-^{1}H COSY LR (400MHz, C_5D_5N) of IIIa90



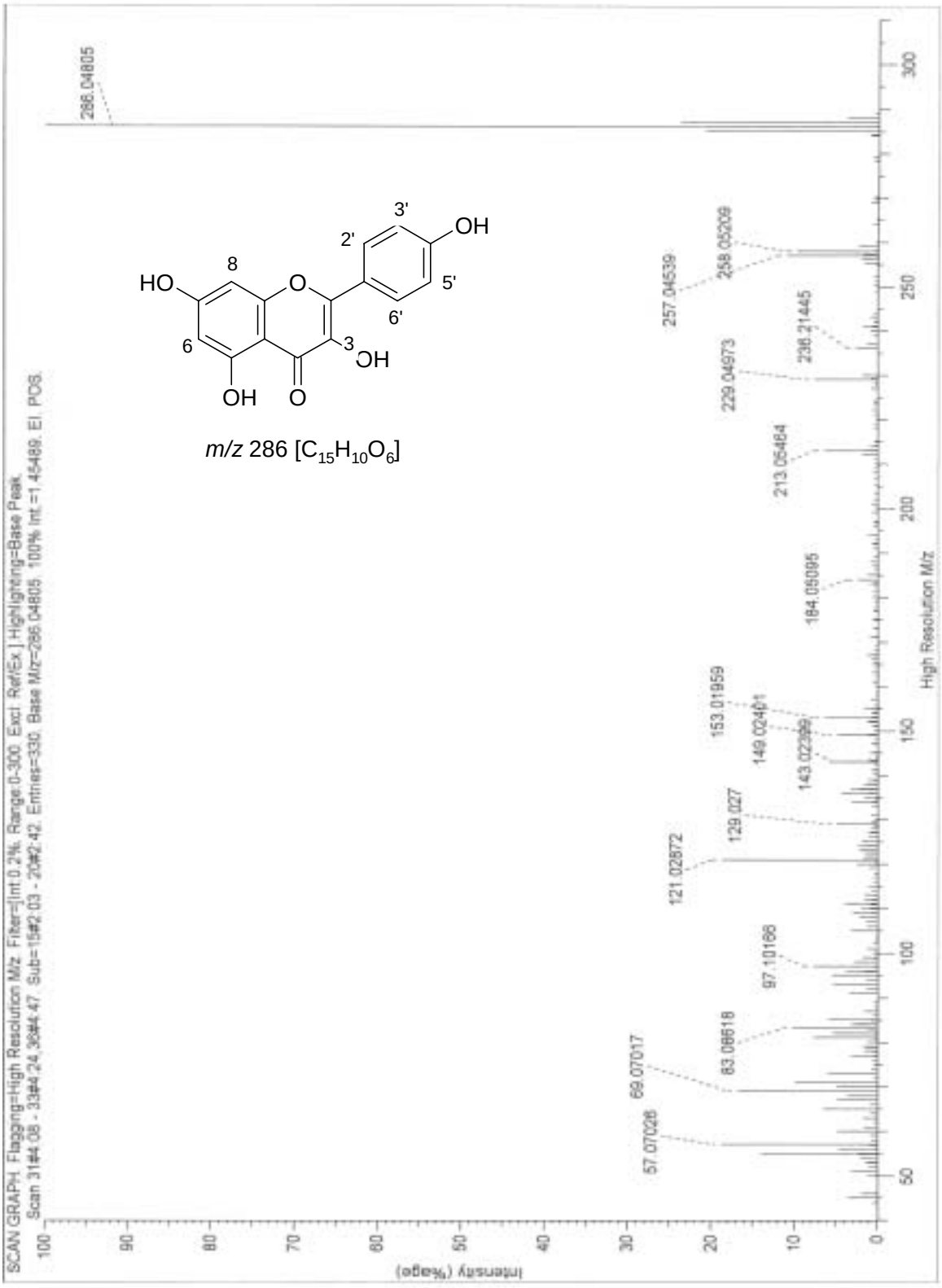
Spectrum 6.19: HREIMS of IIIa90



Spectrum 6.20: ^{1}H NMR (300MHz, Acetone-d_6) of Seph 51



Spectrum 6.21: ^{13}C NMR (300MHz, Acetone-d_6) of Seph 51



Spectrum 6.22: HREIMS of Seph 51

CHAPTER 7

7. BIOLOGICAL ASSAYS

7.1 ANTIMICROBIAL ACTIVITY

The MIC values were calculated using the well dilution method as described in chapter 4. All experiments were repeated at least in duplicate, with some compounds repeated at least six times for more accurate results. For all organisms a standard antibiotic, either gentamicin or ampicillin, was included as a positive control. Amphotericin B was included for the fungus. Since all compounds were solubilized in DMSO and made up to final concentration of 200 μg/ml with distilled water, the same quantity of DMSO to water was included as a negative control.

Figures 7.1 and 7.2 show the activity of test compounds against four common pathogens, namely *E. coli*, *S. sonei*, *V. cholerae* and *E. faecalis*. MIC values were calculated by comparing the size of the pellet of test compound dilutions with those of the growth control (Medium + test organism) (Table 7.1).

Table 7.1: MIC values (μg/ml) of isolated compounds using 11 different bacterial species and a fungus.

	CE36	CE46	CE51	IIIa90	IIIa150
S. typhimurium	>100	>100	>100	>100	>100
K. pneumonia	>100	>100	>100	>100	>100
S. faecalis	**50**	50-100	>100	50-100	50-100
M. luteus	100	**25-100**	>100	**25**	**50**
E. coli	50-100	50-100	100	50-100	100
S. aureus	>100	>100	>100	**50**	>100
S. sonei	**50**	**25-50**	>100	**25-100**	**25-50**
V. cholerae	**25-50**	**50**	**50**	**25-50**	**50**
P. aeruginosa	100	100	100	100	100
E. faecalis	**50**	**25-50**	**25**	**25-50**	50-100
B. subtilis	>100	>100	>100	>100	>100
A. niger	>100	>100	>100	>100	>100

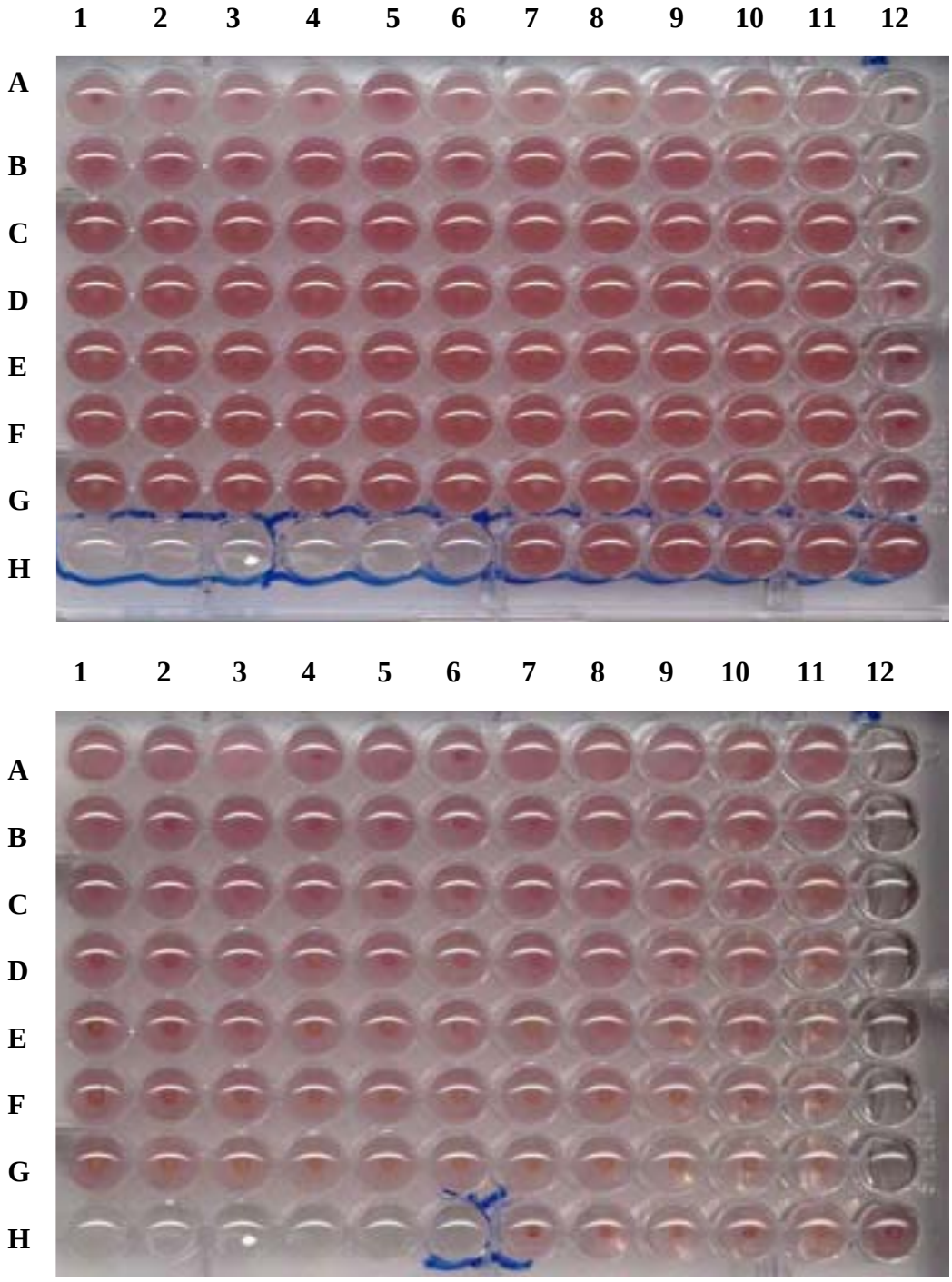


Figure 7.1: MIC plate of *E. coli* (top) and *S. sonei* (bottom) with the concentration in well A = 100µg/ml, B = 50 µg/ml etc. H1-6 represents the sterility control and H7-9 is the growth control with which the compounds were compared. Row 12 is the antibiotic controls, gentamicin (10mg/ml)and ampicillin (250 µg/ml)respectively. The compounds are represented as follows: 1 = IIIa142; 2 = CE37; 3 = CE32; 4 = IIIa150; 5 = IV33; 6 = IIIa105; 7 = CE46; 8 = CE139; 9 = CE36; 10 = IIIa90 and 11 = CE43.

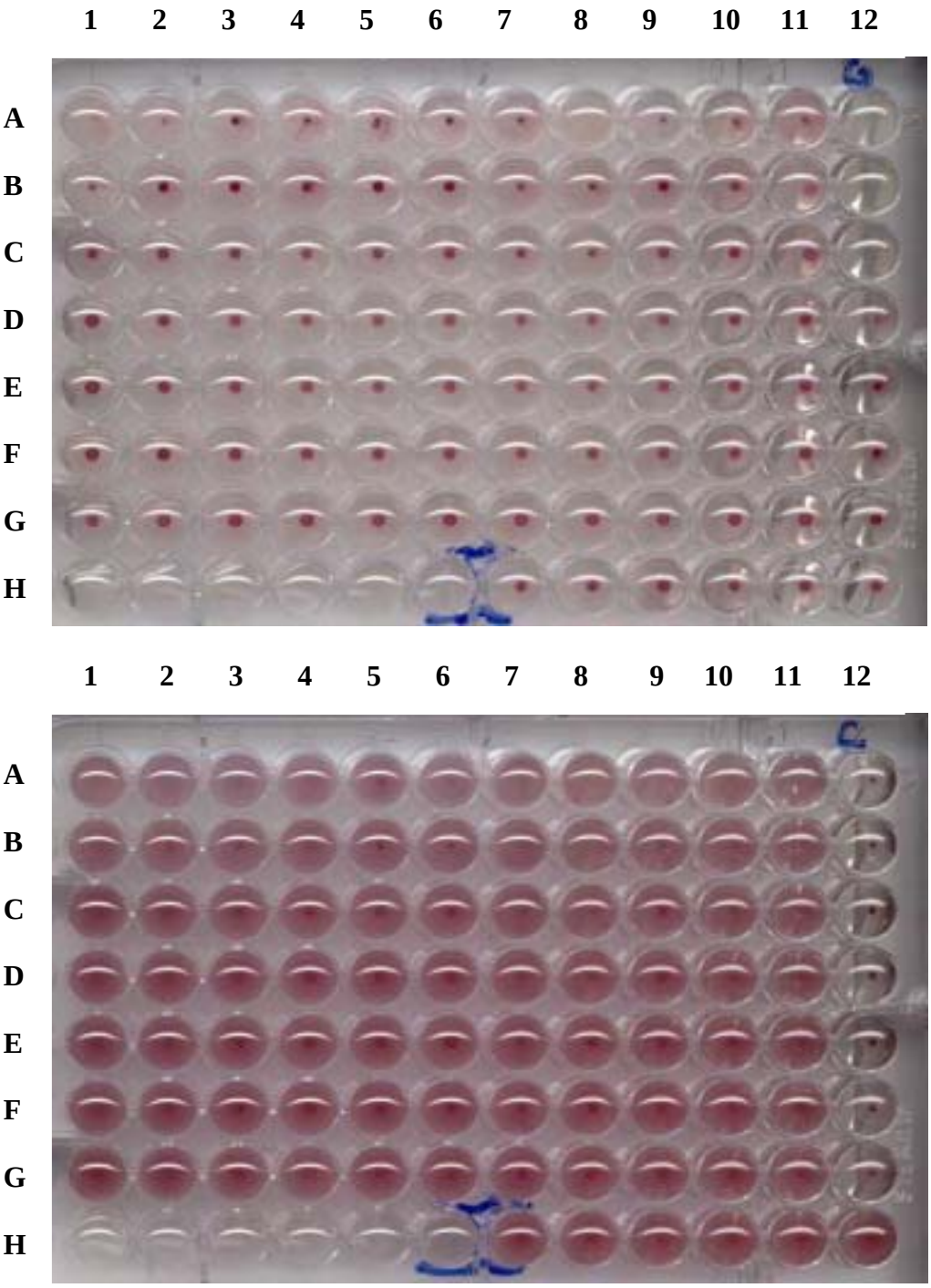


Figure 7.2: MIC plate of *E. faecalis* (top) and *V. cholerae* (bottom) with the concentration in well A = 100µg/ml. H1-6 represents the sterility control and H7-9 is the growth control. Row 12 is the antibiotic positive control, which is gentamicin (10mg/ml) and chloramphenicol (250µg/ml) respectively. The compounds are represented as follows: 1 = IIIa142; 2 = CE37; 3 = CE32; 4 = IIIa150; 5 = IV33; 6 = IIIa105; 7 = CE46; 8 = CE139; 9 = CE36; 10 = IIIa90 and 11 = CE43.

Some of the compounds were identical e.g. CE32, CE36 and CE37 and the MIC range of inhibition is presented (Table 7.1). Other identical compounds were CE43 and CE46; IIIa90, IIIa105 and CE139. Although in some cases no inhibition was apparent, those results showing inhibition were used as these indicated a possibility of antimicrobial activity. DMSO and water did not inhibit growth of organisms.

A. niger gave unpredictable and unreliable results. Some compounds seemed to enhance the growth of the fungus, whereas in other wells, the same compound showed inhibition. Also no growth was observed in wells with very diluted test compounds. For this reason, a member of the same family, *A. fumigatus*, was attempted, which produced clearer results, possibly due to experience developed with the technique procedure. There was no apparent inhibition at the concentrations used (100 mg/ml) and the size and colour of the wells were the same as those of the growth control. Amphoteracin B exhibited an MIC value below 0.78 mg/ml.

Test compounds exhibited similar activities against microorganisms, possibly due to similarities between structures and hence structure activity relationships. There does not appear to be a vast difference in inhibitory activity between Gram-positive and Gram-negative organisms, and it is therefore unclear how these compounds target microbial invasion but is most probably not due to inhibition of cell wall synthesis. With some compounds there appears to be a bacteriostatic rather than bactericidal activity as wells previously showing inhibition had become infected after prolonged incubation periods.

SUMMARY OF RESULTS:

7.1.1 CE36 (5-hydroxy-7,4'-dimethoxyflavone)

This compound exhibited good activity against *V. cholerae* (25-50µg/ml), *S. sonei* (50 µg/ml), *E. faecalis* (50 µg/ml) and *S. faecalis* (50 µg/ml). Not much data regarding the antimicrobial activity of this compound has been recorded but complexes of CuII, NiII, CoII, ZnII, FeII, CrII, CdII and MnII have been synthesized with 5-OH-7,4'-dimethoxyflavone and activity of both the ligand and the complexes have been determined on Gram positive and negative organisms [Wang, 1992].

7.1.2 CE46 (Quercetin-5,3'-dimethylether)

Activity against *V. cholerae* (50µg/ml), *S. sonei* (25-50 µg/ml), *E. faecalis* (25-50 µg/ml) and *M. luteus* (25-100 µg/ml) was recorded. No antimicrobial data regarding this compound could be sourced in the literature.

7.1.3 CE51 (Rhamnazin)

Thic compound did not appear to be very active except in *V. cholerae* (50µg/ml) and *E. faecalis* (25 µg/ml). No literature relating to this compound's antimicrobial activity was sourced.

7.1.4 IIIa90 (Rhamnocitrin)

Activity was recorded against *V. cholerae* (25-50µg/ml), *S. sonei* (25-100 µg/ml), *E. faecalis* (25-50 µg/ml), *S. faecalis* (50-100 µg/ml) and *M. luteus* (25 µg/ml), making this compound quite active. This was the only flavonoid to show activity against *S. aureus* at 50 µg/ml.

Antibacterial activity has been tested against *Streptococcus mutans, Actinomyces viscosus, Porphyromonas gingivalis* and *Prevotella intermedia* [Cai, 1996]

7.1.5 IIIa150 (Genkwanin)

Genkwanin is found to accumulate in barley leaves in response to attack [Christensen, 1998]. This phytoalexin is active against *V. cholerae* (50μg/ml), *S. sonei* (25-50 μg/ml), *E. faecalis* (50-100 μg/ml), *S. faecalis* (50-100 μg/ml) and *M. luteus* (50 μg/ml).

7.1.6 Seph51 (Kaempferol)

Although not included in this testing due to widespread studies, kaempferol-3-O-rutinoside has been found to exhibit low antibacterial and antimycotic activities 125 μg/ml against *S. aureus* and 250-500 μg/ml (*C. albicans*) [Bisignano, 2000]. It has also potent growth inhibitory activity against periodontal pathogens *Porphyromonas gingivalis* and *Prevotella intermedia* [Cai, 1996].

7.1.7 CE144 (Apigenin)

Also not included in the testing due to widespread studies, apigenin has been found to be selectively toxic to *S. aureus* including MRSA and methicillin sensitive strains with MICs ranging from 3.9-15.6 μg/ml. In general Gram-negative bacteria are less sensitive to inhibitory action of this phytoalexin than Gram-positive bacteria [Kurosaki and Nishi, 1983] and some studies have demonstrated that apigenin exhibited no activity against some Gram-positive coccal strains, including S*treptococcus epidermidis* and no appreciable activity against bacteria other than *S. aureus*. All MICs were greater than 250 μg /ml [Sato, 2000].

Other studies showed activity against *P. mirabilis* (16 µg/ml), *P.aeruginosa* (8 µg/ml). *S. typi* (128 µg/ml), *E.coli* (128 µg/ml), *E. aerogenes* (4 µg/ml), *E. cloaceae* (4 µg/ml) and *K. pneumoniae* (128 µg/ml). Apigenin has been found to exert a higher activity than apigenin-7-*O*-triglycoside. Glycosilation of apigenin causes a reduction in antibacterial power due to reduction in lipophilia and a diminished ability to penetrate bacterial membrane [Basile, 1999].

7.2. TOXICITY ASSAY

Lactate dehydrogenase (LDH) is a cytosolic enzyme present within all mammalian cells. The normal plasma membrane is impermeable to LDH, but damage to the cell membrane results in a change in the membrane permeability and subsequent leakage of LDH into the extracellular fluid. *In vitro* release of LDH from cells provides an accurate measure of cell membrane integrity and cell viability. As a result, the release of LDH is a popular and reliable test for cytotoxicity in immunological as well as biocompatibility studies (Allen, 1994).
This assay is based upon the ability of LDH to catalyse the reaction:

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{Lactate}^{+} + \text{NAD}^{+}$$

Changes in optical absorbance, measured at 340 nm, reflect changes in NAD^+ and therefore the level of LDH in the test compound.

For comparative purposes, a positive control is required which causes substantial cell membrane damage. The compound included in this study was LPC (lysophosphatidylcholine), which destroys the cell membrane causing maximum release of LDH. Negative controls included a water control and DMSO + water control. The concentration of the test compounds used in this assay was 100 µg/ml, the highest concentration used to test antimicrobial activity and chosen for this reason.

The main aim was to show whether these compounds could be selectively toxic against micro-organisms and spare the host cells.

The percentage LDH release was calculated by subtracting the time after 1, 5 and 10 minutes from time 0, dividing by time 0 x 100.

i.e. Time 0 = 0.775 Time after 1 min = 0.675
Therefore 0.775-0.675 = 0.1/0.775 x 100 = 12.9%

These values were plotted on a graph (Figure 7.3) for comparative purposes.

Table 7.2: The average percentage LDH released from test compounds after 1, 5 and 10 minutes.

	1	5	10
Water	8	33	48
Water + DMSO	5	19	27
LPC	35	76	77
CE36	11	39	47
CE46	4	17	25
CE51	8	34	47
IIIa90	5	20	30
IIIa150	7	34	43

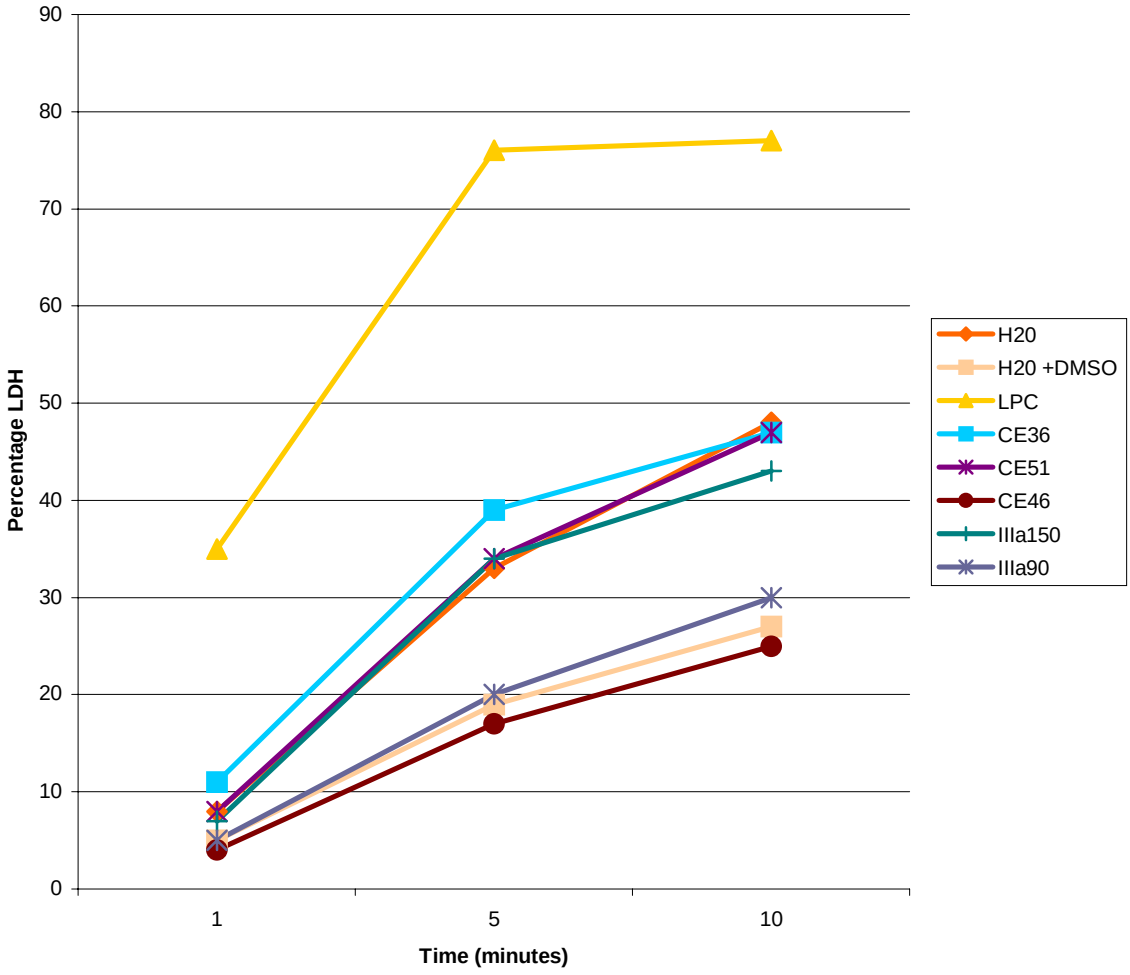


Figure 7.3: The average percentage LDH released by LPC, water, water + DMSO and 5 test compounds

According to these results it is apparent that most of the compounds have similar toxicity profiles to that of water. Since water in large volumes can cause lysis of cells, this could possibly explain the increase in LDH levels. DMSO + water was used as solvent for the compounds and did not appear to influence the cells to any large extent. Rhamnocitrin (IIIa90) and quercetin-5,3'-dimethylether (CE46) had similar profiles to that of the solvent, whereas genkwanin (IIIa150) and rhamnazin (CE51) were close to that of water.

By assaying LDH leakage from LLC-PK1 cells in culture, a study was carried out to clarify whether flavonoid compounds ameliorate renal cellular injury. Genkwanin had virtually no effect. Yokozawa [1999] suggested that the difference in activity is largely decided by the number and position of phenolic hydroxyl groups linked to structural backbone.

Compound CE36 had in some cases shown values very close to those obtained with LPC and since its influence on cell lysis was greater than that of water, therefore it is presumed to be potentially toxic.

7.3 ANTIOXIDANT / ANTI-INFLAMMATORY RESPONSES

Inflammation is a series of events that occur in response to various injuries to living tissues. It is characterised by local vascular changes and infiltration of leukocytes, especially granulocytes in acute inflammation. Granulocytes exhibit an increase in chemotaxis, degranulation and phagocytosis, and the activity is controlled in time and intensity so that healing takes place as soon as possible. Luminol-dependant chemiluminescence (CL) has been recognised as a useful tool for evaluating the phagocytic activity of granulocytes and lymphocytes. Changes in counts of luminol-dependant CL can represent the effects of various drugs on the functions of granulocytes [Ozaki, Y, 1984].

Luminol is converted to an excited amonophthalate ion in the presence of oxidising compound and this reaction emits blue light measured by the chemilumninometer. The chemical basis of the chemiluminescence reaction is not known in every detail but superoxide anion and the myeloperoxidase product hypochlorite (HOCl) are necessary for generating luminol amplified chemiluminescence [Dahlgren as quoted by Wiik, 1996].

Lymphocytes were used primarily due to ease of isolation and greater stability in comparison to neutrophils whose life-span is considerably shorter. A blank (cells + luminol + zymosan) was included as negative control and mefenamic acid (100 mg/ml), a non-steroidal anti-inflammatory drug, as positive control. Cells were stimulated with opsonised zymosan as described in Chapter 4.

Results (Figure 7.4) show, as expected, the blank control emitting the greatest percentage of light (in millivolts) due to superoxide production. The solvent (DMSO + water) had virtually no effect on the free radical production and therefore did not interfere with activity of the test compounds. Mefenamic acid is a commercially available anti-inflammatory and analgesic preparation with moderate anti-inflammatory activity. It is expected to reduce inflammatory components suppressing the reaction. All compound were compared to this positive control.

Genkwanin (IIIa 150) showed greater suppression of the curve than mefenamic acid and was assayed as an *in vitro* antioxidant by its ability to inhibit tert-butyl hydroperoxide initiated chemiluminescence of mouse liver homogenates (IC50 > 1 microM) [Fraga, 1987]. It makes a promising substance to be investigated as a water-soluble protector against lipid peroxidation and other free radical-mediated cell injury. CE46 had a similar activity to genkwanin but no information regarding its activity could be sourced in the literature.

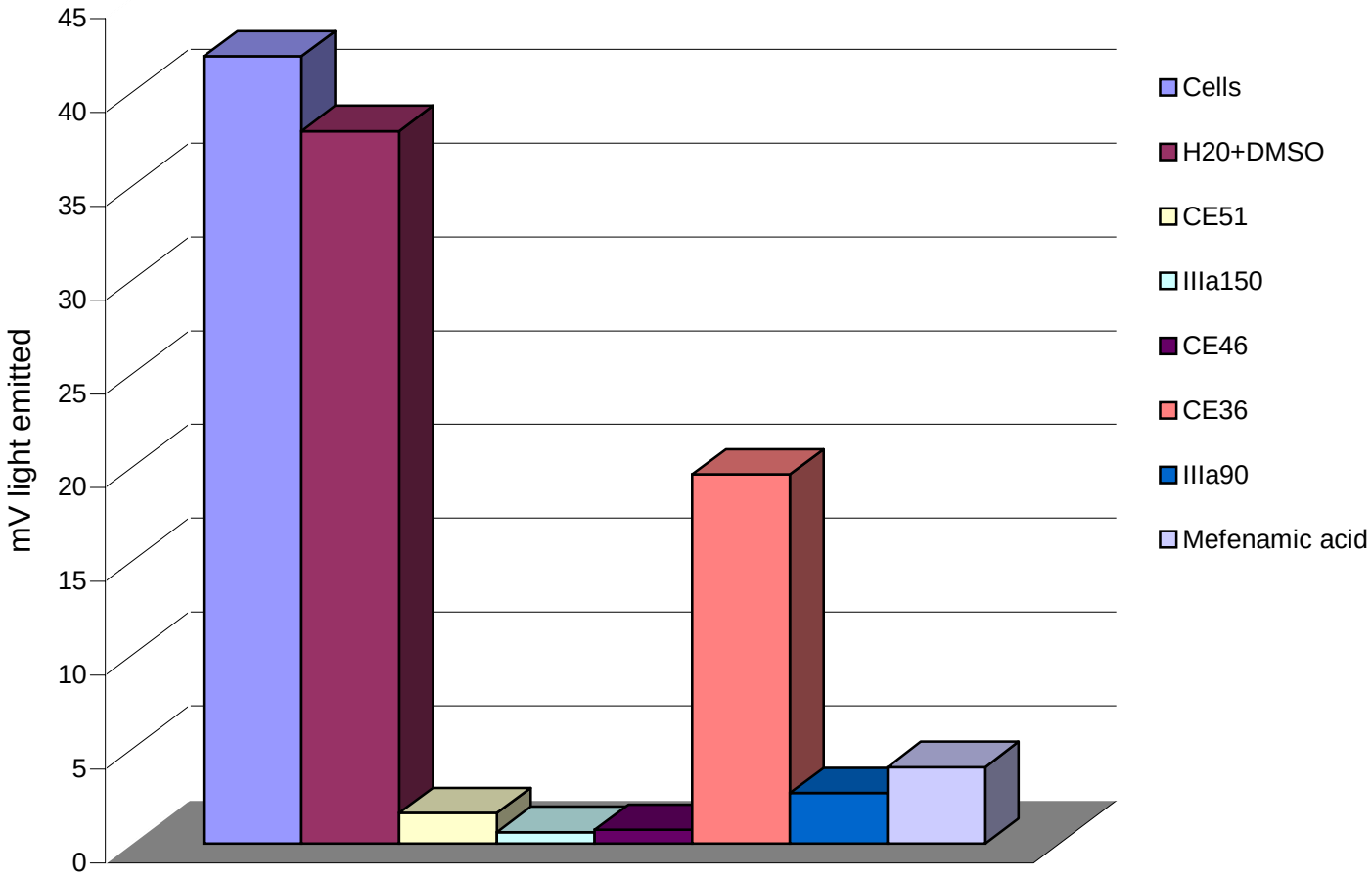


Figure 7.4: Free radical production (measured in millivolts light emitted) after 30 minutes produced by lymphocytes after stimulation with zymosan and test compounds

Both IIIa90 (rhamnocitrin) and CE51 (rhamnazin) showed strong antioxidant effects confirming the results of Manez [1999] and Yun [2000]. Rhamnocitrin was found to exert anti-inflammatory action and exhibit effectivity against 12-O-tetradecanoylphorbol-13-acetate-induced ear oedema in mice, although lacking activity against arachidonic acid-induced oedema [Manez, 1999]. Rhamnazin significantly inhibited lipid peroxidation in rat liver microsome [Yun, 2000].

Although radical production was inhibited by CE36 it had the weakest activity and is therefore not regarded as having good antioxidant activity. A compound not tested was Seph51 (kaempferol) which is already known to have a remarkably high ROO(*)-scavenging activity. It is suggested that a diet rich in these radical scavengers would reduce the cancer-promoting action of ROO(*) and consequently the carcinogenic potentials of oxygen-related radicals may be suppressed [Sawa, 1999].

CHAPTER 8

8. DISCUSSION AND CONCLUSION

In the course of this study a number of secondary metabolites were extracted from *Combretum erythrophyllum.* These compounds are not novel structures but are reported for the first time in this species. Although apigenin has previously been isolated in *C. apiculatum* [Katerere, unpublished 2001] none of the other compounds appear to have been isolated from Combretaceae. All the compounds isolated were flavonoids i.e. three flavones and four flavonols and since Rogers [1998] managed to isolate cycloartanes from *C. erythrophyllum,* one needs consider why this biased isolation took place. One reason might lie in the solvents used in initial extraction of crude material as well as in the solvent/solvent extraction. Also many samples, which might have contained other compounds were not analysed due to insufficient quantities or were not clean enough for spectroscopic analysis and subsequently discarded. Each of the procedures used in the extraction and characterisation of these compounds is discussed below.

8.1 EXTRACTION AND ISOLATION PROCEDURE

In order to test the activity of extracts, the extract must be dissolved in a solvent that will not inhibit the growth of the test organism. This may cause complications if the active fractions are lipophilic compounds that are insoluble or only sparingly soluble in water. According to Malone [1983], a major problem arises from the injection of imperfectly soluble extracts or solvent solubilized materials into what should be a purely aqueous isotonic pharmacological system. Acetone and DMSO have been used to dissolve lipophilic compounds because these solvents are miscible with water. DMSO is apparently not as toxic to bacteria as acetone but the high boiling point [189°C] makes it very difficult to recover the active compound from a DMSO solution. Dried extracts are frequently not soluble enough in DMSO.

Acetone was investigated as solvent for extraction of crude material as it is miscible with water, is volatile and has low toxicity to bioassays used [Eloff, 1998]. Another advantage of acetone is the ease of removal from fractions collected within a relatively short period.

Snyder and Kirkland [1979] grouped solvents in 8 groups depending on the dipole as well as their ability to act as proton-donors or –acceptors. Acetone forms part of group VIa. Other solvents in this same group that are miscible with water are dioxane and to a lesser degree ethyl acetate and may be useful as extractants for dried plant material.

8.1.1 Analysis of extracts

To investigate the number of components in the extracts several TLC systems were developed. In order to obtain well separated components it is essential to select the right mobile phase and it was decided to use primarily three systems: 2A:3MDC for separation of polar components; BEA for non-polar and CEF for both polar and non-polar components. EMW was also attempted in the separation of the highly polar components. Each of the four systems was employed at different stages of extraction.

8.1.2 Solvent/solvent extraction

This procedure involves the separation of compounds with different polarities and since the desired compounds will be soluble in either one or another solvent, this technique is meant to “clean” the solution by removing contaminants.
Although hexane extracted the largest percentage of the dry weight, it contained the fewest inhibitory compounds giving the false impression that isolation of those compounds would be a simple procedure. Hexane is usually employed as a defatting solvent to remove unwanted waxes and oils from the crude material, which may interfere with further analysis, making further separation easier by removing a large portion of “unwanted” material.

It is possible that since acetone was used as initial extraction of crude material and not hexane, as is usually done; many compounds may not have been extracted resulting in primarily flavonoid isolation.

8.1.3 Column Chromatography

Column chromatography is the most commonly used preparative technique to isolate chemical compounds. Initially it was thought essential to develop a good eluting system in order to separate components optimally; therefore much care was taken to develop a good eluting system with the hexane fraction. A combination of acetone:DCM (1:1) and hexane/DCM (1:1) was initially chosen as the former separated the polar and the latter the non-polar components and both components are volatile and easily removed from the fractions. After combination of fractions a second column using BEA as mobile phase was used to separate fraction C. Since benzene is a toxic substance, care was taken to work only in the fume-hood, which made the extraction procedure and drying of samples tedious.

The chloroform fraction contained many antimicrobial compounds as seen with the bioautography and was subsequently chosen for separation via column chromatography. Although much care was taken with the hexane fraction to develop a good mobile system it was decided to remove non-polar components with a solvent such as hexane and then gradually introduce another solvent e.g. dichloromethane in small quantities to 100%. This achieved a good separation. It is sometimes necessary to include a third solvent to elute non-mobile components and therefore methanol was gradually added towards the end of separation with both the hexane and chloroform fractions. Care was used that the concentration of methanol did not increase above 50% in some cases as it has a tendency to dissolve silica gel, which subsequently interferes with structural elucidation. Another problem encountered with methanol was the difficulty in drying of the extracts.

Active fractions CE36, CE46 and CE51 were eluted with 10% hexane in DCM; Seph51 and CE144 with 5% methanol in DCM and IIIa90 and IIIa150 with 10% methanol in DCM.

The different stationary phases used in this study were silica gel, Sephadex and Toyopearl, size-exclusion gels. The reason for these three phases was primarily experimental in order to determine the best effect on separation. The best separations were achieved with silica and Sephadex. Toyopearl was easy to use since it came in a suspension and easy to clean but the separation was poor.

Problems encountered with column chromatography were not so much in the procedure involved but in the resulting fractionation. Some compounds appeared to be more complex after passing them through a column than they had been prior to separation. Columns II and III were based on selected fractions obtained from column I, and column IIIa on the first four fractions of column III. The results should have depicted a separation of the compounds as presented on TLC but in some cases more compounds appeared despite the use of the same mobile phases for elution. This increase in compounds could be due to an increase in the concentration of the solution applied to the TLC plate although Rf values of compounds from column IIIa differed dramatically from the parent column. It might also be possible that decomposition of compounds had taken place and artefacts had formed which contributed to a more complex chromatogram.

8.1.4 Preparative TLC

Preparative TLC was carried out on sample 2C of the hexane fraction because of its relative low complexity as seen with TLC. Although its MIC value lay in the order of 0.18 mg/ml, the mass was calculated at 120 mg and was selected for PTLC. Seven fractions were scraped off and eluted first with acetone and then 1% acetic acid in methanol to ensure full compound recovery. MICs were calculated using *S. aureus* as test organism and lay in the order of 0.09 – 1.31 mg/ml.

Fraction VII was not tested since the extract smelled very strongly of acetic acid and the component could not be recovered. Although all extracts were fairly active, the complexity was still too high for structural elucidation and since the masses ranged between 6 and 21 mg, it was impractical to purify them further. No further experiments with these extracts were carried out.

PTLC was also attempted to purify some extracts obtained from the chloroform fraction but most of these separations did not prove fruitful, as the resulting fractions were never clean enough for further analysis. The only separation, which resulted in NMR and MS analysis was that of fraction C3, which was relatively clean before application to the PTLC plate, however still no structure could be elucidated for this sample.

8.1.5 GC/MS

This type of analysis requires that the sample to be tested be converted to gas under the influence of a high temperature (215°C). Fraction 8 of the hexane fraction had begun to precipitate in solution and was considered a good candidate for GC/MS. Since no prior idea of structure was known, it was decided, under recommendation, to use a steroid analysis. Both the supernatant and precipitate were tested and compared with similar results. Although one steroid structure was seen with the precipitate, it had disappeared upon silinization with TMSI. Silinization converts polar compounds to non-polar compounds so they may be picked up and analysed by the apparatus. Disappearing of compounds could be due to either unbinding of the compound or misanalysis of original structure. Many of the compounds appeared to be long chain fatty acids, which explains why hexane is used as a defatting solvent. Several fatty acids were identified, including octacosane, tetradecanoic acid, stearic acid and eicosanoic acid. Since these compounds were not pure, bioactivity could not be tested and more focus was placed on the chloroform fraction. Waxes and fatty acids tend to precipitate under reduced temperatures and some exhibit high antimicrobial activity possibly indicating that the major source of antimicrobial compounds in *C.*

erythrophyllum might lie in the waxy, outer layers of the leaf material and warranting further investigation into this area.

8.1.6 HPLC

HPLC was used to determine the purity of the Toyopearl isolated compounds. In preparation for application onto the reverse phase column, samples were spotted on RP-TLC plates and eluted with varying ratios of methanol:water. Seeing that the best separation was obtained with 10% water/methanol, this was the choice eluting solvent for the HPLC analysis. All the samples were dissolved in acetone and since mobile phases interfere with compound analysis it is essential to determine where these peaks will appear in relation to the compound peaks. Various flow rates and sample dilutions were tested in an attempt to obtain the optimal separation but with little success. Identification of the compound peaks was not possible since the solvent peaks absorbed at similar wavelengths and therefore impossible to differentiate between them. Another problem was the small quantity of sample, which retarded further experimentation and this type of analysis was subsequently abandoned.

8.2 IDENTIFICATION

Identification of compounds usually involves a combination of various techniques including nuclear magnetic resonance (NMR), mass spectrometry (MS), ultraviolet (UV) and infrared (IR) spectrometry. Other ways of confirming the identification of the compound include calculation of Rf values in different solvent systems and determination of the melting point. In this study NMR was used as a tool for identifying the structure and confirmed with ^{1}H-^{1}H-COSY-LR and MS. Rf values were calculated in some cases using CEF, BEA and 2A:3MDC as the mobile phases. UV and IR were not required as structural elucidation was complete with the techniques used.

8.2.1 Nuclear magnetic resonance (NMR)

The most general technique is ^{1}H NMR spectroscopy but with advances and greater availability of ^{13}C NMR, the past few years has seen an increase in ^{13}C NMR. This technique provides information on the carbon skeleton of the molecule and is regarded as complementary to ^{1}H NMR since it relates more closely to the structural environment of the flavonoid nucleus [Wollenweber, 1988].

^{13}C NMR performed in this study was a DEPT experiment, which failed to detect all the carbon resonances and therefore values were not obtained for all compounds. This didn't affect the structural elucidation since ^{1}H NMR was more than effective in determining the compound structures.

^{1}H-^{1}H-COSY-LR is Long Range COrrelation SpectrometrY designed to give a two dimensional pattern of the protons in relation to one another. This technique was of value in confirming the positions of methoxyl groups on the A- and C-ring.

All the compounds sent for NMR had precipitated out of solution after column chromatography had been performed and the test-tubes had been left to dry. In total 54 samples were sent for analysis and seven compounds were identified, all of them flavonoids (three flavones and four flavonols). Most of the compounds were replicas or mixtures and some were not clean enough for structural elucidation.

8.2.2 Mass Spectrometry (MS)

High Resolution Electron Impact Mass Spectrometry (HREIMS) serves as a valuable aid in determining the structures of flavones and flavonols, especially when only very small quantities are available. Most aglycones are sufficiently volatile at probe temperatures of 100-300°C to allow for successful MS without derivatization [Wollenweber, 1980]. Kingston [1973] suggested that this type of mass spectrometry provides more structural information than chemical ionization and must therefore remain the method of choice for these compounds.

HREIMS was used to confirm the structures identified with NMR and led to the characterisation of seven flavonoids.

8.3 FOCUS ON FLAVONOIDS

Flavonoids were isolated by Albert Szent-Gyorgi, MD of Hungary and researched during the 1940s and '50s [Berkoff, 1998]. They are 15-carbon compounds consisting of two aromatic rings A and B and an oxygen containing heterocyclic ring C. Depending on the oxidation level of ring C, they are classified into several groups, i.e. chalcones, flavanones, flavones, isoflavones and flavonols, and are collectively known as the “yellow pigments” and the coloured “anthocyanin pigments” [Ibrahim, 2000]. The widely occurring flavonols, i.e. kaempferol, quercetin and myricetin, do not contribute to the yellow colour since they are more or less colourless at the pH values normally found in cells and are often abundant in white, ivory or cream coloured petals. Flavonols are therefore isolated from a range of yellow-petalled plants in order to assess their contribution, if any, to flower colour. A significant proportion of yellow-petalled plants that have been examined contain both carotenoid and flavonoid, pigments produced by quite unrelated biosynthetic pathways. It is also clear that for a flavonol to contribute to yellow flower colour it must possess some structural feature absent from the commonly-occurring flavonols [Harborne, 1965].

Flavonoid compounds may undergo further enzymatic hydroxylation, methylation, glycosylation, sulfonation, acylation and/or prenylation reactions, thus resulting in the immense diversity of flavonoid structures which amount to 12 subcategories [Berkoff, 1998] and more than 5000 identified compounds that are found in nature [Ibrahim, 2000].

Flavonoids are present mainly as glycosides, which are water-soluble and located in cell sap but Wollenweber [1980] suggests that an increasing number of free aglycones are being found in various plant species.

8.3.1 Biosynthesis

Enzymatic and genetic studies, coupled with the use of efficient methods for isolation and identification of flavonoids, have allowed the elucidation of the pathways involved in the biosynthesis of the different groups of flavonoids [Ibrahim, 2000].

All flavonoids derive their carbon skeletons from two basic compounds, malonyl-CoA (synthesised from the glycolysis intermediate acetyl-CoA and carbon dioxide) and the CoA ester of a hydrocinnamic acid [Figure and Table 8.1]. Both of these precursors are derived from carbohydrates [Heller and Forkmann, 1988]. The aromatic ring B and its adjacent 3-carbon side-chain are derived from L-phenylalanine via the shikimate pathway, whereas ring A is formed by the head-to-tail condensation of three acetate units via the polyketide pathway that is proposed for the biosynthesis of phloroglucinol and resorcinol derivatives [Ibrahim, 2000] leading to the formation of a C15 chalcone intermediate [Heller and Forkmann, 1988].

Flavonoids, aurones and other diphenylpropanoids are derived from the C-15 chalcone intermediate and the first flavonoid, naringenin, is formed by stereospecific action of chalcone isomerase on this compound. Oxidative rearrangement of this flavonone yields an isoflavone, genistein, which is catalysed by isoflavone synthase. Introduction of a double bond between C-2 and C-3 of the flavonone leads to the abundant class of flavones of which the first and most simple one is apigenin. Dihydroflavonols (dihydrokaempferol) are formed by direct hydroxylation of flavanones in the 3-position, which is catalysed by flavanone 3-hydroxylase.

Dihydroflavonols are biosynthetic intermediates in the formation of flavonols, catechins, proanthocyanidins and anthocyanidins. The large class of flavonols (e.g. kaempferol) is formed by introduction of a double bond between C-2 and C-3 of the dihydroflavonols [Heller and Forkmann, 1988].

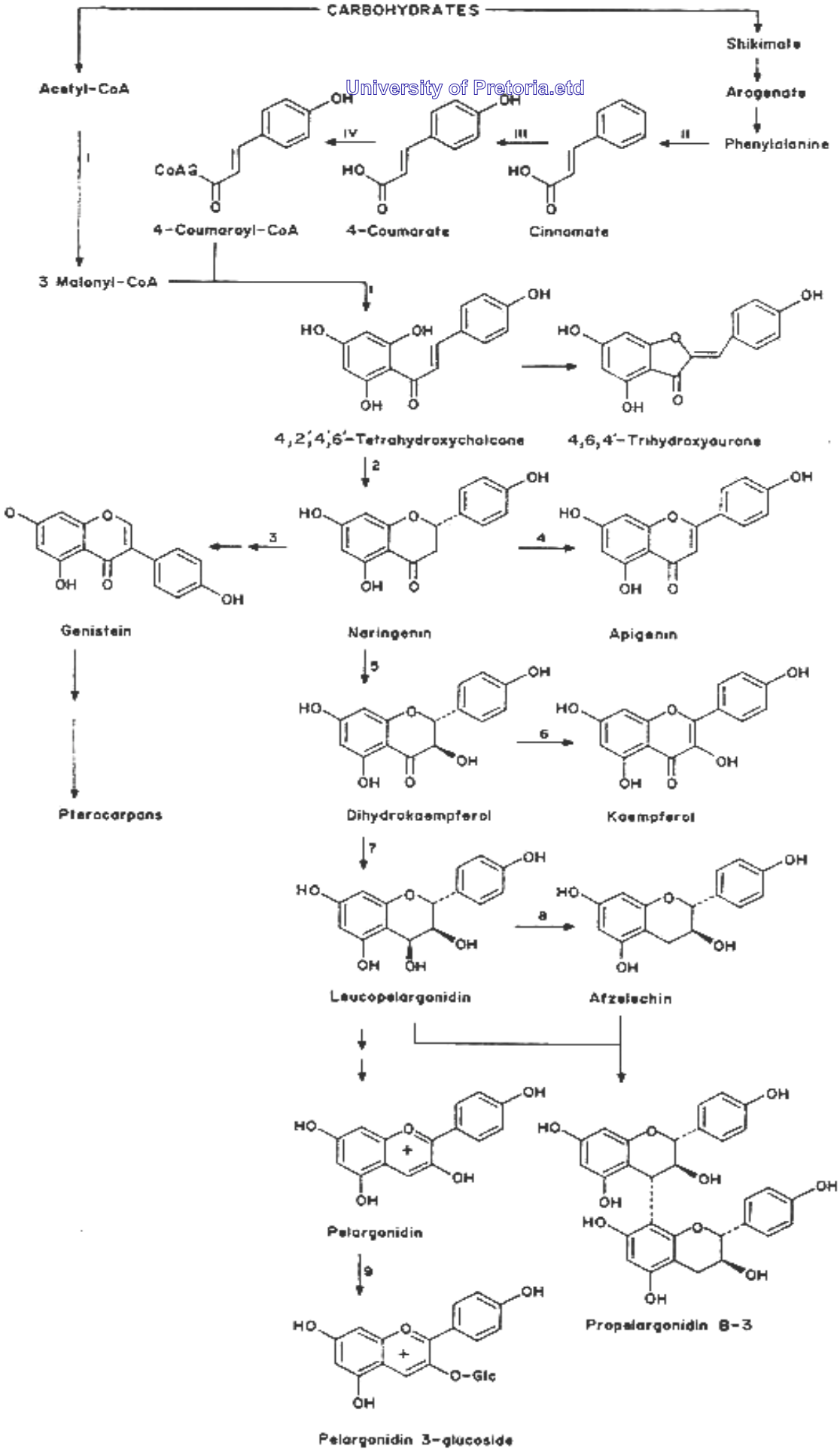


Figure 8.1: Biosynthesis pathway of flavonoids [From Heller & Forkman, 1988]

Table 8.1: List of enzymes leading to various flavonoid classes [adapted from Heller and Forkmann, 1988]

Enzymes
Non-flavonoid precursors
I Acetyl-CoA carboxylase
II Phenylalanine ammonia-lyase
III Cinnamate 4-hydroxylase
IV 4-Coumarate: CoA ligase
Flavonoid classes
1 Chalcone synthase
2 Chalcone isomerase
3 2-Hydroxyisoflavanone synthase
4 Flavone synthase
5 (2S)-Flavanone 3-hydroxylase
6 Flavonol synthase
7 Dihydroflavonol 4-reductase
8 Flavan-3,4-cis-diol 4-reductase
9 Anthocyanidin/flavonol 3-O-glucosyltransferase

8.3.1.1 Pathways to precursors of flavonoid formation:

Acetyl –CoA carboxylase catalyses the reaction in the presence of ATP and Mg^{2+} and has been extensively studied in relation to fatty acid biosynthesis [Lowenstein as quoted by Heller and Forkmann, 1988].

A great number of phenolic compounds in plants are derived from the hydrocinnamic pathway and in many plants specific classes can be stimulated by exogenous factors. UV irradiation for example induces flavonoid glycoside synthesis in parsley cell cultures whereas furanocoumarin phytoalexins are formed on treatment of parsley cells with fungal cell wall preparations (so-called elicitors). In soybean and many other legumes, the same elicitors give rise to the formation of isoflavonoid phytoalexins and *Arachis* of stilbenoids.

The second enzyme of the hydrocinnamic acid metabolism, cinnimate 4-hydroxylase, is located at the endoplasmic membrane and consists essentially of a cytochrome P-450 and NADPH-cytochrome P-450 reductase. Cytochrome P-450 inhibitors such as ketokonazole inhibit this enzyme.

Evidence suggests that 4-coumarate is the main or even exclusive physiological precursor for flavonoids in many plants [Heller and Forkmann, 1988].

8.3.1.2 Individual steps to flavonoid classes

Chalcone synthase is regarded as the key enzyme of flavonoid biosynthesis with the intermediate product being the chalcone naringenin. Chalcone synthases from several plants are more or less strongly inhibited by the flavanone formed in the reaction. A possible role for this effect may be in protecting the cell from phytotoxic concentrations of this product.

With a few exceptions, the various flavonoid classes are derived from a flavanone intermediate. Flavanones are formed from chalcones by isomerization and only flavanones with an (*S*)-configuration act as substrates for the enzymes of the flavonoid pathway. Spontaneous isomerization of some chalcone to flavanone *in vivo* may lead to formation of moderate amounts of anthocyanins leading to an orange colour in the flowers.

In certain plant species desaturation of flavanone to flavone is catalysed by a microsomal enzyme requiring NADPH as cofactor. Both the parsley and the flower enzyme catalyse the reaction from (2*S*)-naringenin to apigenin and (2*S*)-eriodictyol to luteolin. The mechanism of the double-bond formation is still unclear. It is suggested that a 2-hydroxyflavanone is formed in the first step and that water is eliminated via a dehydratase.

Flavanone 3-hydroxylase requires 2-oxoglutarate, Fe^{2+} and ascorbate as cofactors and is rather unstable under normal conditions. Flavonol synthesis most probably proceeds via a 2-hydroxy intermediate with subsequent dehydration similar to the scheme proposed for the conversion of flavanones to flavones. Such intermediates have been shown spontaneously to eliminate water, giving rise to the respective flavonols. Dihydrokaempferol and dihydroquercetin are substrates for the *in vitro* formation of kaempferol and quercetin respectively [adapted from Heller and Forkmann, 1988].

8.3.2 Biological roles

The most powerful antioxidants are to be found among the flavonoids and 18 flavonoids are now acknowledged as having 20 times the potency of vitamin C and 50 times the potency of vitamin E. Flavonoids are essential for processing vitamin C and are needed to maintain capillary walls as well as protect against infection. Rutin is an example of a flavonoid that helps increases capillary strength and ensures fast healing. A deficiency can result in a tendency to bruise easily [Berkoff, 1998].

Research supports the antioxidant and antibacterial properties of flavonoids and their ability to reduce the risk for developing heart disease, possibly due to inhibition of oxidation of low-density lioprotein (LDL). They also inhibit inflammation by decreasing the release of inflammatory mediators and by stabilizing cell membranes [Berkoff, 1998].

Many possess antifungal properties, especially the nonpolar polymethylated flavones and the prenylated isoflavones as well as various chalcones and flavanones. Some exhibit antiallergic and anti-inflammatory activity; and 3-methylquercetin is recognised as a potent, selective inhibitor of viral RNA synthesis.

The pterocarpan phytoalexins, which have antimicrobial activities and characteristic of members of Leguminosae are included in soybean, alfalfa and chickpea [Ibrahim, 2000]. Gallates (in green tea) have also shown powerful inhibition in the growth of staphylococcus [Berkoff, 1998].

They play an important role in plant growth and development, inhibit the activity of certain enzymes, affect cellular protein phosphorylation and act as regulators of hormonal transport, as determinants of pollen germination and functionality, as stress metabolites in response to wounding and nutrient deficiencies, as potent scavengers of free radicals and in the protection against UV radiation [Ibrahim, 2000].

Genistein and daidzein are examples of two powerful antioxidants and cancer inhibitors, proven to lower the risks for breast and prostrate cancer [Berkoff, 1998].

Flavonoids can also act as signalling molecules that regulate the induction and expression of Rhizobium nodulation genes that are involved in nitrogen fixation by leguminous plants. Several flavonoid compounds have been implicated in plant-insect interactions, in which they act as feeding stimulants, feeding deterrents and oviposition stimulants with the potential role of improving host-plant resistance to herbivory [Ibrahim, 2000].

There are currently no dosage or toxicity levels set for flavonoids and an average American diet contains about 1000 mg per day [Berkoff, 1998].

8.3.2.1 Impact on mammalian biology

Where flavonoid structure-activity relationships have in most cases been detected, they have been implicated for immunity, inflammation and carcinogenicity. *In vitro* they inhibit the activity of a number of mammalian enzymes including histidine decarboxylase, alleviating histamine-induced gastric acid secretions; hyaluronidase, responsible for the breakage of glucosaminidic bonds involved in tumour cell invasiveness and hypersensitivity phenomena; the catecholamine-metabolising enzyme COMT resulting in adrenaline-sparing effect; lens aldose reductase, implicated in the pathogenesis of cataracts in diabetic animals; protein kinase C (serine/threonine phosphorylation) involved in a wide range of cellular activities including tumour promotion, mitogenesis, inflammation and T lymphocyte functions; and lipoxygenase which metabolises arachidonic acid released from membrane phospholipids to vasoactive leukotrienes that are involved in signal transduction and cell division processes related to immune systems and activated by hormones, neurotransmitters and growth.

Certain flavonoids exert antitoxic and hepatoprotective effects on X-irradiation-induced capillary permeability of rat intestine with the promise of use of related compounds as antagonists in the treatment of liver diseases and those associated with vascular permeability and capillary fragility.

Some of the endocrine effects include those of quercetin and hesperitin in reducing the sensitivity of membrane calcium ATPase to thyroid-hormonal stimulation of human RBCs and that of phyto-oestrogenic activity of the clover isoflavione formononetin, which competitively antagonises oestradiol binding to cytoplasmic oestrogen receptors thus causing infertility syndrome in sheep.

Most flavonoids with a free 3-hydroxyl group such as kaempferol and quercetin are mutagenic in different strains of *Salmonella typhimurium, E. coli* and *Saccharomyces cerevisiae*, but do not appear mutagenic in mammals. Quercetin has been reported to inhibit many biochemical events associated with tumour promotion such as alteration of protein kinase C and lipoxygenase activities. In general they inhibit carcinogenesis by acting as “blocking agents” via (1) inhibiting metabolic P450-mediated activation of the carcinogen to its reactive intermediates, (2) inducing the enzymes involved in detoxification of the carcinogen, and/or (3) binding to reactive forms of the carcinogen. They are known to inhibit several biochemical events associated with the transformation of non-malignant fibroblasts to sarcoma cells [Ibrahim, 2000].

8.3.4 Pharmacokinetics (adapted from Williamson, 2000]

Flavonoids and cinnamates are secondary metabolites synthesised by plants for defensive purposes i.e. they act as defence against UV light, protect against pathogen attack and are involved in repair of injury as well as play important structural roles in the plant cell wall. Most plants store phenolics attached to a hydrophilic moeity such as a sugar. This renders them more soluble and easily handled by the plant but less biologically active. Certain phenolics are particularly bioactive and have pronounced effects on mammalian cells including antioxidant activity, modulation of gene expression, enzyme inhibition and receptor binding. These effects have led to the belief that a diet rich in fruit and vegetables contribute to health. There still remain large gaps in our knowledge of uptake and metabolism of dietary phenolics and particularly on the biological effects of the phenolic metabolites.

8.3.3.1 ABSORPTION

Many phenolic aglycones are hydrophobic and passively pass through biological membranes. Linkage to a sugar or organic acid increases the water solubility and limits passive diffusion, therefore if phenolic aglycones are absorbed in the small intestine some form of transport mechanism must exist. Flavonols and flavones are absorbed from ingested food and appear rapidly in the plasma (<0.7h). This means that absorption occurs primarily in the small intestine although additional absorption from the colon may still occur. Some are absorbed more rapidly than others indicating that an attached sugar will affect the rate of absorption as the sugar can vary in type and position. It may be possible for the flavonol glycosides to be selectively absorbed in the gut. There is an interaction of some flavonol glycosides with the sodium-dependant glucose transporter (SGLT1) of the rat small intestine. They might however interact by inhibiting SGTL1 but not themselves be transported across the membrane. There is also considerable interspecies variation in SGLT1

properties and therefore conflicting date suggests that further evidence is required to determine the role of sugar transporters in flavonoid glycoside uptake.

8.3.3.2 METABOLISM

The presence of quercetin glucosides have been reported in plasma but positive proof of identification has not yet been provided. The first stage of metabolism is likely to be deglycosylation of the flavonoid glycoside, which is essential if further metabolism is to occur. This could take place either outside the cell, in the gut lumen or inside the enterocyte after transport. The cytosolic β-glucosidase present in cell-free extracts of human small intestine and liver can hydrolyse carious phenolic glucosides.

Once absorbed, phenolics will be metabolised by phase-I or phase-II enzymes. Cytochrome P450 mono-oxygenase dependant activities that may be involved include hydroxylation and demethylation. Two or more hydroxyl groups in the B-ring prevented further hydroxylation. Demethylation is observed when a methyl group is present in the 4’ position but not in the 3’ position. Several investigations of quercetin metabolism in rate have found 3’- and 4’-methylquercetin in the bile and/or the urine. The 3’-position (relative to 4’) for methylation appears to be the preferential site for catechol-O-methyltransferase activity with a ratio of >2:1. There is no evidence for methylation at any other hydroxyl groups of the flavonoids apart from the catechol grouping on the B-ring.

The liver plays a major role in the metabolism, however certain flavonoid glucosides are deglycosylated by human intestinal mucosa at a rate potentially higher than that of the liver. Conjugation with glucuronide or sulphate is the most likely metabolic routes. Conjugation increases solubility and increases molecular mass, important for excretion in the bile. Sulphation is the major pathway for many at low concentrations, although this pathway can easily become saturated, whereas glucuronidation will be dominant at higher concentrations.

8.3.3.3 EXCRETION

The major route for excretion of flavonol conjugates is probably through the bile back into the small intestine. Flavonol glucuronides will reach the colon where gut microflora release the aglycone leading to reabsorption and enterohepatic circulation.

Pharmacokinetic knowledge is extremely important if flavonoids are to be used in the treatment of disease. In microbial infections they need to be adequately absorbed and distributed to the site of infection where they are required to have selective working against the invading microorganism without damaging host cells. Excretion routes may determine whether the flavonoid will for example be effective in urinary tract infections.

Of all seven flavonoids isolated in this study, all showed varying degrees of inhibition against some of the selected organisms. Bioassays performed were all *in vitro* and hence the selectivity of these compounds for microorganisms in relation to host cells is not known. Since they do not appear toxic, it is necessary to determine their pharmacokinetic profiles in test animals at a later stage.

8.3.4 Presence of flavonoids in Combretaceae

The order Myrtales including Myrtaceae, Combretaceae and Thymeleaeceae contains 17 known flavones and 1 flavonol [Wollenweber, 1988]. With regard to accumulation of free aglycones in certain families and/or genera, Combretaceae has been found to have 1 flavone and 3 flavonols [Wollenweber, 1980] of which a few examples are given on the following page.

Terminalia arjuna	(1) 5,7,2',4'-OMe flavone present in fruit
	(2) 5,7,2',4'-OMe arjunone in fruits
Calicopteris floribundus	(3) 3,4-OH-3,6,7,8-OMe flavone
	[Calicopterin (Thapsin) in leaf]
	(4) 3,6,7,8,4'-OMe in leaf
	(5) 3,6,7,8,3'-OMe in leaf

Recently apigenin was identified in *C. apiculatum* [Katerere, unpublished 2001] but no known reports on isolated flavonoid compounds have been recorded in *C. erythrophyllum* and it is assumed that this is a first report of such findings.

Certain substitutions of the aromatic rings of flavonoids are more common in certain families than in others and the frequencies of the substitutions at each of the carbon atoms is shown in Table 8.1.

Table 8.1: Frequencies of the classical O- and C-substitutions at each of the ten substituted carbon atoms of the flavone/flavonol nucleus [adapted from Wollenweber, 1988].

	Frequencies (%)									
Substitutions	C-3	C-5	C-6	C-7	C-8	C-2'	C-3'	C-4'	C-5'	C-6'
Flavones										
H	99	10	46	10	57	76	53	30	78	94
OH	0	70	10	43	8	15	22	32	6	2
OMe	0	20	41	47	34	9	25	37	16	4
C-Me	1		3		1					
Flavonols										
H	0	7	48	1	56	85	44	14	70	100
OH	38	79	10	51	11	9	31	45	16	0
OMe	62	14	34	48	28	6	25	41	14	0
C-Me			8		5					

At C-3, hydroxyl and methoxyl substitutions are of equal frequency in ferns, Betulaceae, Fabaceae and Saxifragaceae; 3-methoxyl derivatives are more abundant in Asteraceae, Cistaceae and Geraniales and dominant in Didiereaceae, Laminaceae and Solanaceae. Hydroxylation at C-5 is commonly present among flavonols. 6-hydroxyl substitution is poorly represented in most taxa but in contrast 6-O-methylation appears to be an important element of flavone diversity. 6-C-Methyl derivatives are characteristic for Myrtales and ferns. There are reports of 7-deoxyflavonols in Rutaceae and especially Myrtales. Substitution at C-8 as absent or rare except for the flavonols from Asteraceae, Lamiaceae, Rutinaceae and Solanaceae. Lamiaceae, Myrtales and Solanaceae are remarkable for the presence of 2'-O-substitution in about 20% of flavonols. They are important since 40-50% of the flavonols from these taxa do not have 4'-O-substituents. O-substitution at C-5' is rare in flavonols and 6'-O-substitution is mainly found in Laminaceae. The C-6 position of flavonols is rarely if ever O-substituted in Cistaceae, Geraniales and Solanaceae. In contrast it is most commonly substituted in Asteraceae and Didiereaceae. O-substitution is rare in flavonols, overall frequency being 20% [Wollenweber, 1988].

Of the seven flavonoids identified in this study, all three flavones have a hydrogen group at C-3 and all four flavonols are hydroxylated. At C-5, all flavonoids are hydroxylated except for the flavonol CE46 and all have a hydrogen group at C-6. Hydroxyl substitutions at C-7 are seen in one flavone and two flavonols, the others being methoxyl substitutions. Hydrogen groups are present in all the flavonoids at C-8, C-2' and C-3' and hydroxyl groups in all flavonols at C-5'. Two flavones (apigenin and genkwanin) have hydroxyl substitutions at C-5'. At C-6' all flavonoids have a hydrogen group.

8.4 BIOASSAYS

8.4.1 Antimicrobial activity

Since phenolic substances generally have significant antimicrobial activity, it is assumed that their function in tissues where they accumulate might be to provide chemical barriers to invading micro-organisms. Swain postulated that the methylated, lipophilic flavonoids are especially suitable as protection against microorganisms because of their ease in penetration of the bacterial membranes [Wollenweber, 1980].

Flavonoids were reported by scientists in some studies to be more active against Gram-negative bacteria contrary to other literature reports indicating that Gram-positive are selectively inhibited by flavonoids and isoflavonoids derived from plants. Basile [1999] suggests that the pattern of selectivity towards Gram-positive bacteria is not restricted to compounds from plants but is a general observation amongst most antibiotics. All isolated flavonoids in this study were found to inhibit some of the selected bacteria to varying degrees and did not exhibit greater activity against either Gram-positive nor –negative organisms. Activity confirms the ethnobotanical use of this plant.

8.4.2 Cytotoxicity

Flavonoids are consumed in great quantities from the foods we eat and there are no reported toxicity levels for these compounds. Many flavonoids have however been found to be toxic to cancer cells and are currently being investigated for this use. They are believed to be implicated in the lower risk of some forms of cancer observed in Asian countries due to their capacity to control cell proliferation, act on certain regulatory enzymes as protein kinases or topoisomerases [Lopez-Lazaro, 2000a].

Genkwanin was reported to be cytotoxic to human nasopharynx carcinoma (IC_{50} 30.6 μM). Their hydroxyl groups at C-5 and C-4' and methoxy groups at C-7 and C-3' are important for inhibition of calf thymus topoisomerase I activity [Zahir, 1996]. Activity against human promyelocytic leukaemia (HL-60) cells lay in the order of 18.3 μM (ED_{50}) [Suh, 1995].

Two new rhamnazin glycosides were found to stabilize the cleavage complex human DNA topoisomerase I at concentrations in the 100-250 μM range acting as topoisomerase I poisons and a test against 3 human cancer cell lines: TK-10 (renal adenocarcinoma), MCF-7 (breast adenocarcinoma) and UACC-62 (melanoma) produced a dose-dependant inhibition of cell growth at concentration in the 10^{-6} to 10^{-4} M and was the most cytotoxic of all flavonoids and extracts tested [Lopez-Lazaro, 2000].

8.4.3 Antioxidant/anti-inflammatory

Many flavonoids exhibit remarkably high radical-scavenging activity indicating perhaps that a diet rich in these flavonoids would reduce cancer-promoting actions of these radicals and possibly other diseases and conditions caused by oxygen-related radicals [Sawa, 1999].

8.5 SUMMARY OF RESULTS

CODE	IDENTITY	MIC (µg/ml) GRAM +	MIC (µg/ml) GRAM -	ANTIOXIDANT ACTIVITY	TOXICITY
CE144	Apigenin	NT	NT	NT	NT
IIIa150	Genkwanin	25 - >100	50 - > 100	GOOD	NON-TOXIC
CE36	5-hydroxy-7,4'-dimethoxyflavone	50 - > 100	25 - > 100	POOR	POTENTIALLY TOXIC
Seph51	Kaempferol	NT	NT	NT	NT
IIIa90	Rhamnocitrin	25 - > 100	25 - > 100	STRONG	NON-TOXIC
CE51	Rhamnazin	25 - > 100	50 - > 100	STRONG	NON-TOXIC
CE46	Quercetin-5,3'-dimethylether	25 - > 100	50 - > 100	GOOD	NON-TOXIC

NT = NOT TESTED

8.6 CONCLUSION

The aim of this study was to isolate and identify the antimicrobial components from *Combretum erythrophyllum* leaf extracts and confirm the ethnobotanical use of this plant. In comparison to other *Combretum* species, *C. erythrophyllum* appears far more complex and to isolate all antimicrobial compounds is a challenging task. This is primarily because most of the compounds isolated (even those not identified) have a potential to be antimicrobial in the correct dose. Waxes isolated from the hexane fraction are strongly antimicrobial but cannot be utilised in medicine due to the non-specificity for host and bacterial cells as well as their poor pharmacokinetic profile.

In this study seven flavonoids were identified of which three were flavones, namely apigenin, genkwanin and 5-hydroxy-7,4'-dimethoxyflavone and four were the flavonols kaempferol, rhamnocitrin, rhamnazin and quercetin-5,3'-dimethylether. All these compounds are reported for the first time in *C. erythrophyllum*. Although these flavonoids did exhibit moderate antimicrobial activity, it was hardly the anticipative results. Many of these compounds are produced in response to infection (phytoalexins) and quantities produced are very small making isolation difficult. For this reason large quantities of crude material is required for proper evaluation.

As tropical forests are destroyed and tribal people acculturated, our ability to discover new pharmaceutical agents is seriously being compromised. There has been a decline in natural product research over the past few decades, combined with a reduction in global plant biodiversity [Balick, 1990]. Plants remain an untapped reservoir of potentially useful chemical compounds as drugs, as unique templates that could serve as a starting point for synthetic work, or as tools for understanding the biological processes better [Farnsworth, 1984]. Baker [1984] suggests that the question of importance is whether the traditional screens of pharmaceutical industries are adequate to ensure the detection of novel substances with novel mechanisms of action. The answer in many cases is NO. For this reason ethnobotanical studies are of increasing importance and with further work, *Combretum erythrophyllum* could contribute more towards medical science than just supplying shade on a sunny day.

CHAPTER 9

9. REFERENCES

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Williamson, E.M., Okpako, D.T., Evans, F.J. (1996) Selection, preparation and pharmacological evaluation of plant material. In: Pharmacological Methods in Phytotherapy Research Vol. 1. John Wiley and Sons Ltd., Chichester, England

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APPENDIX A

```
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Operator    : TIM
Acquired    : 25 Aug 2000    9:46      using AcqMethod STEROID5
Instrument :     GC/MS Ins
Sample Name: FRACTION 8
Misc Info   : Higher concentration
Vial Number: 1
```

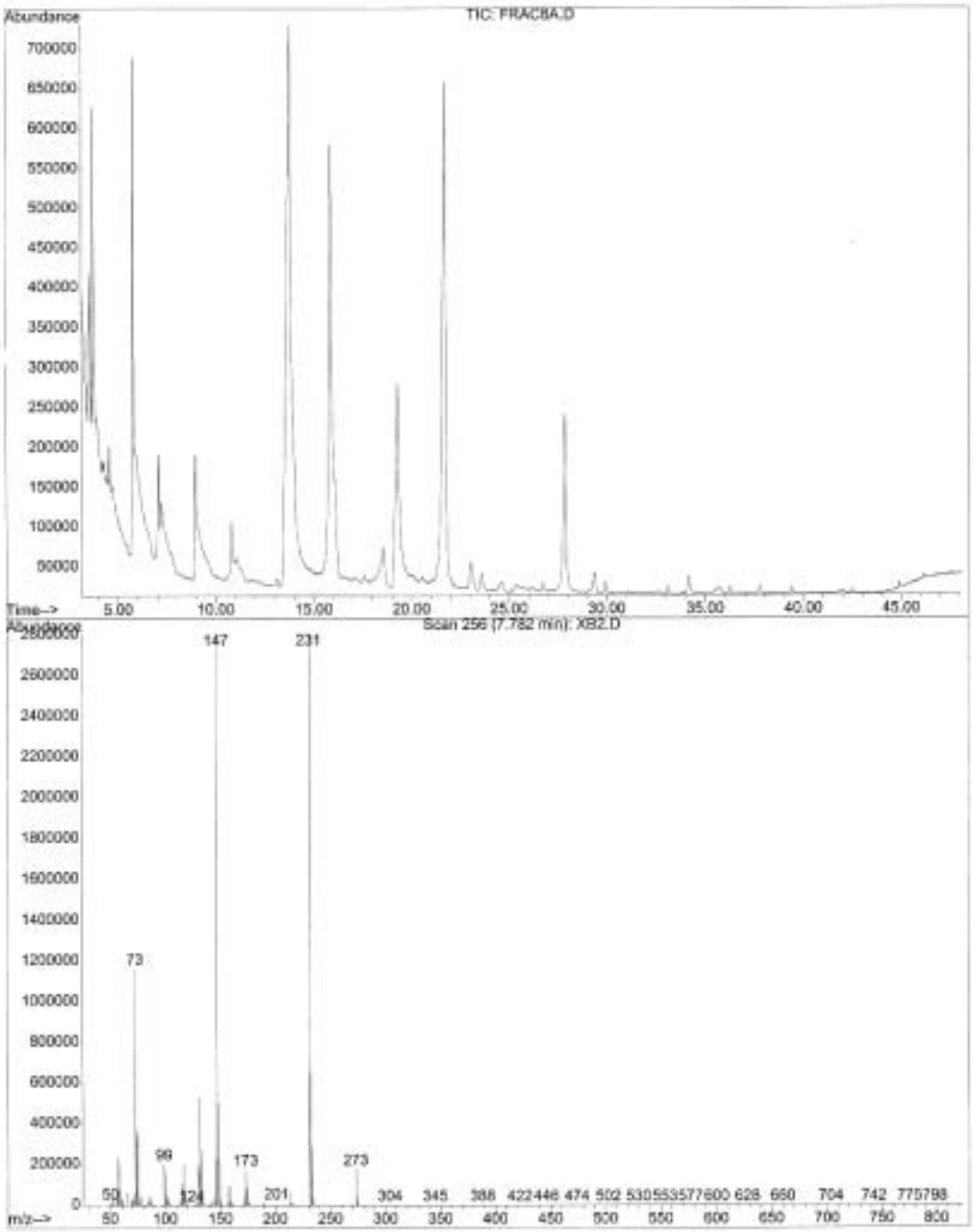

Figure A1: GC/MS spectrum of the whole Fraction 8 precipitate

```
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                   INIC ACID $$ n-Tetradecanoic acid $$ neo-Fat 14 $$
                   Univol U 316S $$ n-Tetradecoic
```

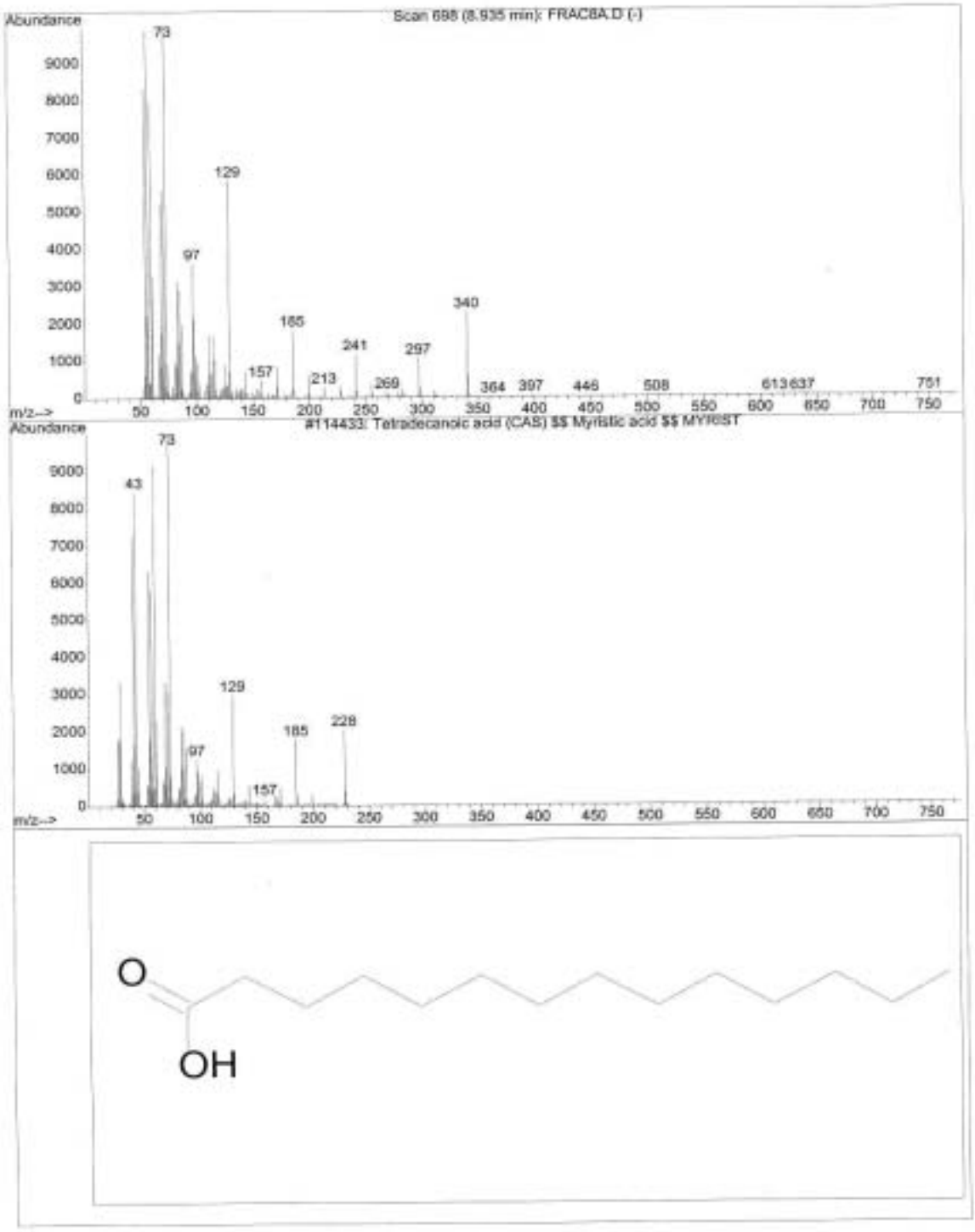

Figure A2: GC/MS data of one possible compound isolated from the Fraction 8 precipitate depicting a 95% probability of tetradecanoic acid

```
Library Searched : C:\DATABASE\WILEY275.L
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                   canoic acid $$ PD 185 $$ NAA 173 $$ Vanicol $$ Kam
                   3000 $$ Kam 1000 $$ Kam 2000 $
```

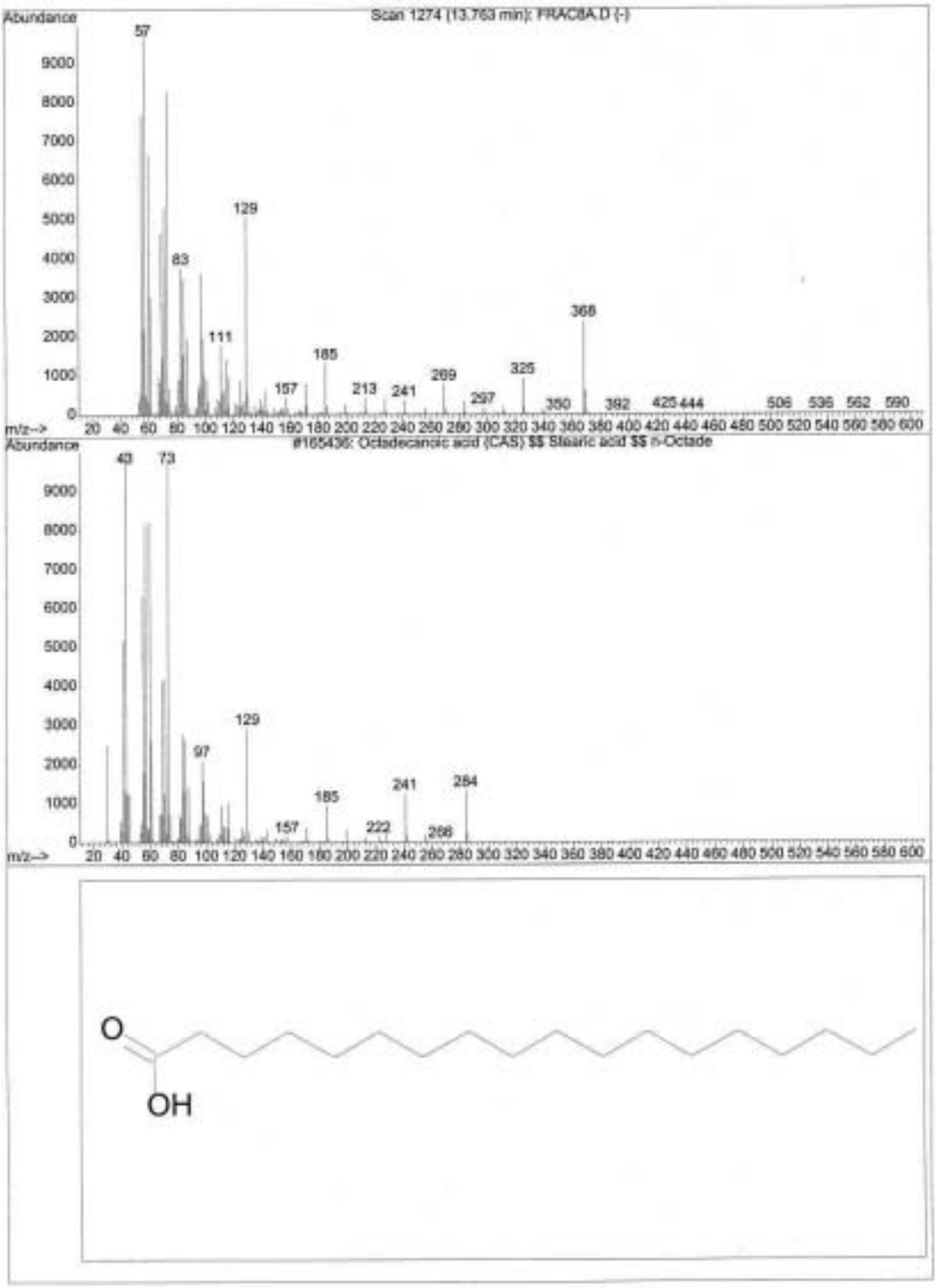

Figure A3: GC/MS data of one possible compound isolated from the Fraction 8 precipitate depicting a 64% probability of octadecanoic acid

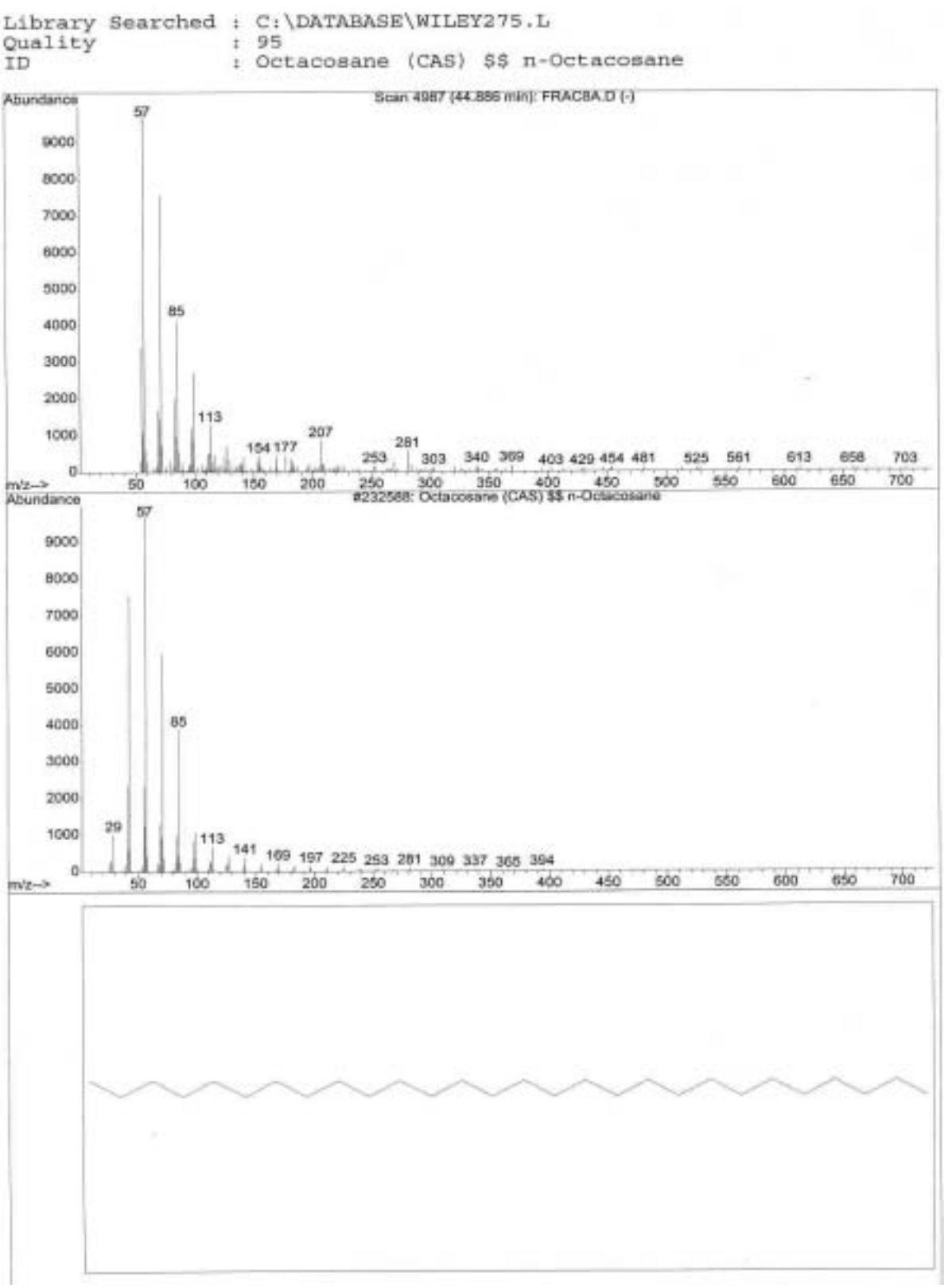

Figure A4: GC/MS data of a possible compound isolated from the Fraction 8 precipitate showing a 95% probability of the alkane octascosane

```
Library Searched : C:\DATABASE\WILEY275.L
Quality          : 25
ID               : Pregnan-20-one, 3,11-dihydroxy-, (3.beta.,5.beta.,1
                   1.beta.)- (CAS) $$ 3.BETA.,11.BETA.-DIHYDROXY-5.BET
                   A.-PREGNAN-20-ONE
```

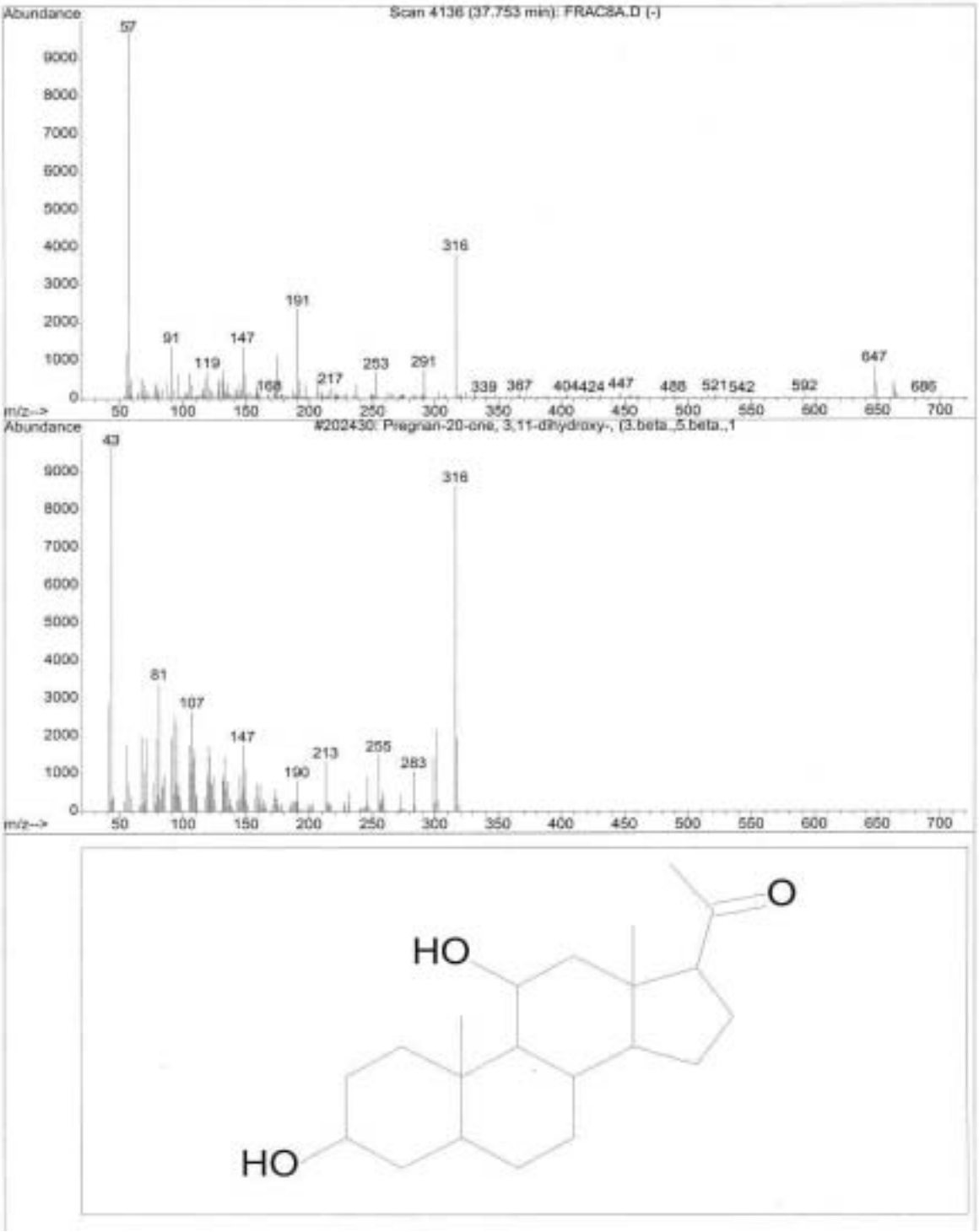

Figure A5: GC/MS data of Fraction 8 precipitate showing a steroid structure (25% probability)

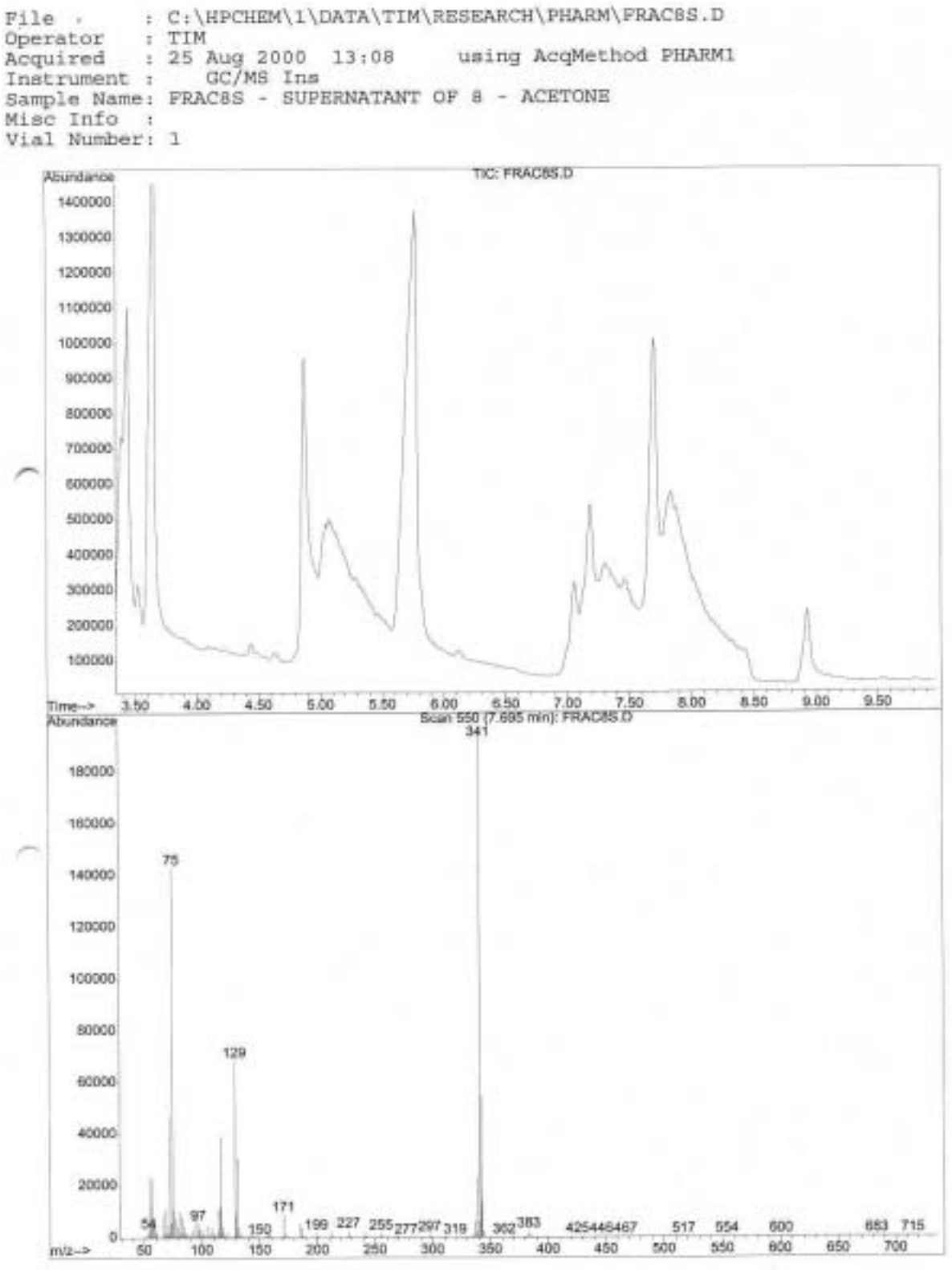

Figure A6: GC/MS data of the Fraction 8 supernatant

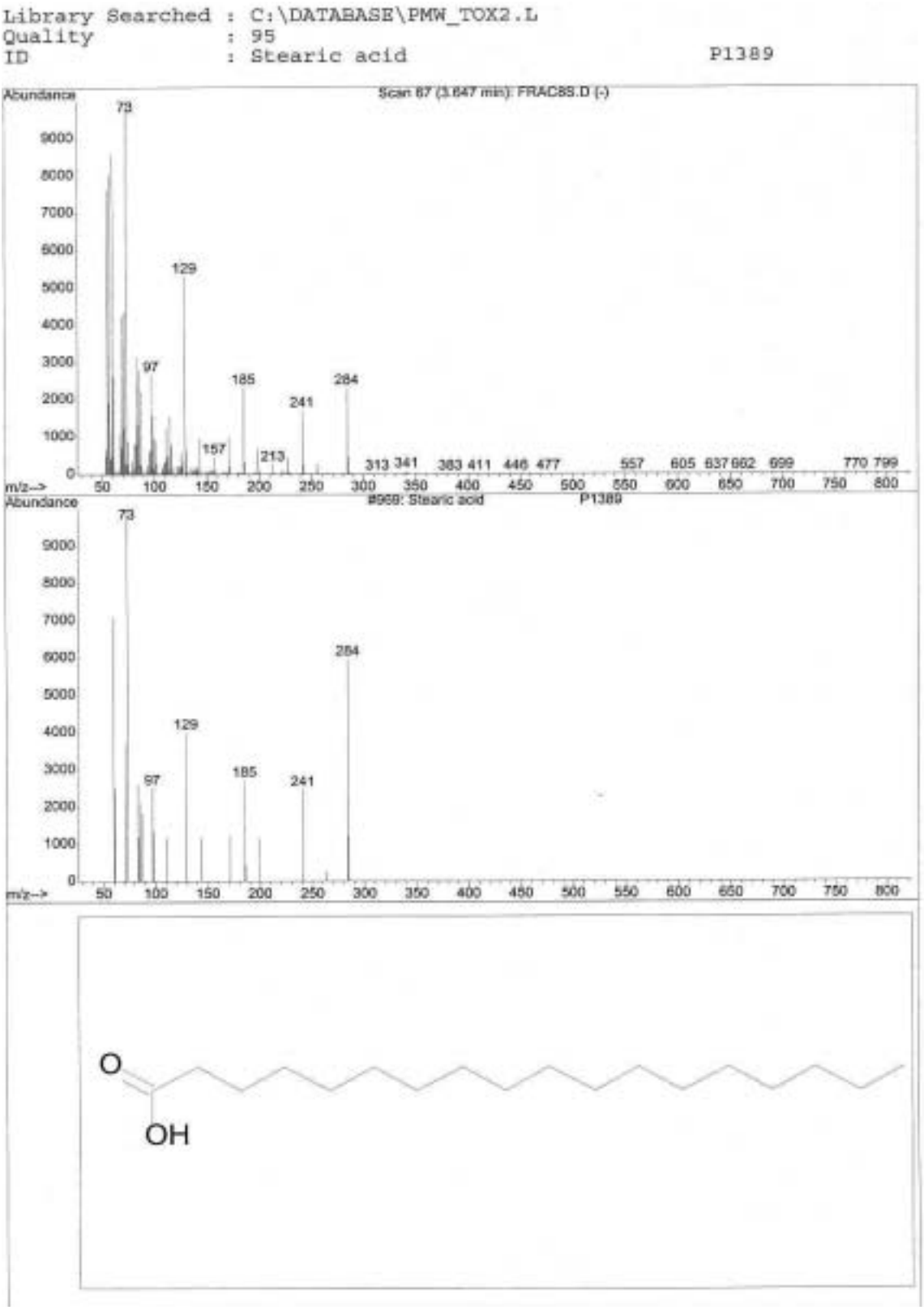

Figure A7: GC/MS data of one possible compound isolated in the supernatant of Fraction 8 depicting a 95% probability of stearic acid

```
File       : C:\HPCHEM\1\DATA\TIM\RESEARCH\PHARM\FRAC8A1.D
Operator   :
Acquired   : 29 Aug 2000    8:51     using AcqMethod STEROID5
Instrument :
  GC/MS Ins
Sample Name: frac8a1
Misc Info  : Silinized frac8 - TMSI
Vial Number: 1
```

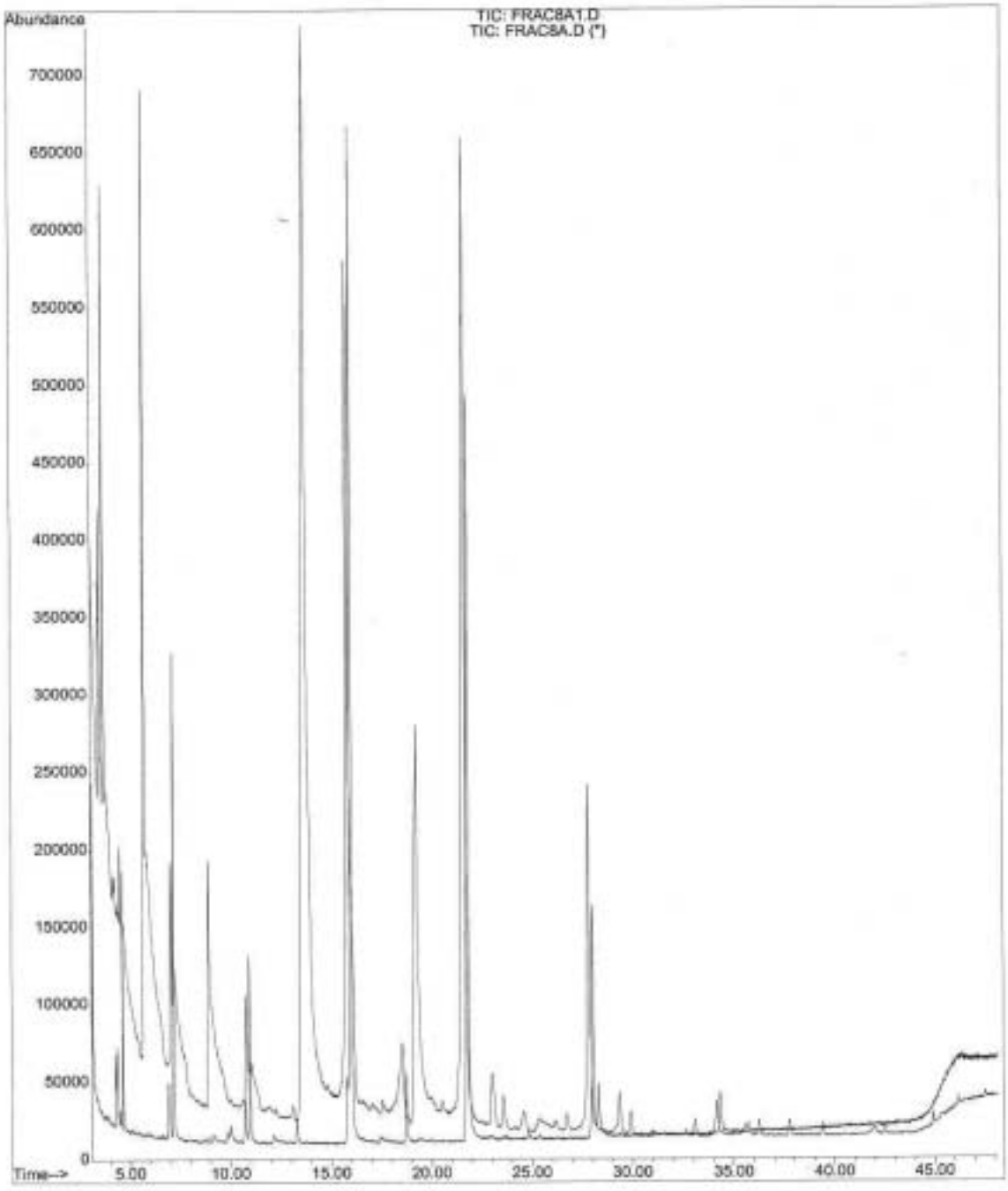

Figure A8: GC/MS showing the precipitate of Fraction 8 superimposed on the silinized precipitate

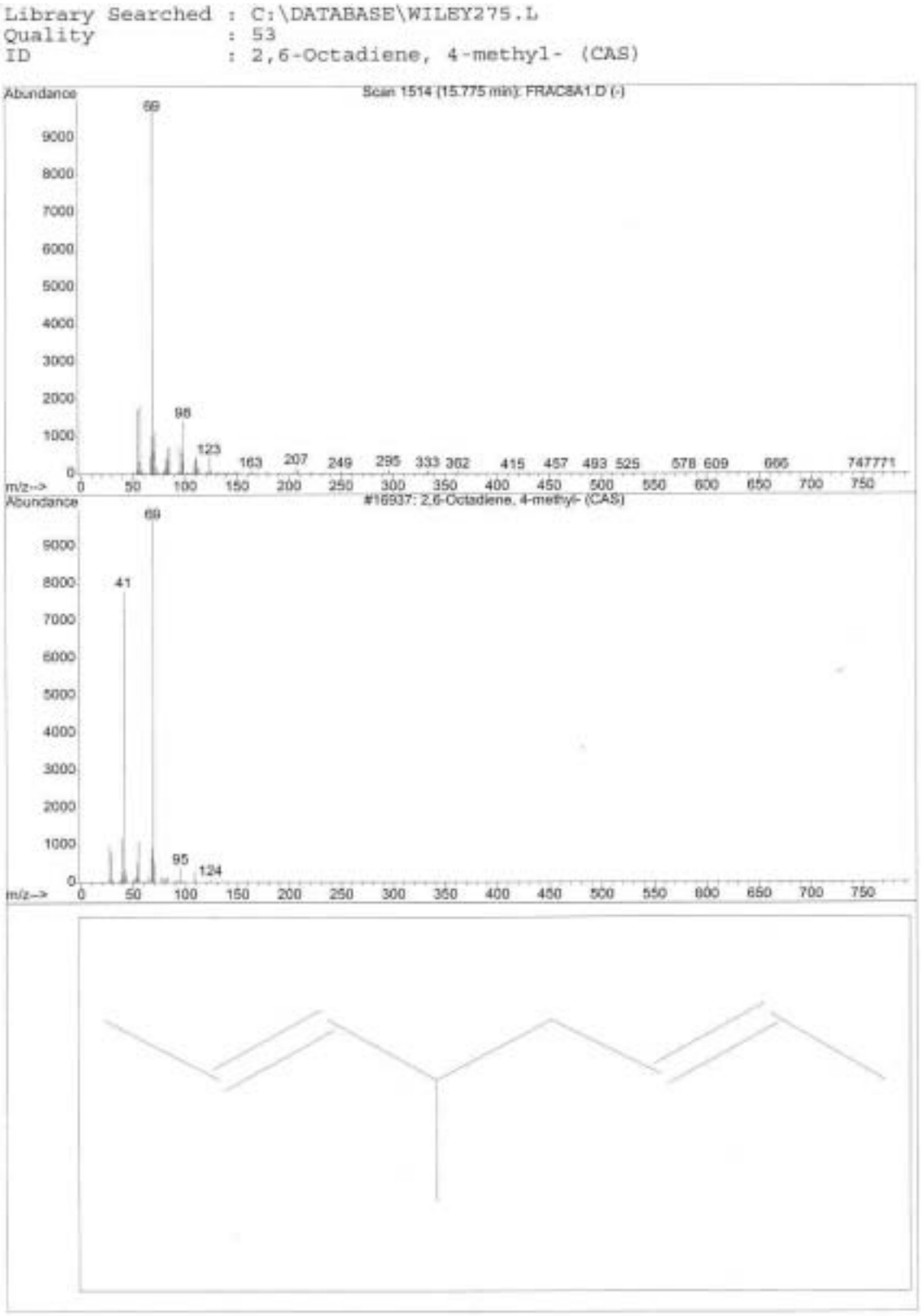

Figure A9: GC/MS data of one possible compound isolated in the silinized precipitate of Fraction 8 depicting a 53% probability of an alkene

```
Library Searched : C:\DATABASE\WILEY275.L
Quality          : 78
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                   TRIMETHYLSILYL ARACHIDIC ACID $$ Arachidic acid, tr
                   imethylsilyl ester
```

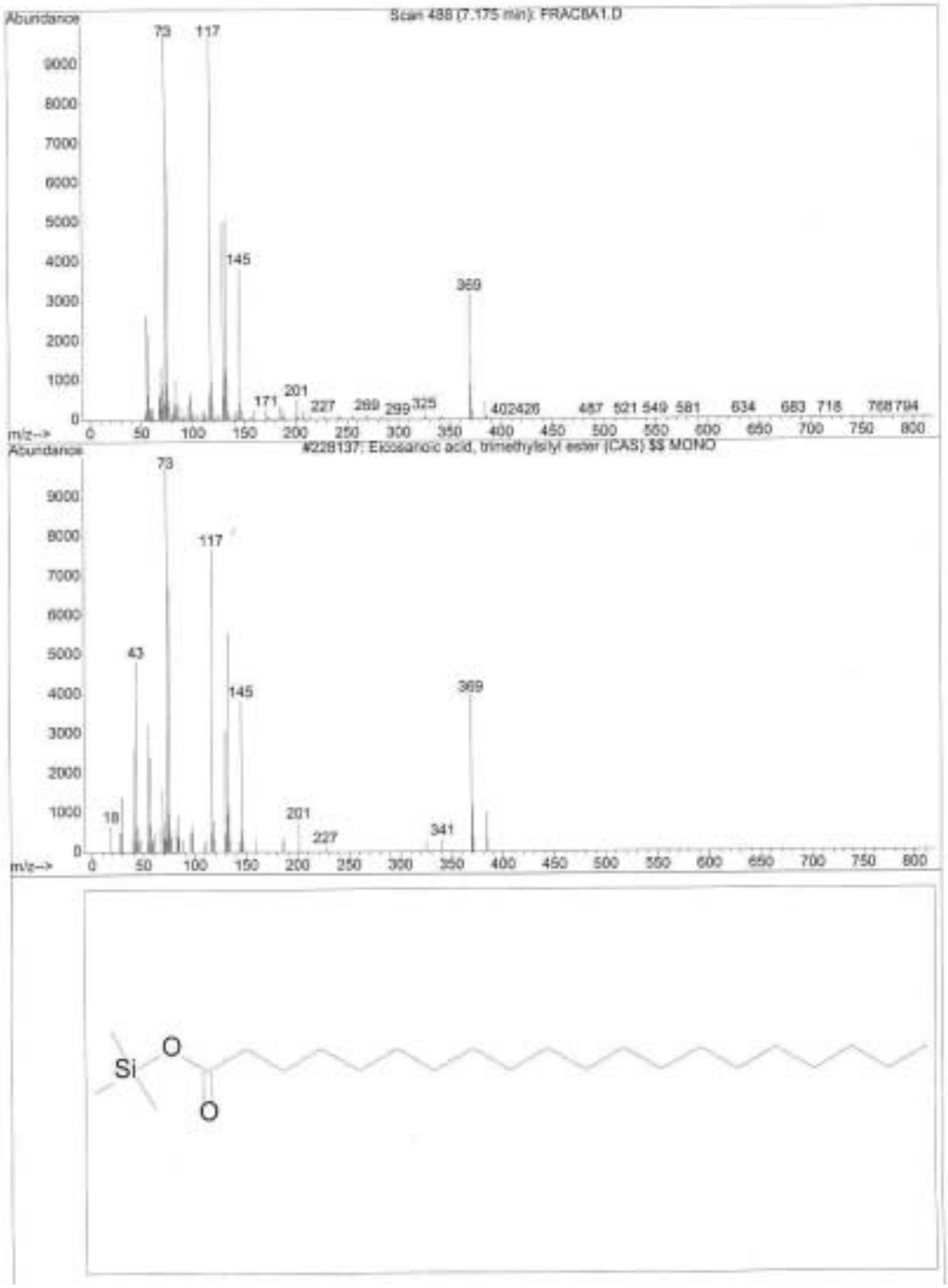

Figure A10: GC/MS data of one possible compound isolated in the silinized precipitate of Fraction 8 depicting a 78% probability of eicosanoic acid

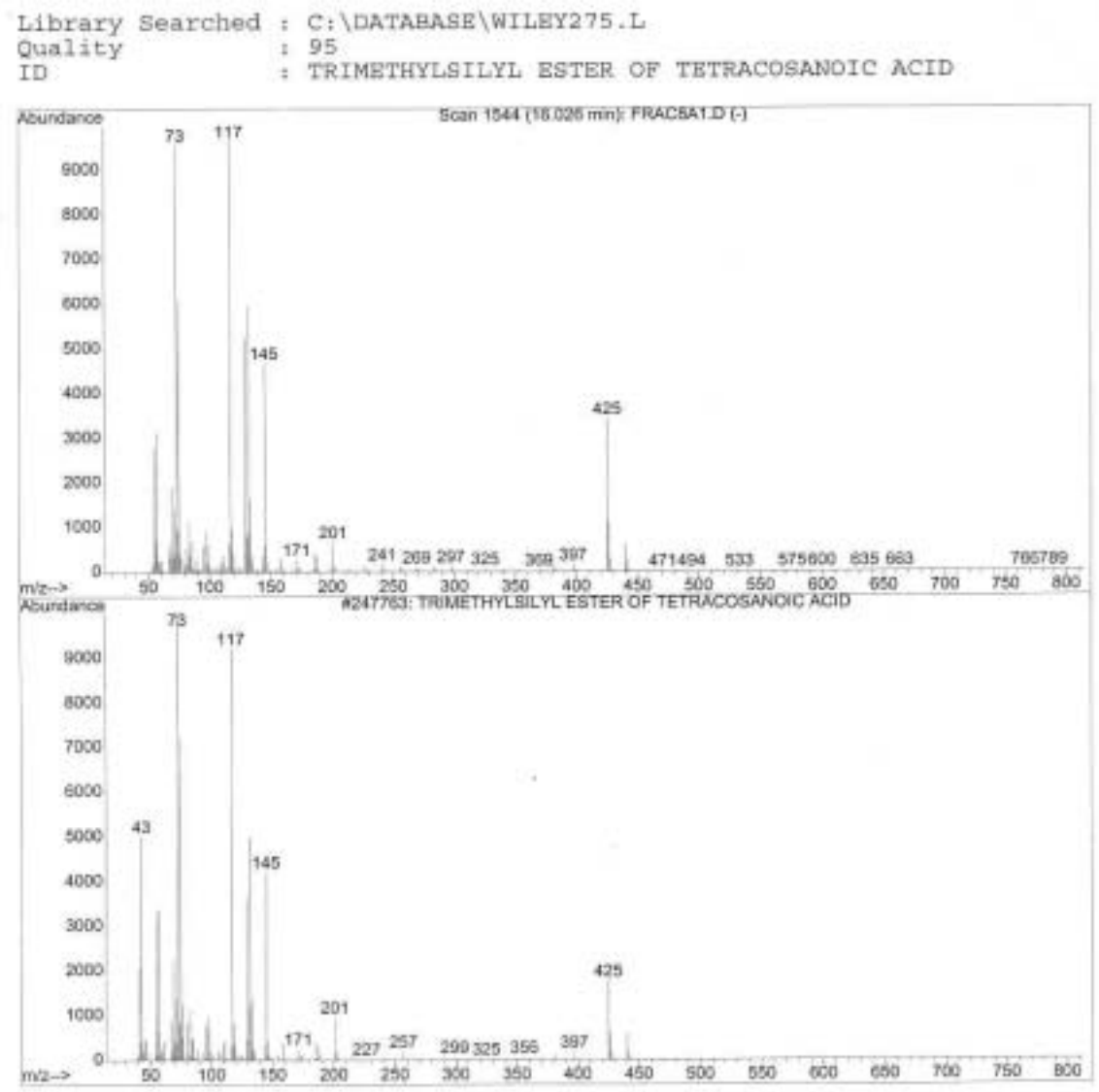

Figure A11: GC/MS data of a possible compound isolated in the silinized precipitate of Fraction 8 depicting a 95% probability of an ester of tetracosanoic acid

APPENDIX B

Injection Date : 2001/02/09 01:58:38 PM
Sample Name : acetone test Vial : 6
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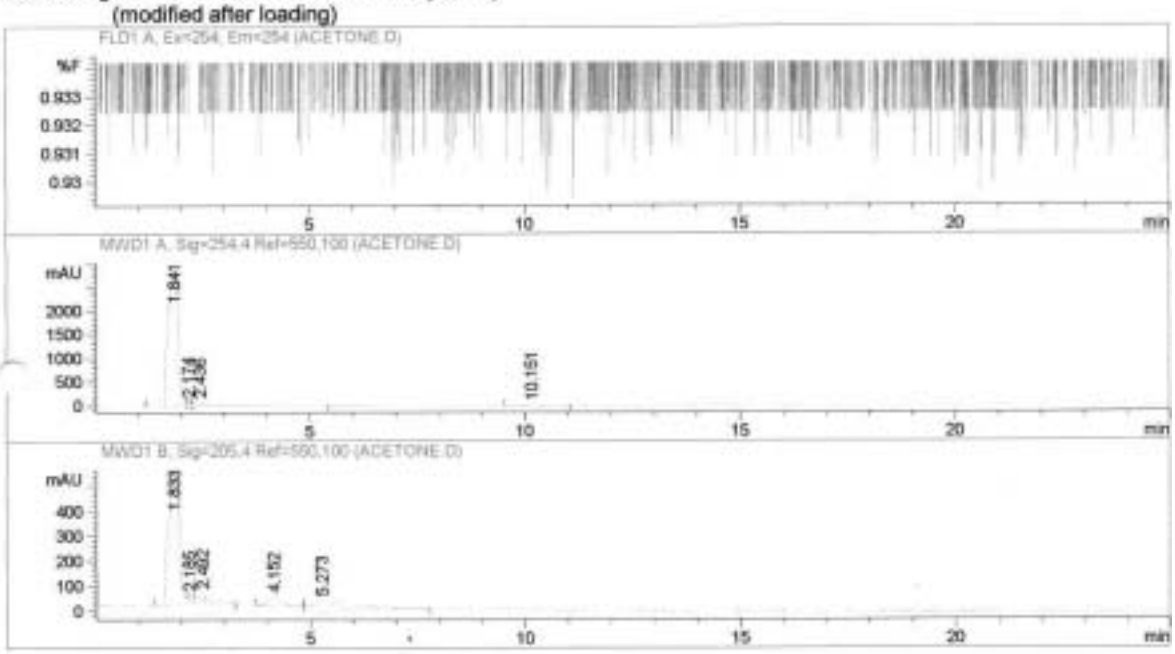

Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
.lution : 1.0000
Sample Amount : 10.00000 [ng/ul] (not used in calc.)

Signal 1: FLD1 A, Ex=254, Em=254

Signal 2: MWD1 A, Sig=254,4 Ref=550,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.841	PV	0.2718	4.71329e4	2867.14868	93.6749
2	2.174	VV	0.1092	438.22711	66.89174	0.8710
3	2.436	VB	0.5411	2680.06567	63.41471	5.3265
4	10.151	BB	0.6168	64.18256	1.32582	0.1276

Totals : 5.03154e4 2998.78096

Signal 3: MWD1 B, Sig=205,4 Ref=550,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.833	BV	0.2816	8851.36035	512.25281	73.6833
2	2.185	VV	0.1197	285.48730	32.11817	2.3765
3	2.492	VB	0.3300	1064.31470	41.11828	8.8599
4	4.152	PB	0.3401	670.81012	31.03953	5.5842
5	5.273	BP	0.6842	1140.73071	20.27998	9.4960

Totals : 1.20127e4 636.80877

Figure B1: HPLC data of acetone run at a flow rate of 1.5 ml/min

Injection Date : 2001/02/09 11:14:25 AM
Sample Name : methanol Vial : 4
Acq. Operator : nataly
Acq. Method : C:\HPCHEM\1\METHODS\NATALY.M
Last changed : 2001/02/09 09:54:40 AM by nataly
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Last changed : 2001/02/09 11:40:30 AM by nataly
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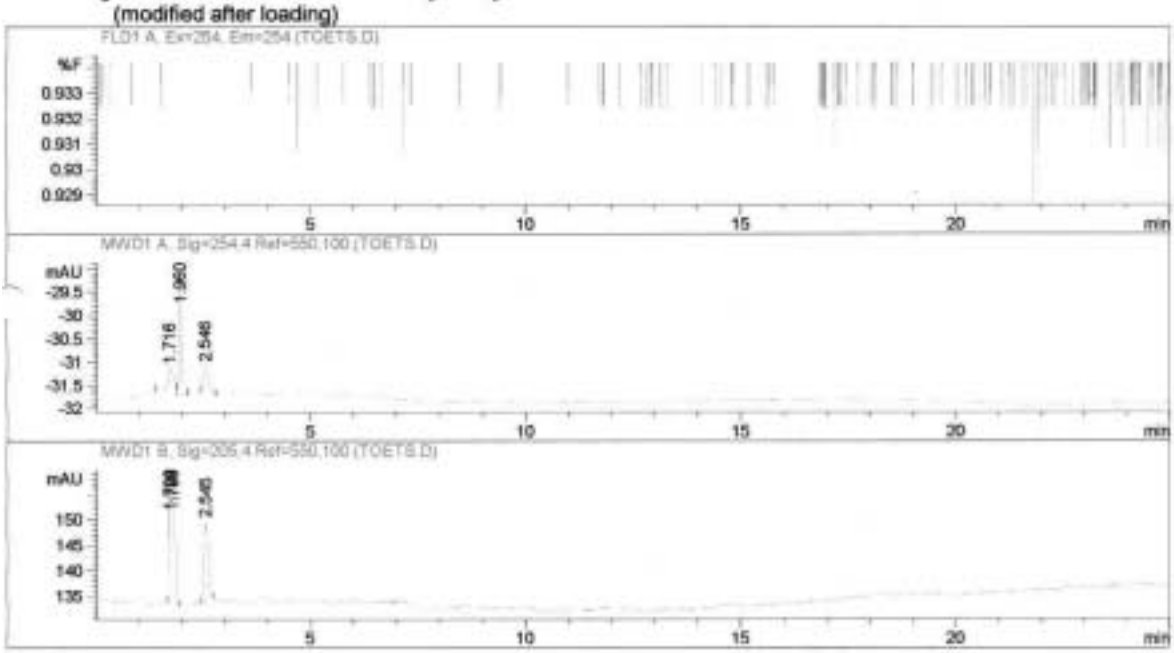

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Area Percent Report

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Sorted By : Signal
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Dilution : 1.0000
Sample Amount : 10.00000 [ng/ul] (not used in calc.)

Signal 1: FLD1 A, Ex=254, Em=254

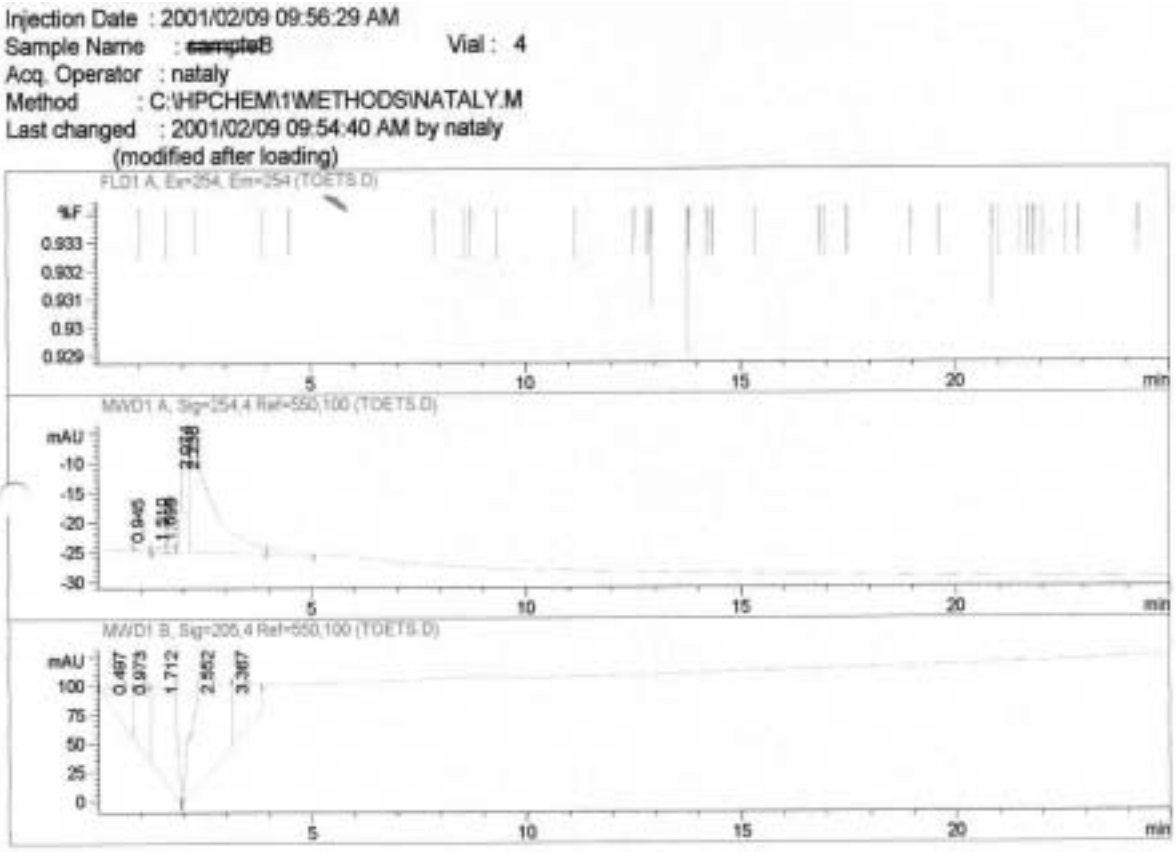
Signal 2: MWD1 A, Sig=254,4 Ref=550,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.716	BV	0.1831	7.38356	5.34787e-1	34.1738
2	1.960	VB	0.0540	9.58527	2.66080	44.3642
3	2.546	BB	0.1201	4.63705	5.62845e-1	21.4620
Totals :				21.60589	3.75843	

Signal 3: MWD1 B, Sig=205,4 Ref=550,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.709	BV	0.0448	59.86104	21.28381	18.8665
2	1.786	VB	0.0871	148.46460	25.05907	46.7917
3	2.545	PB	0.1056	108.96241	15.57163	34.3418
Totals :				317.28805	61.91450	

Figure B2: HPLC data of methanol run at a flow rate of 1.5 ml/min



Injection Date : 2001/02/09 09:56:29 AM
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Acq. Operator : nataly
Method : C:\HPCHEM\1\METHODS\NATALY.M
Last changed : 2001/02/09 09:54:40 AM by nataly
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Area Percent Report

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rted By : Signal
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Dilution : 1.0000
Sample Amount : 10.00000 [ng/ul] (not used in calc.)

Signal 1: FLD1 A, Ex=254, Em=254

Signal 2: MWD1 A, Sig=254,4 Ref=550,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	0.945	VP	0.1616	7.04754	5.80712e-1	0.7002
2	1.510	VV	0.2169	13.19358	8.70076e-1	1.3108
3	1.699	VV	0.1360	10.36509	1.04252	1.0298
4	2.074	VV	0.1908	243.72751	19.36458	24.2143
5	2.236	VB	0.4780	732.20880	18.48874	72.7449

Signal 3: MWD1 B, Sig=205,4 Ref=550,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	0.497	BV	0.4618	1086.69458	31.24513	9.3295
2	0.973	VV	0.3413	1333.60535	51.64213	11.4493
3	1.712	VP	0.4289	2984.38452	90.46104	25.6215
4	2.552	VV	0.7035	4560.66357	86.44473	39.1542
5	3.367	VB	0.4553	1682.61987	46.81979	14.4456

Figure B3: HPLC data of purified water run at a flow rate of 1.5 ml/min

Injection Date : 2001/02/09 11:45:48 AM
Sample Name : sample B Vial : 5
Acq. Operator : nataly
Acq. Method : C:\HPCHEM\1\METHODS\NATALY.M
Last changed : 2001/02/09 11:40:30 AM by nataly
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\NATALY.M
Last changed : 2001/02/09 12:12:25 PM by nataly
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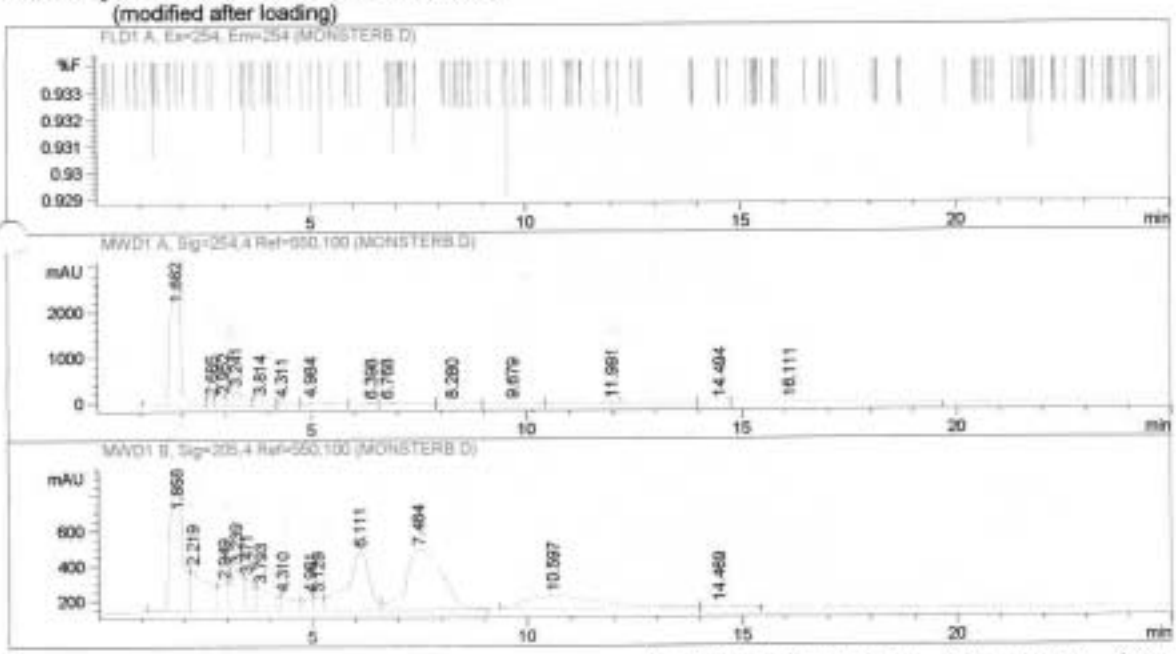

Area Percent Report

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Sample Amount : 10.00000 [ng/ul] (not used in calc.

Signal 1: FLD1 A, Ex=254, Em=254

Signal 2: MWD1 A, Sig=254,4 Ref=550,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.862	BV	0.1978	4.67141e4	2996.96582	72.3573
2	2.685	VV	0.1394	980.42694	95.77476	1.5186
3	2.952	VV	0.1904	1912.69397	144.49033	2.9626
4	3.241	VV	0.3377	6756.17139	287.98752	10.4649
5	3.814	VV	0.3506	2436.35278	100.59360	3.7738
6	4.311	VV	0.3531	1029.00806	38.34810	1.5939
7	4.984	VV	0.5365	1553.67346	37.10403	2.4065
8	6.398	VV	0.4281	414.12177	12.84757	0.6414
9	6.768	VB	0.5113	529.69379	12.89420	0.8205
10	8.280	BV	0.5178	313.77634	8.21145	0.4860
11	9.679	VV	0.7476	293.11731	4.77907	0.4540
12	11.991	VV	1.1302	994.80328	11.26577	1.5409
13	14.494	VV	0.5082	88.54098	2.20624	0.1371
14	16.111	VB	1.5368	543.86914	4.18332	0.8424

Hewlett Packard 1050 HPLC 2001/02/09 12:12:52 PM nataly

Signal 3: MWD1 B, Sig=205,4 Ref=550,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.858	BV	0.2474	1.53135e4	772.00037	19.2821
2	2.219	VV	0.3564	6691.14355	234.81612	8.4252
3	2.949	VV	0.2252	2381.81274	156.79865	2.9991
4	3.239	VV	0.2672	4585.38916	244.09071	5.7737
5	3.471	VV	0.1780	2535.64624	182.59360	3.1928
6	3.793	VV	0.3677	3535.30273	130.65259	4.4515
7	4.310	VV	0.3031	1839.65771	78.00828	2.3164
8	4.961	VV	0.2344	1190.57874	72.95084	1.4991
9	5.129	VV	0.1642	901.63892	77.24799	1.1353
10	6.111	VV	0.5414	1.23268e4	328.80681	15.5213
11	7.484	VB	0.8142	1.95920e4	339.04996	24.6693
12	10.597	BP	1.3691	8399.85547	73.07578	10.5767
13	14.469	BP	0.2698	125.19398	5.75437	0.1576

Figure B4: HPLC data of Fraction B, dissolved in acetone, eluted with 10% water in methanol and run at a flow rate of 1.5 ml/min

APPENDIX C

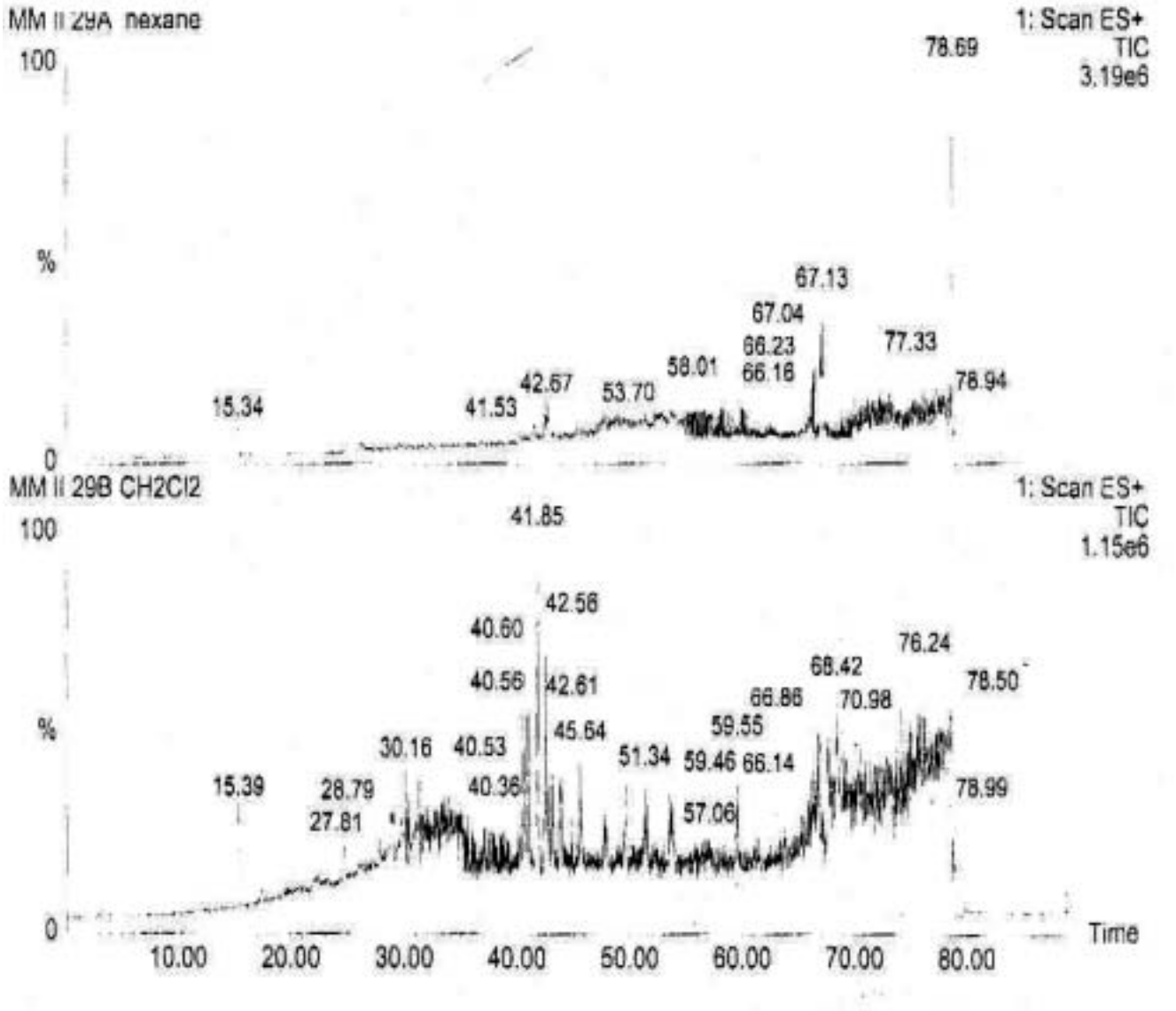

Figure C1: Preparative HPLC of the hexane (top) and chloroform (bottom) fractions following solvent/ solvent extraction

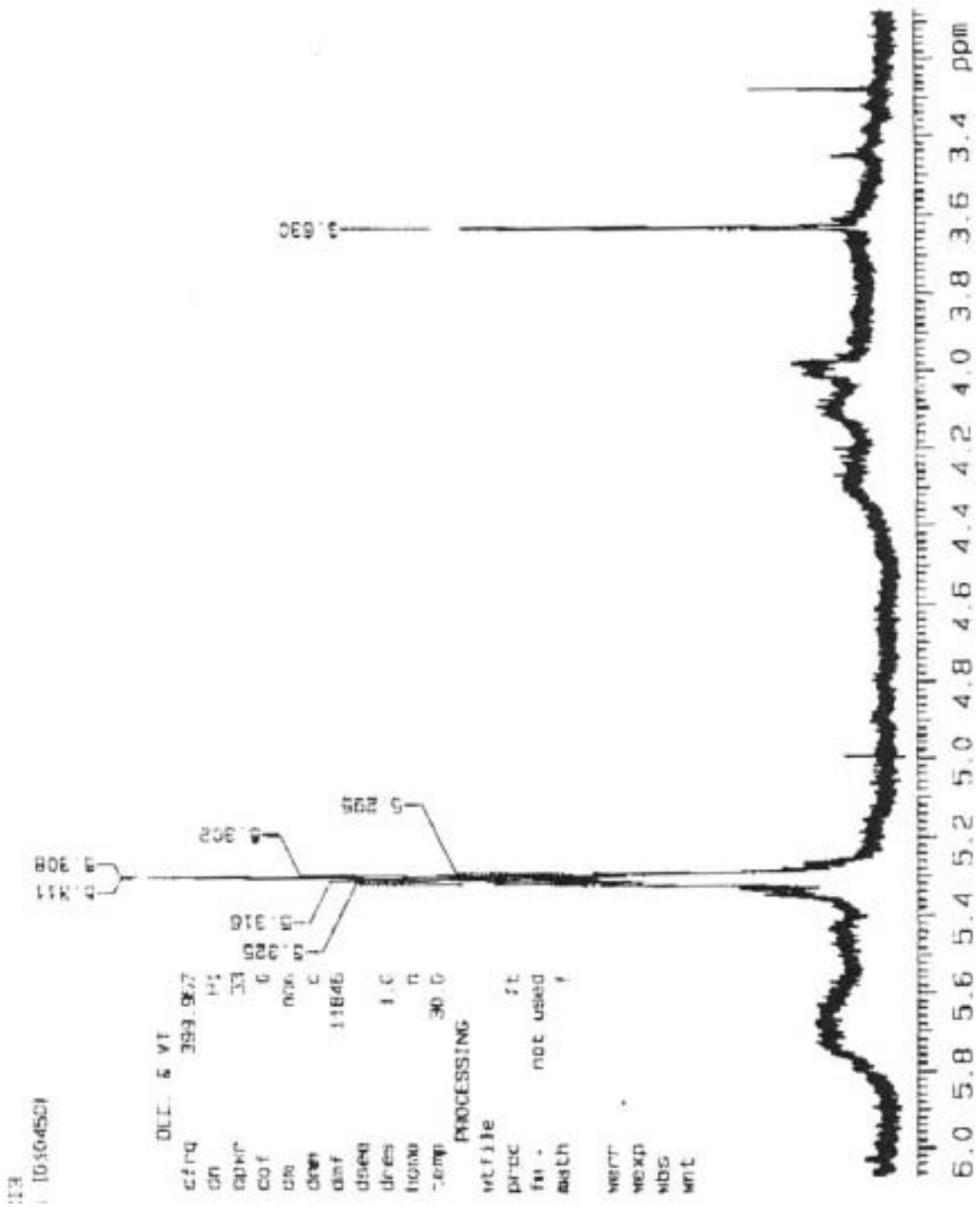

Figure C2: ^{1}H NMR (400MHz, $CDCL_3$) of fraction B2

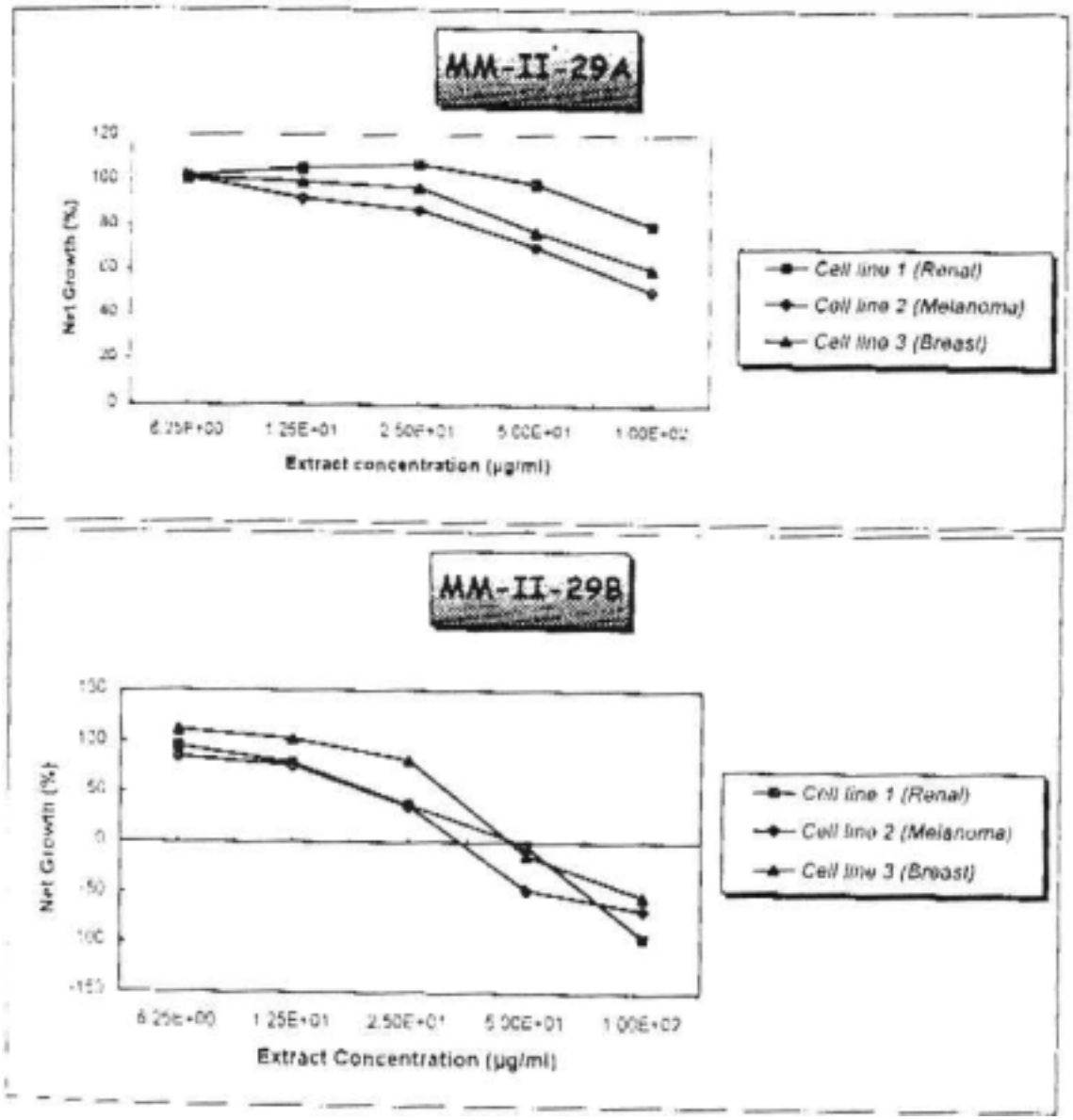

Figure C3: Cytotoxicity of the hexane (top) and chloroform (bottom) fractions on 3 cell lines i.e. renal, melanoma and breast. The chloroform fraction shows inhibition of all three cells lines

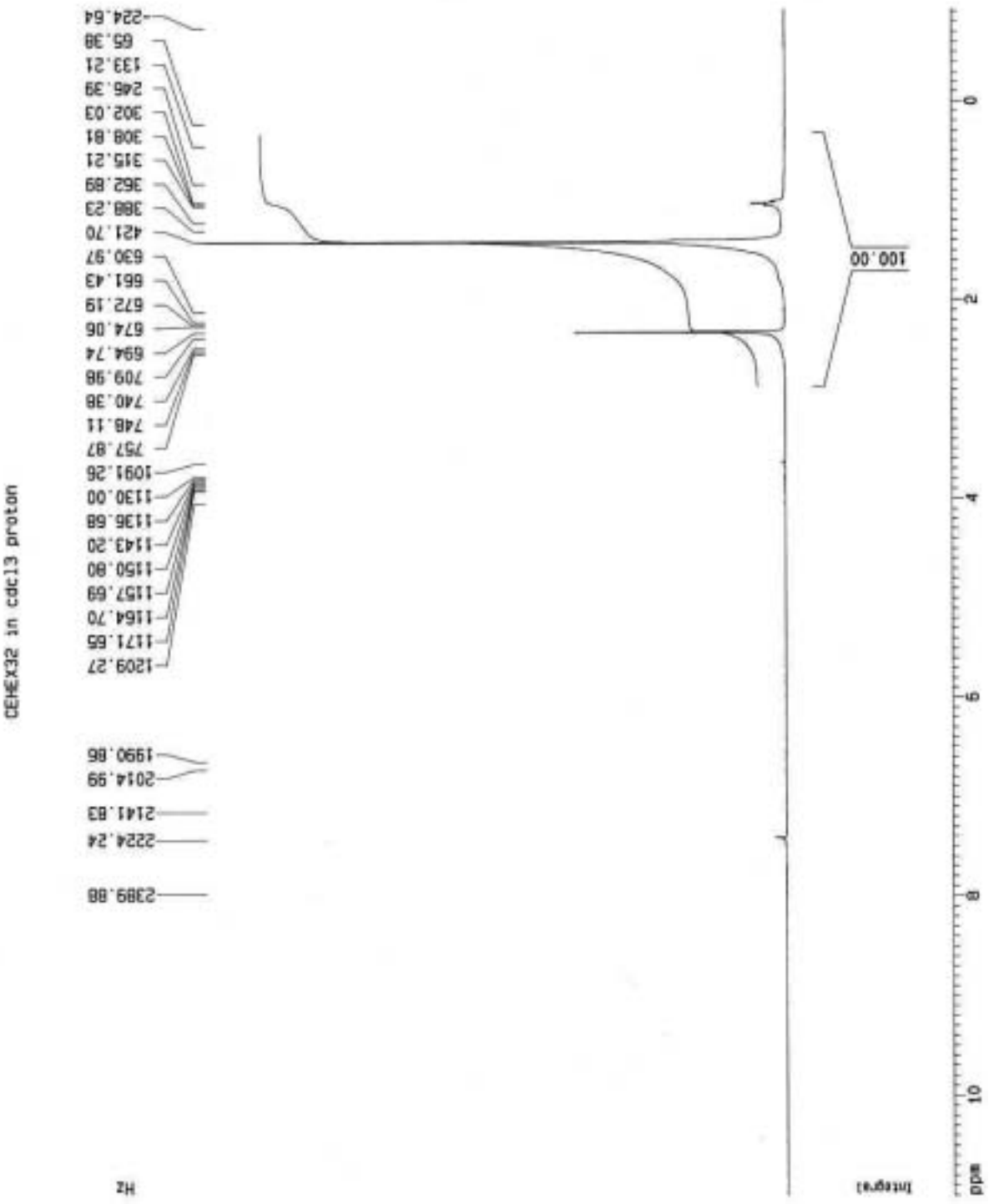

Figure C4: ^{1}H NMR (300MHz, $CDCl_3$) of an isolated compound from the hexane fraction

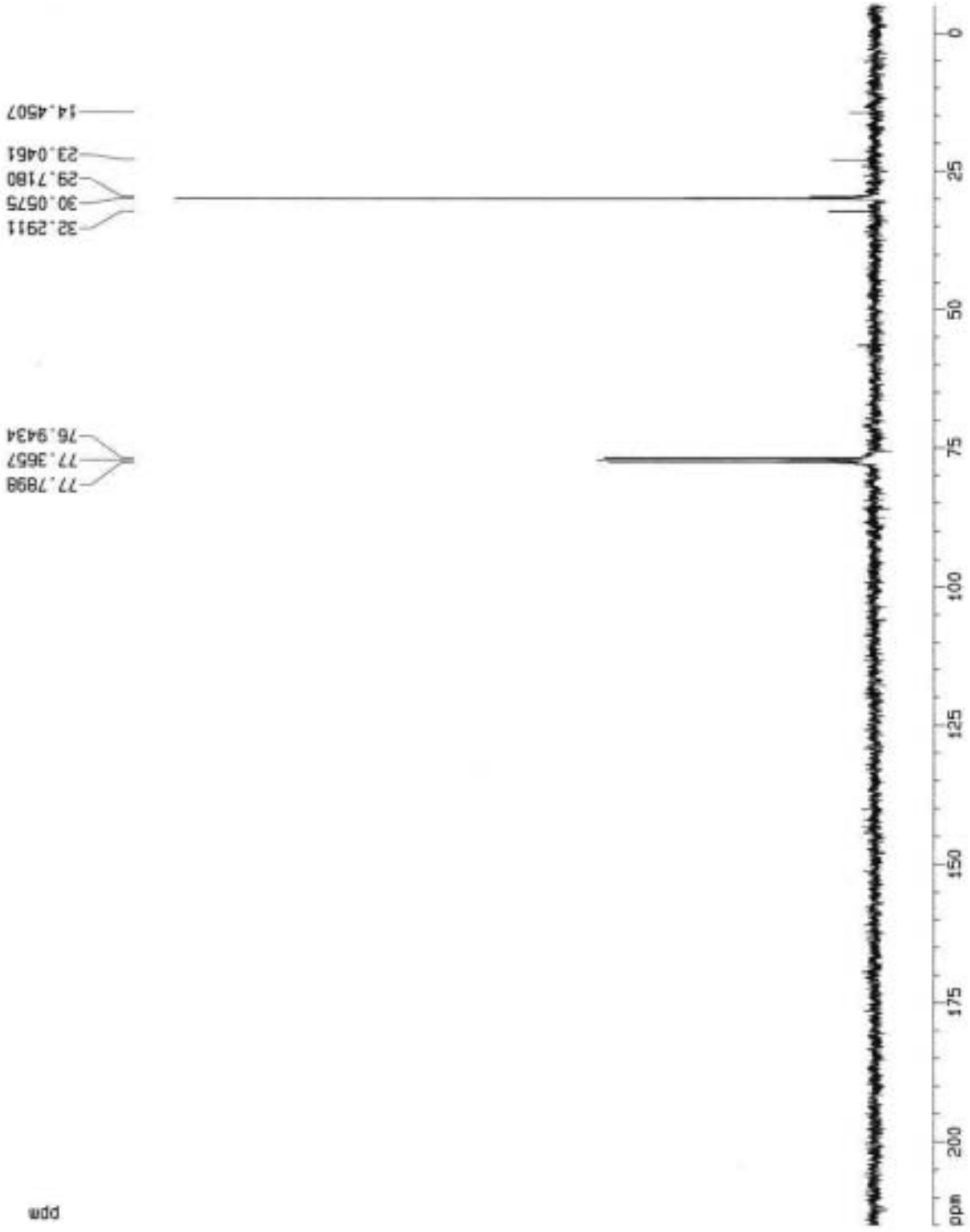

Figure C5: ^{13}C NMR (75MHz, $CDCl_3$) of an isolated compound from the hexane fraction

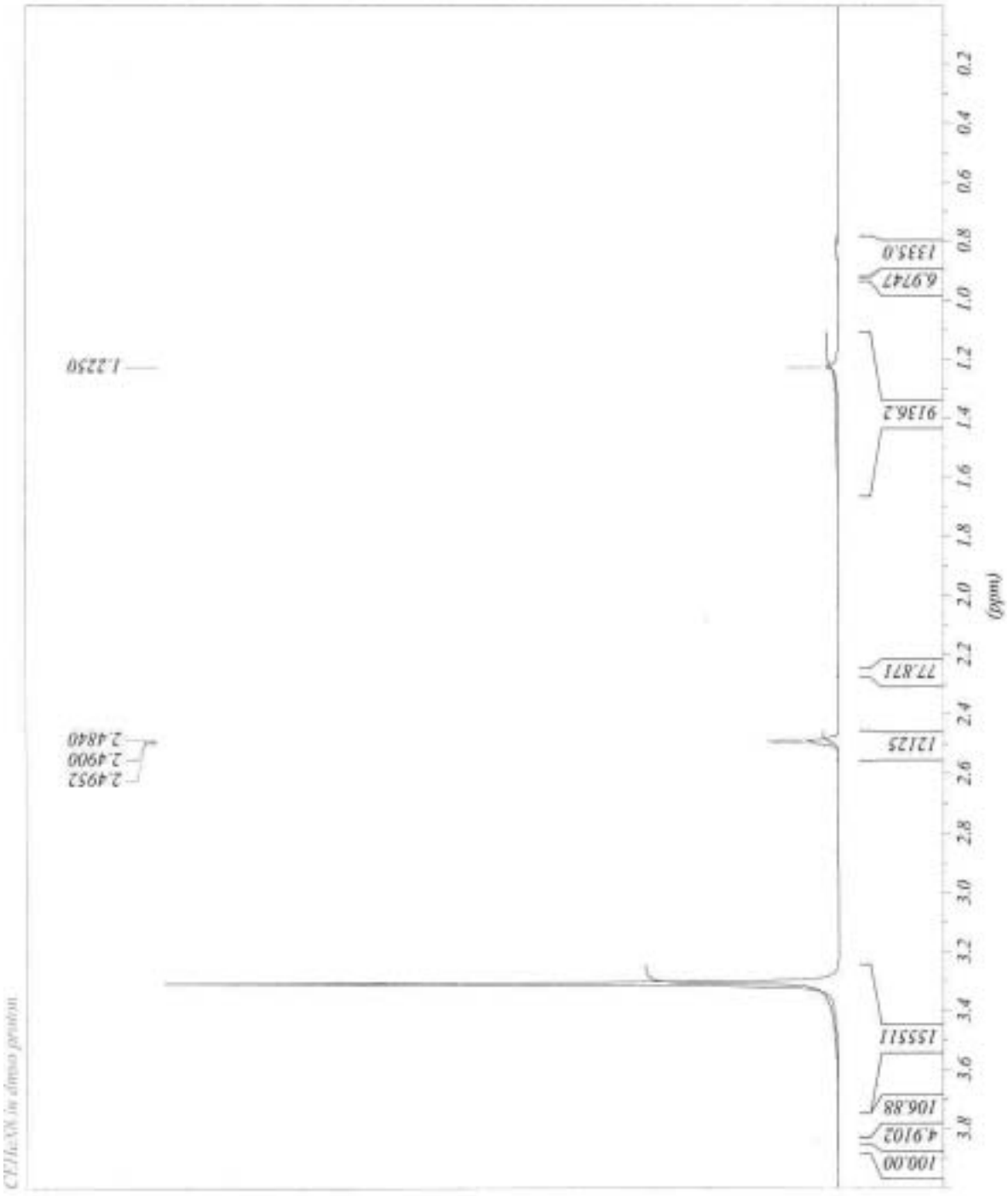

Figure C6: ^{1}H NMR (300MHz, DMSO-d_6) of an isolated compound from the hexane fraction

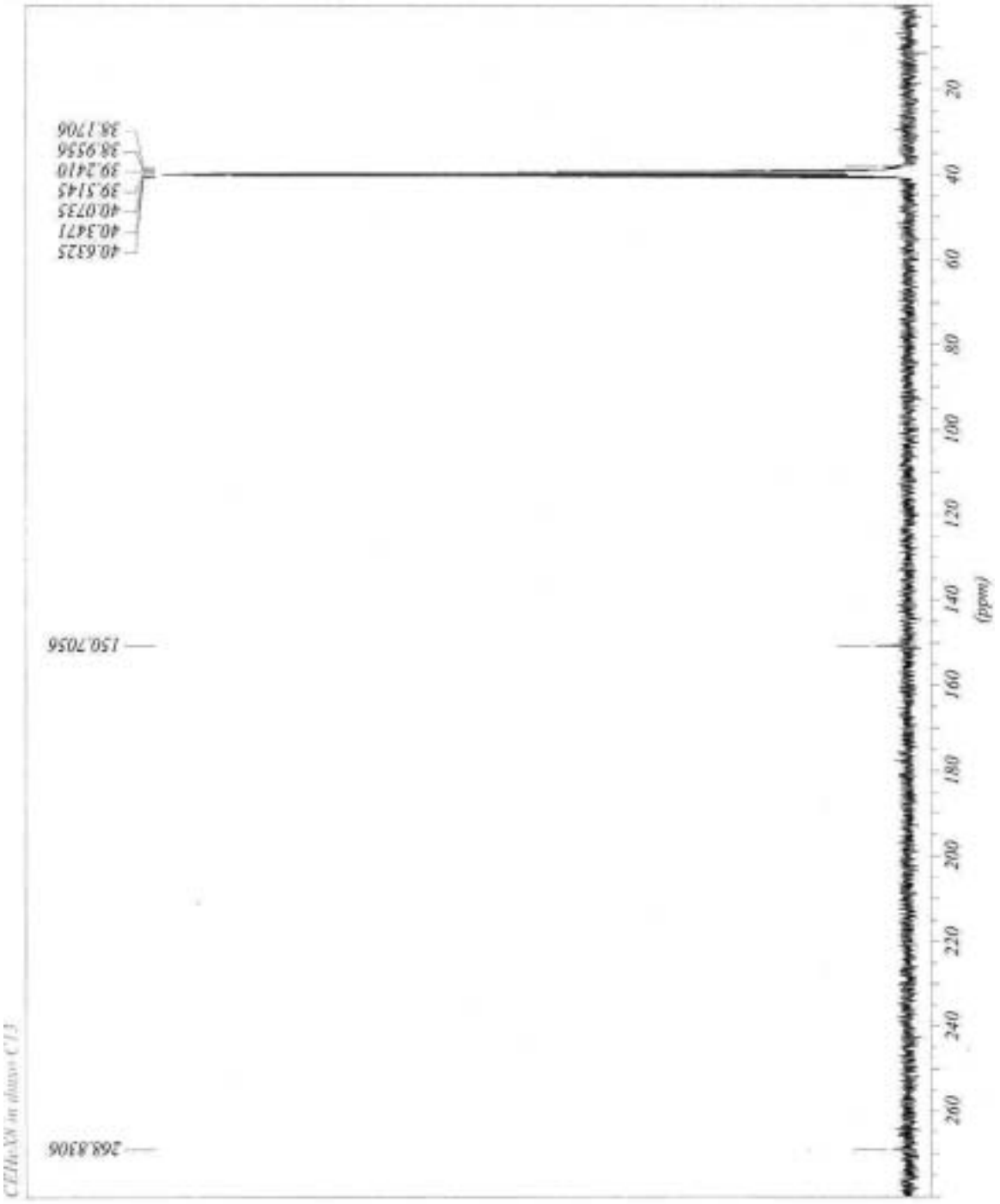

Figure C7: ^{13}C NMR (75MHz, DMSO-d_6) of a isolated compound from the hexane fraction