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INVESTIGATION OF ANTIBACTERIAL COMPOUNDS PRESENT IN *COMBRETUM WOODII* DUEMMER

JAMES OLUSANYA FAMAKIN
B. Pharm (O.A.U, Ile-Ife, Nigeria)

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Promoter : Prof J. N. Eloff
Co-Promoter : DR D. R. P. Katerere

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University of Pretoria etd – Famakin, J O (2002)

PREFACE

The experimental work described in this dissertation was carried out by me in the Department of Pharmacology, Faculty of health science, University of Pretoria, Pretoria, under supervision of Prof. J. N. Eloff and Dr. D. R. P. Katerere.

These studies represent the work done by the author and have not otherwise been submitted in any form of degree or diploma to any other University. Where use has been made of the work of others it is duly acknowledged in the text.

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James Olusanya Famakin

University of Pretoria etd – Famakin, J O (2002)

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University of Pretoria etd – Famakin, J O (2002)

TABLE OF CONTENTS

	Page
Preface	i
Acknowledgements	ii
Table of contents	iii
List of tables	vi
List of figures	vii
Glossary of abbreviations	xii
Abstract	1
Samevatting	3
Chapter 1	4
1. Introduction	4
1.1 Resistance to antibiotics –A world-wide problem	4
1.2 Possible solution	4
1.3 Medicinal Plants-As source of Antibacterial drugs.	5
1.3.1 Plants as sources of medicines.	5
1.3.2 Plant and Antibacterial production	7
1.4 Antimicrobial activity of some plant constituents-An overview	10
1.4.1 Tannins	10
1.4.2 Flavones, flavonoids, and flavonols	11
1.4.3 Terpenoids and essential oils	11
1.4.4 Alkaloids	12
1.5. Combretaceae	14
1.5.1 Introduction.	14
1.5.2 Taxonomy	14
1.5.3 Use of Combretaceae in traditional medicine	14
1.5.4 Ethnopharmacology of Combretaceae	17
1.5.5 Phytochemistry of Combretaceae	18
1.5.6 Selection of *C. woodii*	18
1.6 An overview of methods that have been employed to isolate compounds from plants	21

1.6.1 Plant materials 21
1.6.2 Extraction techniques 21
1.6.3 Isolation and Analysis of Constituents 22
1.6.4 Assay of plant extracts 24
1.7 Problem statement and Hypothesis 26
1.8 Aim and Objectives of the study. 26
Chapter 2 27
2 Materials and Methods 27
2.1 Plant Material 27
2.2 Preparation and extraction 27
2.3 TLC analysis of extract 27
2.4 Bioautographic assay 28
2.4.1 Preparation of TLC plates for bioautography 28
2.4.2 Preparation of bacteria 28
2.4.3 Bioassay method 28
2.5 Determination of minimum inhibitory concentration 29
2.5 1 Dilution of extract 29
2.5.2 Addition of bacteria 29
2.6 Solvent-Solvent fractionation 30
2.6.1 Analysis and bioautographic assay fractions 32
2.7 Isolation of bioactive compounds 32
2.7.1 TLC analysis 32
2.7.2 Isolation by column chromatography 32
2.7.3 Bioassay work on isolated compounds 33
2.8 Spectroscopic analysis of isolated compounds 34
Chapter 3 34
Results and discussion on extraction 34
3.1 Introduction 34
3.1.1 Quantity extracted with initial extractants 34
3.1.2 Profiling of extracts 36
3.1.3 Bioautography 40

University of Pretoria etd – Famakin, J O (2002)

3.1.4 Quantity of antibacterial compound present and MIC values of extract 42
3.2 Solvent-Solvent extraction 45
3.2.1 Extraction of *C. woodii* and solvent-solvent fractionation 46
3.2.2 Complexity of fractions 47
3.2.3 Bioautography of fractions 50
3.2.4 Antibacterial activity of fractions 53
Chapter 4 58
Results and discussion on isolation of compound 58
4.1 Isolation of active compound 58
4.1.1 Method development 58
4.1.2 Column chromatography of chosen fraction 58
4.1.3 Analysis of collected fractions by TLC. 60
4.1.4 Combination of collected fractions 64
4.1.5 TLC analysis of CF1 and column chromatography 65
4.1.6. Minimum inhibitory concentration of isolated active compound 69
4.1.7 Bioautography of isolated active compound 70
Chapter 5 72
Spectroscopic analysis of isolated active compound 72
Discussion and Conclusion 80
Appendix 1 83
References. 84

University of Pretoria etd – Famakin, J O (2002)

LIST OF TABLES

		Page
Table 1	Major mechanisms of resistance to antimicrobial agents	5
Table 2	Some plant-derived preparations for medicinal use	7
Table 3	The subgeneric classification of genus *Combretum* in South Africa	15
Table 4	Traditional uses of some *Combretum* species	16
Table 5	Type of phytochemicals extracted by different solvents used	22
Table 6	Examples of colour detected under UV for some phytochemical preparation.	23
Table 7	Pharmacopoeia methods for microbiological assay of some antibacterial agents	25
Table 8	Quantity in mg extracted using various solvent systems	35
Table 9	Percentage of total extracted from *C. woodii* leaves after different periods	35
Table 10	Quantity extracted, MIC values in mg/ml and total activity in ml of *C.woodii* per gram leaves extracted with hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethyl acetate, tetrahydrofuran, acetone, ethanol, methanol and water.	43
Table 11	Quantity [in g] and percentage of total initial mass [140g] of *C. woodii* leaves extracted by each solvent-solvent fractionation process.	47
Table 12	The minimum inhibitory concentration in mg/ml of different fractions obtained by solvent-solvent extraction of *C. woodii* leaves	54
Table 13	MIC in mg/ml and total activity in ml of acetone extract of *C. woodii* leaves in different fractions obtained by solvent-solvent fractionation	55
Table 14	Quantity in mg of grouped fractions from column chromatography of CF1	68
Table 15	MIC values in mg/ml of CF1b compared with ampicillin and chloramphenicol	70
Table 16	^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) spectra data for isolated compounds. Data obtained in $CDCl_3$	72
Table 17	Typical chemical shifts of hydrogen attached to various types of functional groups	83

University of Pretoria etd – Famakin, J O (2002)

LIST OF FIGURES

		Page
Fig. 1	Synthesis of plant secondary compounds	9
Fig. 2	Structure of a condensed tannin	11
Fig. 3	Structure of a flavone	12
Fig. 4	Structure of a terpenoid	13
Fig. 5	Structure of an alkaloid	13
Fig. 6	*Combretum woodii* from which leaves were collected in Lowveld National Botanical Garden	20
Fig. 7	Distribution of *C. woodii* in Southern Africa	20
Fig. 8	The procedure used for the solvent-solvent fractionation of the components in *C. woodii* leaves extracts.	31
Fig. 9	Quantity (mg) extracted from 0.5 g of powdered dried leaves of *C. woodii* with 5 ml of hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethyl acetate, tetrahydrofuran, acetone, ethanol, methanol and water.	36
Fig. 10	Separation of components present in 50 µg of 10 different extracts with BEA as eluent and vanillin-sulphuric acid spray reagent. Lanes from left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethylacetate, tetrahydrofuran, acetone, ethanol, methanol and water.	37
Fig. 11	Separation of components present in 50 µg of 10 different extracts using CEF as eluent and vanillin-sulphuric acid spray reagent. Lanes from left to right : hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethylacetate, tetrahydrofuran, acetone, ethanol, methanol and water.	37
Fig. 12	Separation of components present in 50 µg of 10 different extracts using EMW as eluent and vanillin-sulphuric acid spray reagent. Lanes from left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethylacetate, tetrahydrofuran, acetone, ethanol, methanol and water.	38
Fig. 13	Separation of compound present in 50 µg of 10 different extracts with BEA as eluent and *p*-anisaldehyde-sulphuric acid spray reagent. Lanes from left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethylacetate, tetrahydrofuran, acetone, ethanol, methanol and water.	38
Fig. 14	Separation of compounds present in 50 µg of 10 different extracts using CEF as eluent and *p*-anisaldehyde-sulphuric acid spray reagent. Lanes from left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride,	

ethylacetate, tetrahydrofuran, acetone, ethanol, methanol and water. 39

Fig.15 Separation of compounds present in 50 µg of 10 different extracts using EMW as eluent and *p*-anisaldehyde-sulphuric acid spray reagent. Lanes from left to right hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethylacetate, tetrahydrofuran, acetone, ethanol, methanol and water. 39

Fig. 16 Bioautograms of *C. woodii* leaves extracted by 10 different extractants. TLC developed in BEA (left) and EMW (right) and sprayed with *S. aureus* culture, incubated overnight then sprayed with INT. Growth inhibition indicated by colourless zone on TLC plates. Lanes from left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethyl acetate, tetrahydrofuran, acetone, ethanol, methanol and water. 41

Fig. 17 Bioautograms of *C. woodii* leaves extracted by 10 different extractants. TLC developed in BEA (left) and EMW (right) and sprayed with *E. faecalis* culture, incubated overnight then sprayed with INT. Growth inhibition indicated by colourless zone on TLC plates. Lanes from left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethyl acetate, tetrahydrofuran, acetone, ethanol, methanol and water. 41

Fig. 18 Total antibacterial activity of *C. woodii* extracts on four test organisms. From left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethyl acetate, tetrahydrofuran, acetone, ethanol, methanol and water. 44

Fig. 19 Total antibacterial activity of *C. woodii* extracts on four tested organisms 44

Fig. 20 Quantity extracted in each solvent fraction by solvent-solvent fractionation process 46

Fig. 21 Separation of components present in the different fractions obtained by solvent-solvent extraction by BEA and sprayed with vanillin-sulphuric acid. Lanes from left to right: hexane, chloroform, carbon tetrachloride, 35 % methanol in water, butanol and water fractions. In each case 50 µg was chromatographed. 48

Fig. 22 Separation of components present in the different fractions obtained by solvent –solvent extraction by CEF and sprayed with vanillin-sulphuric acid. Lanes from left to right: hexane, chloroform, carbon tetrachloride, 35 % methanol in water, butanol and water fractions. In each case 50 µg was chromatographed. 48

Fig. 23 Separation of components present in the different fractions obtained by solvent - solvent extraction by EMW and sprayed with vanillin-sulphuric acid. Lanes from left to right: hexane, chloroform, carbon tetrachloride, 35 % methanol in water, butanol and water fractions. In each case 50 µg was chromatographed. 49

Fig. 24 Separation of components present in the different fractions obtained by

University of Pretoria etd – Famakin, J O (2002)

solvent –solvent extraction by BEA, CEF and EMW (from left to right) and sprayed with vanillin-sulphuric acid. Lanes at the bottom in each TLC plate from left to right: insoluble water fractions (IW), insoluble butanol fractions (IB) and insoluble 35 % water in methanol fractions and interphase in chloroform (GR). 49

Fig. 25 Bioautogram of acetone extract of *C. woodii* leaves separated into different fractions by solvent-solvent extraction. TLC plate developed in BEA and sprayed with S.aureus culture incubated overnight and then sprayed with INT. Growth inhibition indicated by colourless zones on TLC plate. Lane from left to right: chloroform, carbon tetrachloride, 35 % methanol in water, butanol and water fractions. 50

Fig. 26 Bioautogram of acetone extract of *C. woodii* leaves separated into different fractions by solvent-solvent extraction. TLC plate developed in CEF and sprayed with S.aureus culture incubated overnight and then sprayed with INT. Growth inhibition indicated by colourless zones on TLC plate. Lane from left to right: chloroform, carbon tetrachloride, 35% methanol in water, butanol and water fractions. 51

Fig. 27 Bioautogram of acetone extract of *C. woodii* leaves separated into different fractions by solvent-solvent extraction. TLC plate developed in EMW and sprayed with S. aureus culture incubated overnight and then sprayed with INT. Growth inhibition indicated by colourless zones on TLC plate. Lane from left to right: chloroform, carbon tetrachloride, 35% methanol in water, butanol and water fractions. 51

Fig. 28 Bioautogram of acetone extract of *C. woodii* leaves separated by CEF (left) and EMW (right) and *E. faecalis* as a test organism. White or yellowish brown areas indicated growth inhibition. 52

Fig. 29 Biautogram of acetone extract of *C. woodii* leaves separated by CEF (left) and EMW (right) and *E. coli* as a test organism. White or yellowish brown areas indicated growth inhibition. 53

Fig. 30 Bioautogram of acetone extract of *C. woodii* leaves separated by CEF (the left) and EMW (right) and *Ps. aeruginosa* as a test organism. White or yellowish brown areas indicated growth inhibition. 53

Fig. 31 Total antibacterial activity to four test organisms of *C. woodii* leaves extracted with solvent fractions. Fractions from left to right: hexane (H), chloroform ($CHCl_3$), carbon tetrachloride (CCl_4), 35% water in methanol (35% W/M), butanol (B) and water (W). 56

Fig. 32 The relative quantities obtained by solvent-solvent fractionation and the relative antibacterial activities of the different fractions. Solvent fractions from the top to the bottom: hexane (H), chloroform ($CHCl_3$), carbon tetrachloride (CCl_4), butanol, 35% water in methanol (35% W/M) and water (W). 56

University of Pretoria etd – Famakin, J O (2002)

Fig. 33	TLC of chloroform fraction using different ratios of chloroform and ethyl acetate combination as solvent systems. From left to right: ratios 2 : 1, 1 :2, 1 :1, 1 : 5. Lines indicate fluorescing compounds.	59
Fig. 34	TLC of chloroform fraction using different ratios of chloroform and tetrahydrofuran combination as solvent systems. From left to right: ratios 1.1, 1.2. Lines represent fluorescing compounds.	59
Fig. 35	TLC of chloroform fraction using different ratios of chloroform and methanol combination as solvent systems. From left to right: ratios 1: 2, 2: 1. Lines represent fluorescing compounds.	60
Fig. 36	TLC of every fourth fraction collected separated by CEF and sprayed with vanillin-sulphuric acid reagent. Lanes from left to right: fractions 4, 8, …, 60, 64.	61
Fig. 37	TLC of collected fractions separated by CEF and sprayed with vanillin-sulphuric acid reagent. Lanes from left to right: fractions: 1, 2, 3, ……, 24, 25.	61
Fig. 38	TLC of collected fractions separated by CEF and sprayed with vanillin-sulphuric acid reagent. Lanes from left to right: fractions 26, 27, 28, …., 53, 54.	62
Fig. 39	TLC of column chromatography fractions separated by CEF and sprayed with vanillin-sulphuric acid reagent. Lanes from left to right: fractions 55, 56, ….., 78, 79.	62
Fig. 40	TLC of collected fractions separated by EMW and sprayed with vanillin-sulphuric acid reagent. Lanes from left to right: fractions 26, 27, 28,…., 53, 54.	62
Fig. 41	TLC of collected fractions separated by EMW and sprayed with vanillin-sulphuric acid reagent. Lanes from left to right: fractions 107, 108, …, 122, 123.	63
Fig. 42	TLC of collected fractions separated by CEF and sprayed with a vanillin-sulphuric acid reagent. Lanes from left to right: fractions 4, 5, …11, 12.	63
Fig. 43	TLC of some of CF2 extracted into three different solvent using CEF (left) and EMW (right) as the solvent systems. From left to right: acetone (ACN), chloroform (CHC), and methanol (MEOH) components. Lines indicate fluorescing compounds.	65
Fig. 44	Separation of CF1 by different solvent systems using vanillin as the spray reagent. The TLC plates from left to right were developed in acetonitrile : water (1 :.1), 5% acetonitrile in water, 2% acetonitrile in water and 1% acetonitrile in water. Lines represent fluorescing compounds.	66
Fig. 45	Separation of CF1 by different solvent systems using vanillin-sulphuric acid	

University of Pretoria etd – Famakin, J O (2002)

as the spray reagent. The TLC plates from left to right were developed in different chloroform and ethyl acetate combinations (2:1, 1:5, 1:3, 4:1). 66

Fig. 46 Separation of CF1 by different solvent systems using vanillin-sulphuric acid as the spray reagent. The TLC plates from left to right were developed in methanol:water (1:1), 5% methanol in chloroform and 1% methanol in water. 66

Fig. 47 TLC separation of fractions obtained from column chromatography of CF1 by CEF and using vanillin-sulphuric acid spray reagent. From left to right: fractions 1, 2, …, 29, 30. 68

Fig. 48 TLC separation of grouped fractions from column chromatography of CF1 by CEF and using vanillin-sulphuric acid spray reagent. From left to right: A, B, ……, K, L. 69

Fig. 49 Bioautograms of CF1b (left) and CF2C (right). TLC developed in EMW and sprayed with growing *S. aureus* culture and later with INT. Brownish-yellow colour indicated growth inhibition zone. 71

Fig. 50 Proposed structure for isolated compound 74

Fig. 51 The isolated active compound and its fragmentation into two tropylium ions. 74

Fig. 52 The isolated active compounds with its two aromatic rings labeled as 'A' and 'B'. 75

University of Pretoria etd – Famakin, J O (2002)

GLOSSARY OF ABBREVIATIONS

1	ACN	Acetone (extractant)
2	ATCC	American type Culture Collection
3	B	Butanol (extractant or fraction)
4	^{13}C	Carbon 13
4	BEA	Benzene:Ethanol:Ammonium hydroxide [36:5.4:4]
5	CC	Column chromatography
6	CCl_4	Carbon tetrachloride (extractant or fraction)
7	CEF	Chloroform:Ethyl acetate:Formic acid [5:4:1]
8	CF	Column chromatography fraction
9	$CHCl_3$	Chloroform (extractant or fraction)
10	EA	Ethyl acetate extractant.
11	*E. coli*	*Escherichia coli*
12	EE	Diethyl ether (extractant)
13	*E. faecalis*	*Enterococcus faecalis*
14	EMW	Ethanol : methanol : water [40:5.4:4]
15	ET	Ethanol (extractant)
16	H	Hexane (extractant or fraction)
17	^{1}H	Proton
18	I	Isopropyl ether
19	IB	Insoluble butanol fraction
20	INT	*p*-iodonitrotetrazolium violet
21	IW	Insoluble water fraction
22	IWM	Insoluble 35% water in methanol (fraction)
23	M	Methanol extractant
24	MDC	Methylene dichloride
25	MS	Mass spectroscopy
26	35% W/M	35% water in methanol (fraction)
27	NMR	Nuclear magnetic resonance
28	*Ps. aeruginosa*	*Pseudomonas aeruginosa*
29	R_f	fractional movement of a solute band, relative to the distance moved by solvent front
30	*S. aureus*	*Staphylococcus aureus*
31	THF	Tetrahydrofuran extractant
32	TLC	Thin layer chromatography
33	UV	Ultra-violet light
34	W	Water (extractant)

University of Pretoria etd – Famakin, J O (2002)

ABSTRACT

Dried ground leaves of *Combretum woodii* were extracted with 10 different solvents (hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethyl acetate, tetrahydrofuran, acetone, ethanol, methanol and water) to determine the best extractant for isolating and characterizing any compound(s) with antibacterial activity present. The antibacterial activity of all the extracts was tested against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis*. All the extracts, with exception of the water extract, inhibited the growth of *S. aureus* and *E. faecalis* using bioautography of thin layer chromatography plates. Two major inhibitory compounds with R_f values of 0.74 and 0.88 were visible on the bioautograms of extracts sprayed with *S. aureus* and *E. faecalis* respectively in ethanol:methanol:water (40:5.4:4) solvent systems. There were at least three more polar inhibitory compounds against *E. faecalis* separated in benzene:ethanol:ammonium hydroxide (36:4:0.4) solvent system.

According to thin layer chromatography using *p*-anisaldehyde-sulphuric acid as spray reagent, most solvents extracted at least seven compounds but water extracted only one visible compound.

Tetrahydrofuran, methylene dichloride, and acetone extracted the largest quantity. The methylene dichloride and acetone extracts had the highest antibacterial activity against all the four test organisms. However, acetone was selected for extraction of *C. woodii* dried ground leaves because of its relatively low toxicity to test organisms and the ease of removal after extraction.

Acetone extracted 11% of 140 g of dried ground leaves. Group separation by solvent-solvent extraction was applied to the acetone extract. The complex extract was simplified by separating into six fractions and an interphase. The highest number of non-polar compounds was in the hexane fraction, followed by carbon tetrachloride and chloroform fractions. The highest quantity of extract, 32%, was also in the hexane fraction followed by chloroform (25.6%), butanol (11.7%), water (7.2%), 35% water in methanol (6.5%), and carbon tetrachloride (6.4%) fractions. The carbon tetrachloride fraction had the most complex mixture of compounds. The six fractions obtained inhibited the four test organisms to different degrees. Most of the bioactive compounds were in the chloroform and hexane fractions. The chloroform fraction had the highest relative antibacterial activity (almost 33 times higher than the water fraction). Generally, *S. aureus* was the most sensitive, followed by *E. faecalis*, *Ps. aeruginosa* and *E. coli*. There were at least six growth inhibitors of pathogenic

bacteria. A major active compound with R_f value of 0.67 in chloroform:ethylacetate:formic acid (20:16:4) and 0.74 in ethanol:methanol:water (40:5.4:4) solvent systems was present in all the fractions (except water fraction). Attempts were made to isolate and characterize this major active compound.

The chloroform fraction was subjected to silica gel 60 (63–200 µm) column chromatography using a chloroform and ethyl acetate mixture and 10% methanol in acetone to elute the column fractions. Further TLC analyses and column chromatographic procedures on the collected fractions led to the isolation of this compound. This was identified by nuclear magnetic resonance and mass spectroscopy as combretastatin B5 (2', 3', 4-trihydroxyl, 3, 5, 4'-trimethoxybibenzyl) previously isolated from the seeds of *C. krausii.* This compound has been found to have antimitotic activity. The closely related combretastatin A4, the first of a new class of anticancer agents, is currently undergoing clinical trials.

The antibacterial activity of combretastatin B5 showed significant activity against *S. aureus*, *Ps. aeruginosa*, *E. faecalis* and slight activity against *E. coli*. The MIC values of the isolated active compound for *S. aureus* was 16 µg/ml, which compares favourably to the MIC values of 80 µg/ml and 160 µg/ml for ampicillin and chloramphenicol in this test respectively.

The results obtained validate the use of *Combretum* species for the bacterial infections in traditional medicine. Further work, needs to be done to investigate the possible clinical value of combretastatin B5 and isolate and characterize other antibacterial compounds in *C. woodii.*

University of Pretoria etd – Famakin, J O (2002)

SAMEVATTING

Om te bepaal wat die beste ekstraheermiddel is vir gedroogde *C. woodii* blare is, is tien vloeistowwe (heksaan, di-isopropieleter, dietieleter, metileendichloried, etielasetaat, tetrahidrofuraan, asetoon, etanol, metanol en water) gebruik om antibakteriese verbindings te isoleer en karakteriseer. Die toetsorganismes was *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis*. Volgens bio-outografieresultate, het al die ekstrakte behalwe die waterekstrak die groei van *S. aureus* and *E. faecalis* onderdruk. Daar was twee belangrike inhibeerders met R_f waardes van 0.74 en 0.88 in bio-outogramme ontwikkel in etanol:metanol:water (40:5.4:4). Daar was ten minste nog drie meer polêre inhibeerders van *E. faecalis* volgens bio-outogramme ontwikkel in benseen:etanol:ammoniumhidroksied (36:4:0.4). Met die uitsondering van die waterekstrak kon ten minste sewe verbindings na dunlaagchromatografie (DLC) en spuit met *p*-anysaldehied-swawelsuur aangetoon word.

Tetrahidrofuraan, metileen dichloried en asetoon het die grootste hoeveelheid ge-ekstraheer en laasgenoemde twee het die hoogste antibakteriese aktiwiteit teen die vier toetsorganismes gehad. Asetoon is gekies as ekstraheermiddel omdat dit 'n relatiewe lae toksisiteit vir die toetsorganismes gehad het en maklik verwyder kon word na ekstraksie.

Asetoon het 11% van die 140 g gedroogde fyngemaalde blare ge-ekstraheer. Die ekstrak is deur vloeistof-vloeistof groepskeiding vereenvoudig na ses fraksies en 'n interfase. Die grootste getal nie-polêre verbindings was in die heksaanfraksie gevolg deur die koolstoftetrachloried- en chloroformfraksies. Die grootste hoeveelheid, 32% van die totaal, was ook in die heksaanfraksie gevolg deur die chloroform- (25.6%), butanol- (11.7%), water- (7.2%), 35% water in metanol- (6.5%), and koolstoftetrachloriedfraksies (6.4%). Die koolstoftetrachloriedfraksie het die mees komplekse samestelling gehad en al ses fraksie het die groei van die toetsorganismes tot 'n mindere of meerdere mate geïnhibeer. Oor die algemeen was *S. aureus* die sensitiefste gevolg deur *E. faecalis, Ps. aeruginosa* en *E. coli*. Daar was ten minste ses groei-inhibeerders van die bakterieë teenwoordig. Die sterkste inhibeerder het 'n R_f-waarde van 0.67 in chloroform:etielasetaat: mieresuur (20:16:4) and 0.74 in etanol:metanol:water (40:5.4:4) gehad. Hierdie verbinding was teenwoordig in al die fraksies behalwe die waterfraksie. Pogings is aangewend om hierdie verbinding te isoleer.

Die komponente van die chloroformfraksie is deur silikagel chromatografie (silika gel 60 (63-200 µm) met 'n gradiënt van chloroform-etielasetaat en later 10% metanol in asetoon geskei. Die suiwer verbinding is deur verdere DLC analise en kolomchromatografie geïsoleer. Die verbinding is deur kernmagnetiese resonansspektroskopie en massaspektroskopie geïdentifiseer as combretastatin B5 (2',3',4-trihidroksiel, 3,5,4'-trimethoksiebibensiel) wat voorheen geïsoleer is uit die saad van *C. krausii*. Hierdie verbinding het antimitotiese aktiwiteit gehad. Die naverwante combretestatin A4, die eerste van 'n nuwe klas antikanker agense ondergaan tans kliniese proewe.

Combrestatin B5 het sterk antibakteriese aktiwiteit teen *S. aureus*, *Ps. aeruginosa*, *E. faecalis* en laer aktiwiteit teen *E. coli* gehad.. Die MIC-waardes vir *S. aureus* was 16 µg/ml, teenoor MIC waardes van 80 µg/ml en 160 µg/ml vir ampisillien en chlooramfenikol in hierdie eksperimente.

Die resultate ondersteun die etnobotaniese gebruik van *Combretum* spesies vir bakteriese infeksies. Verdere werk behoort uitgevoer te word op die moontlike kliniese waarde van combretastatin B5 en die isolering van ander antibakteriese verbindings in *C. woodii*.

University of Pretoria etd – Famakin, J O (2002)

CHAPTER 1

1 Introduction

1.1 Resistance to antibiotics-A world-wide problem

Resistance to antimicrobial agents is recognized at present as a major global public health problem (Iwu et al., 1999). Infective diseases account for approximately one-half of all deaths in tropical countries. In industrialized nations, despite the progress made in the understanding of microorganisms and their control, incidents of epidemics due to drug resistant microorganisms and the emergence of hitherto unknown disease-causing microbes, pose enormous public health concerns.

The introduction of penicillin 50 years ago was followed by an extraordinary period of discovery, exuberant use, and predictable obsolescence (Calvin, 1993). Resistant bacterial strains have emerged and have spread throughout the world because of the remarkable genetic plasticity of the microorganisms, heavy selective pressures of use, and the mobility of the world population. The widespread use and misuse of antibiotics led to emergence of antibiotic-resistant bacteria. Bacteria have become resistant to antimicrobial agents because of chromosomal changes or exchange of genetic materials via plasmids and transposons among other adaptations (Table 1).

Streptococcus pneumonia, Streptococcus pyogenes and *Staphylococci*, the organisms that cause respiratory and cutaneous infections, and member of the Enterobacteriaceae and Pseudomonas families, the organisms that cause diarrhoea, urinary infection, and sepsis, are now resistant to virtually all of the older antibiotics (Harold, 1992). Despite the availability of a wide range of antibiotics (e.g. penicillins, cephalosporins, tetracycline, amino-glycosides, monobactams, carbapenems, macrolides, streptogranins and dihydrofolate reductase inhibitors), people die in hospitals because of resistant bacterial infections.

1.2 Possible solution

Due to emergence of drug resistant bacteria, the search for new antibacterial compounds with improved activity is necessary. Many indigenous plants are used in treating bacterial related diseases (Carr, 1988). Only a small fraction of these indigenous plants has been investigated. Scientific examination of plants used traditionally in bacteria infection seems to be a logical step of exploiting the antimicrobial compounds, which may be present in plants. Plant based antimicrobials represent a vast untapped source of medicines. Plant-based antimicrobials have enormous therapeutic potential (Cowan, 1999). They are supposedly effective in treatment of

infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Iwu et al., 1999)

Table 1 Major Mechanisms of Resistance to Antimicrobial agents (Jacoby and Archer, 1991)

Type of Resistance and Antimicrobial class	Specific Resistance Mechanism
Quinolones	Altered DNA gyrase
Rifampicin	Altered RNA polymerase
Sulfonamides	New drug-insensitive dihydropteroate synthase
Tetracycline	Ribosomal protection
Trimethoprim	New drug-insensitive dihydrofolate reductase
Vancomycin	Altered cell wall stem peptide
Detoxifying enzyme	
Aminoglycosides (amikacin, gentamicin, Kanamycin, netilmicin, tobramycin)	Acetyltransferase, nuceotidyl-transferase, phosphotransferase
β-Lactam antibiotics (Carbapenems, Cephalosporins, monobactams, Penicillins)	β-Lactamase
Chloramphenicol	Acetyltransferase
Decreased Uptake	
Diminished permeability	
β-lactam antibiotics, chloramphenicol, quinolones, tetracycline, trimethoprim	Alterations in outer membrane proteins
Active efflux	
Erythromycin	New membrane transport system
Tetracycline	New membrane transport system

1.3 Medicinal Plants-As source of Antibacterial drugs

1.3.1 Plants as sources of medicines

The use of medicinal plants is widespread (Farnsworth, 1991). The production of medicines and the pharmacological treatment of diseases, began with the use of herbs (Tyler, 1997). Life saving and essential drugs from medicinal plants such as morphine, digoxin, aspirin, emetine, and ephedrine were introduced into modern therapeutics several centuries ago. However, plants have been used as drugs for over millenia by human beings.

University of Pretoria etd – Famakin, J O (2002)

Other than for purposes of scientific inquiry, plants historically have served as models in drug development for three reasons:

First, each plant is a unique chemical factory capable of synthesizing large numbers of highly complex and unusual chemical substances. In the United States of America alone, about 25% of prescription drugs contain active principles that are still extracted from higher plants and there is increasing popularity in the use of plant-derived preparations (Farnsworth and Morris, 1976). It has also been estimated by the World Health Organization (WHO) that about 80% of the population of the developing countries rely exclusively on plants to meet their health care needs (Farnsworth et al., 1985).

Second, the biologically active substances derived from plants have served as templates for synthesis of pharmaceuticals. Such compounds may have poor pharmacological and toxicological profiles.

Third, many highly active secondary plant constituents have been instrumental as pharmacological tools to evaluate physiological processes (Farnsworth, 1984). There are numerous illustrations of plant-derived drugs. Some selected examples are presented below (Table 2).

The isoquinoline alkaloid emetine obtained from the underground part of *Cephaelis ipecacuanha* and related species has been used for many years as an amoebicidal drug as well as for the treatment of abscesses resulting from *Escherichia histolytica* infections. Another important drug of plant origin with a long history of use is quinine. This alkaloid occurs in the bark of the cinchona tree. Apart from its usefulness in the treatment of malaria, it can be used to relieve nocturnal leg cramps (Iwu et al., 1999).

Similarly, higher plants have also played important roles in cancer therapies. Recent examples include the antileukaemic alkaloids, vinblastine and vincristine, which were both obtained from *Catharantus roseus* (Nelson, 1982) and combretastatins from *Combretum caffrum* (Pettit et al., 1987). In the last two decades a series of stilbenes and dihydrostilbenes (the combretastatins) with potent cytototoxic activity and acidic triterpenoids and their glycosides with molluscicidal, antifungal, antimicrobial activity have been isolated from species of *Combretum* (Rogers, 1989). Other antineoplastic agents include taxol and several derivatives of camptothecin from *Taxus brevifolia* and *Camptotheca acuminata* respectively (Monroe and Mansukkh, 1996).

Table 2 Some plant-derived preparations for medicinal use

Active compound	Origin	Application
Ephedrine	*Ephedra sinica*	Bronchodilator
Egortamine	*Ergot spp*	Migraine remedy
Hyoscyamine	*Hyoscyamus niger*	Anticholinergic
Ipatropium	*Hyoscyamus niger*	Bronchodilator
Morphine	*Papaver somniferum*	Analgesic
Penicillin	*Penicillin spp*	Antibiotic
Physostigmine	*Physostigma venenosum*	Cholinesterase inhibitor
Pilocarpine	*Pilocarpus jaborandi*	Glaucoma remedy
Quinidine	*Cinchona pubescens*	Anti arrhythmic
Quinine	*Cinchona pubescens*	Antimalarial
Reserpine	*Rauwolfia serpentina*	Antihypertensive
Salicin	*Salix spp*	Anti-inflammatory
Scopolamine	*Datura stramonium*	Antispasmodic
Sennoside A+ B	*Cassia angustifolia*	Laxative
Theophylline	*Camellia sinensis*	Bronchodilator
Vinblastine	*Catharantus roseus*	Antineoplastic

1.3.2 Plant and antibacterial production.

An antibiotic has been defined as a chemical compound derived from or produced by living organisms, which is capable, in small concentrations of inhibiting the growth of micro-organisms (Evans, 1989). This definition limited antibiotics to substances produced by microorganisms but the definition must now be extended to include similar substances present in higher plants.

Plants have many ways of generating antibacterial compounds to protect them against pathogens (Kuc, 1990). External plant surfaces are often protected by biopolymers e.g. waxes, and fatty acid esters such as cutin and suberin. In addition, external tissues can be rich in phenolic compounds, alkaloids, diterpenoids, steroid glycoalkaloids and other compounds, which inhibit the development of fungi and bacteria (Kuc, 1985). Cell walls of at least some

monocotyledons also contain antimicrobial proteins, referred to as thionins. (Carr and Klessig 1989; Bohlman et al., 1987).

Plant cells containing sequestered glycosides release them when ruptured by injury or infection. These glycosides may have antimicrobial activity against the invading pathogens or may be hydrolyzed by glycosidases to yield more active aglycones. In the case of phenolic compounds, these may be oxidized to highly reactive, antimicrobial quinones and free radicals (Kuc, 1985; Dean and Kuc, 1987). Thus, damage to a few cells may rapidly create an extremely hostile environment for a developing pathogen. This rapid, but restricted disruption of a few cells after infection can also result in the biosynthesis and accumulation of phytoalexins, which are low molecular weight antimicrobial compounds, which accumulate at sites of infection (Kuc, 1985; Carr and Klessig, 1989; Bailey and Mansfield, 1992; Dean and Kuc, 1987). Some phytoalexins are synthesized by the malonate pathway others by the mevalonate, or shikimate pathways, whereas still others require participation of two or all three of the pathways. Phytoalexins are degraded by some pathogens and by the plant; thus they are transient constituents and their accumulation is a reflection of both synthesis and degradation rates.

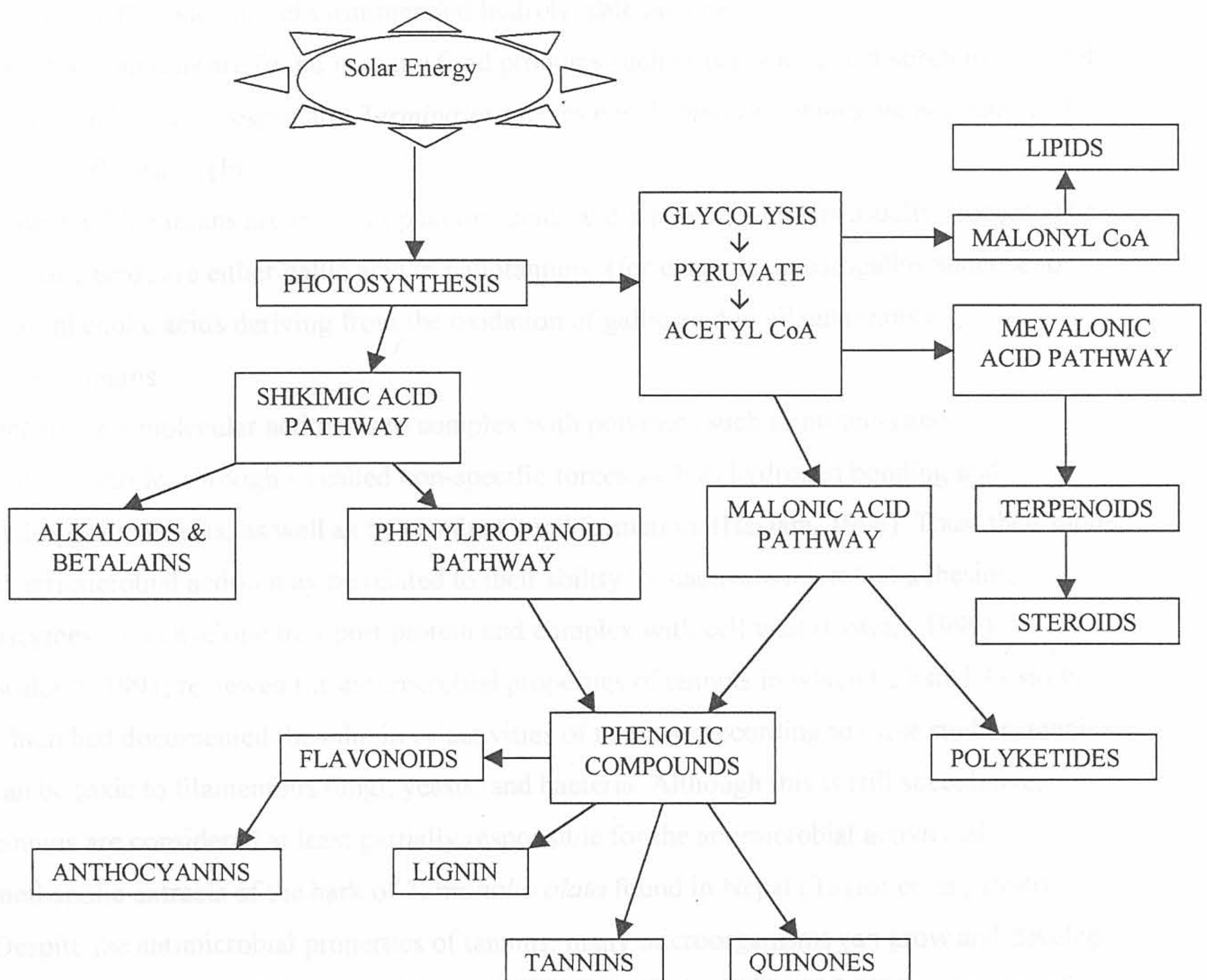
Biopolymers are also often associated with the phytoalexin accumulation at the site of injury or infection. These biopolymers include: lignin, a polymer of oxidized phenolic compounds; callose, a polymer of β-1, 3-linked glucopyranose; hydroxyproline-rich glycoproteins, and suberin. They provide both mechanical and chemical restriction of development of pathogens (Kuc 1985; Carr and Klessig, 1989; Rao and Kuc, 1990).

The macromolecule produced after infection or certain forms of physiological stress includes enzymes, which can hydrolyse the walls of some pathogens including chitinases, β-1,3-glucanases and proteases (Carr and Klessig, 1989; Boller, 1987; Rao and Kuc, 1990). Unlike the phyoalexins and structural biopolymers, the amounts of these enzymes increase systemically in infected plants even in response to localized infection. These enzymes are part of a group of stress or infection-related proteins commonly referred to as pathogenesis-related (PR) proteins. The function of many of these proteins is unknown. Some may be defense compounds while others may regulate the response to infection (Carr and Klessig, 1989; Boller, 1987; Rao and Kuc, 1990).

Another group of systemically produced biopolymer defense compounds comprises the peroxidases and phenoloxidases (Hammerschmidt et al., 1982; Rao and Kuc, 1990). Both can oxidize phenols to generate protective barriers to infection, including lignin. Phenolic

oxidation products can also cross-link to carbohydrates and proteins in the cell walls of plants and fungi to restrict further microbial development (Stermer and Hammerschmidt, 1987). Peroxidases also generate hydrogen peroxide, which is strongly antimicrobial. Associated with peroxidative reactions after infection is the transient localized accumulation of hydroxyl radicals and super oxide anion, both of which are highly reactive and toxic to cells. Plants therefore have several mechanisms to counter antimicrobial attack. Some of the antimcrobial compounds in plants may be exploited for use against bacterial diseases in man.

Fig. 1 Synthesis of plant secondary compounds.



University of Pretoria etd – Famakin, J O (2002)

1.4 Antimicrobial activity of some plant constituents- An overview

Some plant secondary metabolites which are synthesized by photosynthesis via various pathways have antibacterial activities [Fig. 1].

1.4.1 Tannins

Tannins are water-soluble polyphenols, which differ from other natural phenolic compounds in their ability to precipitate proteins such as gelatin from solution (Bruneton, 1995). They differ in that respect from simpler phenols such as catechol, pyrogallol, gallic acid, catechin and other flavanols. Tannins are commonly found in a large array of higher plant species of both herbaceous and woody types. They accumulate in large amounts (often more than 10 % of the dry weight) in particular organs or tissues, which can be almost any plant: bark, wood, leaves, fruits or roots (Haslam, 1989). They are classified in two groups according to their structures, proanthocyanidins (condensed tannins) for example, procyanidin trimmer, and prodelphinidin trimmer and hydrolysable tannins.

Proanthocyanidins are found in many food products such as tea, cocoa, and sorghum of carob pods. Combretaceae especially *Terminalia* species e.g. *Terminalia oblongata* is a source of condensed tannins [Fig. 2].

Hydrolysable tannins are esters of phenolic acids and a polyol, which is usually glucose. The phenolic acids are either gallic acid in gallotannins, (for example, hepatogalloylglucose) or other phenolic acids deriving from the oxidation of gallic acid in ellagitannins e.g. pedunculagins.

One of their molecular actions is to complex with polymers such as proteins and polysaccharides through so called non-specific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation. (Haslam, 1996). Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport protein and complex with cell wall (Cowan, 1999). Scalbert, 1991, reviewed the antimicrobial properties of tannins in which he listed 33 studies which had documented the inhibitory activities of tannins. According to these studies, tannins can be toxic to filamentous fungi, yeasts, and bacteria. Although this is still speculative, tannins are considered at least partially responsible for the antimicrobial activity of methanolic extracts of the bark of *Terminalia alata* found in Nepal (Taylor et. al., 1996). Despite the antimicrobial properties of tannins, many microorganisms can grow and develop on tannin-rich materials by several detoxification mechanisms. These include secretion of tannin-binding polymers, tannin-resistant enzymes, oxidation of tannins, siderophores, and

tannin biodegradation (Scalbert, 1991). Further, tannins are non-specific in their activity and present absorption problems.

2, 3- (S)-Hexahydroxydiphenoyl-4, 6-(S, S)-gallagylglucose (punicalagin) from *Terminalia oblongata*

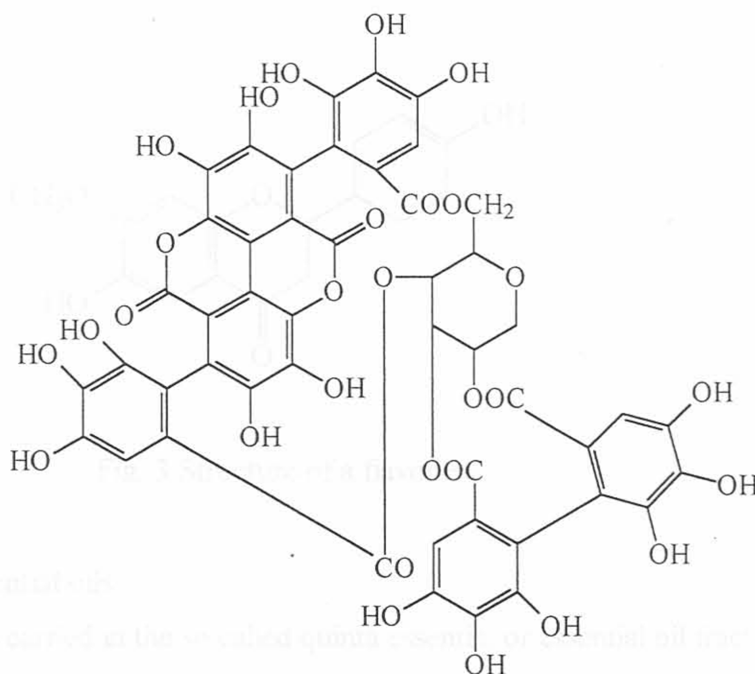


Fig. 2 Structure of a condensed tannin

1.4.2 Flavones, flavonoids and flavonols

Flavones are phenolic structures containing one carbonyl group [Fig. 3]. The addition of a 3-hydroxyl group yields a flavonol. Flavonoids are also hydroxylated phenolic substances but occur as a C_6-C_3 unit linked to an aromatic ring. They are found in fruits and vegetables essential for processing vitamin C and needed to maintain capillary walls. They may aid in protecting against infections (Mosby Medical Encyclopedia, 1997). Almost all are water-soluble; they are responsible for the colour of flowers, and fruits and sometimes leaves. Biflavonoids are dimers of flavonoids. The majority of natural biflavonoids are dimers of flavones and flavonones.

Since they are known to be synthesized by plants in response to microbial infections, they have been found in vitro to be effective antimicrobial substances against a wide range of microorganisms (Cowan, 1999). Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls.

University of Pretoria etd – Famakin, J O (2002)

Flavonoids isolated from the leaves of *C. micranthum* have been shown to have antimicrobial activity against both Gram positive and Gram negative microorganisms (Rogers and Verrotta, 1996).

Flavone from *Terminalia arjuna*

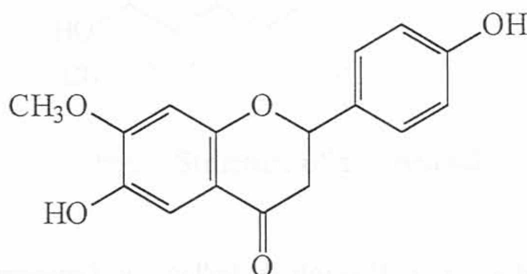


Fig. 3 Structure of a flavone.

1.4.3 Terpenoids and essential oils

The fragrance of plants is carried in the so called quinta essentia, or essential oil fraction. These oils are secondary metabolites that are highly enriched in compound based on an isoprene structure. They are called terpenes, their general chemical structure is $C_{10}H_{16}$, and they occur as diterpenes, triterpenes and tetraterpenes (C_{20}, C_{30}, and C_{40}), as well as hemiterpenes (C_5) and sesquiterpenes (C_{15}). When the compounds contain additional elements, usually oxygen, they are termed terpenoids.

Terpenoids are synthesized from acetate units, and as such, they share their origins with fatty acids. They differ from fatty acids in that they contain extensive branching and are cyclized. Examples of common terpenoids are methanol and camphor (monoterpenes) and farnesol and artemisin (sesquiterpenoids).

Terpenes or terpenoids are active against bacteria and fungi (Taylor et. al., 1996). The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds. A rich variety of triterpenoid acids has been isolated from *C. molle* and *C. imberbe* (Rogers and Verotta, 1996) [Fig. 4].

Pentacyclic terpenoid from *C. molle*

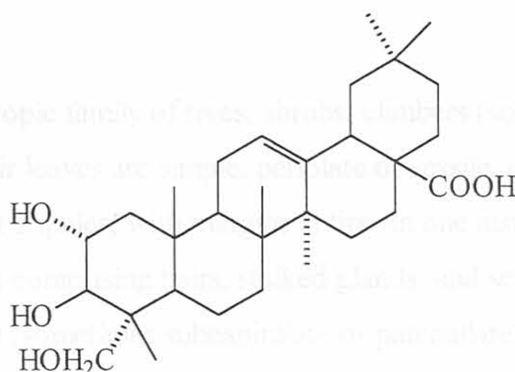


Fig. 4 Structure of a terpenoid

1.4.4 Alkaloids

Heterocyclic nitrogen compounds are called alkaloids [Fig. 5]. Indo quinoline alkaloids, the active principals in *Cryptolepsis sanguinnolenta* has been shown to inhibit Gram negative bacteria and yeast (Silva, 1996). Additional studies have shown this plant to have bactericidal activity. Recent *in vitro* studies have shown activity against bacteria specifically, enteric pathogens, most notably *E. coli* (but also *Staphylococcus*, *Pseudomonas*, *Salmonella*, *Shigella*, *Streptococcus* and *Vibrio* spp) and some activity against *Candida* spp (Sawer, 1995). Indo-quinolizidine alkaloids and glycoalkaloids and saponins, the essential constituents of *Naclea latifolia* have antibacterial activity against Gram positive and Gram negative bacteria and antifungal activity (Iwu, 1993). They are most effective against *Cornebacterium diptheriae, Streptobacillis sp.*, *Streptococcus* sp., *Neisseria* sp., *Pseudomonas aeruginosa, Salmonella* sp. (Deeni 1991).

Berberine

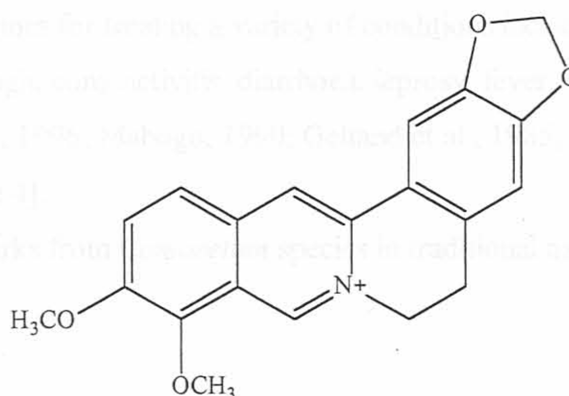


Fig. 5 Structure of an alkaloid.

University of Pretoria etd – Famakin, J O (2002)

1.5 Combretaceae

1.5.1 Introduction

The Combretaceae is a pantropic family of trees, shrubs, climbers (sometimes spinose) and mangrove (Carr, 1988). Their leaves are simple, petiolate or sessile, opposite, alternate, verticillate, whorled, without stipules, with margins entire (in one instance sometimes crenulate), with indumentum comprising hairs, stalked glands, and scales. The inflorescences are axillary, terminal, spicate (sometimes subcapitulate or paniculate). The flowers are sessile, or pedicellate, bisexual or bisexual and male on the same inflorescence. The receptacles are usually in two parts, the lower containing the ovary, the upper terminating in four or five sepals. The number of petals may be four or five or absent. The stamen may be eight or ten. The style are centrally situated on disc (Carr, 1988).

1.5.2 Taxonomy

The Combretaceae family belongs to the order Myrtales consisting of 18 genera. The two largest genera in Africa include *Combretum* with about 370 species and *Terminalia* with about 200 species (Lawrence, 1951). The other genera are smaller; e.g. *Calopyxes* and *Buchenavia* comprise 22 species each, *Quesqualis* 16, *Angioeissis* 14, *Conocarpus* 12 and *Pteleopsis* 10 species (Rogers and Verotta, 1996). The genus *Combretum* has two subgenera, these being subgenus *Combretum* and sub genus *Cacoucia*. *C. woodii*, a member of subgenus *Combretum* was investigated in this study [Table 3].

1.5.3 Use of Combretaceae in Traditional medicine

Many species of Combretaceae are widely distributed in Southern Africa. These plants are used in traditional medicines for treating a variety of conditions including pneumonia, syphilis, colds, chest cough, conjunctivitis, diarrhoea, leprosy, fever, wound dressings, and mumps (Hutchings et al., 1996; Mabogo, 1990; Gelfand et al., 1985; Watt and Breyer-Brandwijk, 1962) [Table 4].

The use of leaves and barks from *Combretum* species in traditional medicine is widespread.

University of Pretoria etd – Famakin, J O (2002)

Table 3 The subgeneric classification the genus *Combretum* in South Africa according to Carr 1988.

Combretum **Loefl**	
Subgenus *Combretum*	**Subgenus *Cacoucia***
Section *Hypocrateropsis*	**Section *Lasiopetala***
C. celastroides	*C. obovatum*
C. imberbe	**Section Conniventia**
C. padoides	*C. microphyllum*
Section *Combretastrum*	*C. paniculatum*
C. umbricola	*C. platypetalum*
Section *Angustimarginata*	Section *Oxystachya*
C. caffrum	*C. oxystachytum*
C. erythrophyllum	**Section *Megalantherum***
C. kraussili	*C. wattii*
C. vendae	**Section *Poivrea***
C. woodii	*C. bracteosum*
Section *Macrostigmatea*	*C. mossambicense*
C. engleri	
C. kirkii	
C. mkuzense	
Section *Metallicum*	
C. collinum	
Section *Glabripetala*	
C. fragrans	
Section *Spathulipetala*	
C. zeyheri	
Section *Ciliatipetala*	
C. albopunctatum	
C. apiculatum	
C. edwardsii	
C. maggii	
C. molle	
C. petrophilum	
C. psidioxides	
Section *Fusca*	
C. coriifolium	
Section *Breviramea*	
C. hereroense	
Section *Elaeagnoida*	
C. elaeagnoides	

Table 4 Traditional uses of some *Combretum* species

Combretum	**Medicinal uses**
C. apiculatum	Snakebite, Scorpion bite, Bloody diarrhoea, leprosy, Abdominal disorders, Conjunctivitis.
C. erythropyllum	Fattening tonic for dogs. To reduce the size of vaginal orifice, Infertility, Venereal disease, To facilitate labour.
C. fragrans	Chest coughs, Syphilis, Aphrodisiacs.
C. hereroense	Bilhariziasis, Heart disease, Heartburns, Body pains, Stomach complaints.
C. kraussii	Appetite stimulant, Cleaning of urinary system.
C. microphyllum	Lunacy, Lucky charm.
C. molle	Snake bite, Stomach ache, Abortion, Wound dressing, Fattening of infants, Abdominal pains, Diarrhoea, To stop bleeding after birth, Convulsions, Backache, Headache.
C. zeyheri	Gallstones, Bloody diarrhoea, To arrest menstrual flow, Embrocation, Scorpion bite.

1.5.4 Ethnopharmacology of Combretaceae

Species of Combretaceae contain compounds with potential antimicrobial properties (Eloff, 1999). There is a large variation in the chemical composition and antibacterial activity among different genera and species in the Combretaceae. Seven species of Combretaceae used in traditional medicine in West Africa have been investigated for their antifungal activity against the pathogenic fungi. Phytochemical screening revealed that these plants are particularly rich in tannins and saponins, which might be responsible for their antifungal activity (Baba-Moussa et al., 1999).

Leaf extracts of *Combretum padoides, C. celestroides, C. hereroense, C. obovatum* and *C. zeyheri. C. erythrophyllum, C. paniculatum, C. edwarsii, C. apiculatum, C. imberbe* have been shown to have some activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Serratia marcescens, Mycobacterium phlei, Saccharomyces cerevisiae* (Alexander, 1992).

University of Pretoria etd – Famakin, J O (2002)

The ethnopharmacological use of *Combretum zeyheri* against diarrhoea and eye infections has been ascribed to its antibacterial activity towards Gram- positive microbes (Breytenbach and Malan, 1989).

Combretum erythrophyllum has been shown to possess many antibacterial compounds and some of these had activities higher than chloramphenicol and ampicillin (Martini and Eloff, 1998). Eloff (1999) also found that all the leaf extracts from 27 Southern African members of the Combretaceae including *Combretum woodii* exhibited antibacterial activity against *Staphylococcus aureus, Escherichia coli, Enterococcus feacalis and Pseudomonas aeruginosa*.

The leaves of *Combretum molle* and *Combretum imberbe* have been shown to have molluscicidal activity against *Biomphalaria glabrata* snails (Rogers and Verotta, 1996).

1.5.5 Phytochemistry of Combretaceae

Members of the family are often tanniferous and produce ellagic and gallic acids and frequently proanthocyanins. They are sometimes cyanogenic and often accumulate triterpenoids, especially as saponins (Hutchings et al., 1996).

Chemical studies of the *Combretum* genus have yielded acidic triterpenoids and their glycosides (*C. molle, C. padoides, C. eleagnoides*), phenanthrenes (*C. hereroense, C. molle, C. apiculatum, C. caffrum*), amino acids (*C. zeyheri*), and stilbenes (*C. caffrum*)(Pellizzoni et al., 1992).

A series of closely related bibenzyls, stilbenes and phenanthrenes have been isolated from *C. caffrum*, a member closely related to *C. woodii*. Some of these stilbenes have been found to be potent antimitotic agents, which inhibited both tubulin polymerization and binding of colchine to tubulin. These stilbenes are called combretastatins. Combretastatin A-4 has been shown the most potent cancer cell growth inhibitor of the series (Petit et al., 1995). Combretastatins A-4, A-5, A-6, were also found to inhibit growth of *Neisseria gonorrhea* (Petit et al., 1995).

The anti-inflammatory and molluscicidal compounds mollic acid β-D-glucoside and imberbic acid have been isolated from *C. molle* and *C. imberbe* respectively (Pegel and Roger 1976, Roger, 1988). The saponin, jessic acid linked to α-L-arabinose has been found in *C. eleagnoides* leaves (Osborne and Pegel, 1984). Panzini et al., 1993 also identified jessic acid 3-0-β-D-xylopyranoside from the fruit of *C. molle*.

University of Pretoria etd – Famakin, J O (2002)

The presence of poly-cis prenols of chain length 20 - 60 isoprene units or longer in leaves of plants belonging to Combretaceae family has been shown to be the common feature of this group in most cases the polyprenols occurred in the form of fatty acid esters. The polyprenols in *Combretum molle* are in form of free alcohols (Kulcitsky et al., 1996). The aerial parts and fruits of *C. zeyheri* have been found to contain ursolic acid, and a compound named as CZ 34 and L-3. (3-aminomethylphenyl) alanine. (Breytenbach et al., 1985, Nwauluka et al., 1975). Flavonoids have been isolated from *C. micranthum* leaves (Rogers and Verotta, 1996). The fruits of *Terminalia cheluba* have yielded complex esters of gallic acid, corilagin (Haslam, 1989).
With the exception of the simple indole alkaloids harman and eleagnine isolated from the roots of *G. senegalensis*, there have been no other reports of alkaloids from the Combretaceae. (Rogers and Verotta, 1996).

1.5.6 Selection of *C. woodii*

C. woodii belongs to the subgenus *Combretum*, the same section of very important medicinal plants such as *C. caffrum*, *C. erythrophyllum*. The activities of *Combretum* species are ascribed mainly to stilbenoids (combretastatins), triterpenoids and flavonoids (Roger, 1996). Triterpenoids and saponins are well known for their antimicrobial and anti-inflammatory activity (Bruneton, 1995). The water, acetone and ethyl acetate extracts of *C. woodii* leaves have been shown to have anti-inflammatory activity (McGaw et. al., 2001). However, there has not been any report in the literature on the antibacterial compounds that are present in *C. woodii*. It contains antibacterial compound chemically different to those in *C. erythrophyllum*, according to our preliminary results (Eloff 1998, unpublished data). It was selected for this study because of these reasons.
C. woodii Duemmer is a deciduous tree or shrub with a height of 8 - 12 m [Fig. 6].
It is found in South Africa: Transvaal, Kruger national park, Natal, Ndumu, Mkuze game reserve,) and Swaziland: Lebombo, Nhlemen [Fig. 7].
It grows in steep rocky slopes, canyon margins, ravines, sand forest, dry forest, closed forest, riverine woodland, rocky hillsides, mountain grassland, and at low to medium altitudes (up to 1200 m).
Common names in South Africa are Bastard Forest Bush willow, Basterbosvaderlandswilg (Afrikaans), iWaphu (Zulu), Mbondvo sehlatsi (Siswati).

University of Pretoria etd – Famakin, J O (2002)

The bark is mostly smooth light grey (almost white) and a slightly darker, biscuit-tinged grey. In places, however, the outer covering lifts in large flakes, exposing a biscuit to ginger sub-surface, which is also smooth.
Leaves are often alternate on extending shoots but usually opposite to sub- opposite when born on new lateral twigs. Lamina shape varies somewhat. It may be broadly elliptic with base bluntly tapered and apex rounded or broadly elliptic to obovate with a rounded taper to the base and the apex bluntly acuminate with slight attenuation or elliptic, tapering to the base and acuminate and attenuate at the apex.
The size is also variable, from 65 x 25 mm with petiole 2.5 mm to 150 mm x 75 mm and an 8 mm petiole. Calculated average dimensions are 79 x 38 x 6 mm. The leaf keel is arched and the lamina is vee-ed about the midrib. There can be considerable marginal undulations. Lamina texture is papyraceous. The upper surface of the leaf is medium green with the underside appreciably paler. Foliage may develop yellow and red coloration in the autumn and (during flowering) leaves near inflorescences are sometimes markedly blanched. Venation on the upper surface is flush to slightly rise and a yellowish green. Main and lateral veins can be readily discerned but secondary venation is sometimes indistinct.
The inflorescences are borne singly in axis along (and particularly near the terminal of short, new, leafy, lateral shoots) from August to first week in December. Spikes may be glabrous except for small light- coloured scales, dense to rather obscure, with in some cases scattering hairs, or puberulous. Petals are spathulate, glabrous, whitish and are about 1 mm long. Filamens are exserted up to 4.5 mm. Anthers, yellowish brown, are, 0.8 mm long. The style projects up to 3 mm and has an apical swelling. The disc has whitish hairs, sometimes long and projecting to the same extent as the petals.
The fruit is 4-winged (occasionally 5- or 6-winged) with outline broadly elliptic to sub-circular. Some fruit has dense, minute, yellowish to light brown scales evenly over the body and wings, but scales are usually closely spaced on the body and are more scattered on the wings. With the unripe fruit, there is a tingling mainly on the body but spread to the wings. When ripe, wings are biscuit- coloured and the body a light brown color. The seed is cigar shaped up to 12 mm long and 3.5 mm across at its greatest diameter. There are four longitudinal grooves, the testa is appreciably wrinkled and the colour is cinnamon.

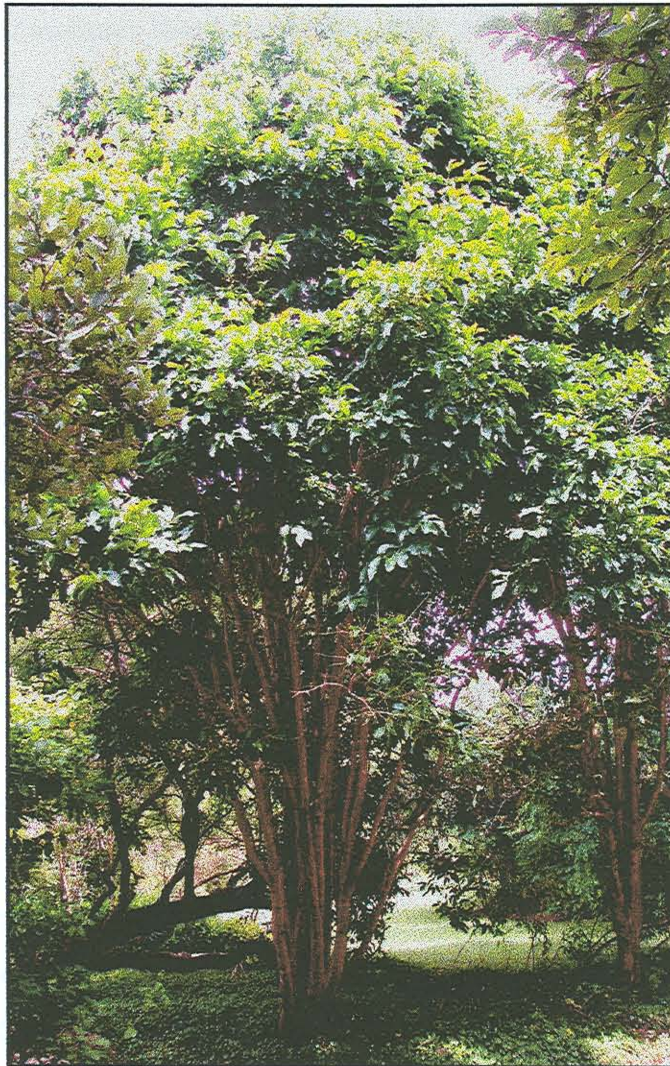


Fig. 6 *Combretum woodii* from which leaves were collected in Lowveld National Botanical Garden

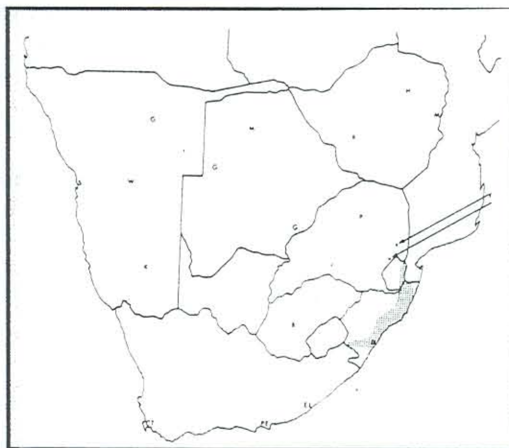


Fig. 7 Distribution of *C. woodii* in Southern Africa

1.6 An overview of methods that have been employed to isolate compounds from plants.

1.6.1 Plant materials.

Fresh or dried plant material can be used as a source of extract for secondary plant metabolites. It is preferable to use dried material because:

- Plant material not rapidly dried may be attacked by fungi, which could alter the chemical composition of the components drastically.
- The time delay between collecting plant material and processing it makes it difficult to work with fresh material because differences in water content may affect solubility or subsequent separation by solvent-solvent extraction.
- There are fewer problems associated with large-scale extraction of dried plant material than with fresh material.
- Dried plant materials are mostly used by traditional healers, so it may be necessary to follow their method of plant harvesting and preparation.

1.6.2 Extraction techniques

An important factor governing the general and specific method used in an extraction is the type of phytochemical groups that are to be extracted [Table 5]. The main groups of compound to be considered are fixed oils, fats and waxes, volatile or essential oils, carotenoids, alkaloids, glycosides, aglycones, phenolic compounds, polysaccharides and proteins.

Many workers have used different extractants while screening for antimicrobial activity of medicinal plants. These include, for instance, 80% ethanol solution (Vlietinck et. al., 1995), ethanol-water (50:50,v/v)(Baba-Mousa et al., 1999), methanol (Taylor et al., 1995), petroleum ether, chloroform, ethanol, methanol and water (Salie et. al., 1996). Many scientists employ Soxhlet extraction of dried plant material using solvents with increasing polarity. This may be suitable for compounds that can withstand the temperature of the boiling solvent, but cannot be used for thermolabile compounds. The problem can be overcome by extracting under reduced pressure. Acetone is an ideal solvent for use in extractions. It dissolves many hydrophilic and lipophilic components. It is miscible with water, volatile and has low toxicity (Eloff, 1997).

Different phytochemical groups are extracted by different extractants according to Houghton and Raman (1998) [Table 5]

i 17405762
b16348217

Table 5 Type of phytochemicals extracted by different solvents (Houghton and Raman, 1998)

Polarity	**Solvent**	**Phytochemicals extracted**
Low	Hexane	Waxes, Fats, Volatile oils
Low	Chloroform	Alkaloids, Aglycones, Volatile oils
Medium	Diethyl ether	Alkaloids, Aglycones
Medium	Ethyl acetate	Alkaloids, Aglycones, Glycosides
Medium	Acetone	Alkaloids, Aglycones, Glycosides
Medium	Ethanol	Glycosides
Medium	Methanol	Sugars, Amino acids, Glycosides
High	Water	Sugars, Amino acids, Glycosides
High	Aqueous acid	Sugars, Amino acids, Bases
High	Aqueous alkali	Sugars, Amino acids, Acids.

1.6.3 Isolation and analysis of constituents

The isolation and analysis of phytochemical constituents or fractions with antibacterial activity are carried out by means of thin layer chromatography (TLC), column chromatography, nuclear magnetic resonance spectroscopy (NMR), and mass spectroscopy. TLC is used for qualitative analysis of crude extract for identification of isolated constituents present in an extract by comparison with reference substances and /or data in the literature. For a given substance, a useful parameter to measure is its retardation factor or the Rvalue on TLC. This is the ratio of the distance from the baseline (point of application) to the center of the zone divided by the distance from the baseline to the solvent front.

Ultraviolet detection of UV active compounds on TLC plates at UV light (254 nm and 366 nm) can be carried out [Table 6].

Table 6 Examples of colours detected under UV for some phytochemical preparation according to Wagner and Bladt (1996).

Type of compound	**UV-254nm**	**UV-365nm**
Alkaloids	Pronounced quenching of some alkaloids of indoles, quinolines, isoquinolines, and purines. Weak quenching e.g. atropine alkaloids	Blue, blue-green or violet fluorescence. Yellow fluorescence e.g. Colchicine, berberine.
Flavonoids	All flavonoids cause fluorescence quenching. Caffeic acid, its derivatives and isoflavones show quenching	Dark yellow, green or blue fluorescence depending on the structure type Caffeic acid, its derivatives and isoflavones fluoresce blue
Triterpenes and Essential oils	Compound containing at least two conjugated double bonds quench fluorescence and appear as dark zones against light green fluorescent background of the TLC plate	No characteristic fluorescence of terpenoids and propylphenols is noticed
Saponins	No saponins are detectable	No detection

Column chromatography separates constituents of a mixture according to the polarity as well as several other factors. Adsorption chromatography using silica gel as a stationary phase is used for analysis of components.

Nuclear magnetic resonance spectroscopy (NMR) essentially provides a means of determining the structure of an organic compound by measuring the magnetic moments of its hydrogen atoms. In most compounds, hydrogen atoms are attached to different groups (as –CH_2, -, -CH_3-, -CHO, -NH_2-, -CHOH-, etc.) and the NMR spectrum provides a record of the number of hydrogen or carbon atoms in these different situations. It can give any direct information on the nature of skeleton of the molecule; this must be obtained in the first

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instance by application of other spectral techniques. NMR spectroscopy will be used for structural elucidation of isolated compounds in the fractions.

Therefore, both proton and carbon NMR are employed for structural elucidation of the pure compounds isolated.

Mass spectroscopy has a value, in that with only microgram amounts of material, it can provide an accurate molecular weight and it may yield a complex fragmentation which is usually characteristic of (and may identify) that particular isolated compound.

1.6.4 Assay of plant extracts

Most workers use agar diffusion assays to determine the antibacterial activity of plant extracts. The technique works well with defined inhibitors (Hewit and Vincent, 1989), but when examining extracts containing unknown components, there are problems leading to false positive and false negative results (Eloff, 1998). The antimicrobial effect may be inhibited or increased by extrinsic factors or contaminants. The agar type, salt concentration, incubation temperature, and molecular size of the antimicrobial component influence results obtained with agar diffusion assays (Marsh and Goode, 1994). This technique also does not distinguish between bactericidal and bacteristatic effects and minimum inhibitory concentration (MIC) cannot be determined. An alternative technique most widely used in general microbial assay is serial dilution of the extract in a number of test tubes followed by the addition of the test organism to determine to minimum inhibitory concentration for the test organism using turbidity as an indication of growth. This technique requires relatively large quantities of extracts and is therefore not useful in bioassay guided isolation of antimicrobial compounds (Eloff, 1998).

Eloff, 1998 developed a micro-dilution technique using 96-well micro plates and tetrazolium salts to indicate bacteria growth. He found that *p*-Iodonitrotetrazolium violet (0.2 mg/ml) gave better results than tetrazolium red or thiazolium blue. The method is quick, worked well with *Staphylococcus aureus*, *Enterococcus faecalis, Pseudomonas aeruginosa,* and *Escherichia coli* and with non- aqueous extracts from many different plants.

This method is useful in screening plants for antimicrobial activity and for the bioassay-guided isolation of antimicrobial compounds from plants (Eloff, 1998).

Some antibacterial agents are assayed by agar diffusion method and / or turbidimetry [Table 7].

University of Pretoria etd – Famakin, J O (2002)

Table 7 Pharmacopoeia methods for microbiological assay of some antibacterial agents

Antibiotic	British Pharmacopoeia (1980)	United States CFR (1980)
Amoxycillin	-	agar diffusion assay
Bactracin	agar diffusion assay	-
Capreomycin	agar diffusion assay	turbidimetry
Carbenicillin	-	agar diffusion assay
Cephalexin	-	agar diffusion assay
Chloramphenicol	agar diffusion assay	turbidimetry
Clindamycin	agar diffusion assay	-
Cloxacillin	agar diffusion assay	-
Doxycycline	agar diffusion assay	turbidimetry
Erythromycin	agar diffusion assay	agar diffusion assay
Gentamicin	agar diffusion assay	agar diffusion assay
Neomycin	agar diffusion assay, turbidimetry	agar diffusion assay
Polymyxin B	agar diffusion assay	agar diffusion assay
Rifampicin	-	agar diffusion assay
Streptomycin	agar diffusion assay, turbidimetry	agar diffusion assay, turbidimetry
Tetracycline	agar diffusion assay, turbidimetry	turbidimetry
Tobramycin	agar diffusion assay, turbidimetry	turbidimetry
Vancomycin	agar diffusion assay	agar diffusion assay

Bioautography is another method of studying antimicrobial activity. Among the numerous *in vitro* methods for studying the antimicrobial activity of plant extracts, bioautography has found widespread applications, especially for the detection of new compounds in complex plant extracts. The methods allows for the detection of spots of growth inhibition of cultures directly on the extract thin layer chromatography plate previously dispersed with a broth culture containing the microorganism.

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1.7 Problem statement and Hypothesis

The growing number of resistant strains of microbial pathogens is a worldwide problem, which justifies the search for new antibiotics from indigenous plants.

Many *Combretum* species are used in traditional medicine for diseases related to bacterial infections, *Combretum woodii* contains different antibacterial compounds from other *Combretum* species examined in our laboratory (Eloff, 1998, unpublished data) hence the motivation to investigate *C. woodii* for antibacterial activity.

By applying standard bioassay guided procedures, I will be able to isolate and characterize one or more of these compounds.

1.8 Aim and Objectives of the study

To isolate and characterize antibacterial compounds in *Combretum woodii* by:

- Extracting the compounds from with several extractants.
- Testing the biological activity of the extracts against the common pathogens.
- Determining the best preliminary group separation technique.
- Fractionating the compound(s) responsible for activity using a bioassay guided process.
- Isolating the antibacterial compound(s).
- Determining its biological activity the chemical structure of the isolated compound(s).

CHAPTER 2
MATERIALS AND METHODS

2.1 Plant material

Leaf material was collected from two trees growing in the Lowveld National Botanical Garden, Nelspruit [Fig. 6]. The origin of each tree is recorded in the database of the botanical garden and voucher material from each tree was deposited in the garden's herbarium (Eloff, 1999).

2.2 Preparation and Extraction.

Leaves were dried under shade at room temperature (20 ^{0}C) and milled into a fine powder with a Junkel and Kunkel model A10 mill. The powder was stored at room temperature in the dark in tightly closed glass containers.

In a preliminary extraction, 0.5 g each of finely ground plant material was extracted in 5 ml in each of 10 solvents of varying polarity: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethyl acetate, tetrahydrofuran, acetone, methanol and water with vigorous shaking. The extracts were decanted after centrifuging at 5300 x g for 5 minutes.

The process was repeated twice more. The extractants used were of technical grade (Merck). The solvents were removed under a cold air stream at 7 ^{0}C. The extracts were weighed and re-dissolved in acetone to yield 10 mg/ml solutions.

2.3. TLC analysis of extracts

Thin layer chromatography was used to determine the composition of extracts. A quantity of 50 µg of extract was separated by TLC (Merck, Kieselgel 60 F_{254}) using the following solvent systems developed in our laboratory:

Benzene:ethanol:ammonium hydroxide (BEA) (36:4:0.4)

Ethylacetate:methanol:water (EMW) (40:5.4:4)

Chloroform:ethylacetate:formic acid (CEF) (20:16:4)

A 5 µl of the extract solution was applied by micropipette 1 cm from the bottom of the TLC plates and allowed to develop in solvent in the solvent systems. The development of the chromatograms was carried out in a closed, saturated TLC tank. Separated components were visualized under visible and ultraviolet light (254 nm and 360 nm, Camac Universal UV lamp TL-600). TLC plates were sprayed with one of the following reagents (Stahl, 1969):

- *p*-anisaldehyde-sulphuric acid (a freshly prepared mixture of 1 ml *p*-anisaldehyde,

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18 ml ethanol, 1 ml sulphuric acid) for detection of sugars, steroids, terpenes, etc (Smith 1937 as referred to by Stahl, 1969)

- vanillin-sulphuric acid (0.1 g vanillin, 28 ml methanol, 1 ml sulphuric acid) for detection of higher alcohols, phenols, steroids and essential oils (Stahl, 1969).

The plates were heated at 105 ^{0}C until the development of colour was complete.

2.4 Bioautographic assay

The bioautography procedure described by Begue and Kline (1972) was used.

2.4.1 Preparation of TLC plates for bioautography

The TLC plates were prepared in duplicate (3 x 2) and developed in different mobile systems above. Chromatography plates were first dried overnight under a stream of air to remove the remaining TLC solvents that might kill the bacteria before spraying the plates. Duplicate plates were sprayed with the vanillin spray reagent or with bacteria.

2.4.2 Preparation of bacteria

A 10 ml of highly dense fresh bacteria culture was added into two centrifuge tubes and centrifuged at 5300 x g for 20 minutes to concentrate the bacteria. The supernatant was discarded and the pellet at the bottom of the tube was visible. The pellet was resuspended in 2 – 4 ml of fresh Mueller Hilton broth.

2.4.3 Bioassay method

The dried chromatographic plates were sprayed with a concentrated suspension of actively growing cells of *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 25922), *Escherichia coli* (ATCC 27853) or *Enterococcus faecalis* (ATCC21212).

The selection of test organisms was based on recommendation of the National committee for clinical laboratory standard (NCCLS) that these are bacteria strains for clinical Laboratory Standards. (Waitz et al., 1992) These species of bacteria are also the major cause of nosocomial infections in hospitals (Sacho et. al., 1993). The plates were sprayed until they were wet and opaque, before being incubated overnight at 38 ^{0}C in a clean chamber at 100 % relative humidity. After overnight incubation, plates were sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (Sigma Chemicals). Inhibition of growth was indicated by clear zones on chromatogram after incubating for about one hour (Begue and Kline, 1972).

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2.5 Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) values were determined by INT microplate bioassay (Eloff, 1998). The test organisms used are the same as those used above for bioautography. The work was done within the Laminar flow cabinet to prevent the contamination of the working culture.

2.5.1. Dilution of extract

The 96-well microplate was labeled with sample code after deciding on two-fold serial dilution of eight times (landscape model) or 12 times (portrait model). Distilled water (100 μl) was placed in each of the wells with a Socorex multichannel micropipette to the first well in the relevant series. This reduced the concentration in this well to 50% of the original concentration. The pipette tip was rinsed between applications with a small quantity of water. After all extracts have been diluted in the first well, the multiple channel pipette was used to remove 100 μl from the first well and place the content into next well. The plunger was pushed up and down three to four times to ensure that the contents of the well were properly mixed. The concentration in this well was 25% of the original extract concentration, the next well 12.5%, etc.

The process was repeated all the way to the bottom of the plate. The first 100 μl from the last row of wells was discarded to ensure that all the wells contain 100 μl of the extract. The first column will then have a series of two-fold dilutions of extract number one.

2.5.2 Addition of bacteria

The working culture of the bacteria was prepared ahead to get active bacteria culture required. The working culture was grown for 4 – 6 hours at 37 ^{0}C and then stored in the fridge for up to10 days before using (Eloff, 1998c). The 100 μl of the relevant bacteria culture was placed in each of the wells and mixed by squirting the bacteria into wells. This resulted in 25% of the original extract concentration in the first row, 12.5% in the second row, etc.

The microplates were incubated overnight in the incubator at 37 ^{0}C. Then, 40 μl of 0.2 mg/ml *p*-iodonitrotetrazolium (INT) solution was added to each row with a multichannel micropipette. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis* cultures were used in the microplates. The microplates were examined for colour changes after 30, 60 and 120 minutes of incubation. The *E. faecalis* cultures took much longer to react, up to 16 hours.

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The lowest concentration where growth is inhibited was recorded. The minimum inhibitory concentration (MIC) of the extract was calculated from the original concentration of the extracts.

2.6 Solvent-Solvent fractionation

This method was used to simplify the extract into different fractions. The solvent-solvent group separation procedure used by the USA National Cancer Institute as described by Suffness and Douros (1979) was adopted with minor modifications. The acetone extract of *C. woodii* leaves was fractionated into solvent of different polarities. The protocol is depicted diagrammatically below [Fig. 8].

2.6.1 Analysis and bioautographic assay of fractions

The thin layer chromatography and the bioautography assay of fractions obtained from solvent-solvent extraction were carried out as explained in the section 2.5 above. The MIC values of different fractions obtained were similarly determined.

2.7 Isolation of bioactive compounds

The best fraction from solvent-solvent extraction was subjected further to silica gel column chromatography.

2.7.1 TLC analysis

The TLC analysis of chosen fraction was used to select the best solvent system that could be employed as mobile phase for column chromatography. Thin layer chromatography was also carried out on the chosen fraction in different solvent systems [Section 4.1.1].

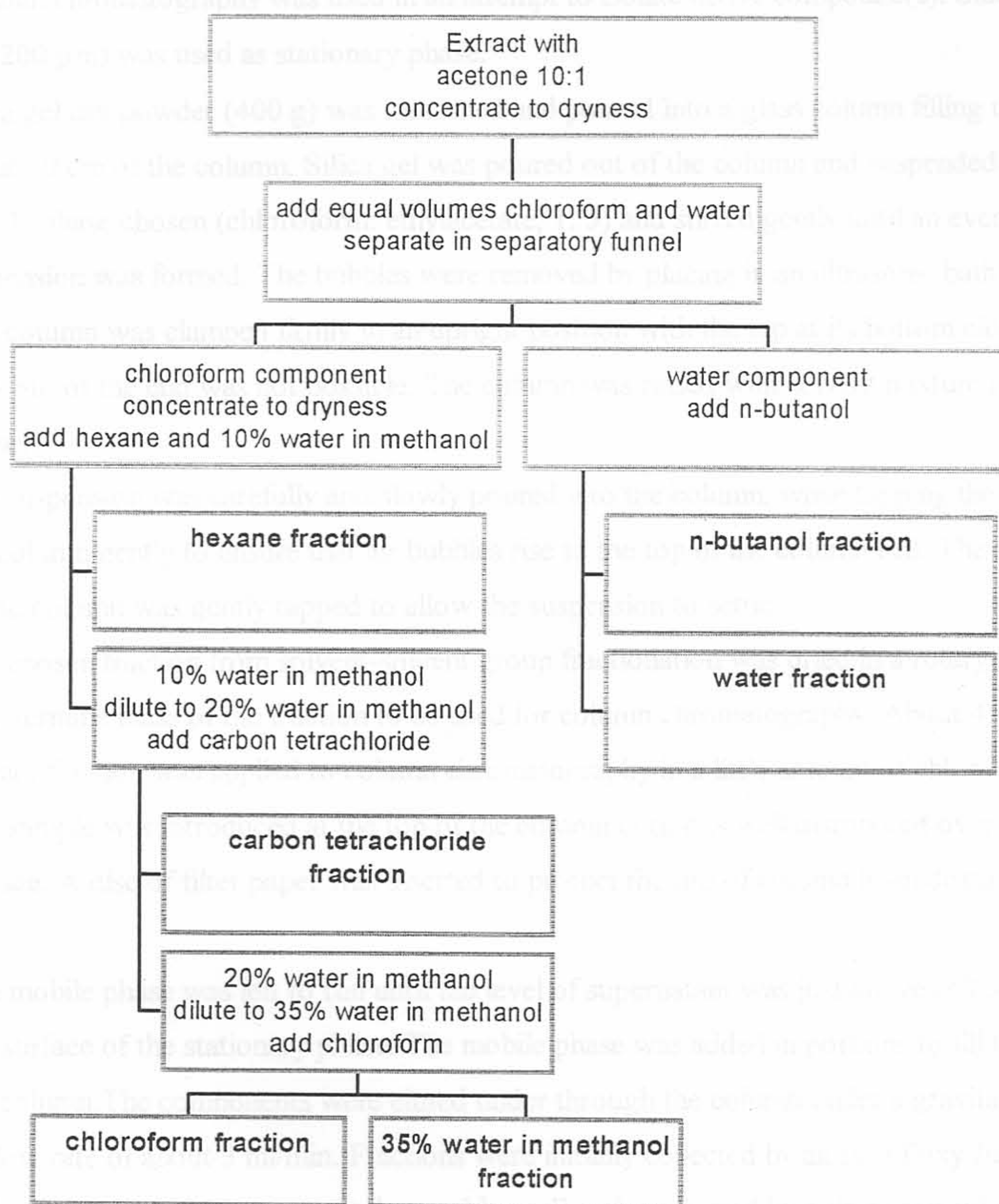

Fig. 8 The procedure used for the solvent-solvent fractionation of the components in the *C. woodii* leaves extracts.

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2.7.2 Isolation by Column Chromatography

Column chromatography was used in an attempt to isolate active compound(s). Silica gel 60 (63-200 µm) was used as stationary phase.

Silica gel dry powder (400 g) was measured and poured into a glass column filling up to about 70 cm of the column. Silica gel was poured out of the column and suspended in the mobile phase chosen (chloroform: ethylacetate, 1: 5) and stirred gently until an even suspension was formed. The bubbles were removed by placing in an ultrasonic bath.

The column was clamped firmly in an upright position with the tap at its bottom closed so that flow out of the end was not possible. The column was rinsed with solvent mixture (mobile phase).

The suspension was carefully and slowly poured into the column, while tapping the wall of the column gently to ensure that air bubbles rise to the top of the column bed. The bottom end of the column was gently tapped to allow the suspension to settle.

The chosen fraction from solvent-solvent group fractionation was dried in a rotary evaporator to determine mass of the fraction to be used for column chromatography. About 4 g of the extract fraction was applied to column chromatography in a little amount of chloroform

The sample was introduced at the top of the column until it is well distributed over the whole surface. A disc of filter paper was inserted to protect the top of column from disturbance.

The mobile phase was left to run until the level of supernatant was just above (<3 mm) at the top surface of the stationary phase. The mobile phase was added in portions to fill the top of the column.The components were eluted under through the column under a gravitational force at flow rate of about 3 ml/min. Fractions were initially collected by an Isco Foxy Junior collector into 16 x125 mm test tubes and later directly collected by culture test tubes (25 x 150 mm). About 500 ml of 10% methanol in acetone was gradually introduced into the column after about 1500 ml to elute components which could not moved by chloroform : ethylacetate mixture.

As the separation was completed, the test tubes were placed in the fume cupboard under a stream of air to concentrate the fractions for bioassay and further analysis by TLC.

Approximately 160 test tubes were collected in separating the chloroform fraction. Various TLC analyses were done on the fractions to isolate the active compound.

The isolated compounds were purified by recrystallization in different solvents of varying polarities.

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2.7.3 Bioassay work on isolated compounds

Bioautography was carried out on the purified compounds. MIC values were determined using all the four tested organisms in order to compare the strength of inhibition in comparison to standard antibiotics (ampicillin and chloramphenicol) used as controls.

2. 8 Spectroscopic analysis of isolated compounds

The samples were weighed and dissolved in deuterated chloroform for NMR analysis. Both ^{1}H- and ^{13}C-NMR experiments were carried out at Medical University of Southern Africa on a 300 MHz Varian NMR machine (Oxford instruments).

The isolated compounds were also sent to Cape Technickon for mass spectrometric analysis on a VG70-SEQ instrument.

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CHAPTER 3

RESULTS AND DISCUSSION ON EXTRACTION

3.1 INTRODUCTION

Many species of sub genus Combretum are used in traditional medicine as aqueous decoctions or infusions, with the herbs boiled or soaked in water and then administered (Carr, 1988). This process must be mimicked as closely as possible if the extracts are to be the subject of further biological or chemical scientific studies, particularly if the purpose of these is to validate traditional use.

The aim of extraction is to test the plant material for the presence of compounds with antibacterial activity.

However, many extractants can be used to see if any of these extractants can selectively extract antibacterial compounds. Therefore, it is necessary to select extraction methods appropriate to the bioassay and extract out all the compounds present from the plant. This can be achieved by extracting with a range of solvents of different polarities. Thus, non-polar constituents are extracted out by non- polar solvents and polar materials by polar solvents. Most workers investigating plants, however, use one or three extractants (Eloff, 1998a).

In order to test for the activity of extracts, the solvent used must not inhibit the growth of the test organisms. Most biological assays are carried out in water, therefore problems arise if the active compounds are only lipid soluble. An alternative is to use a water miscible general solvent such as dimethylsulphoxide (DMSO) to dissolve non- polar solvents prepared with more lipophilic solvents.

Eloff (1998a), found out that acetone gave the best result, due to its low toxicity to test organisms, when compared with methanol, methylene dichloride and a methanol- chloroform mixture as extractants of *C. erythrophyllum* powdered leaves. Because acetone also dissolves many hydrophilic and lipophilic components, is miscible with water and volatile and has a low toxicity to bioassay used, it is a useful extractant (Eloff, 1998).

In general, the larger the variety of compounds that are extracted by the extractant, the better the chance that biologically active components will also be extracted.

3.1.1 Quantity extracted with initial extractants

In initial work with the plant, the extraction on 500 mg of finely ground leaf material with 5 ml of 10 different solvents took place on a rotating shaker in three successful stages,

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decanting between each stage. Tetrahydrofuran (THF) (11%) extracted largest quantity of the material followed by methylene dichloride (MDC) (10.6%), acetone (ACN) (9.6%), ethanol (ET) (9%), ethylacetate (EA) (6.4%), methanol (M) (5.8%), diethyl ether (EE) (4%), diisopropyl ether (I) (3.4%), and hexane (H) (2%) and water (W) (0.8%) in order of decreasing extracted quantity. Water extracted a minimal quantity of the material [Table 8] Although, tetrahydrofuran extracted slightly higher initial percentage than acetone [Table 9], but acetone was selected for extraction of compounds from the leaves of *C. woodii*.

Table 8 Quantity in mg extracted from 500 mg of *C. woodii* using various solvents and re-extracting twice.

	H	I	EE	MDC	EA	THF	ACN	ET	M	W
1st	8	14	18	44	29	47	43	36	23	2
2nd	1	2	2	7	2	6	4	7	4	1
3rd	1	1	0	2	1	2	2	2	2	1
Total	10	17	20	53	32	55	48	45	29	4
% extracted	2	3.4	4	10.6	6.4	11	9.6	9	5.8	0.8

Table 9 Percentage of the total extracted from *C. woodii* leaves after different periods

	1st	2nd	3rd
H	80	10	10
I	82	12	6
EE	90	10	0
MDC	83	13	4
EA	91	6	3
THF	86	11	4
ACN	90	8	2
ET	80	16	4
M	79	14	7
W	50	25	25

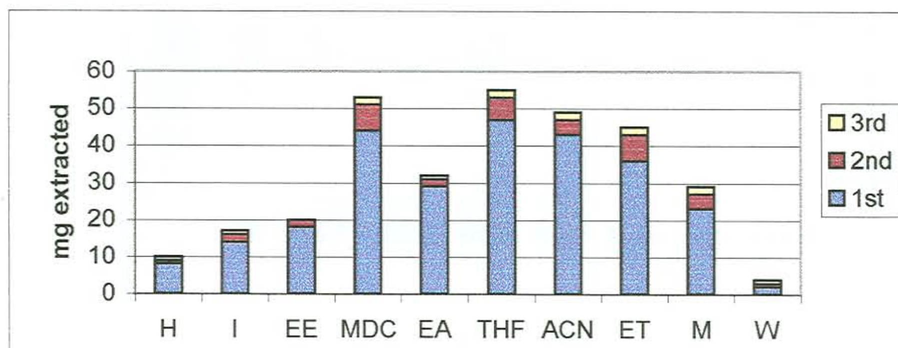

Fig. 9 Quantity (mg) extracted from 0.5 g of powdered dried leaves of *C. woodii* with 5 ml of hexane (H), diisopropyl ether (I), diethyl ether (EE), methylene dichloride (MDC), ethyl acetate (EA), tetrahydrofuran (THF), acetone (ACN), ethanol (ET), methanol (M) and water (W). Value for first extraction at the bottom, second in the middle and third at the top.

3.1.2 Profiling of extracts

In order to investigate the complexity of extracts from different extractant, three TLC solvent systems developed in our laboratory were employed.

Because acetone extracted a large quantity of the plant material and is easier to work with than other solvents for reasons given earlier, it was used for reconstituting the extracts. A concentration of 10 mg/ml was used. Each of the extract (5 µl) was analyzed in duplicate by TLC using BEA, CEF, and EMW as eluents. The TLC plates were sprayed with the vanillin-sulphuric acid and *p*-anisaldehyde-sulphuric acid spray reagents.

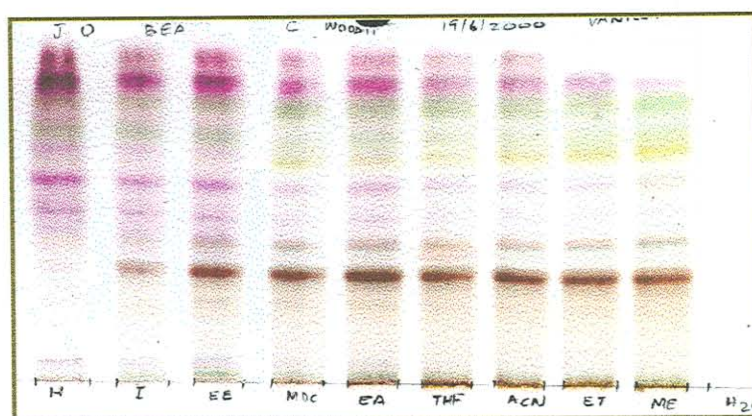

Fig. 10 Separation of components present in 50 µg of 10 different extracts with BEA as eluent and vanillin-sulphuric acid spray reagent. Lanes from left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethylacetate, tetrahydrofuran, acetone, ethanol, methanol and water.

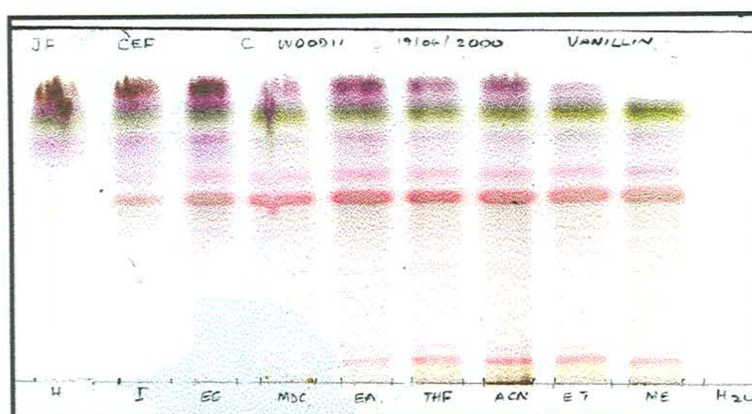

Fig. 11 Separation of components present in 50 µg of 10 different extracts using CEF as eluent and vanillin-sulphuric acid spray reagent. Lanes from left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethylacetate, tetrahydrofuran, acetone, ethanol, methanol and water.

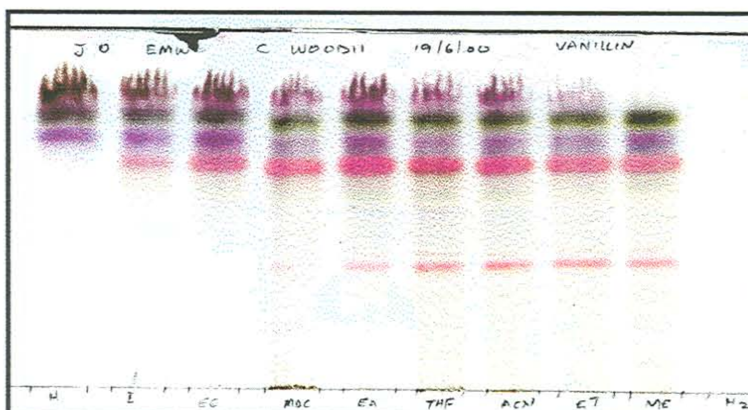

Fig. 12 Separation of components present in 50 µg of 10 different extracts using EMW as eluent and vanillin-sulphuric acid spray reagent. Lanes from left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethylacetate, tetrahydrofuran, acetone, ethanol, methanol and water.

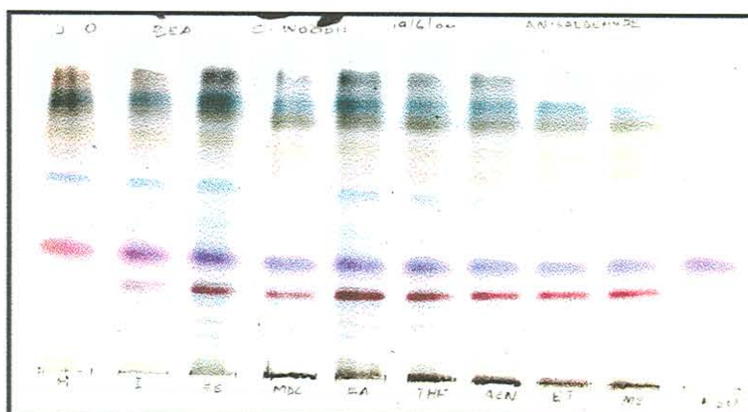

Fig. 13 Separation of compounds present in 50 µg of 10 different extracts using BEA as eluent and *p*-anisaldehyde-sulphuric acid spray reagent. Lanes from left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethylacetate, tetrahydrofuran, acetone, ethanol, methanol, and water.

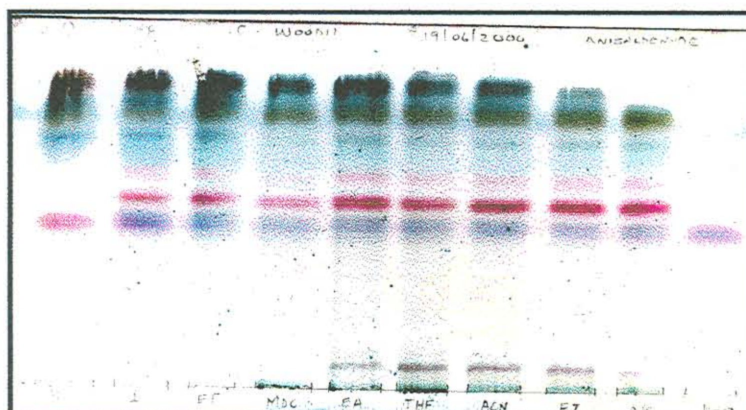

Fig. 14 Separation of compounds present in 50 µg of different extracts using CEF as eluent and *p*-anisaldehyde-sulphuric acid spray reagent. Lanes from left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethylacetate, tetrahydrofuran, acetone, ethanol, methanol, and water.

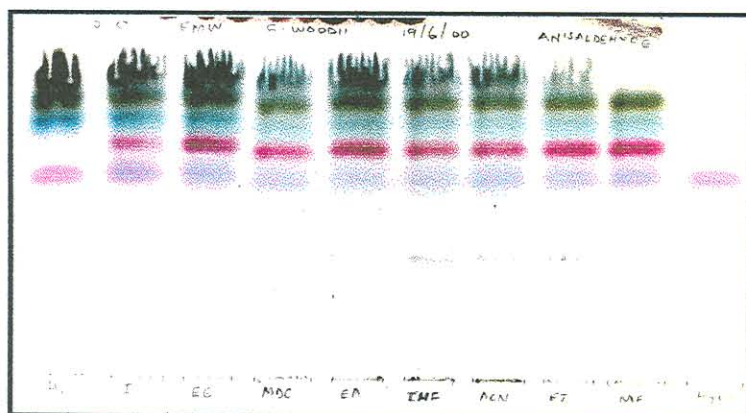

Fig. 15 Separation of compounds present in 50 µg of different extracts using EMW as eluent and *p*-anisaldehyde-sulphuric acid spray reagent. Lanes from left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethylacetate, tetrahydrofuran, acetone, ethanol, methanol, and water.

All solvents used with the exception of water extracted at least seven different compounds. The water extract showed only one coloured compound after spraying with *p*-anisaldehyde. Although, the quantities extracted by different solvents from 500 mg of plant material were different, separation of the same quantity (50 µg) of the extract by TLC indicated little

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difference in the composition. The situation was however different with water and hexane extracts [Fig. 10, 11, 12, 13, 14].

Many more non- polar compounds were visible using BEA as eluent. TLC with intermediate polar eluent CEF and polar eluent EMW revealed that less of the polar compounds were extracted by hexane, diisopropyl ether, and diethyl ether.

The fact that polar solvents such as ethanol and methanol also extracted similar concentrations of non-polar compounds suggested the presence of saponins compounds with polar and non-polar ends, which solubilize in polar solvents (Bruneton, 1995).

Although with water extract *p*-anisaldehyde spray reagent showed a compound, more compounds were visible with vanillin spray reagent. Therefore, I decided to use vanillin-sulphuric acid spray reagent in the future work.

3.1.3 Bioautography

The bioautography technique worked well with *S. aureus* and *E. faecalis*, but not as well with the *E. coli* and *Ps. aeruginosa*, because the zones of inhibition were easily seen. Many of the components extracted did not have antibacterial activity, although there were more than one antibacterial compound. The antibacterial activity resided in polar compounds, but different compounds inhibited different bacteria.

In BEA, *S. aureus* was inhibited by only one major compound [Fig. 16] while *E. faecalis* was inhibited by at least three compounds [Fig. 17].

There were two major antibacterial compounds with R_f values of 0.74 and 0.88, which inhibited the growth of *S. aureus* and *E. faecalis* respectively when EMW was used as the solvent system.

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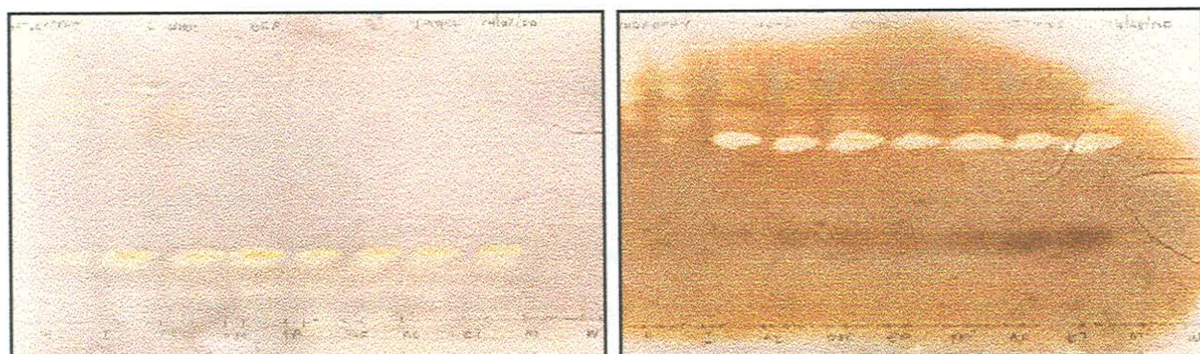

Fig. 16 Bioautograms of *C. woodii* leaves extracted by 10 different extractants. TLC developed in BEA (left) and EMW (right) and sprayed with *S. aureus* culture, incubated overnight then sprayed with INT. Growth inhibition indicated by lighter or colourless zones on TLC plates. Lanes from left to right: hexane (H), diisopropyl ether, (I), diethyl ether (EE), methylene dichloride (MDC), ethyl acetate (EA), tetrahydrofuran (THF), acetone (ACN), ethanol (ET), methanol (M) and water (W).

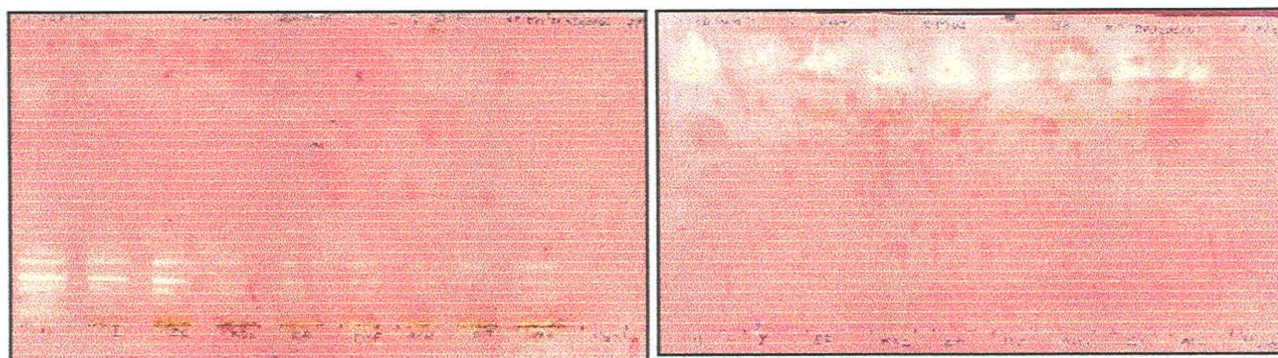

Fig. 17 Bioautograms of *C. woodii* leaves extracted by 10 different extractants. TLC developed in BEA (left) and EMW (right) and sprayed with *E. faecalis* culture, incubated overnight then sprayed with INT. Growth inhibition is indicated by colourless zone. Lane from left to right: hexane (H), diisopropyl ether (I), diethyl ether (EE), methylene dichloride (MDC), ethyl acetate (EA), tetrahydrofuran (THF), acetone (ACN), ethanol (ET), methanol (M) and water (H_2O).

The best separation was obtained with CEF and EMW solvent systems. However, it was difficult to remove formic acid in CEF solvent systems, which is quite toxic to bacteria, from the TLC plates. Therefore, the plates for CEF solvent system were allowed to dry for two days before spraying with bacteria.

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3.1.4 Quantity of antibacterial compound present and MIC values of extracts.

To determine whether *C. woodii* is a promising source of antibacterial compounds, not only the MIC of the extract but also the quantity present in the plant is important. Because the MIC value is inversely related to the quantity of antibacterial compounds present, an arbitrary measure of the quantity of antibacterial compounds present was calculated by dividing the quantity extracted in milligrams from 500 mg leaves by the MIC value in $mgml^{-1}$. The unit of this arbitrary measure is ml / 500 mg and if multiplied by 2 changes to mlg^{-1}. This value indicates the volume to which the biologically active compounds present in one gram of dried plant material can be diluted and still kill bacteria (Eloff, 1999).

The MIC values for all the extracts ranged between 0.05 and >2.5 mg/ml. The highest average total activity was found with methylene dichloride and acetone as extractants and the lowest activity with water as extractant.

The intermediate polarity extractants, methylene dichloride, tetrahydrofuran with the exception of diisopropyl ether and diethyl ether, had high activity [Table 10].

Methylene dichloride, tetrahydrofuran and acetone extracted most antibacterial compounds. The bio-active compounds may therefore have an intermediate polarity.

Methylene dichloride and tetrahydrofuran are not miscible with water, in contrast to acetone; the use of these extractants complicates the handling of extracts.

Since acetone extracted the bioactive compound in large quantity and is easier to handle than other solvents as stated earlier on, it was used as extractant in further work. The acetone extract had a high and equal activity on *E. faecalis* and *E. coli*, but lower activity on *Ps. aeruginosa* and *S. aureus.*

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Table 10 Quantity extracted, MIC values in mg/ml and total activity in ml of *C. woodii* per gram leaves extracted with hexane (H), diisopropyl ether (I), diethyl ether (EE), methylene dichloride, (MDC), ethylacetate (EA), tetrahydrofuran (THF), acetone (ACN), ethanol (ET), methanol (M), and water (W).

	H	I	EE	MDC	EA	THF	ACN	ET	M	W
Total quantity in mg present in 1g	16	28	36	88	58	94	86	72	46	4
MIC										
E. faecalis	0.16	0.04	0.04	0.04	0.04	0.08	0.04	0.31	0.63	> 2.5
S. aureus	1.25	0.63	0.63	0.16	0.08	0.31	0.31	0.31	0.31	> 2.5
Ps. aeruginosa	0.04	0.16	0.31	0.31	0.16	0.16	0.16	> 2.5	> 2.5	> 2.5
E. coli	0.08	0.08	0.04	0.04	0.04	0.04	0.04	0.04	0.31	>2.5
Average	0.38	0.23	0.26	0.14	0.08	0.15	0.14	>0.79	>0.94	> 2.5
TOTAL ACTIVITY										
E. faecalis	100	700	900	2200	1450	1175	2150	232	73	<1.6
S. aureus	13	44	57	550	725	303	277	232	148	<1.6
Ps. aeruginosa	400	175	116	284	363	588	538	<1.6	<1.6	<1.6
E. coli	200	350	900	2200	1450	2350	2150	1800	148	<1.6
Total activity for all organisms	713	1269	1973	5234	3988	4416	5115	2266	371	<6.4

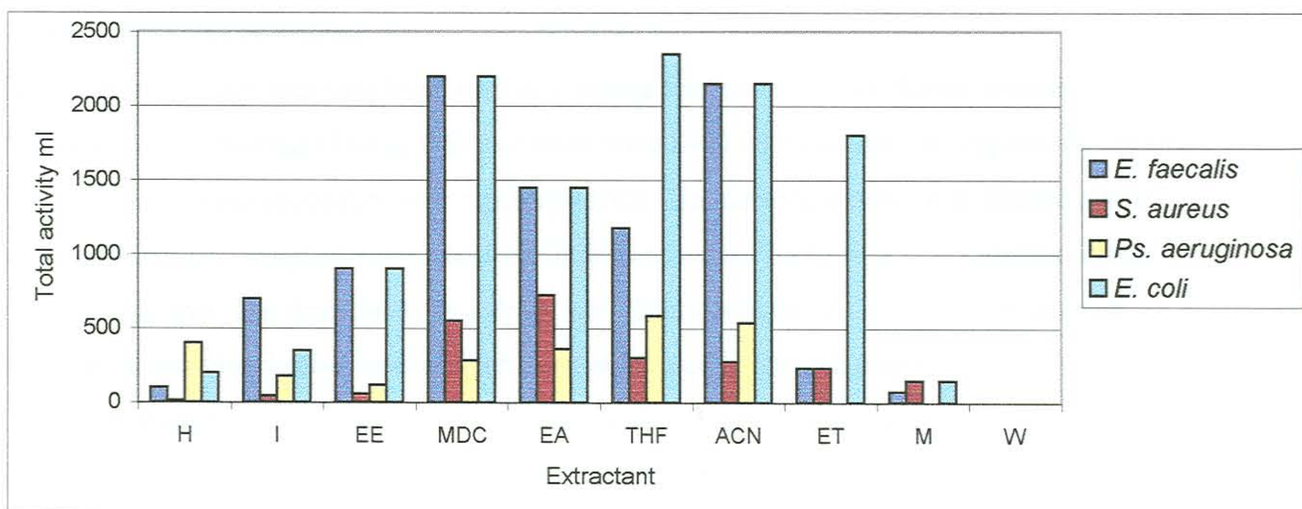

Fig. 18 Total antibacterial activity of *C. woodii* extracts with 10 different extractants on four test organisms. From left to right: hexane (H), diisopropyl ether (I), diethyl ether (EE), methylene dichloride (MDC), ethyl acetate (EA), tetrahydrofuran (THF), acetone (ACN), ethanol (ET), methanol (M) and water (W).

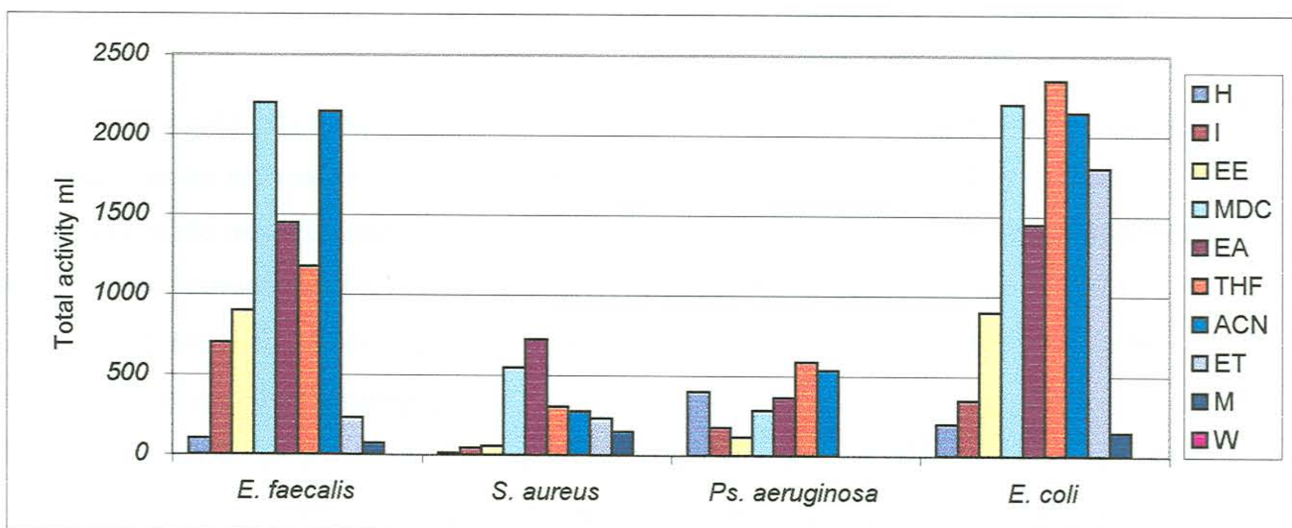

Fig. 19 Total antibacterial activity of *C. woodii* extracts on four test organisms.

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3.2 Solvent–Solvent Extraction

The complex extract was simplified by fractionating into solvents of different polarities. Powdered plant material (140 g) was extracted with 1400 ml of acetone by vigorously shaking for 30 minutes. This procedure was repeated thrice. The acetone extract was filtered and taken to dryness in a pre-weighed round-bottom flask in a Buchi PE120 rotary evaporator under reduced pressure. The dried acetone extract was redissolved in 100 ml each of water and chloroform resulting in two layers which were separated from each other.

The water fraction was extracted with an equal volume of butanol in a separatory funnel to yield the water and butanol fractions. The chloroform fraction was taken to dryness in a rotary evaporator under reduced pressure and dissolved in 1:1 mixture of hexane and 10% water in methanol.

The hexane fractions were recovered with a separatory funnel. The 10% water in methanol extract was diluted to 20% water in methanol by adding 0.125 ml of water for every ml of 10% water in methanol and extracted with carbon tetrachloride to yield carbon tetrachloride fraction. The 20% water in methanol extract was diluted to 35% methanol in water by adding 0.2308 ml of water for every ml of 20% water in methanol and extracted with chloroform to yield the chloroform fraction and the 35% methanol in water fractions. In all cases, equal volumes of the solvents were used and the extraction was repeated with a small volume three more times or until the colour was extracted. All extracts were taken to dryness in a rotatory evaporator under reduced pressure. The weights of extracts were determined by subtracting the weight of the round bottom flask on the rotary evaporator before from its weight after with dry fractions. Extracts were re-dissolved in acetone or solvent in which they are soluble in pre-weighed amber flasks with tight sealing lids and the concentration of each fraction was determined before chromatography.

Thin layer chromatography (5 µl of 20 mg/ml of extract was done on Merck TLC plates using the same solvent systems developed in our laboratory. Separated components were visualized under visible and ultra-violet light (254 and 360 nm, Camac Universal UV lamp TL-600). TLC plates were sprayed with vanillin-sulphuric acid spray reagent.

Bioautography were carried out for all the six fractions obtained. The minimum inhibitory concentration (MIC) values of fractions obtained by solvent-solvent extraction were also determined.

3.2 1. Extraction of *C. woodii* and solvent-solvent fractionation.

The aim of solvent - solvent fractionation is to determine the extent to which the extracts can be fractionated using a mild technique without reducing the antibacterial activity.

The group separation of extracts using different solvents of wide range of polarities for simplifying extract enhanced the isolation of antibacterial compounds from the complex crude extract of *C. woodii* leaves.

Acetone extracted about 11% (15.60 g) of starting plant material. The quantity fractionated in each solvent mixture by each solvent from acetone extract of crude plant extract was calculated after drying off the solvent in the pre-weighed glass flask. The highest percentage of the acetone extract was in hexane (32%) and chloroform (25.6%) fractions. Water and 35% water in methanol fractions had the lowest percentage. This gave a similar result obtained in *C. erythrophyllum* investigated previously in our laboratory (Martini and Eloff, 1998). A gummy residue was obtained in the interphase when hexane and 10% water in methanol was added to chloroform component. The quantity of the gummy residue was determined. About 4.1% of the acetone extract was lost during the simplification process after adding the quantity of extract in each solvent fraction [Fig. 20].

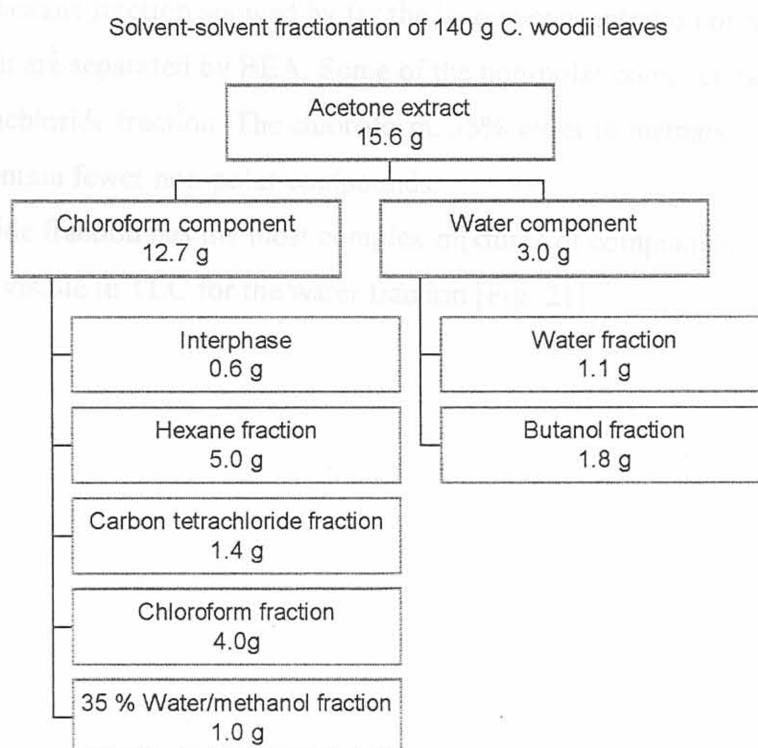

Fig. 20 Quantity extracted in each solvent fraction by solvent-solvent fractionation process

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Table 11 Quantity [in g] and percentage of total initial mass [140 g] of *C. woodii* leaves extracted by each solvent in solvent-solvent fractionation process.

Fraction	Mass	% of total initial mass
H	5.001	3.6
W	1.123	0.8
B	1.831	1.3
35% W/M	1.009	0.7
CCl_4	1.406	1.0
$CHCl_3$	3.998	2.9

3.2.2 Complexity of fractions.

The complexity of each solvent fraction was determined by TLC in the three solvent systems developed in our laboratory. The different fractions were dissolved in acetone to yield 20 mg/ml solution before chromatography.

As expected, the hexane fraction showed by far the largest concentration of non- polar compounds, which are separated by BEA. Some of the non-polar compounds were also found in the carbon tetrachloride fraction. The chloroform, 35% water in methanol, butanol and water fractions contain fewer non-polar compounds.

Carbon tetrachloride fraction has the most complex mixtures of compounds. No separated components were visible in TLC for the water fraction [Fig. 21].

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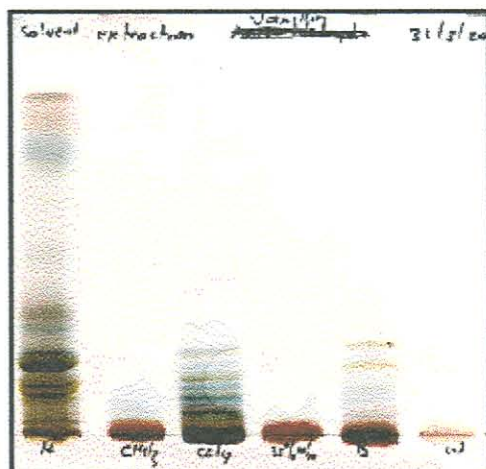

Fig. 21 Separation of components present in the different fractions obtained by solvent-solvent extraction by BEA and sprayed with vanillin-sulphuric acid. Lanes from left to right: hexane, chloroform, carbon tetrachloride, 35% water in methanol, butanol and water fractions. In each case 50 μg was chromatographed.

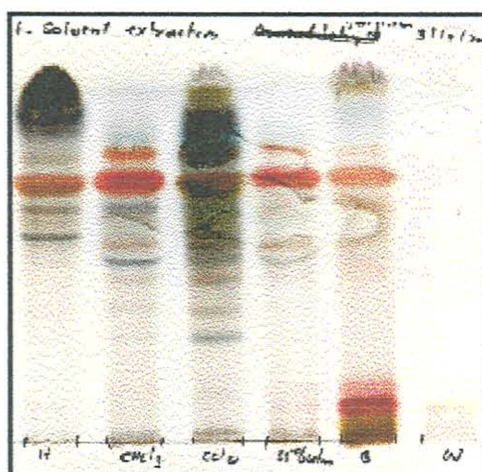

Fig. 22 Separation of components present in the different fractions obtained by solvent-solvent extraction by CEF and sprayed with vanillin-sulphuric acid. Lanes from left to right: hexane, chloroform, carbon tetrachloride, 35% water in methanol, butanol and water fractions. In each case 50 μg was chromatographed.

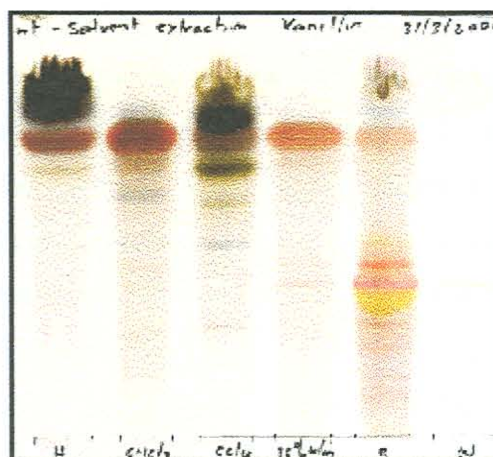

Fig. 23 Separation of components present in the different fractions obtained by solvent-solvent extraction by EMW and sprayed with vanillin-sulphuric acid. Lanes from left to right: hexane, chloroform, carbon tetrachloride, 35% water in methanol, butanol and water fractions. In each case 50 µg was chromatographed.

Some components of water, butanol and 35% water in methanol did not dissolve in acetone. The quantity of water, butanol and 35% water in methanol fractions, which were insoluble in acetone, were 302 mg, 920 mg and 850 mg respectively. These components were dissolved in methanol prior to chromatographing. The gummy residue discussed earlier was dissolved in chloroform and analyzed by TLC with all the other fractions [Fig. 24].

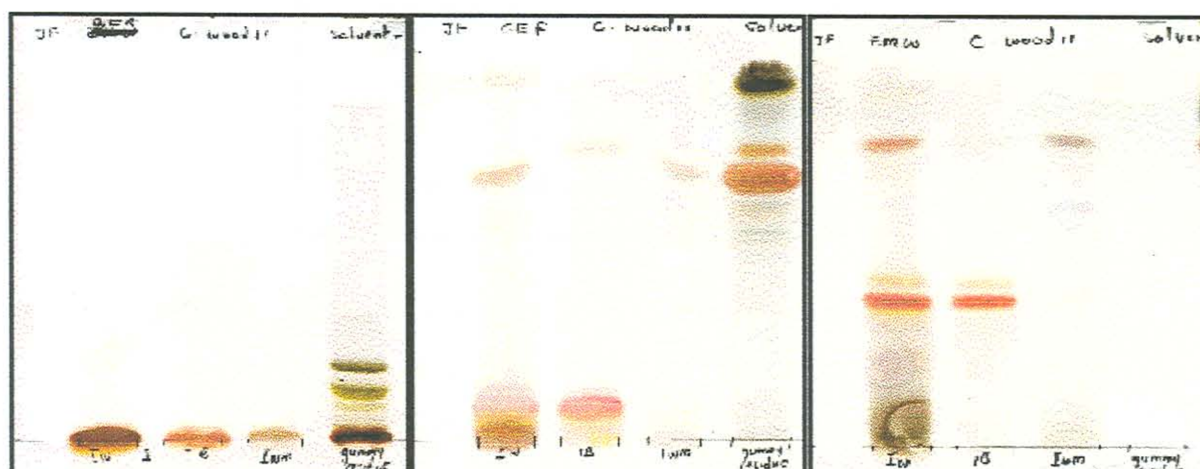

Fig. 24 Separation of components present in the different fractions obtained by solvent-solvent extraction by BEA, CEF, and EMW (from left to right) and sprayed with vanillin-sulphuric acid. Lanes at the bottom in each TLC plate from left to right, insoluble water fractions (IW), insoluble butanol fractions (IB), and insoluble 35% water in methanol fractions (IWM) and gummy residue (interphase).

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The acetone insoluble butanol fractions had one major intermediate polar compound. The interphase (gum residue) contains both polar and non-polar compounds separated by TLC. Based on the colours formed in response to the spray reagent, a tentative identification of flavonoids (orange-yellow) and triterpenoids (purple) in *C. woodii* leaves can only be deduced until all the compounds present are isolated and identified (Carr and Rogers, 1987). However, a red compound with the R_f value of 0.74 was very prominent on TLC plate for hexane, chloroform, carbon tetrachloride, 35% water in methanol and butanol fractions using EMW as solvent system [Fig. 23].

3.2.3 Bioautography of fractions

The bioautography was carried out using all the four test organisms. The bioautography technique worked better with *S. aureus* than with all the other three organisms. Nevertheless, the presence of growth inhibitors were seen with all the four cultures.

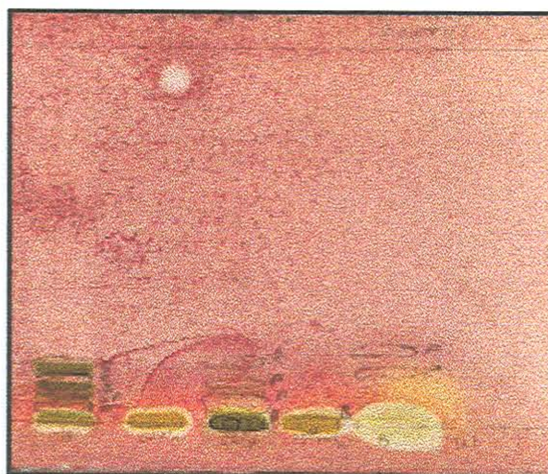

Fig. 25 Bioautogram of acetone extract of *C. woodii* leaves separated into different fractions by solvent-solvent extraction. TLC plate developed in BEA and sprayed with *S. aureus* culture incubated overnight and then sprayed with INT. Growth inhibition indicated by colourless zones on TLC plate. Lanes from left to right: chloroform, carbon tetrachloride, 35% methanol in water, butanol and water fractions.

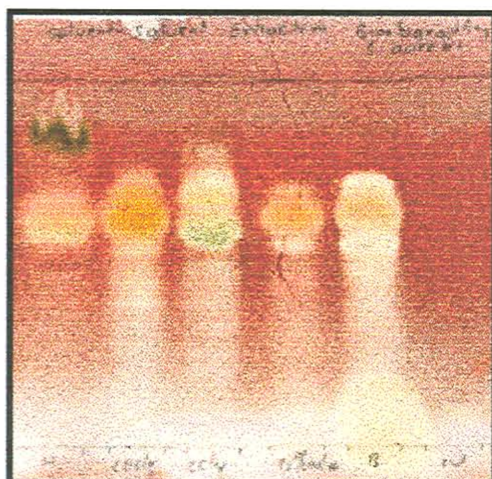

Fig. 26 Bioautogram of acetone extract of *C. woodii* leaves separated into different fractions by solvent-solvent extraction. TLC plate developed in CEF and sprayed with *S. aureus* culture incubated overnight and then sprayed with INT. Growth inhibition indicated by colourless zones on TLC plates. Lanes from left to right: chloroform, carbon tetrachloride, 35% water in methanol, butanol and water fractions.

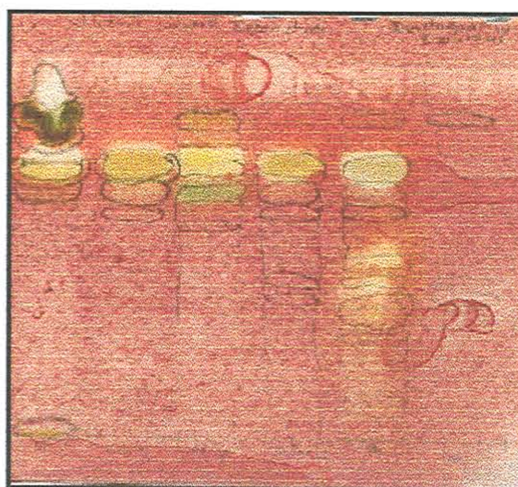

Fig. 27 Bioautogram of acetone extract of *C. woodii* leaves separated into different fractions by solvent-solvent extraction. TLC plate developed in EMW and sprayed with *S. aureus* culture incubated overnight and then sprayed with INT. Growth inhibition indicated by colourless or lighter zone on TLC plate. Lanes from left to right: chloroform, carbon tetrachloride, 35% water in methanol, butanol and water fractions.

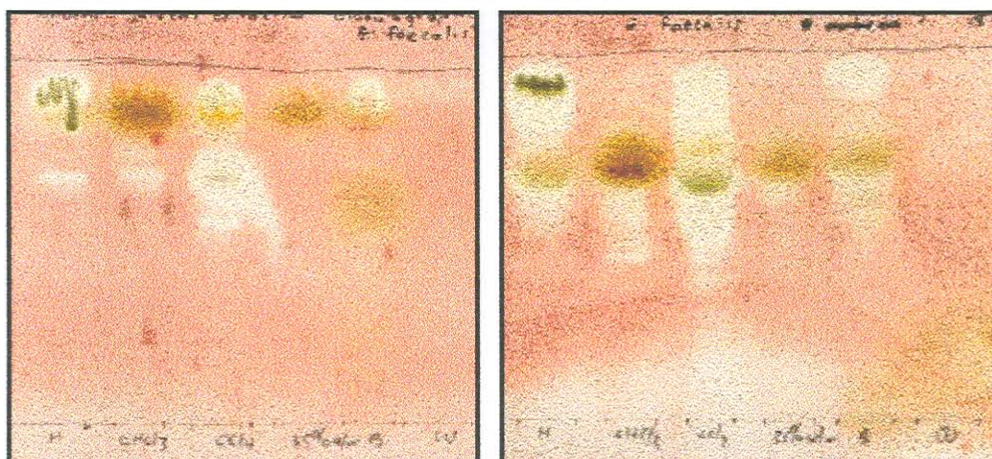

Fig. 28 Bioautograms of the acetone extract of *C. woodii* leaves separated by CEF (left) and EMW (right) and *E. faecalis* as the test organism. White or brownish yellow areas indicate growth inhibition. Lanes from left to right: hexane, chloroform, carbon tetrachloride, 35% water in methanol, butanol and water fractions.

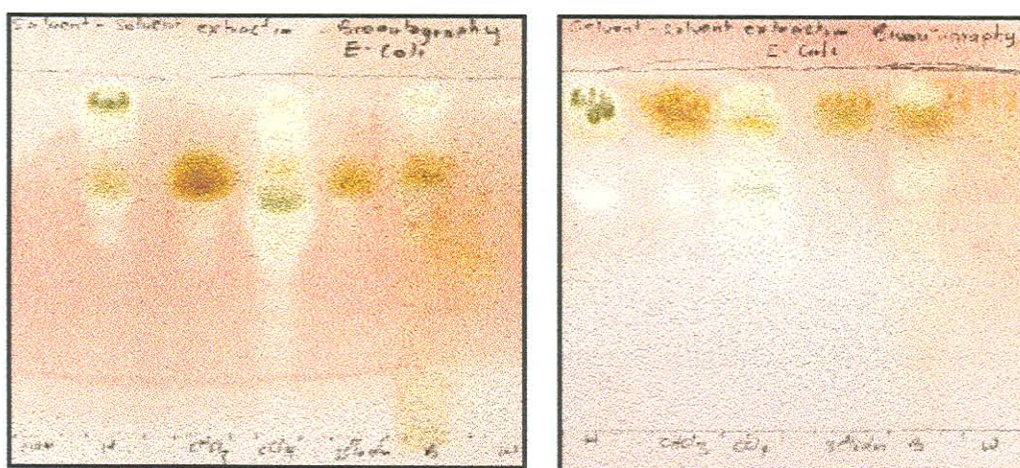

Fig. 29 Bioautograms of the acetone extract of *C. woodii* leaves separated by CEF (left) and EMW (right) and *E. coli* as the test organism. White or brownish yellow areas indicate growth inhibition. Lanes from left to right: hexane, chloroform, carbon tetrachloride, 35% water in methanol, butanol and water fractions.

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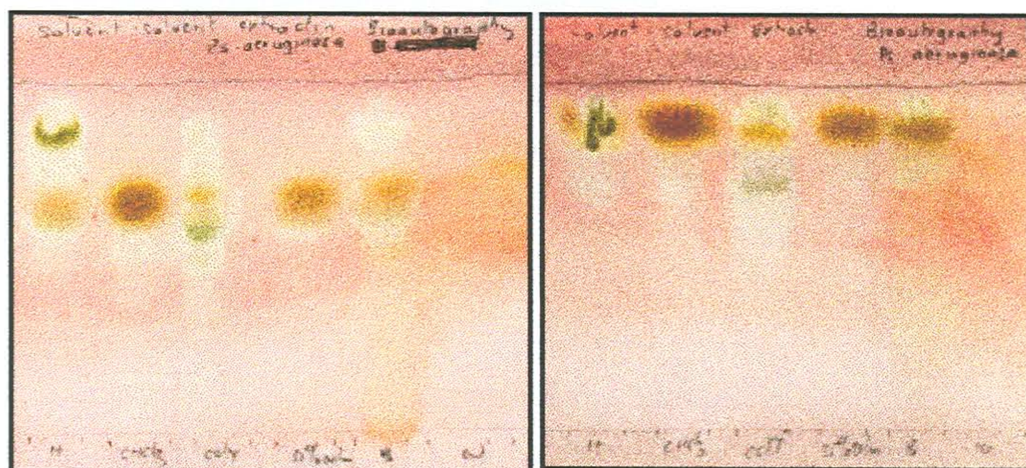

Fig. 30 Bioautograms of the acetone extract of *C. woodii* leaves separated by CEF (left) and EMW (right) and *Ps. aeruginosa* as the test organism. White or brownish yellow areas indicate growth inhibition. Lanes from left to right: hexane, chloroform, carbon tetrachloride, 35% water in methanol, butanol and water fractions.

The acetone extract of *C. woodii* leaves contains more than one inhibitor of pathogenic organisms. There was a major inhibitory compound with R_f value of 0.74 using EMW the solvent system that was present in all the various fractions [Fig. 27]. This antibacterial compound corresponds to the red compounds obtained above [Fig. 23].
The bioautography technique has a drawback in that coloured compounds may mask the growth inhibition of the bacteria. Some of the more polar components have a green, yellow or brown colour and it is possible that some of these compounds may also inhibit the growth of one or more of the test organisms because the colour may mask the absence of a reaction of the bacteria with the INT in the bioautography. This situation happened with *Combretum erythrophyllum* extracts (Martini and Eloff, 1998).

3.2.4 Antibacterial activity of fractions

It was decided to obtain more information on the antibacterial activity of various fractions collected. The fractions were dried in pre-weighed flask and dissolved in acetone to give concentrations of 20 mg/ml solutions. The MIC values were determined by microplate serial dilution method using all the four test organisms.

Table 12 The minimum inhibitory concentration (MIC) in mg/ml of different fractions obtained by solvent-solvent extraction of *C. woodii* leaves

	H	$CHCl_3$	CCl_4	35% W/M	B	W
S. aureus	0.6	0.3	0.1	0.2	0.2	>5.0
E. coli	2.5	2.5	5.0	5.0	5.0	>5.0
Ps. aeruginosa	0.3	0.3	0.3	2.5	2.5	5.0
E. faecalis	0.6	0.1	0.2	0.1	0.6	0.6

All the fractions had antibacterial activity against all the four test organisms. Water fraction had activity against *E. faecalis* but very low activity against all other organisms. *S. aureus* was inhibited most by the carbon tetrachloride fraction. Chloroform and 35% water in methanol fractions are highly active against *E. faecalis.* The fractions generally have lower activity against *E. coli* than all the other organisms.

Both the Gram-positive and Gram-negative (with the exception of *E. coli*) organisms are very sensitive to all the fractions.

The total antibacterial activity of different solvent fractions was determined [Table 13]. The chloroform fraction had the highest activity (almost 33 times higher than the water fraction). Both the Gram-negative organisms and Gram-positive organisms were inhibited by all the fractions, except the water fraction. The Gram-negative organism, *Ps. aeruginosa*, was inhibited to a higher degree in the hexane fraction.

Chloroform fraction inhibited *E. faecalis* more than all the other organisms used. The component in water very little activity against only three bacteria strains. The chloroform fraction inhibited the growth of *S. aureus* and *Ps. aeruginosa* to the same degree.

Although the relative quantity of material present in the hexane fraction was higher than in the chloroform fraction, the relative antibacterial activity of the chloroform fraction was higher. This indicated that the inhibitory activity in *C. woodii* leaves was due to intermediate polar compounds. Many non-polar compounds in the hexane fraction are not active.

Table 13 MIC in mg/ml and total activity in ml of acetone extract of *C. woodii* leaves in different fractions obtained by solvent-solvent fractionation.

	H	$CHCl_3$	CCl_4	35% W/M	B	W
Total quantity obtained in 1g	320	256	64	65	117	72
MIC						
S. aureus	0.6	0.3	0.1	0.2	0.2	> 5.0
E. coli	2.5	2.5	5.0	5.0	5.0	> 5.0
Ps. aeruginosa	0.3	0.3	0.3	2.5	2.5	5.0
E. faecalis	0.6	0.1	0.2	0.1	0.6	0.6
Total activity in ml						
S. aureus	533	853	640	325	585	<5
E. coli	128	102	13	13	23	<5
Ps. aeruginosa	1067	853	213	26	47	14
E. faecalis	533	2560	320	650	195	120
Total activity all organisms	2261	4360	1186	1014	850	134
Distribution of mass and total antibacterial activity						
% of total mass	35.8	28.6	7.2	7.3	13.1	8.1
% of total activity	23.1	44.5	12.1	10.3	8.7	1.4
ratio activity/mass	0.6	1.6	1.7	1.4	0.7	0.2

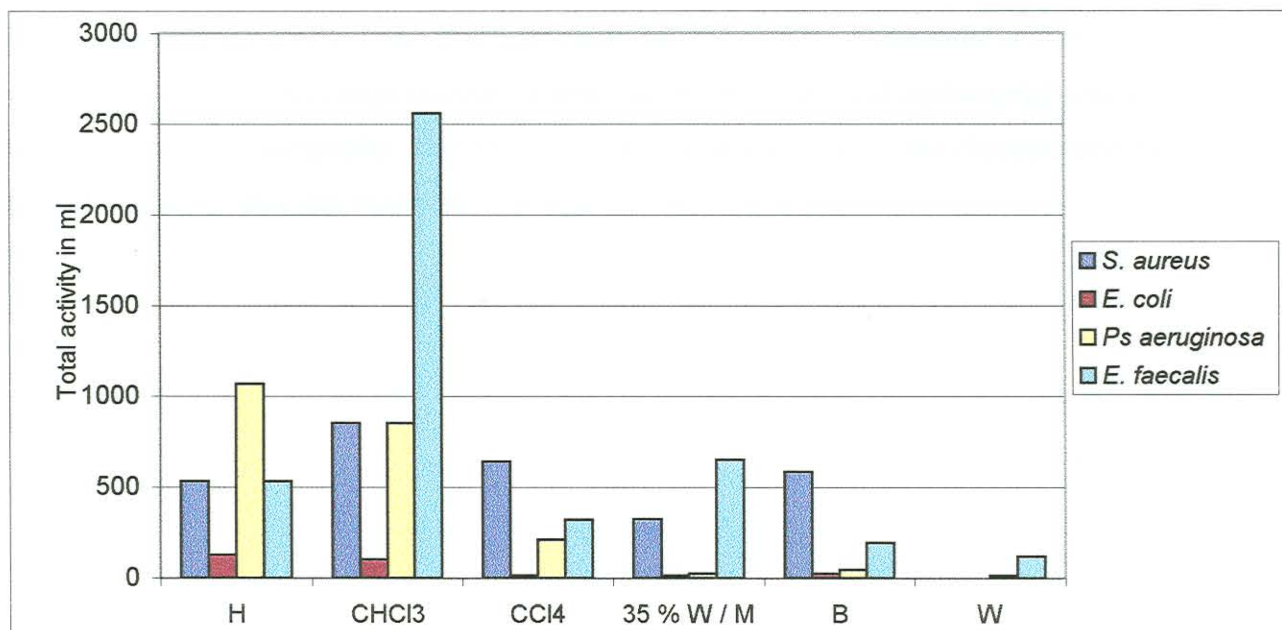

Fig. 31 Total antibacterial activity to four test organisms of *C.woodii* leaves extracted with solvent fractions. Fractions from left to right: hexane (H), chloroform ($CHCl_3$), carbon tetrachloride (CCl_4), 35% water in methanol (35%W/M), butanol (B) and water (W).

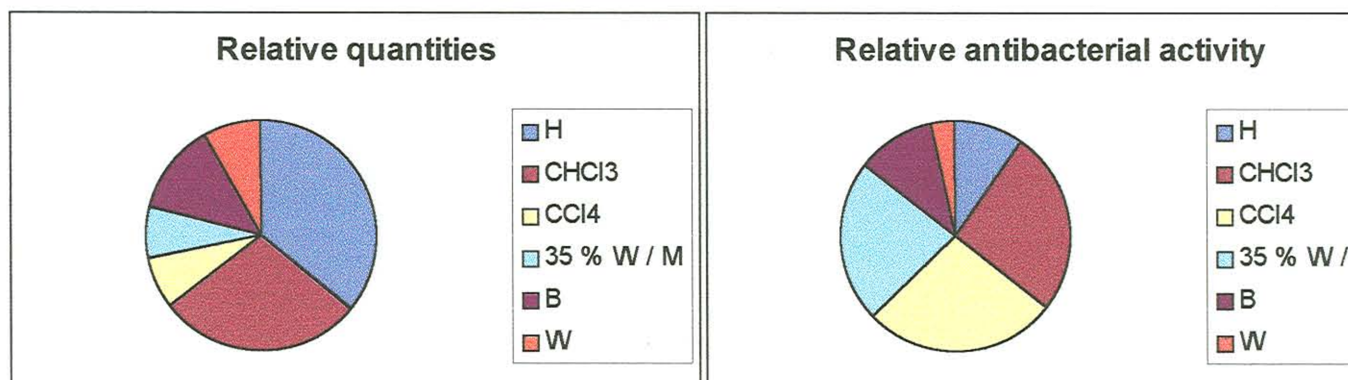

Fig. 32 The relative quantities obtained by solvent-solvent fractionation and the relative antibacterial activities of the different fractions. Solvent fractions from the top to the bottom: hexane (H), chloroform ($CHCl_3$), carbon tetrachloride (CCl_4), butanol (B), 35% water in methanol (35% W/M) and water (W).

In conclusion, there were more than one antibacterial compound in the acetone extract of *C. woodii* leaves. One of the major antibacterial compounds present in the all the solvent fractions was the red compound with R_f value of 0.74 in EMW solvent system.

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Because chloroform fraction was relatively clean, had the highest antibacterial activity to quantity ratio, and was in large quantity, it was chosen for isolation of antibacterial compound using column chromatography. The active red compound was isolated and characterized in the subsequent work using the chloroform fraction.

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CHAPTER 4

RESULTS AND DISCUSSION ON ISOLATION OF COMPOUND

4.1 **Isolation of active compound**

In order to isolate the active compound, further fractionation of the solvent fractions by column chromatography was employed. Various qualitative and quantitative analyses were carried out on the isolated active compound from the column chromatography. The active compound of interest was also examined for antibacterial activity against four test organisms.

4.1.1 Method development

The chloroform fraction from solvent - solvent fractionation of acetone extract of *C. woodii* leaves was selected for isolation of active compound because it showed the highest relative antibacterial activity. It was necessary to select a TLC system, which would give good resolution of components in the chloroform fraction. Various TLC analyses were done on the fraction in an attempt to determine the best solvent system for column chromatography. About 50 µg of the dried chloroform fraction was applied to the TLC plates for this purpose. The following solvent systems were tested: chloroform / ethyl acetate, chloroform / tetrahydrofuran, chloroform / methanol combinations in different ratios [Figs. 33, 34, 35]. These solvents were chosen because of their varying polarities and selectivities (Snyder and Kirkland, 1979).

The separated components were observed under UV light at 254 nm and 365 nm. The compound of interest was identified by the yellow colour in the visible light and and by purple-blue fluorescence at 365 nm.

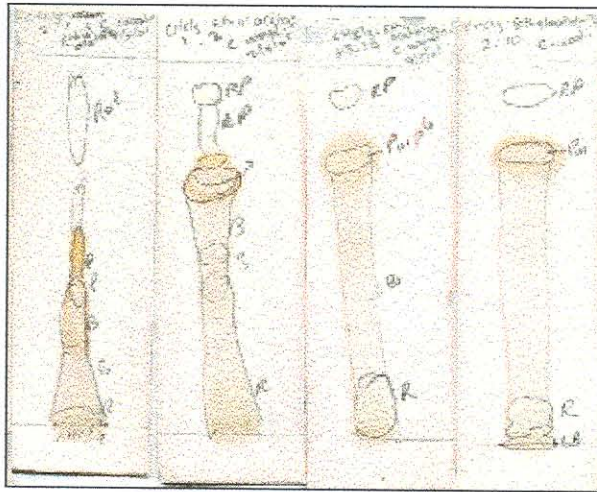

Fig. 33 TLC of chloroform fraction using different ratios of chloroform and ethyl acetate combination as solvent systems. From left to right, ratios 2:1, 1:2, 1:3, 1:5. Lines indicate fluorescing compounds.

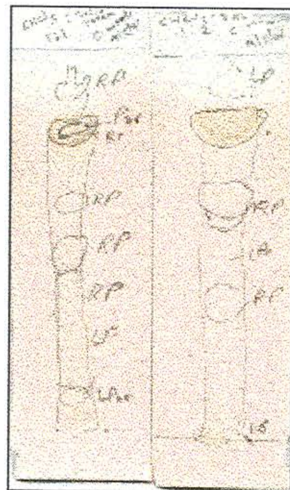

Fig. 34 TLC of chloroform fraction using different ratios of chloroform and tetrahydrofuran combination as solvent systems. From left to right: ratios 1:1, 1:2. Lines represent fluorescing compounds.

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Fig. 35 TLC of chloroform fraction using different ratios of chloroform and methanol combination as solvent systems. From left to right: ratios 1:2, 2:1. Lines represent fluorescing compounds.

The chloroform and ethyl acetate combination in the ratio of 1:5 gave the best resolution and was chosen as the best solvent system for the column chromatography.

4.1.2 Column chromatography of chosen fraction

Silica gel 60 (63-200 μm) was use as the packing material to scale up TLC separation in a glass column of about 5 cm in diameter and 130 cm in length.

The column was eluted using chloroform: ethyl acetate (1:5). About 10% methanol in acetone was later employed to remove components not eluted with the mixture.

4.1.3 Analysis of collected fractions by TLC

Thin layer chromatography was carried out on the fractions collected to determine their complexities. After about 50% of the volume of the eluent evaporated, TLC analysis of every fourth fraction (from fractions 4 to 64) collected was first carried out in CEF solvent system [Fig. 36]. Then all the fractions were analyzed by TLC using about 0.1% of each fraction. Depending on the evaporation of the eluent, 5 – 20 μl was applied. The plates were run in two different solvent systems, chloroform: ethyl acetate: formic acid (CEF) (20:16:4) and ethyl acetate: methanol: water (EMW) (40:5.4:4) as the polarity of fractions collected increased [Figs. 37, 38, 39, 40, 41]. TLC analysis was repeated with fractions 4 to 12 in CEF solvent

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system [Fig. 42]. All the fractions were taken to dryness under a cold stream of air and refrigerated.

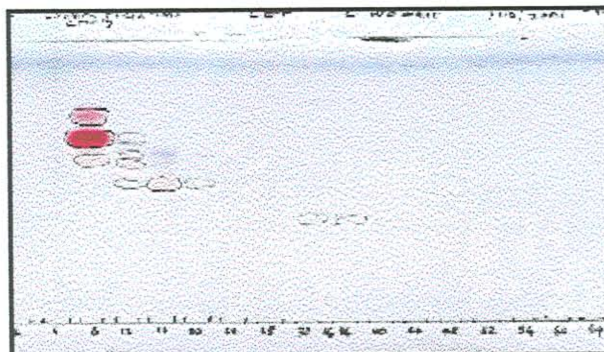

Fig. 36 TLC of every fourth collected fraction separated by CEF and sprayed with vanillin-sulphuric acid reagent. Lanes from left to right: fractions 4, 8,…, 60, 64.

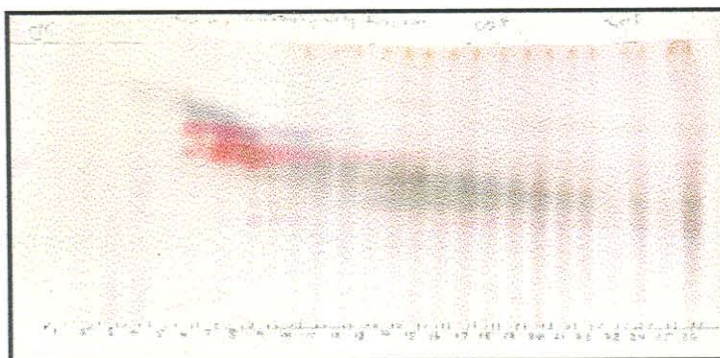

Fig. 37 TLC of collected fractions separated by CEF and sprayed with vanillin-sulphuric acid reagent. Lanes from left to right: fractions 1, 2, 3, …, 24, 25.

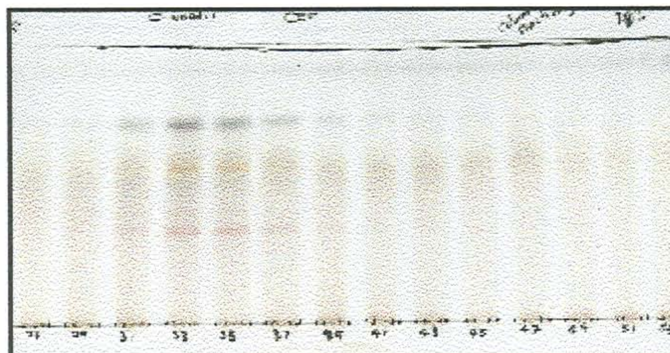

Fig. 38 TLC of collected fractions separated by CEF and sprayed with vanillin-sulphuric acid reagent. Lanes from left to right: fractions 26, 27, 28, …., 53, 54.

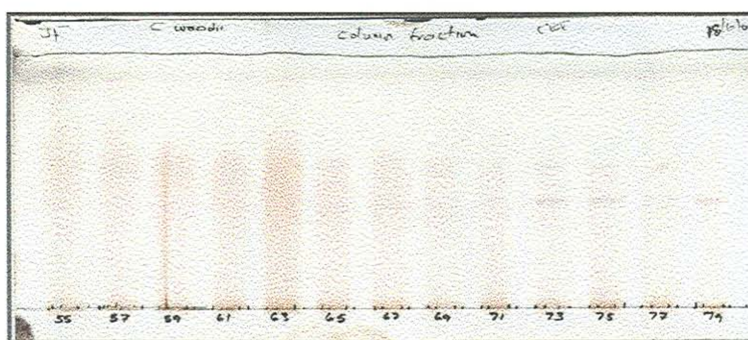

Fig. 39 TLC of column chromatography fractions separated by CEF and sprayed with vanillin-sulphuric acid reagent. Lanes from left to right: fractions from 55, 56, …, 78, 79.

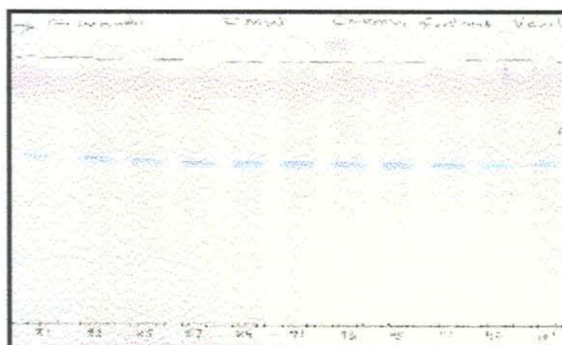

Fig. 40 TLC of collected fractions separated by EMW and sprayed with vanillin-sulphuric acid reagent. Lanes from left to right: fractions 81, 82,…, 100, 101.

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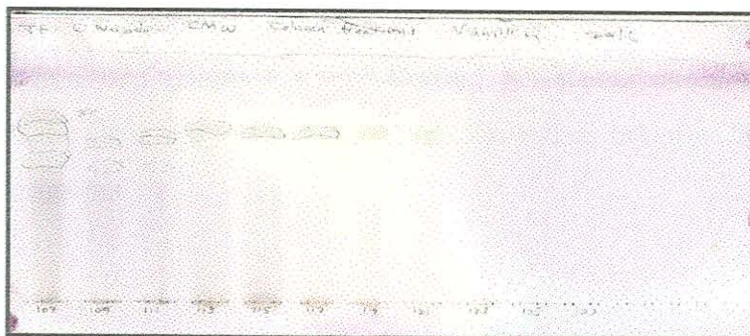

Fig. 41 TLC of collected fractions separated by EMW and sprayed with vanillin-sulphuric acid reagent. Lanes from left to right: fractions 107, 108,…, 122, 123.

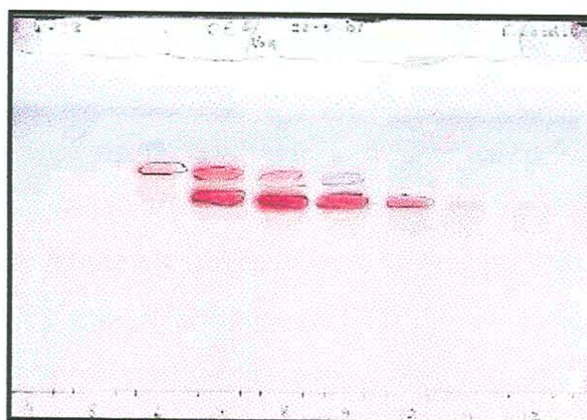

Fig. 42 TLC of collected fractions separated by CEF and sprayed with a vanillin-sulphuric acid reagent. Lanes from left to right: fractions 4, 5, …, 11, 12.

TLC analysis of every fourth fraction collected revealed that only fraction 8 had the compound with similar R_f value of 0.67 in CEF solvent system as the major active red compound in the chloroform fraction [Figs. 26, 36]. However, TLC analysis of every fraction showed that the main antibacterial compound was between fractions 4 and 12. Therefore, fractions 4 to 12 was spotted on TLC plates and developed in CEF solvent system. This helped to locate all the column fractions that contain the major active compound separated by column chromatography from the chloroform fraction of the solvent-solvent extraction [Fig. 42].

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Fractions 81 to 101 had a single component with Rf value of 0.62 in EMW solvent system. These fractions were combined and dried in a pre- weighed flask in a rotary evaporator at 50 ^{0}C. The weight of the combined fractions was 60 mg.

The compound in these fractions was characterized. NMR analysis showed that the compound has a long aliphatic chain and this work was not followed up because this was not the main antibacterial compound.

4.1.4 Combination of collected fractions

From the TLC results, fractions 7, 8, 9 and 10 showed a similar profile, although fractions 7, 8, 9 have more than one component. The quantity of material in fraction 10 was very small. Therefore, it was decided to combine the fractions in order to isolate the active compound in larger quantity. Fractions 6 and 7 were kept in the fridge while fractions, 8, 9 and 10 were combined and taken to dryness in a rotatory evaporator. The mass of the combined fractions was 819 mg.

The combined fractions were dissolved in chloroform. This led to the formation of white sediment. This suspension was then filtered. Both filtrate and the sediment were dried and weighed. The mass of the filtrate and sediment were 409 mg and 405 mg respectively. The filtrate was labeled as 'CF1' and the sediment as 'CF2.'

CF2 was further purified by recrystallization in about 3 ml each of three different solvents. Chloroform, acetone and methanol were used progressively for this purpose. The mass of CF2 that recrystallized from acetone, chloroform, and methanol were 108 mg, 24 mg and 36 mg respectively. Largest quantity of the some of CF2 were obtained from chloroform. The acetone, chloroform and methanol components of CF2 were labeled as 'CF2A', 'CF2C', and 'CF2M' respectively.

CF2A, CF2C were dissolved again in about 5 ml of their respective solvents and about 10 µl of each extract were spotted on TLC plates.

TLC analysis of CF2A, CF2C, and CF2M in CEF and EMW indicated that CF2A and CF2C are relatively pure compounds. Although all the three compounds showed only one compound with EMW in large amount, CF2M had two more fluorescing compounds separated by CEF. [Fig. 43]. Therefore, CF2A and CF2C were sent for NMR analysis.

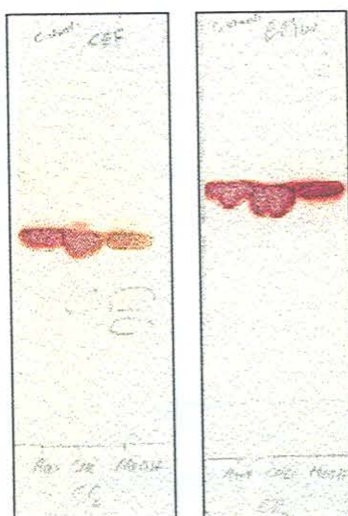

Fig. 43 TLC of some of CF2 that dissolved in three different solvents using CEF (left) and EMW (right) as the solvent systems. From left to right: acetone (ACN), chloroform (CHC), and methanol (MEOH) components. Lines indicate fluorescing compounds.

4.1.5. Analysis of CF1 by TLC and Column chromatography

In order to isolate and characterize the active compound in CF1, TLC analysis was carried out with various solvent systems to determine best solvent for column chromatography. The analysis was done in both normal and reverse phase mode.

The following solvent systems were attempted as eluents: chloroform:ethyl acetate [2:1, 1:5, 1:3, 4:1], acetonitrile:water (1:1), 1% acetonitrile in water, 2% acetonitrile in water , 5% acetonitrile in water, methanol:water (1:1), 5% methanol in chloroform, 1% methanol in chloroform.

5 µl of 10 mg/ml of CF1 in acetone was applied to TLC plates [Figs. 44, 45, 46].

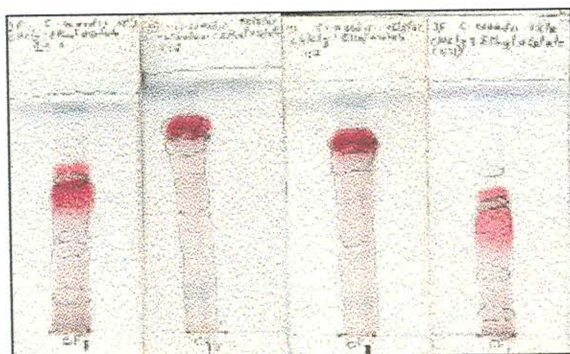

Fig. 44 Separation of CF1 by different solvent systems using vanillin-sulphuric acid as the spray reagent. The TLC plates from left to right were in developed in different chloroform-ethyl acetate combinations (2:1, 1:5, 1:3, 4:1).

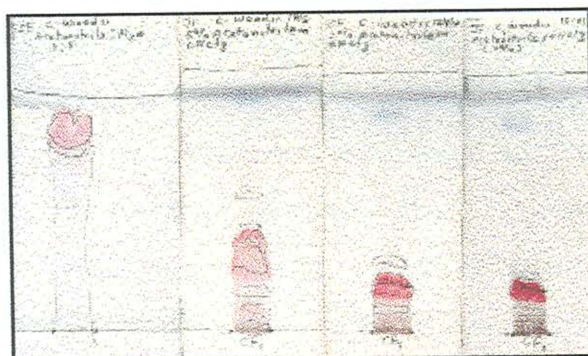

Fig. 45 Separation of CF1 by different solvent systems using vanillin-sulphuric acid as the spray reagent. The TLC plates from left to right were developed in acetonitrile:water (1:1), 5% acetonitrile in water, 2% acetonitrile in water, and 1% acetonitrile in water. Lines represent fluorescing compounds.

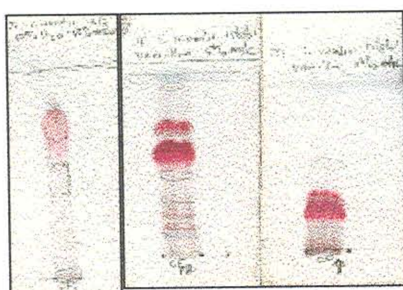

Fig. 46 Separation of CF1 by different solvent systems using vanillin-sulphuric acid as the spray reagent. The TLC plates from left to right were developed in methanol: water. (1:1), 5% methanol in chloroform, and 1% methanol in water.

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Poor separation of CF1 was obtained with normal phase [Fig. 44]. Therefore, it was necessary to carry out TLC with reverse phases [Figs. 45, 46].

Good separation were obtained with chloroform- methanol combination [Fig. 46]. The best separation was obtained with 5% methanol in chloroform. Therefore, different concentrations of methanol in chloroform were subsequently used as mobile phase for column chromatography of CF1.

Dried CF1 (about 410 mg) was dissolved in 2 ml of chloroform and applied to the column (length of column 26 cm) containing about 100 g of silica gel suspended in 5% methanol in chloroform. The column was set up in the same manner as previously explained [Section 2.7.2].

The components were eluted through a column under a gravitational force. A concentration gradient of eluents was used to elute the components through the column, starting with 80 % chloroform in hexane, and gradually with 100% chloroform, 1% methanol in chloroform (M/C), 5% methanol in chloroform, 10% methanol in chloroform, 20% methanol in chloroform, 40% methanol in chloroform and finally with 50% methanol in chloroform.

The fractions (about 20 ml) were collected by hand into test tubes every 3 minutes. About 200 ml of ammonium chloride solution (25%) was used to wash the whole column. Ammonium chloride will removes any basic compound attached to the silica. Approximately 144 test tubes were collected in separating the components. The test tubes were placed in the fume cupboard under a stream of air to concentrate the fractions.

The TLC analysis of column fractions was also carried out. Fractions with the similar components and Rf values from the TLC plates were combined and grouped into A, B, C, D, E, F, G, H, I, J, and K. All fractions were allowed to dry under a stream of air in fume cupboard [Table 14].

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Table 14. Quantity in mg of grouped fractions obtained from column chromatography of CF1

Group	Fractions	Quantity in mg
Group A	4 - 8	105
Group B	9 - 12	102
Group C	14 - 22	39
Group D	24 - 28	28
Group E	30 - 40	11
Group F	42 - 45	12
Group G	46 - 47	17
Group H	49 - 52	23
Group I	54 - 60	33
Group J	62 - 78	15
Group K	122 - 144	10

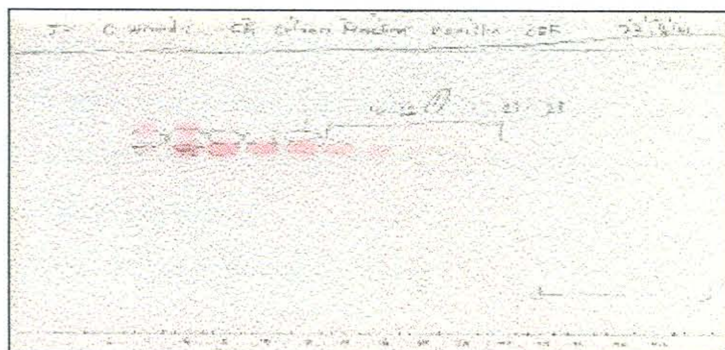

Fig. 47 TLC separation of fractions obtained from column chromatography of CF1 by CEF and using vanillin-sulphuric acid spray reagent. From left to right: fractions 1, 2, …, 29, 30.

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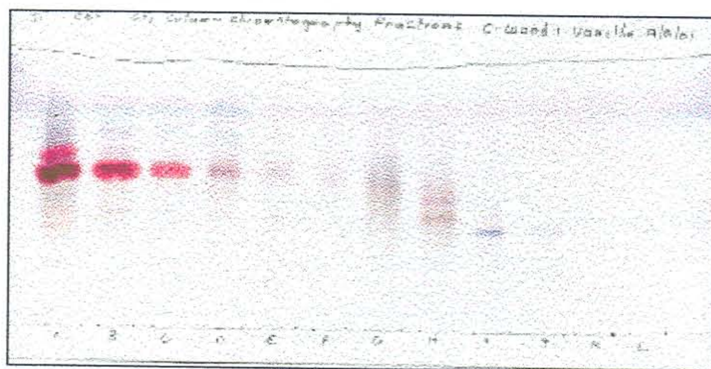

Fig. 48 TLC separation of grouped fractions from column chromatography of CF1 by CEF using vanillin-sulphuric acid spray reagent. From left to right, A, B, …, K, L.

Groups A, B and C were shown to contain the major active compound of R_f value of 0.74 in EMW solvent system but only group B had this active compound as the only component and also in large quantity. Group A had more than one component [Fig. 48]. The amount of this active compound present in group C was very small. Therefore, group B was used for isolation of the active compound.

About 5 ml of chloroform was added to group B and then dried in a rotary evaporator in a pre weighed round bottom flask. The mass of group B that was recovered after evaporating off chloroform was 102 mg. It was labeled as' CF1b' for identification and further analysis.

Although there were more than one antibacterial compound separated in the TLC systems, efforts were made to isolate and characterize only the major active compound having R_f value of 0.74 in EMW as solvent system.

In conclusion, the major active compound was isolated as CF2A, CF2C and CF1b.

4.1.6 Minimum inhibitory concentration of isolated active compound

In order to evaluate the antibacterial activity of isolated active compound CF1b or CF2C, 10 mg of each of the compound was weighed out and reconstituted in 10 ml acetone. The minimum inhibitory concentration (MIC) of each compound was determined twice by microplate serial dilution assay technique outlined in section 2.5. The results obtained for the two experiments for each compound were the same. The values of MIC obtained for the isolated compound were compared with values for ampicillin and chloramphenicol.

University of Pretoria etd – Famakin, J O (2002)

The results are as shown below [Table 15]

Table 15 MIC values in µg/ml of CF1b compared with ampicillin and chloramphenicol.

	CF1b	Ampicillin	Chloramphenicol
S. aureus	16	80	160
E. coli	>250	160	40
Ps. aeruginosa	125	125	125
E. faecalis	125	160	160

CFIb was more active against *S. aureus, Ps. aeruginosa and E. faecalis* than ampicillin and chloramphenicol in this experiment [Table 15]. CF1b has highest activity against *S. aureus*. In other word, the isolated compound had significant activity against *S. aureus, Ps. aeruginosa and E. faecalis*. However, the compound had little activity against *E. coli* with an MIC value greater than 250 µg/ml

4.1.6 Bioautography of isolated compound

Bioautography was carried out to carry out using *S. aureus* as a test organism. There was a single clear zone of inhibition on chromatogram [Fig. 49].

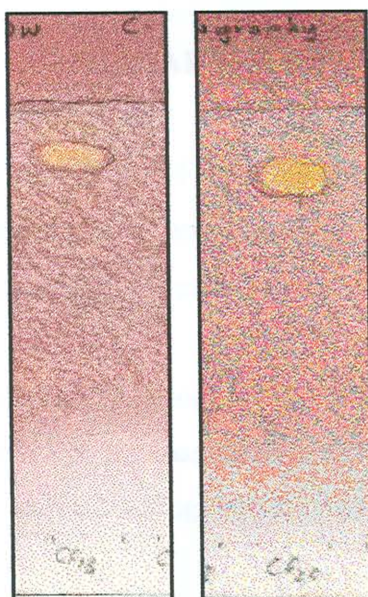

Fig. 49 Bioautograms of CF1b (left) and CF2C (right). TLC developed in EMW and sprayed with actively growing *S. aureus* culture and later with INT. Brownish-yellow colour indicated growth inhibition zone.

Many natural products are sensitive to light, oxygen, water and changes in pH. Many plants contain compounds that prevent such decomposition e.g., antioxidant and these may occur in different aliquots from the biologically active compounds after fractionation.

There was also a possibility that activity of plant extract or fractions was due to synergism between components, which were separated as a result of fractionation. The active components might also remain in the system particularly that a chromatography with a solid phase has been applied.

However, antibacterial activity of isolated compound of interest was retained after the various fractionation processes used.

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CHAPTER 5

SPECTROSCOPIC ANALYSIS OF ISOLATED COMPOUND

The active compounds, 10 mg each of CF2A and CF2C, were dissolved in deuterated chloroform ($CDCl_3$) and sent for NMR analysis with a 300 MHz Varian NMR machine (Oxford instruments). Both compounds gave similar NMR spectra, however CF2C showed higher purity than CF2A.
The active compound isolated as CF1b together with CF2C was sent for NMR and mass spectroscopic analysis. The NMR and mass spectra of both CF1b and CF2C showed that they are similar compounds but isolated through different procedures. CF1b was however, the purest.

Table 16 ^{1}H-NMR (300MHz) and ^{13}C- NMR (75MHz) spectra data for isolated compound (CF1b). Data obtained in $CDCl_3$

Carbon Position	Chemical shift (δ, ppm)	
	^{1}H	^{13}C
1	-	133.4
2	6.40	105.1
3	-	146.8
4	-	132.2
5	-	146.8
6	6.40	105.1
1'	-	121.5
2'	-	142.1
3'	-	132.7
4'	6.54	145.2
5'	-	102.3
6'	6.35	120.1
1a	3.84	36.5
1'a	3.84	32.1
3, 5 OMe	2.82	56.2
4' OMe	2.82	56.1

The ^{1}H-NMR spectrum of CF1b, showed signals for three aromatic protons at 6.5 ppm (1H, d, J= 8.4Hz), 6.40 ppm (2H, s) and 6.36 ppm (1H, d, J=8.4Hz), a signal at 3.84 ppm integrating for three methoxyl groups and a complex multiplet at 2.82 ppm [Spectrum 1]. The complex multiplet arises from an ethane bridge, which is typical of bibenzylic, compounds (Majumder et al, 1999; Katerere, 2001).

Mass spectroscopy confirmed the bibenzylic nature of CF1b. It gave a molecular ion at m/z 320 (41%) corresponding to $C_{17}H_{20}O_6$ and two major fragments at m/z 153 $C_8H_9O_3$ (100%, base peak) and 167 $C_9H_{11}O_3$ (89.9%). The fragments are tropylium derivatives of the phenolic ring which is typical of bibenzyls (Letcher et al., 1972; Katerere, 2001)[Fig. 53]. This suggested that one ring contained two methoxyl groups and one hydroxyl group and the other one methoxyl group and two hydroxyl groups. The exact positions of the hydroxyl and methoxyl groups around the two aromatic rings were ascertained from the chemical shift and the splitting patterns of signals of the aromatic protons. 2D NMR was also attempted but gave equivocal results.

The isolated active compound was divided into aromatic rings arbitrarily labeled as 'A' and 'B' [Fig. 52]. Mass spectroscopy of CF1b gave fragment of m/z 167 ($C_9H_{11}O_3$) representing aromatic ring A. It has two methoxyl and one-hydroxyl functions.

For ring A, ^{1}H-NMR showed a singlet at 6.40 ppm, which correspond to two protons. These are *meta*-coupled and magnetically equivalent and also imply that ring A is probably symmetrical. Therefore, the protons were placed at position 2 and 6. Positions 3, 5, 6 would have to be oxygenated as seen from the mass spectroscopic fragment m/z 167 ($C_9H_{11}O_3$).

The other fragment of m/z 153 ($C_8H_9O_3$) from mass spectroscopy of CF1b represents the aromatic ring B. The proton at 6.54 ppm is *ortho*- coupled to that at 6.35 ppm. This is implied from the coupling constant J=8.4. The protons were placed at 5' and 6' leaving the other positions to be taken up by two hydroxyl and a methoxyl group.

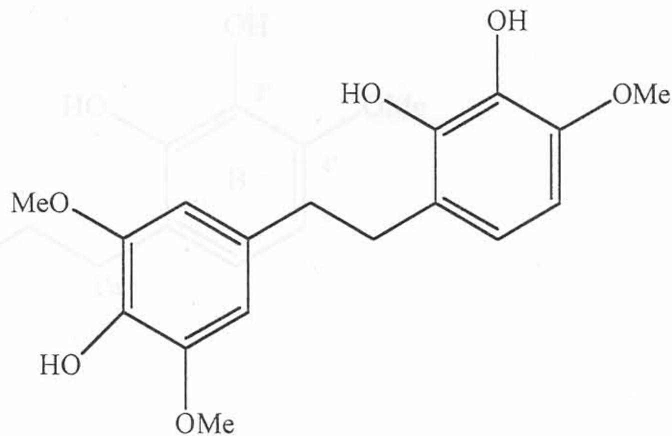

Fig. 50 Proposed structure for isolated active compound.

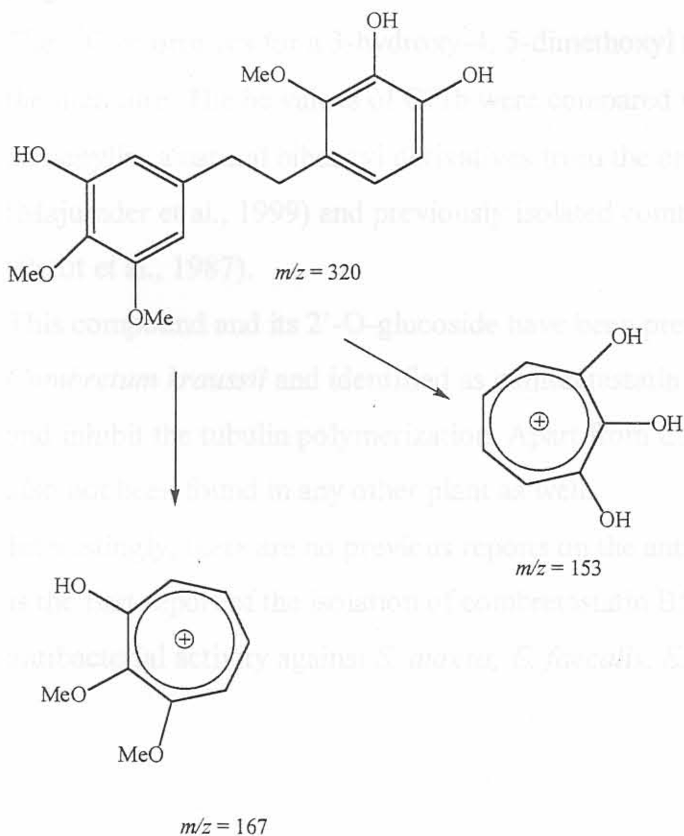

Fig. 51 The isolated active compound and its fragmentation into two tropylium ions.

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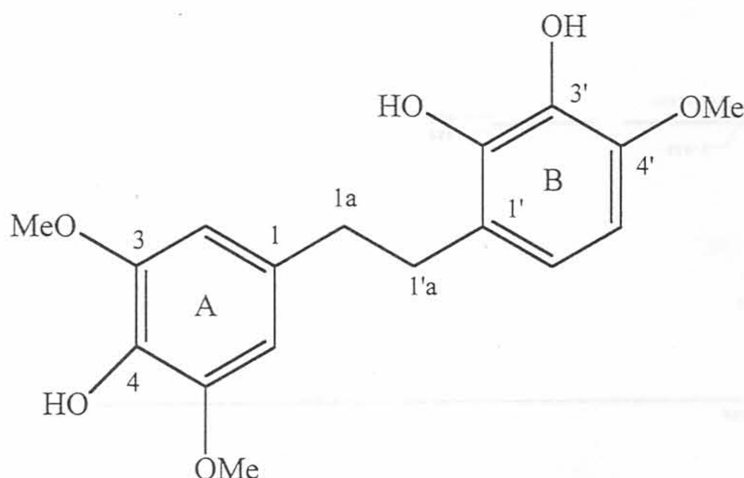


Fig. 52 Isolated active compound with its two aromatic rings labeled as 'A' and 'B'

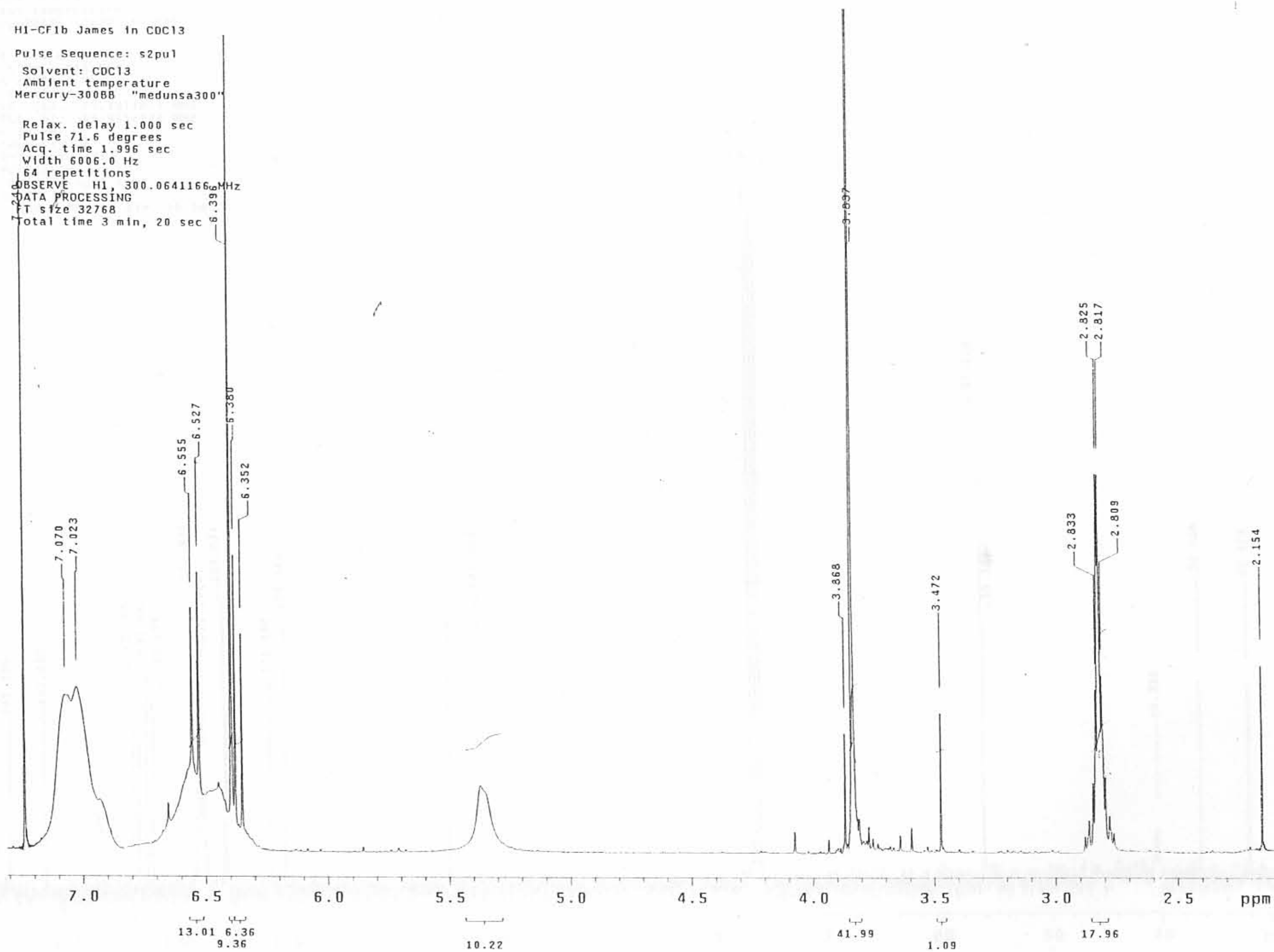
The compound isolated was proposed to be 2', 3', 4-trihydroxyl, 3, 5, 4'-trimethoxybibenzyl [Fig. 50].

The ^{13}C resonances for a 3-hydroxy-4, 5-dimethoxyl ring were compared experimental data in the literature. The δc values of CFIb were compared with structurally related carbon atoms of amoenylin, a natural bibenzyl derivatives from the orchid *Dendrobium amoenumm* (Majumder et al., 1999) and previously isolated combretastatins A-1 and B-I from *C. caffrum* (Pettit et al., 1987).

This compound and its 2'-O-glucoside have been previously isolated from seeds of *Combretum kraussii* and identified as combretastatin B5. They showed *in vitro* cytotoxicity and inhibit the tubulin polymerization. Apart from C. *krausii*, this compound has apparently also not been found in any other plant as well.

Interestingly, there are no previous reports on the antibacterial activity of this compound. This is the first report of the isolation of combretastatin B5 from *C. woodii* as well as its antibacterial activity against *S. aureus, E. faecalis*, *E. coli*, and *Ps. aeruginosa.*

University of Pretoria etd – Famakin, J O (2002)



Spectrum 1 ^{1}H-NMR Spectroscopy of CF1b

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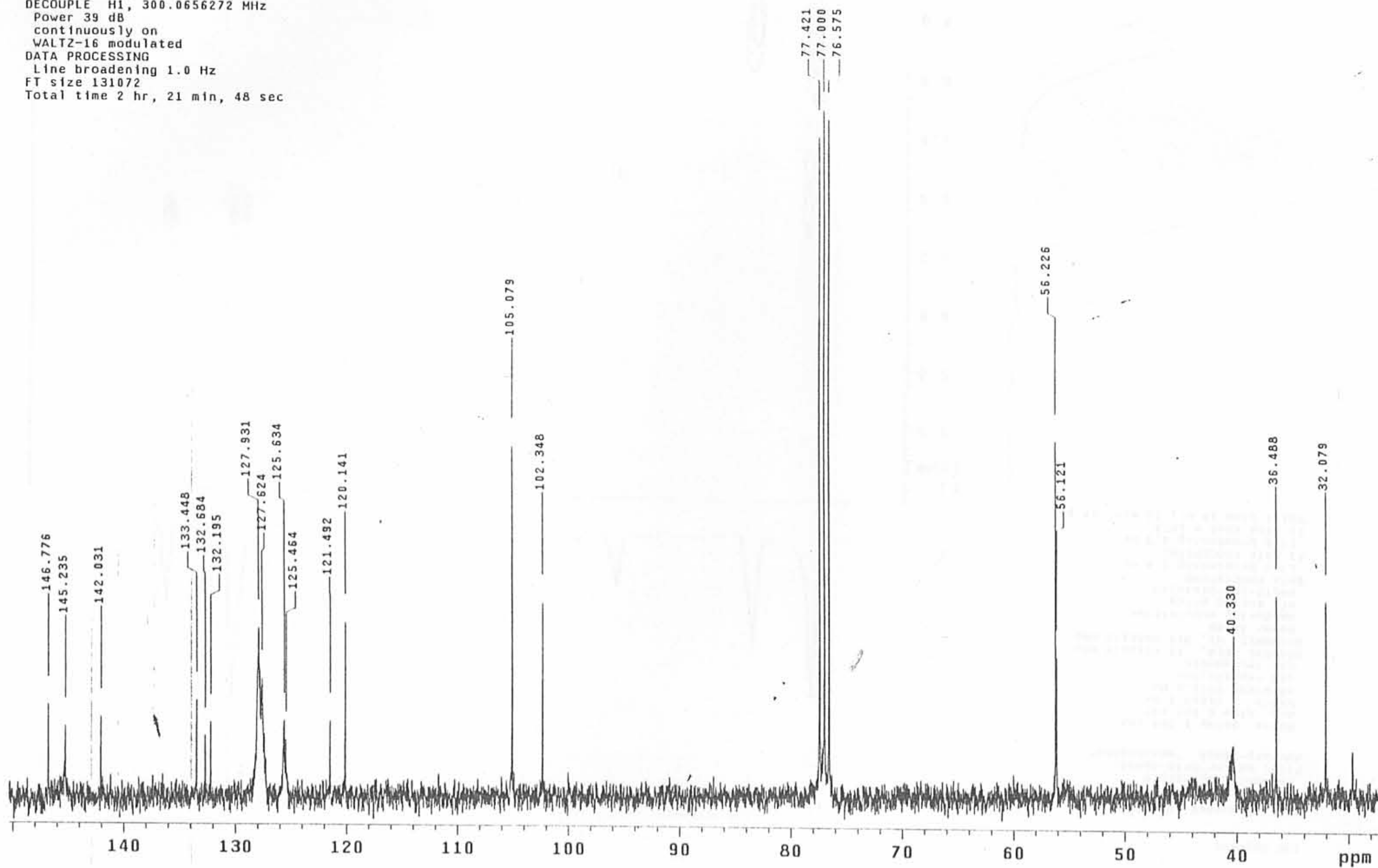
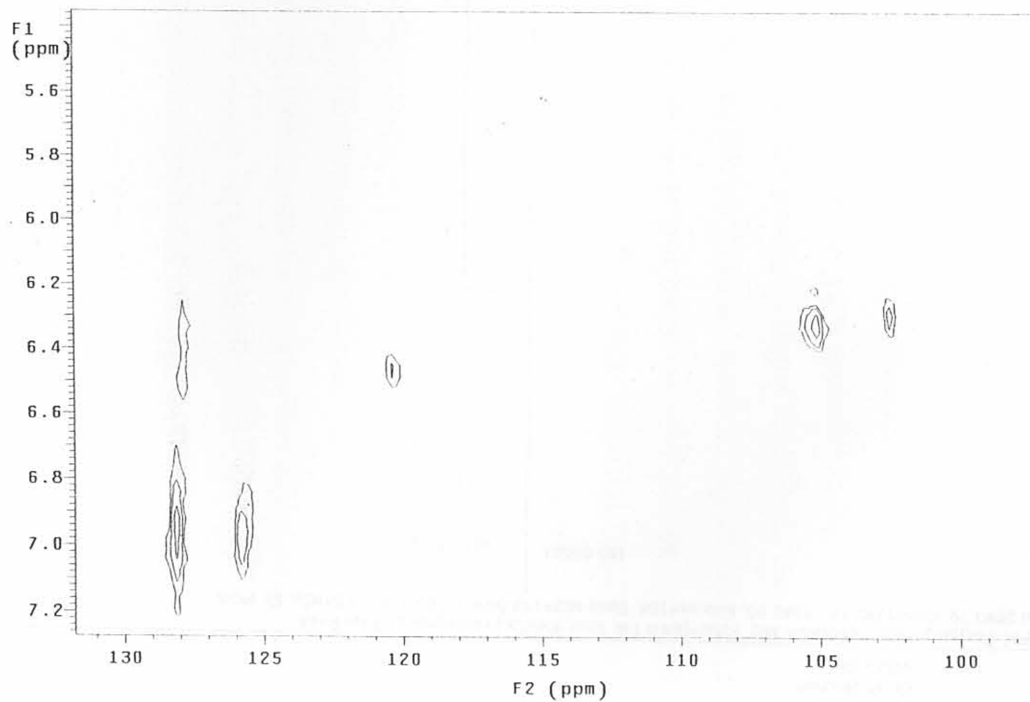


Spectrum 2 ^{13}C-NMR Spectroscopy of CF1b

13C OBSERVE

Pulse Sequence: hetcor
Solvent: CDCl3
Ambient temperature
File: Hetcor-CF1b-James
Mercury-300BB "medunsa300"

Relax. delay 1.000 sec
Acq. time 0.051 sec
Width 20000.0 Hz
2D Width 6006.0 Hz
128 repetitions
256 increments
OBSERVE C13, 75.4511625 MHz
DECOUPLE H1, 300.0656272 MHz
Power 39 dB
on during acquisition
off during delay
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.0 Hz
F1 DATA PROCESSING
Line broadening 0.3 Hz
FT size 2048 x 1024
Total time 10 hr, 23 min, 12 sec



Spectrum 3 2D NMR Spectroscopy of CF1b

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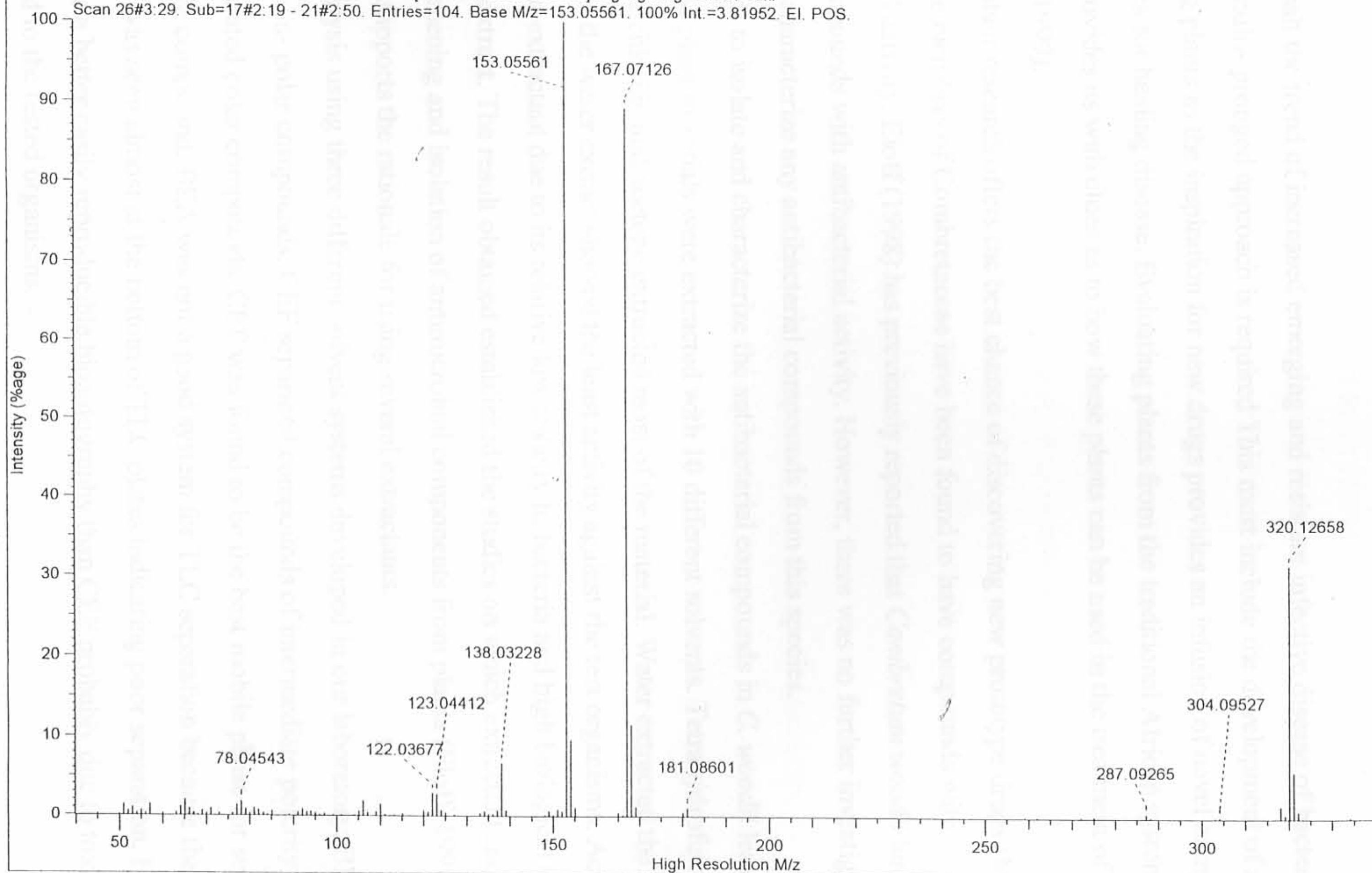

Spectrum 4 Mass spectrometry of CF1b

DISCUSSION AND CONCLUSION

In order to halt the trend of increased emerging and resistant infective disease of bacterial etiology, a multi- pronged approach is required This must include the development of new drugs. Using plants as the inspiration for new drugs provides an infusion of novel compounds or substances for healing disease. Evaluating plants from the traditional African system of medicine provides us with clues as to how these plants can be used in the treatment of disease (Iwu et al., 1999).

Medicinal plant research offers the best chance of discovering new prototype drugs (Malone, 1983). Some members of Combretaceae have been found to have compounds with antibacterial activity. Eloff (1998) has previously reported that *Combretum woodii* leaves contain compounds with antibacterial activity. However, there was no further investigation to isolate and characterize any antibacterial compounds from this species.

In an attempt to isolate and characterize the antibacterial compounds in *C. woodii* leaves, the dried ground plant materials were extracted with 10 different solvents. Tetrahydrofuran and methylene dichloride and acetone extracted most of the material. Water extracted the least quantity and the water extract showed the least activity against the test organisms. Acetone was the best extractant due to its relative low toxicity to bacteria and high biological activity of acetone extract. The result obtained established the studies on which extractant should be used for screening and isolation of antimicrobial components from plants (Eloff, 1998). This result also supports the rationale for using several extractants.

In TLC analysis using three different solvent systems developed in our laboratory, BEA separated non- polar compounds, CEF separated compounds of intermediate polarity and EMW separated polar compounds. CEF was found to be the best mobile phase for separation of the active compound. BEA was not a good system for TLC separation because the active compound was seen almost at the bottom of TLC plates indicating poor separation. However, EMW gave a better easily reproducible bioautography than CEF probably due to toxicity of formic acid to the tested organisms.

Both vanillin- sulphuric acid and *p*-anisaldehyde-sulphuric acid spraying reagents were initially used. However, more compounds were visible with vanillin than with anisaldehyde spray reagent. Vanillin-sulphuric acid spray reagent was therefore routinely used; it appears to be a good spray reagent for detection of the active compound in *C. woodii* leaves. However, not all the compounds that were seen under on UV light were revealed by it, but they were not the compounds with antibacterial activity.

University of Pretoria etd – Famakin, J O (2002)

After solvent- solvent fractionation, most of the activity resided in chloroform and hexane fractions. Bioassay-guided isolations resulted in the identification of the responsible antibacterial compounds in the leaves of this plant. There were many antibacterial compounds shown by bioautography. However, attempts were directed towards isolating one major compound, with R_f value of 0.74 in EMW solvent systems, present in all the fractions (except water fraction) obtained. Column chromatography of the chloroform fraction led to the successful isolation of this compound. This isolated compound exhibited *in vitro* antibacterial activity against all the four test organisms. Both Gram-positive organisms and Gram-negative organisms were sensitive to the compound. *S. aureus* was the most sensitive of all the four tested with an MIC value of 16 µg/ml followed by *E. faecalis* (125 µg/ml) and *Ps. aeruginosa* (125 µg/ml) and *E. coli* (250 µg/ml). Activities on three of the bacteria tested were higher than the standard antibiotics compared.

NMR and mass spectra of the isolated active compound led to its structural elucidation. This antibacterial compound was found to be a bibenzylic compound, 2', 3', 4-trihydroxyl, 3, 5, 4'-trimethoxyl bibenzyl. This compound is also a stilbene. The stilbenes are phytoalexins, which are antimicrobial compounds which accumulates in response to a pathogen (Kuc, 1990) Therefore, the possible role of the bibenzyl in *C. woodii* is to protect the plant against any invading microorganism. This same compound also named as combretastatin B5 previously isolated from seeds of *C. krausii* has been found to be antimitotic (Pellizzoni et. al, 1992). There are no previous reports of its antibacterial activity. Based on the MIC values of this compound, further investigation is necessary for treating ailments caused by bacteria, mostly *S. aureus*, *Ps. aeruginosa* and *E. faecalis*. Infections caused by the *S. aureus* and *Ps. aeruginosa* organisms are amongst the most difficult to treat with conventional antibiotics (Salie et al, 1996).

With the presence of antibacterial compounds, first time isolated in leaves, this plant or isolated constituent, which is accessible, could be an inexpensive additional means for treating bacterial infections

Generally, *C. woodii* leaves contain many antibacterial compounds of which only one was isolated and characterized. The results obtained with *C. woodii* indicated that not only the stability of the biologically active component, but also probably the high concentration of the desired component enhances the applicability of all the techniques used. This, in fact, suggested that *C. woodii* has a high biological activity, since it is impossible to detect antibacterial activity if the quantity of material is limited.

University of Pretoria etd – Famakin, J O (2002)

Because this plant contains many compounds, further work needs to be carried out on isolation and characterization of other antibacterial compounds.

These results validate the ethnobotanical use of many *Combretum* species for bacteria infections.

Furthermore, it is recommended for future work that the spectrum of antimicrobial activity of the active compound isolated be determined by testing its activity against many bacteria and fungi.

APPENDIX 1

Table 17 Typical chemical shifts of hydrogen attached to various types of functional groups.

Type of hydrogen		Chemical shift δ-ppm
Primary alkyl,	RCH_3	0.8 - 1.0
Secondary alkyl,	R_2CH_2	1.2 - 1.4
Tertiary alkyl	R_3CH	1.4 - 1.7
Alkyl fluoride	RCH_2F	4.0 - 4.5
Alkyl chloride	RCH_2F	3.6 - 4.0
Alkyl bromide	RCH_2Br	3.4 - 3.6
Alkyl iodide	RCH_2I	3.0 - 3.3
Vinylic,	$R_2C{=}CH_2$	4.6 - 5.0
Vinylic	R2=CHR	5.2 - 5.9
Allylic	R2C=CHR CH_3	1.6 - 1.9
Acetylene	$RC\equiv CH$	2.5 - 3.1
Acetylene	$RC\equiv CH.CH_3$	1.7 - 2.0
Alcohol	$HOHCH_2R$	3.3 - 4.0
Ether	$ROCH_2R$	3.3 - 3.9
Methylenedioxy	OCHO	5.2 - 5.5
Ketone	$RCOCH_3$	2.1 - 2.7
Aldehdyde	RCHO	9 - 10.0
Aromatic	ArH	6.0 - 8.5
Benzylic	$ArCH_3$	2.2 - 3.0
Alcoholic	OH R-OH	1.0 - 5.5 (broad)
Phenolic	ArOH	4.0 - 12.0
Carboxyl	RCOOH	10.0 - 13.0
Ester	$RCOOCH_3$	2.0 - 2.5
Amino	RNH_2	1.0 - 5.0
Amide	RCONH2	5.0 - 9.0 (very broad)
Nitro	RCH_2NO_2	4.2 - 4.6

University of Pretoria etd – Famakin, J O (2002)

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