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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

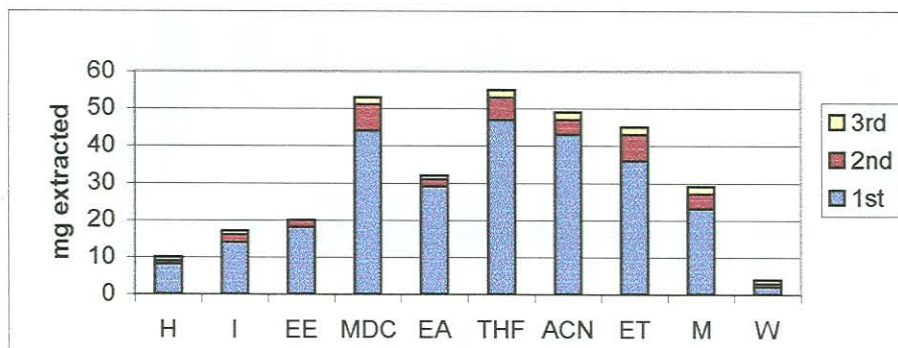

Fig. 9 Quantity (mg) extracted from 0.5 g of powdered dried leaves of *C. woodii* with 5 ml of hexane (H), diisopropyl ether (I), diethyl ether (EE), methylene dichloride (MDC), ethyl acetate (EA), tetrahydrofuran (THF), acetone (ACN), ethanol (ET), methanol (M) and water (W). Value for first extraction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

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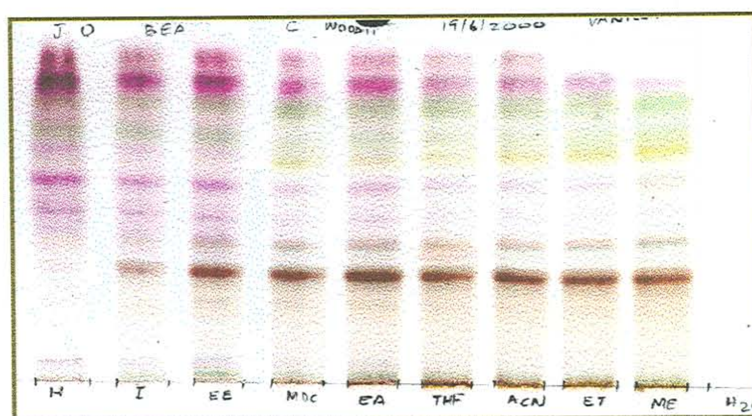

Fig. 10 Separation of components present in 50 µg of 10 different extracts with BEA as eluent and vanillin-sulphuric acid spray reagent. Lanes from left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethylacetate, tetrahydrofuran, acetone, ethanol, methanol and water.

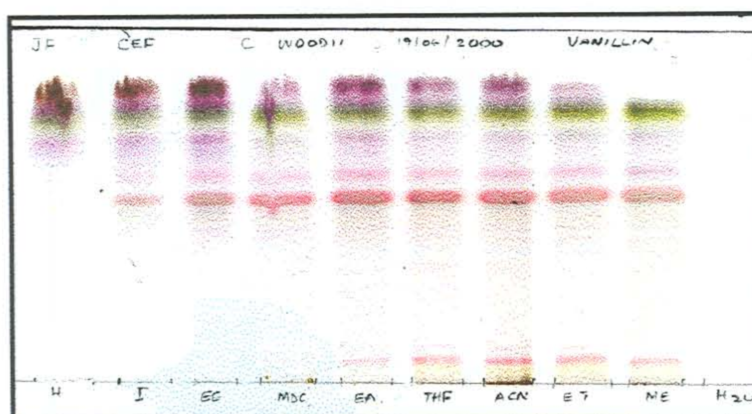

Fig. 11 Separation of components present in 50 µg of 10 different extracts using CEF as eluent and vanillin-sulphuric acid spray reagent. Lanes from left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethylacetate, tetrahydrofuran, acetone, ethanol, methanol and water.

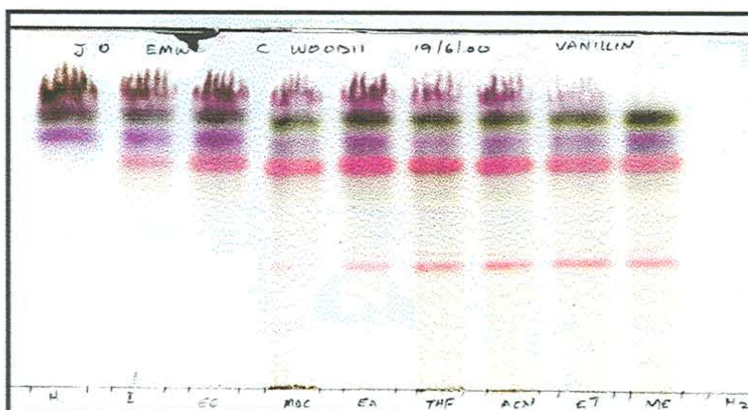

Fig. 12 Separation of components present in 50 µg of 10 different extracts using EMW as eluent and vanillin-sulphuric acid spray reagent. Lanes from left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethylacetate, tetrahydrofuran, acetone, ethanol, methanol and water.

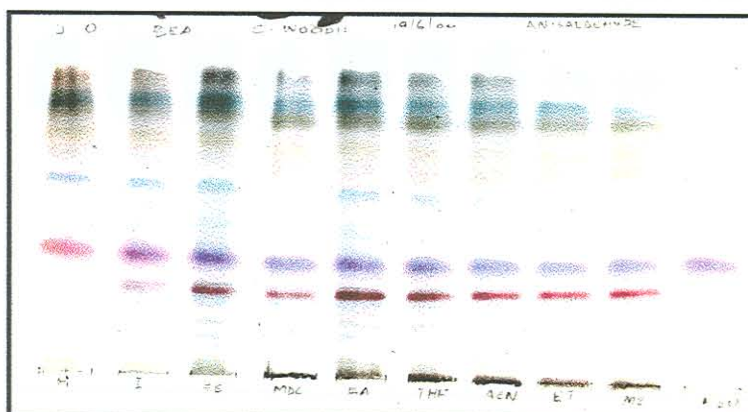

Fig. 13 Separation of compounds present in 50 µg of 10 different extracts using BEA as eluent and *p*-anisaldehyde-sulphuric acid spray reagent. Lanes from left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethylacetate, tetrahydrofuran, acetone, ethanol, methanol, and water.

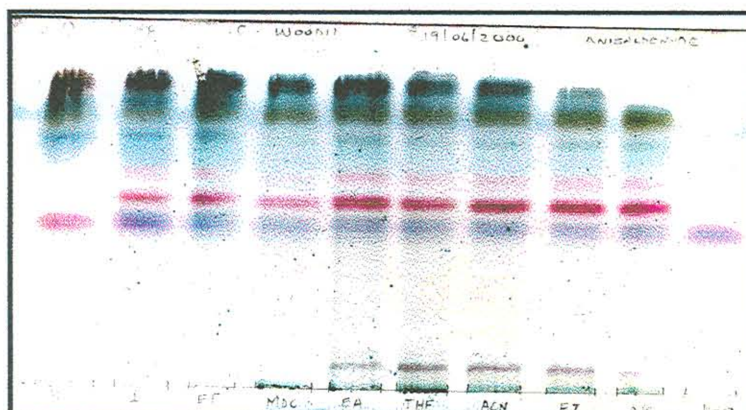

Fig. 14 Separation of compounds present in 50 μg of different extracts using CEF as eluent and *p*-anisaldehyde-sulphuric acid spray reagent. Lanes from left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethylacetate, tetrahydrofuran, acetone, ethanol, methanol, and water.

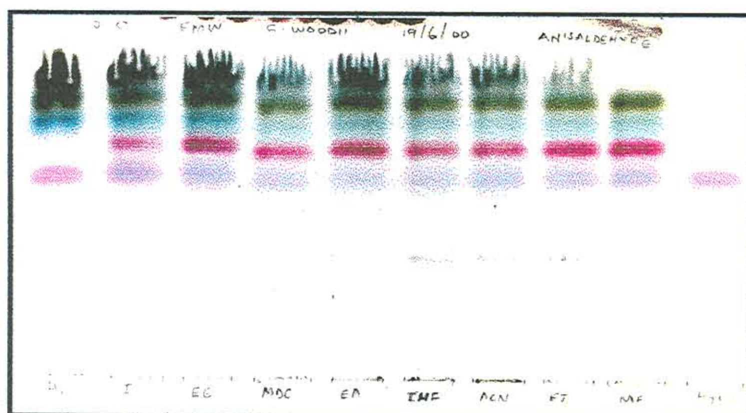

Fig. 15 Separation of compounds present in 50 μg of different extracts using EMW as eluent and *p*-anisaldehyde-sulphuric acid spray reagent. Lanes from left to right: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethylacetate, tetrahydrofuran, acetone, ethanol, methanol, and water.

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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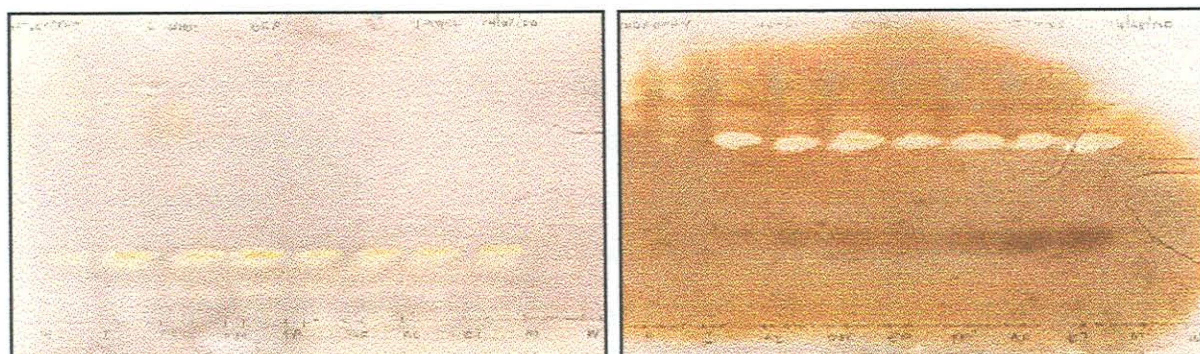

Fig. 16 Bioautograms of *C. woodii* leaves extracted by 10 different extractants. TLC developed in BEA (left) and EMW (right) and sprayed with *S. aureus* culture, incubated overnight then sprayed with INT. Growth inhibition indicated by lighter or colourless zones on TLC plates. Lanes from left to right: 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).

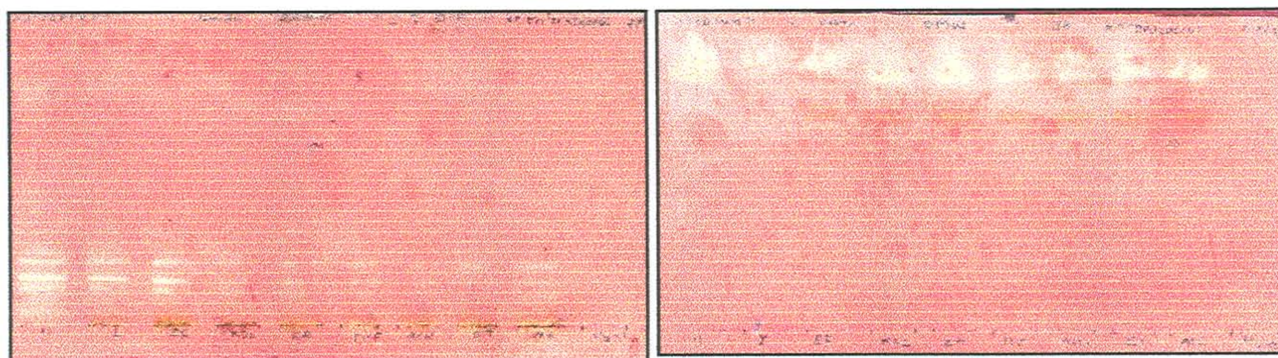

Fig. 17 Bioautograms of *C. woodii* leaves extracted by 10 different extractants. TLC developed in BEA (left) and EMW (right) and sprayed with *E. faecalis* culture, incubated overnight then sprayed with INT. Growth inhibition is indicated by colourless zone. Lane from left to right: hexane (H), diisopropyl 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.

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

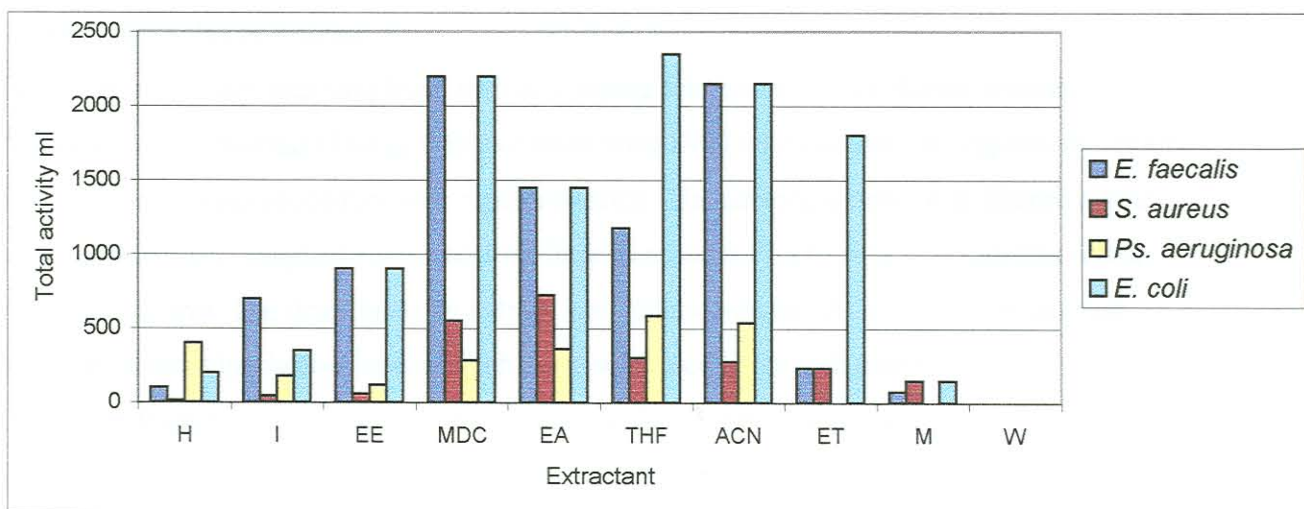

Fig. 18 Total antibacterial activity of *C. woodii* extracts with 10 different extractants on four test organisms. From left to right: hexane (H), diisopropyl ether (I), diethyl ether (EE), methylene dichloride (MDC), ethyl acetate (EA), tetrahydrofuran (THF), acetone (ACN), ethanol (ET), methanol (M)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

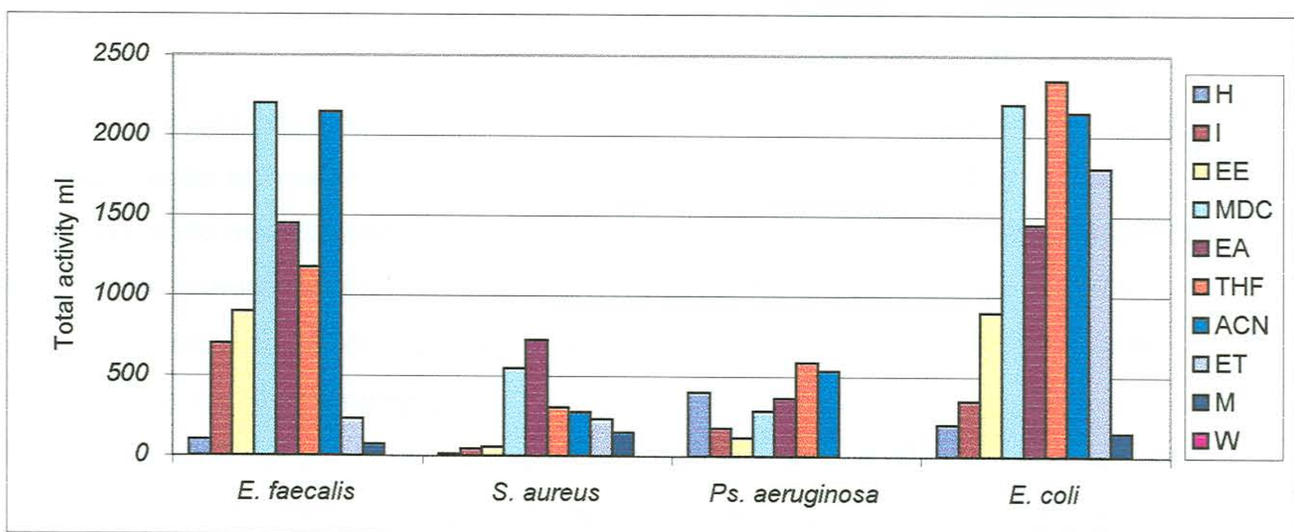

Fig. 19 Total antibacterial activity of *C. woodii* extracts on four test organisms.

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].

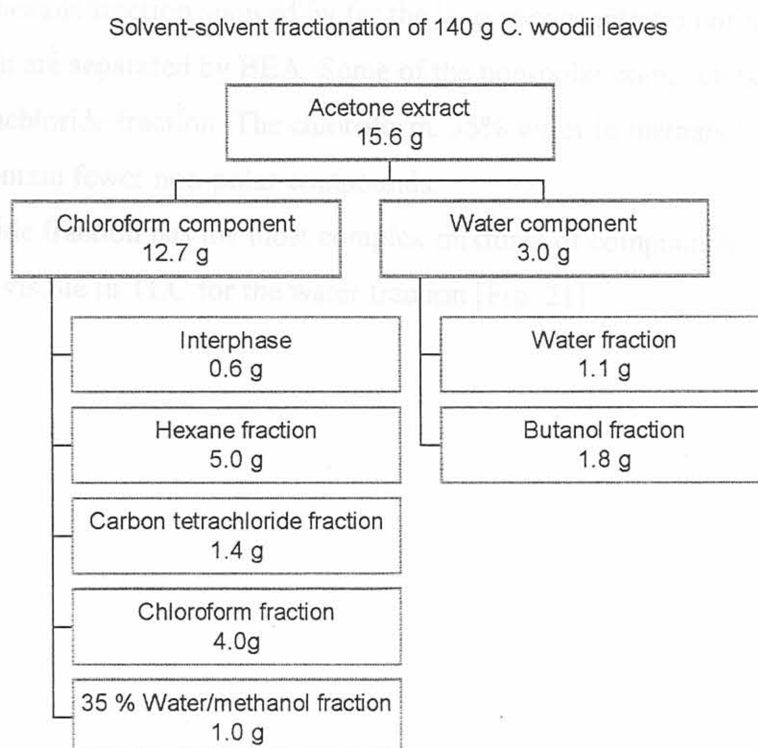

Fig. 20 Quantity extracted in each solvent fraction by solvent-solvent fractionation process

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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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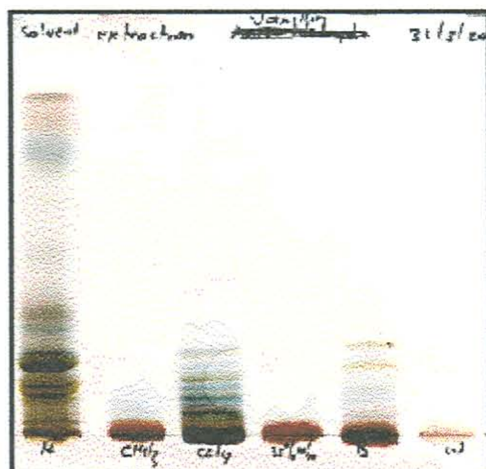

Fig. 21 Separation of components present in the different fractions obtained by solvent-solvent extraction by BEA and sprayed with vanillin-sulphuric acid. Lanes from left to right: hexane, chloroform, carbon tetrachloride, 35% water in methanol, butanol and water fractions. In each case 50 µg was chromatographed.

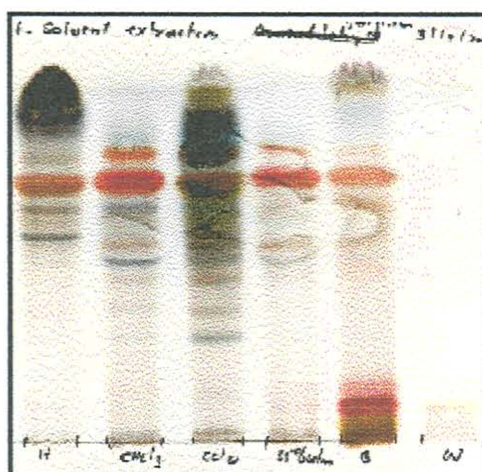

Fig. 22 Separation of components present in the different fractions obtained by solvent-solvent extraction by CEF and sprayed with vanillin-sulphuric acid. Lanes from left to right: hexane, chloroform, carbon tetrachloride, 35% water in methanol, butanol and water fractions. In each case 50 µg was chromatographed.

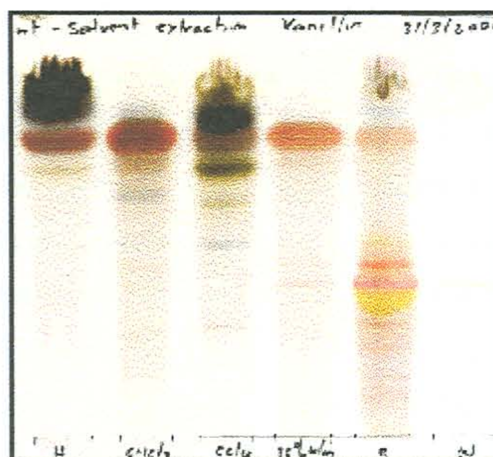

Fig. 23 Separation of components present in the different fractions obtained by solvent-solvent extraction by EMW and sprayed with vanillin-sulphuric acid. Lanes from left to right: hexane, chloroform, carbon tetrachloride, 35% water in methanol, butanol and water fractions. In each case 50 μg was chromatographed.

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].

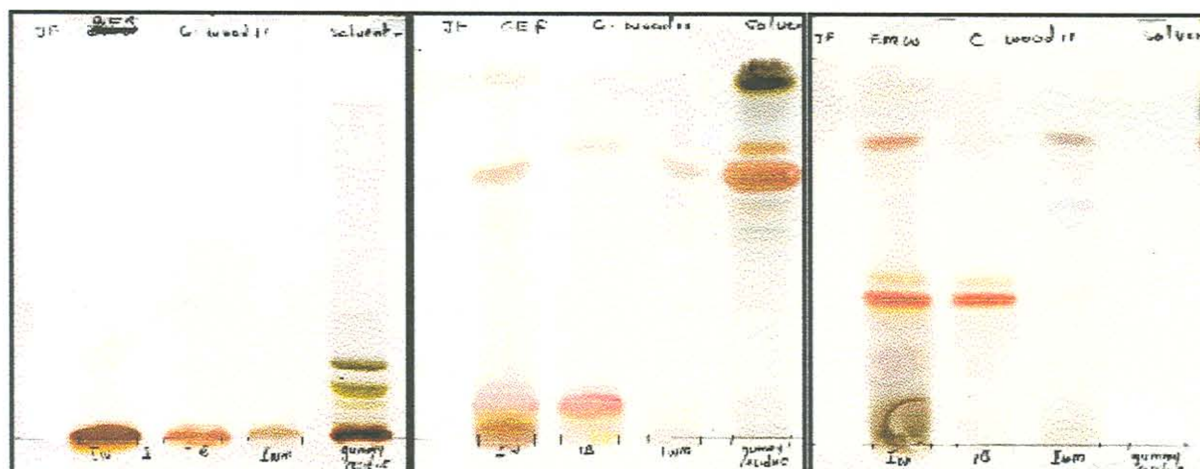

Fig. 24 Separation of components present in the different fractions obtained by solvent-solvent extraction by BEA, CEF, and EMW (from left to right) and sprayed with vanillin-sulphuric acid. Lanes at the bottom in each TLC plate from left to right, insoluble water fractions (IW), insoluble butanol fractions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

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.

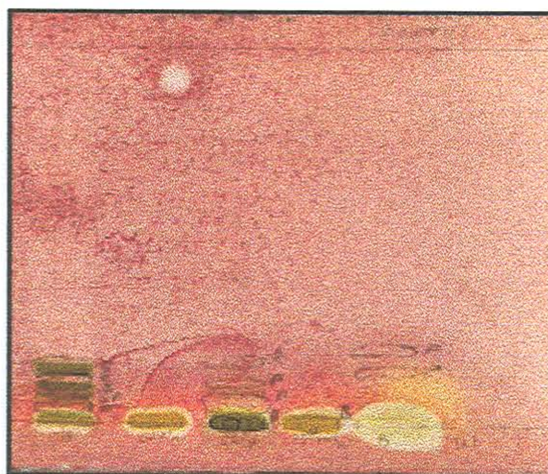

Fig. 25 Bioautogram of acetone extract of *C. woodii* leaves separated into different fractions by solvent-solvent extraction. TLC plate developed in BEA and sprayed with *S. aureus* culture incubated overnight and then sprayed with INT. Growth inhibition indicated by colourless zones on TLC plate. Lanes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

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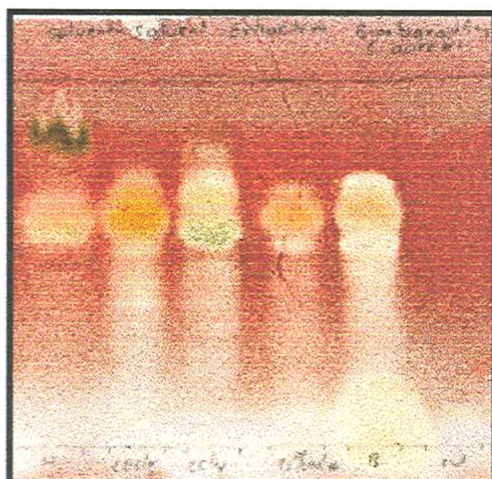

Fig. 26 Bioautogram of acetone extract of *C. woodii* leaves separated into different fractions by solvent-solvent extraction. TLC plate developed in CEF and sprayed with *S. aureus* culture incubated overnight and then sprayed with INT. Growth inhibition indicated by colourless zones on TLC plates.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

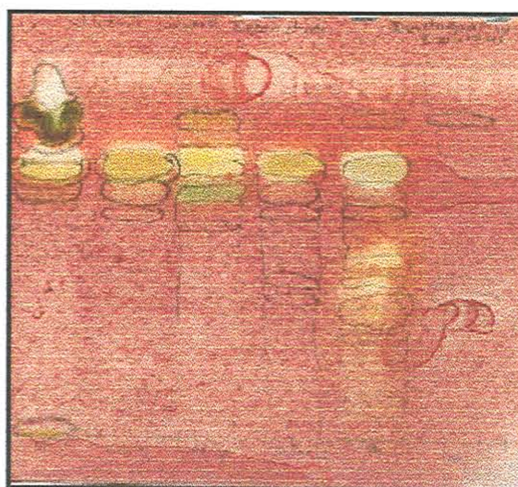

Fig. 27 Bioautogram of acetone extract of *C. woodii* leaves separated into different fractions by solvent-solvent extraction. TLC plate developed in EMW and sprayed with *S. aureus* culture incubated overnight and then sprayed with INT. Growth inhibition indicated by colourless or lighter zone on TLC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

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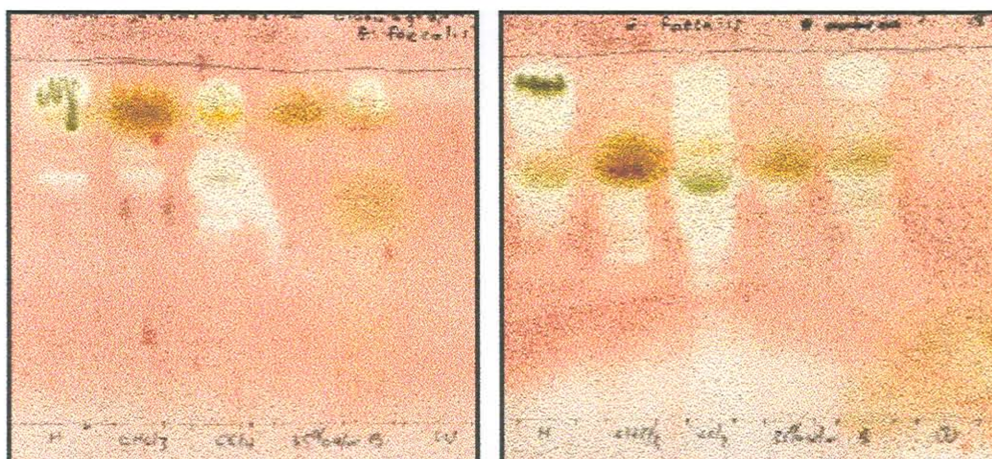

Fig. 28 Bioautograms of the acetone extract of *C. woodii* leaves separated by CEF (left) and EMW (right) and *E. faecalis* as the test organism. White or brownish yellow areas indicate growth inhibition. Lanes from left to right: hexane, chloroform, carbon tetrachloride, 35% water in methanol, butanol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

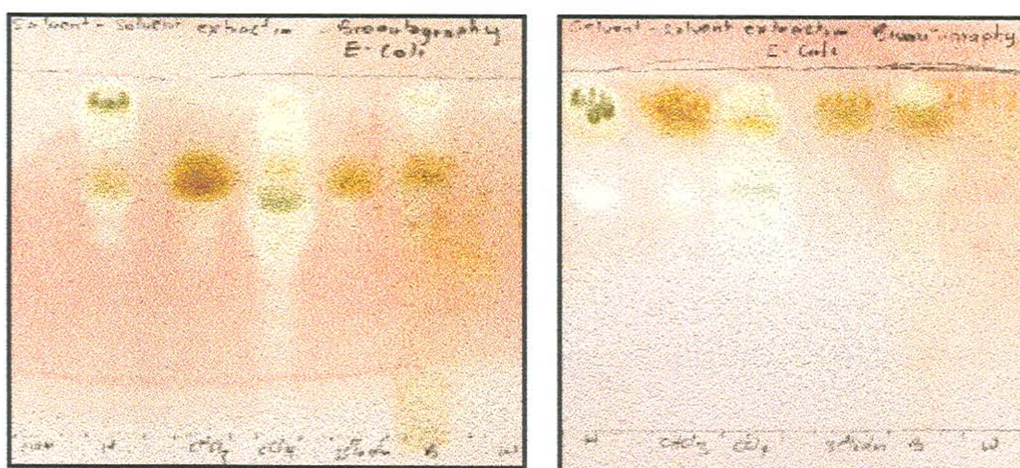

Fig. 29 Bioautograms of the acetone extract of *C. woodii* leaves separated by CEF (left) and EMW (right) and *E. coli* as the test organism. White or brownish yellow areas indicate growth inhibition. Lanes from left to right: hexane, chloroform, carbon tetrachloride, 35% water in methanol, butanol and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

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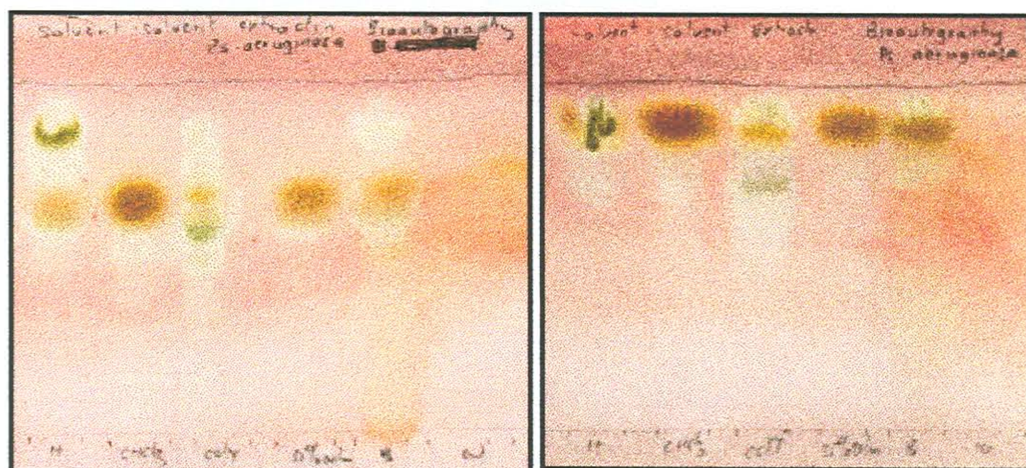

Fig. 30 Bioautograms of the acetone extract of *C. woodii* leaves separated by CEF (left) and EMW (right) and *Ps. aeruginosa* as the test organism. White or brownish yellow areas indicate growth inhibition. Lanes from left to right: hexane, chloroform, carbon tetrachloride, 35% water in methanol, butanol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

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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.

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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

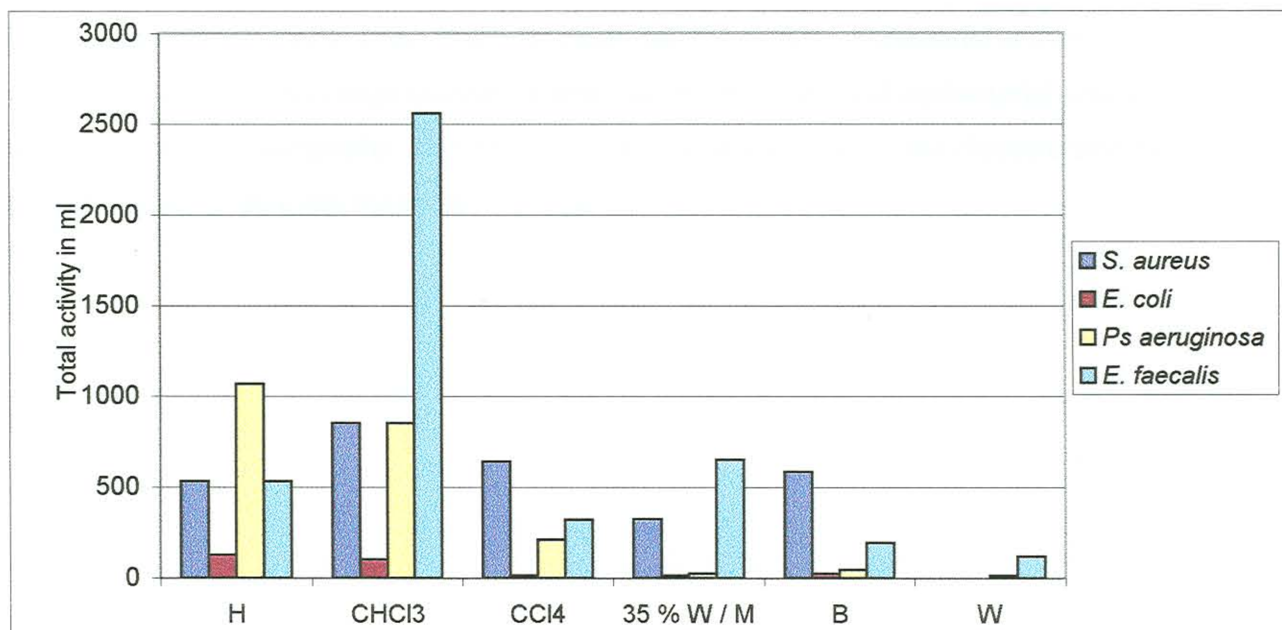

Fig. 31 Total antibacterial activity to four test organisms of *C.woodii* leaves extracted with solvent fractions. Fractions from left to right: hexane (H), chloroform ($CHCl_3$), carbon tetrachloride (CCl_4), 35% water in methanol (35%W/M), butanol (B) and water (W).

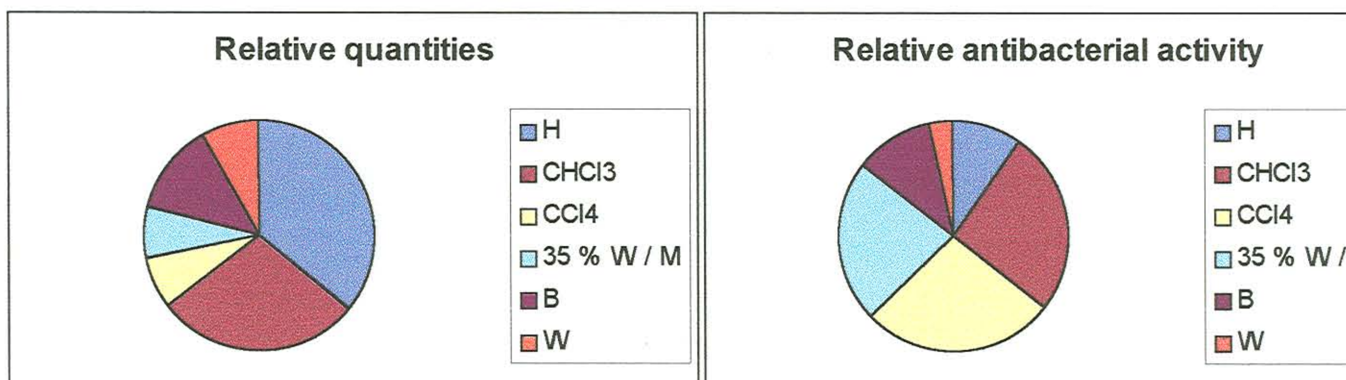

Fig. 32 The relative quantities obtained by solvent-solvent fractionation and the relative antibacterial activities of the different fractions. Solvent fractions from the top to the bottom: hexane (H), chloroform ($CHCl_3$), carbon tetrachloride (CCl_4), butanol (B), 35% water in methanol (35% W/M) and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