CHAPTER 3

RESULTS AND DISCUSSION

3.1 EXTRACTION

3.1.1 Comparing sodium bicarbonate and acetone as extractants

3.1.1.1 Introduction

Rogers (1998) proposed that a hot 1 % $NaHCO_3$ solution should be used for the extraction of compounds from *Combretum* spp. The basis of this extraction procedure is that many of the compounds are acidic and occur on the surface of the leaf. At a low pH, these compounds are insoluble in water. By decreasing the pH, the acidic compounds are changed to water soluble sodium salts and are then extracted into water. Subsequently the water extract is acidified with HCl and the sodium salts are changed to the water insoluble acidic compounds and precipitated out. The process is cheap and it delivers good results.

In a first experiment, I compared acetone, which efficiently removes compounds from finely ground leaves (Eloff 1998a), with sodium bicarbonate, which dissolves outside compounds from intact leaves, as extractants [**Section 2.3** for procedure].

3.1.1.2 Quantity extracted with initial extractants

The extraction on 500 mg intact, dried leaves of *C. microphyllum* was repeated three times. The acetone extract (A) was bright green and yielded 19 mg and the

sodium bicarbonate extract (B) was black and yielded 27 mg from the intact dried leaves.

3.1.1.3 Thin layer chromatography of acetone and bicarbonate extracts

Initially quantities between 100 and 600 μg (5 - 30 μl) were placed on TLC plates. The CEF-eluent was used and the plates were sprayed with the *p*-anisaldehyde spray reagent [**Section 2.4** for eluent and spray reagent formula]. Little fluorescence could be detected under the UV-light at 350 nm.

Later, quantities between 600 and 1 200 μg (20 - 60 μl) were chromatographed using the same eluent. Some plates were sprayed with the vanillin spray reagent. To investigate whether other spray reagents would show better separated components, plates were sprayed after one week with the vanillin, perchloric and phosphoric acid spray reagents. All plates were placed in an oven at 110 °C after spraying for colour development.

Better isolated and more definite bands could be observed (also fluorescence at different wavelengths under UV-light) with higher concentrations of the acetone extract. There was a slight decrease in colour intensity after one week. The vanillin and perchloric spray reagents showed the best separation of compounds for the acetone extract. Neither the higher concentrations used, the different spray reagents nor the time sprayed, gave better results on TLC for the sodium bicarbonate extract [**Fig. 3.1 and 3.2**].

From these results I decided to use the vanillin spray reagent in future work. Because I am primarily interested in isolating bioactive compounds, the antibacterial activity of the extracts was determined next.

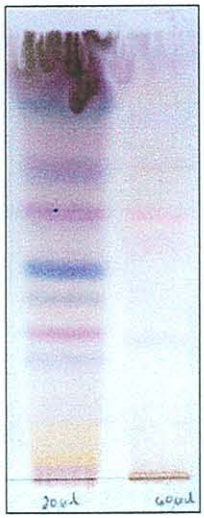

Figure 3.1 Chromatogram showing the separation of compounds present in 380 μg of the acetone extract (left) and 408 μg of the bicarbonate extract (right). The CEF system was used as eluent and the plate was sprayed directly after TLC with the vanillin spray reagent.

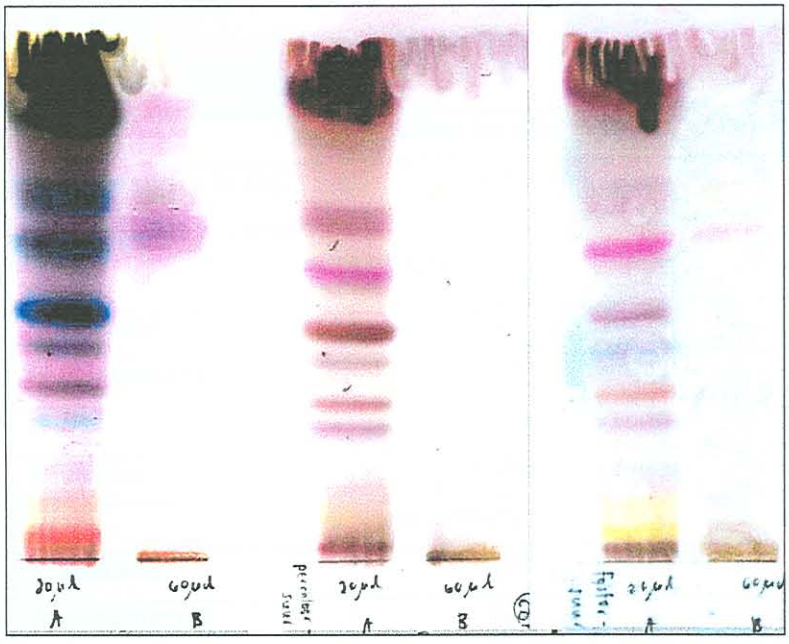

Figure 3.2 Three chromatograms of 380 μg acetone extract (left) and 408 μg bicarbonate extract (right) sprayed one week after chromatography: vanillin spray reagent (left), perchloric spray reagent (centre) and phosphoric spray reagent (right).

3.1.1.4 Minimum inhibitory concentration by INT microplate bioassay of acetone and $NaHCO_3$ extracts

MIC was determined in triplicate by two-fold dilution of extracts beyond the level where no inhibition of growth of the four test organisms occurred [**Section 2.5.1** for procedure].

Growth of bacteria was not easily visible before INT was added due to the inherent turbidity in different wells. After INT addition, growth at low extract concentrations was visible. Acetone extracts inhibited growth most in all four cultures. $NaHCO_3$ fractions inhibited *E. faecalis* and *S. aureus* growth the strongest [**Table 3.1**].

Table 3.1 MIC values in mg/ml and total activity in ml for different test organisms of intact *C. microphyllum* leaves extracted with sodium bicarbonate and acetone.

	Sodium bicarbonate	Acetone
Total quantity in mg extracted from 1 gram	9.1	38
MIC		
E. faecalis	0.07	0.11
P. aeruginosa	0.16	0.23
E. coli	0.16	0.03
S. aureus	0.06	0.01
Total activity		
E. faecalis	130	77
P. aeruginosa	57	37
E. coli	57	283
S. aureus	152	850
Average total activity	99	312

Total activity was calculated by dividing the quantity extracted from 1 gram of leaves with the MIC value (Eloff, 2000). This makes it possible to compare the total quantity of antibacterial activity extracted. Because the quantity extracted per gram is divided by the MIC in mg/ml, the unit of total activity is ml. This represents the volume to which the antibacterial compounds in the extract can be diluted and still inhibit growth of the bacteria.

In a preliminary study (Eloff, 1999), 27 southern African members of Combretaceae were compared with acetone as extractant. The values found for *C. microphyllum* were calculated and are presented in **Table 3.2**.

Table 3.2 MIC values in mg/ml and total activity in ml for different test organisms for *C. microphyllum* leaves extracted with acetone calculated from Eloff (1999). (Total quantity extracted from 1 gram was 62 mg).

Test organisms	MIC	Total activity
E. faecalis	0.8	78
P. aeruginosa	1.6	39
E. coli	0.8	78
S. aureus	0.4	155
Average		88

3.1.1.5 Discussion

The average total activity for fresh *C. microphyllum* acetone extracts calculated from Eloff (1999) was 88 ml, which is similar to the value I found for $NaHCO_3$ (99 ml), but is substantially lower than my average total activity of 312 ml for the acetone extraction of intact leaves. Although the total quantity extracted by acetone from intact leaves was substantially lower (38 mg vs. 62 mg), the MIC values for the extracts of intact leaves were much lower. This indicates that fewer inactive compounds were extracted when intact leaves were used.

Acetone as extractant gave more promising results than sodium bicarbonate. It also compared well with preliminary results found earlier (Eloff, 1999). My acetone extract yielded 38 mg from 1000 mg intact dried leaves by triple acetone extraction and the average quantity obtained from 1000 mg of finely ground leaf material by triple acetone extraction was c. 62 mg (Eloff, 1999). The sodium bicarbonate extract inhibited Gram-positive bacteria to a larger extent than Gram-negative bacteria, but *E. coli* was inhibited much more than *E. faecalis* by the acetone extract. This indicates that there may be some selectivity.

I experienced difficulties in the acid precipitation of the bicarbonate extracts and decided not to continue with sodium bicarbonate as extractant.

Because extracts of *Combretum* spp are complex, I decided to test extractants with varying polarity to determine if I could simplify extracts and/or extract more antibacterial activity with different extractants.

Consequently I decided to continue working on finely ground leaf material. Our experience with other Combretaceae indicated that as little as 50 μg gave good separations on TLC compared to larger quantities required with whole leaves. A major part of the compounds extracted from whole leaves may not have reacted with the spray reagents while contributing to the mass extracted.

3.1.2 Eleven different extractants tested for best screening and isolation of antimicrobial components from *C. microphyllum*

3.1.2.1 Extraction with 11 different extractants

When acetone, methanol, methylene dichloride and a methanol-chloroform-water mixture were used as extractants of *C. erythrophyllum* powdered leaves, acetone gave the best results (Eloff, 1998a). I decided to compare more extractants with

differing polarity on *C. microphyllum* powdered leaves. Finely ground plant material (0.5 g) was extracted with 5 ml of each of the following extractants: hexane (HE), carbon tetrachloride (CCl_4), isopropyl ether (IPE), diethyl ether (EE), methylene dichloride (MDC), tetrahydrofurane (THF), acetone (A), ethanol (E), methanol (M) and water (WA). [Procedure as described in **Section 2.3**].

Later ethyl acetate (EA), with a polarity intermediate between tetrahydrofurane and acetone, was also tested as extractant. The carbon tetrachloride extraction was repeated because it is not possible to collect the extract by centrifugation because the density of carbon tetrachloride is higher than that of the plant material. The extract had to be collected by filtration.

All the extracts were made up to 10 mg/ml in acetone and 5 µl (c. 50 µg) of each extract was analyzed in duplicate by TLC. The BEA and CEF eluents were used. The different extracts chromatographed were arranged more or less from more non-polar to polar extractants [**Fig. 3.3- 3.7**]. Even before spraying the plates it was clear that there were differences in the composition of the different extracts based on the presence of green and yellow substances [**Fig. 3.3**].

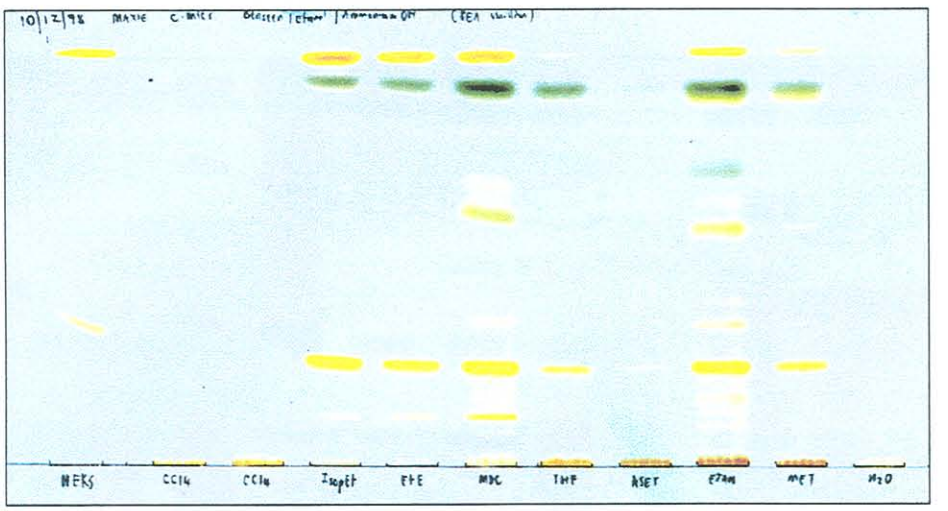

Figure 3.3 Separation of components present in 50 µg of 11 different extracts with BEA as eluent and viewed in daylight before spraying with spray reagent. Lanes from left to right extracted by hexane, carbon tetrachloride [centrifuged], carbon tetrachloride [filtered], isopropyl ether, diethyl ether, methylene dichloride, tetrahydrofurane, ethanol, methanol and water.

Methylene dichloride and ethanol extracted the most compounds. With hexane, carbon tetrachloride, acetone and water, no coloured compounds were visible with the 50 μg applied to the plates.

Plates were susequently sprayed with the vanillin spray reagent. A dark band occurred on the BEA-plate [**Fig. 3.4**]. This might have been due to a problem with the eluent, a dirty TLC tank, a secondary reaction with light or more likely due to absorption of vapours on the TLC plate before chromatography. In subsequent separations we ensured that plates were not exposed to laboratory vapour and the problem was not experienced again.

As could be expected, there were large differences in the compounds extracted. The CEF chromatogram showed distinct bands. The intermediate polarity extractants isopropyl ether, ethyl ether and methylene dichloride extracts gave the best separation, whereas the carbon tetrachloride and tetrahydrofurane extracts gave pronounced streaking. Carbon tetrachloride surprisingly extracted a large quantity of polar compounds [low Rf-value] and small quantity of non-polar compounds in comparison to the more polar isopropyl ether and diethyl ether [**Fig. 3.4, 3.5**].

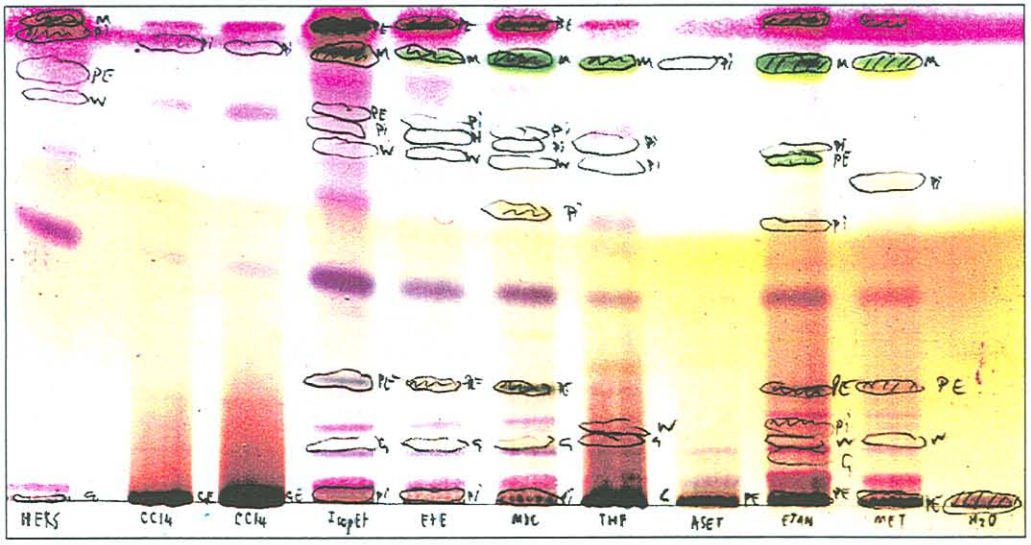

Figure 3.4 Separation of components present in 50 μg of 11 different extracts with BEA as eluent and vanillin as spray reagent. Lanes from left to right as in Figure 3.3.

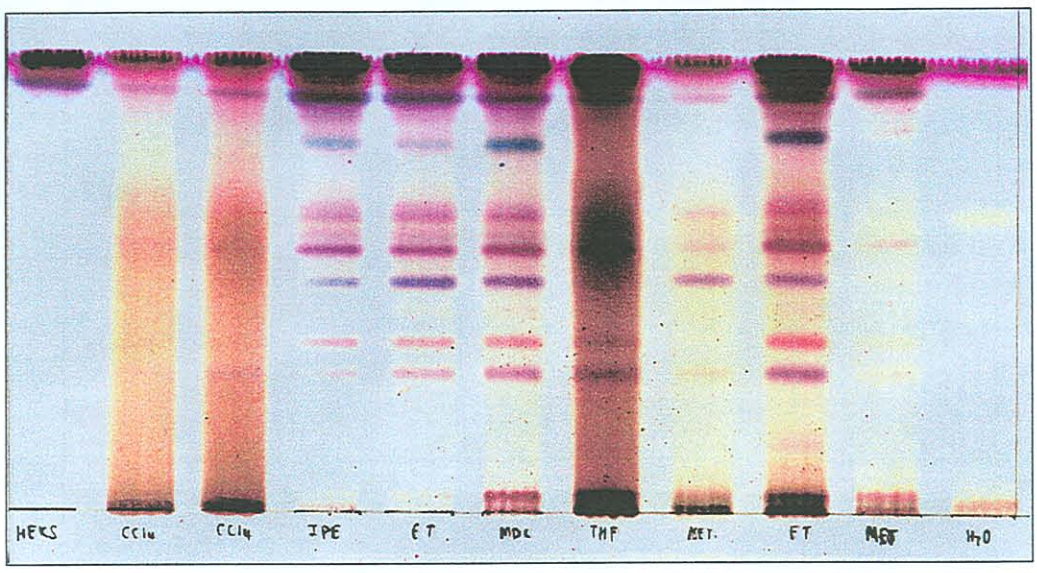

Figure 3.5 Separation of components present in 50 μg of 11 different extracts using the CEF eluent and vanillin spray reagent. Lanes as in Figure 3.3.

The results obtained with acetone as extractant was not in line with results obtained previously in our laboratory and I decided to repeat the extraction and to separate a larger quantity [100 μg] of all extracts [**Fig, 3.6**].

Better chromatograms with more definite bands and better separation were obtained especially with the acetone extract. The separation of the more non-polar compounds, using BEA as eluent yielded many compounds. One would have expected that the more polar extractants would have extracted few of the very non-polar compounds. It has to be kept in mind that the same quantity of all the extracts was chromatographed. If all extracts were made up to the same volume irrespective of the quantity extracted, different results might have been obtained.

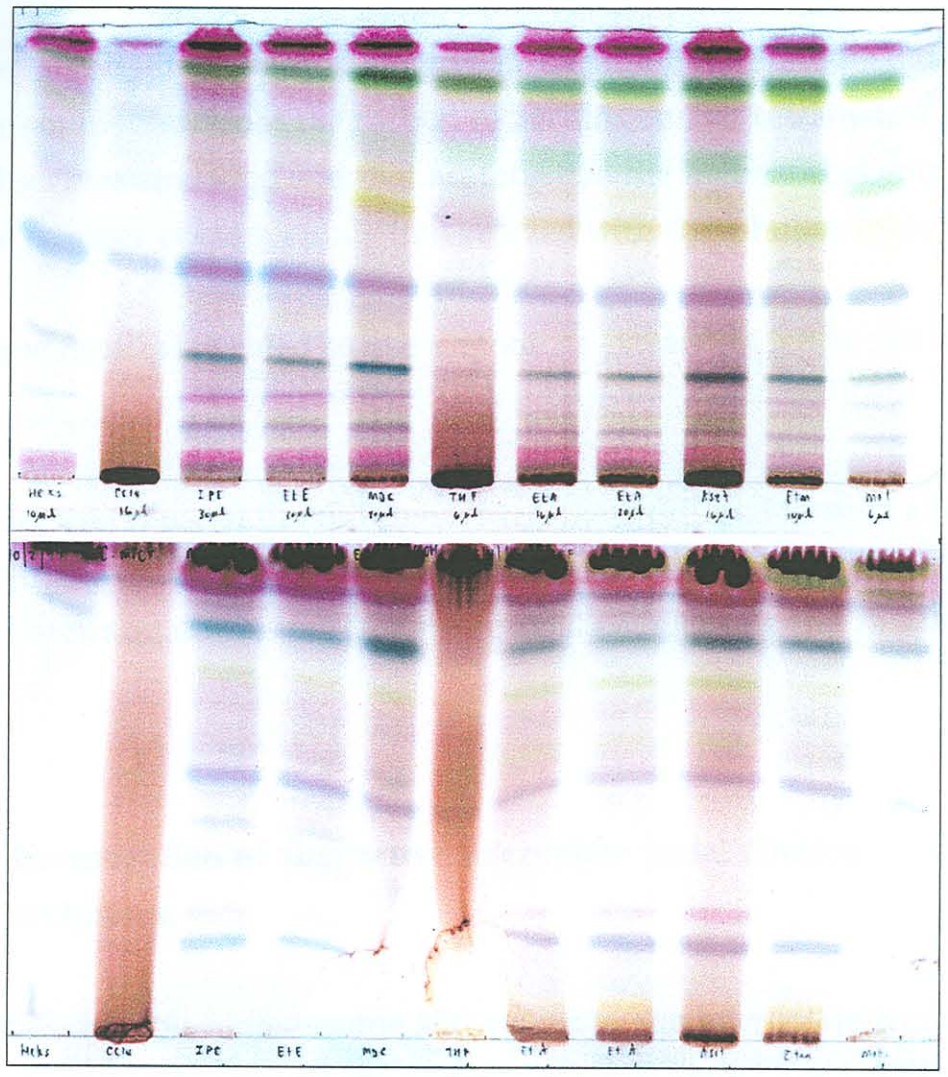

Figure 3.6 Separation of components present in 100 μg of 11 different extracts using the BEA (top) and CEF (bottom) eluents and the vanillin spray reagent. Lanes as in Figure 3.3. No components separated in the water extract [results not shown].

Surprisingly, a relatively polar extractant as ethanol, extracted similar concentrations of non-polar compounds [high Rf-values in normal phase chromatography]. This can probably be explained by the presence of saponin-like components present in the plant material. Similar to a soap with a polar and non-polar end, these compounds could make non-polar compounds soluble in polar extractants. This also explains how relatively non-polar medicinal compounds can be present in aqueous herbal preparations by traditional healers.

TLC with the intermediate polarity eluent CEF indicated that hexane did not extract polar compounds, that the carbon tetrachloride and tetrahydrofurane extracts had serious streaking and that acetone had the highest concentration and most diverse number of compounds that reacted with the vanillin spray reagent.

Based on these results I decided to use acetone as extractant in the rest of this study.

It is surprising that most of the different extractants differed to such a low degree. The important question was however, if the different extractants extracted different quantities of the antibacterial compounds and this was examined next.

3.1.2.2 Investigation of alternative microplate serial dilution technique

It is sometimes difficult to determine MIC values by the microplate technique, because the precipitated form of the extracts of some plants has a dark colour, which masks the red colour of the INT formazan. Because all actively growing bacteria contain catalase, I investigated whether addition of hydrogen peroxide (H_2O_2) would lead to visible oxygen (O_2) production and facilitate the determination of MIC values.

There was vigorous bubbling in the microwells in which the bacteria grew well, but the technique was not as sensitive as the INT technique.

3.1.2.3 Bioassay of extracts found with different extractants

MIC values were determined using the technique as described in **Section 2.5.1**. Gentamycin was used as positive control. (The MIC values for Gentamycin were 4

μg/ml for *E. coli*, 8 μg/ml for *P. aeruginosa* and *S. aureus* and 16 μg/ml for *E. faecalis*.) [**Table 3.3** for MIC values for different test organisms.]

Table 3.3 MIC values in mg/ml and total activity in ml of *C. microphyllum* leaves extracted with 11 different extractants.

	HE	CCl_4	IPE	EE	MD	TH	EA	A	E	M	W
Total quantity in mg extracted from 1 gram	36	48	20	24	106	64	24	32	36	174	48
MIC											
E. faecalis	0.08	0.15	0.21	0.15	0.30	0.28	0.40	0.13	0.07	0.18	1.20
P. aeruginosa	0.63	0.15	0.11	0.15	0.15	0.28	0.20	0.13	0.14	0.18	1.20
E. coli	0.32	0.60	0.05	0.15	0.14	0.20	0.20	0.27	0.07	0.18	1.20
S. aureus	1.25	0.60	0.21	0.30	0.15	0.57	0.20	0.27	0.14	0.18	1.20
Total activity											
E. faecalis	450	320	95	160	353	229	600	246	514	967	15
P. aeruginosa	57	320	182	160	707	229	120	246	257	967	15
E. coli	113	80	400	160	707	457	120	119	514	967	15
S. aureus	29	80	95	80	707	112	120	119	257	967	15

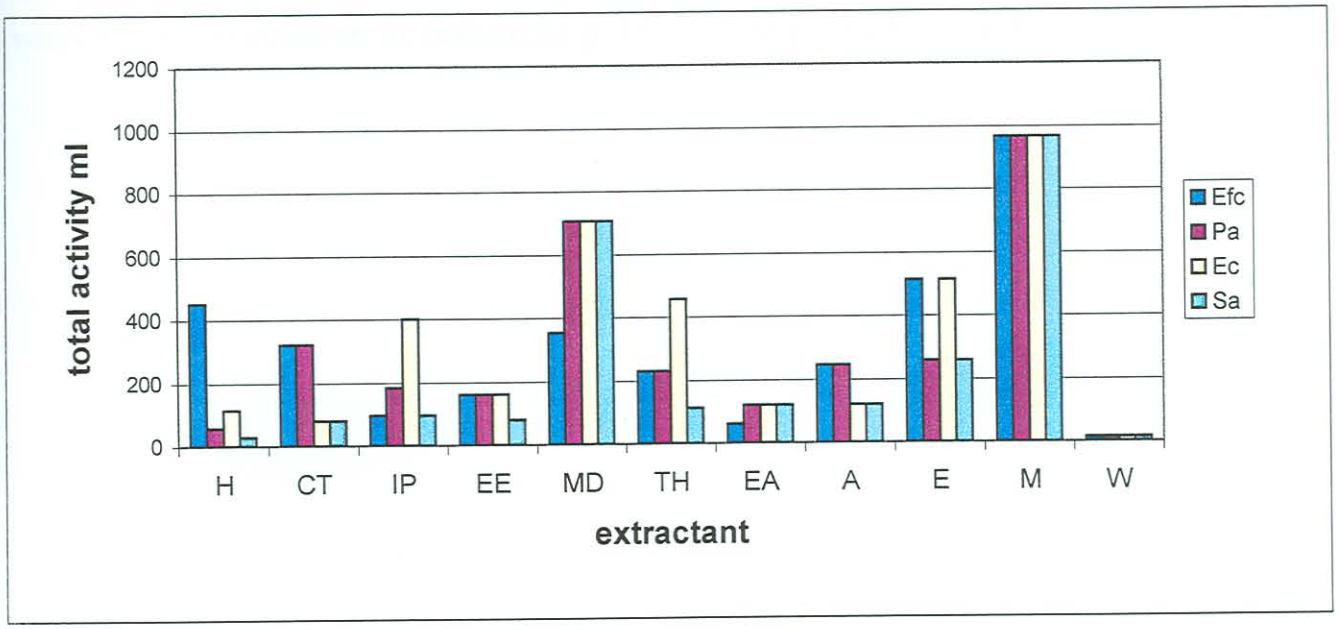

Figure 3.7 Total antibacterial activity to four test organisms of finely ground *C. microphyllum* leaves extracted with 11 different extractants. From left to right: hexane, carbon tetrachloride, isopropyl ether, diethyl ether, methylene dichloride, tetrahydrofurane, ethyl acetate, acetone, ethanol, methanol and water.

3.1.2.4 Discussion

Although methanol and methylene dichloride extracted more activity [**Fig. 3.7**], I decided to continue with acetone as extractant due to its relatively low toxicity to test organisms and good TLC separation of compounds obtained with acetone as extractant and ease of removal from extracts.

Because methylene dichloride is not miscible with water, the use of this extractant complicates the handling of extracts.

Furthermore, methanol may extract polar compounds such as polysaccharides, polyphenols and tannins, which may have antibacterial activity. Major pharmaceutical companies are not interested in these compounds, because most polysaccharides break down to glucose on oral administration. Polyphenols/tannins bind to protein so that if it is absorbed, a large proportion binds to serum and is not bioavailable. These molecules are also very difficult to work with (Farnsworth in comment p. 19 - 20 on paper by Balick, 1994).

3.2 GROUP SEPARATION OF EXTRACTS BY SOLVENT/SOLVENT FRACTIONATION

3.2.1 Introduction

From the results obtained with sodium bicarbonate and acetone as extractants, it appeared that there is substantial selectivity using different extractants. By using 11 different extractants of varying polarity, little evidence of selectivity was however found. Because the extracts are very complex, I investigated whether solvent/solvent fractionation could simplify extracts without reducing antibacterial activity. This is a mild technique, which should not lead to inactivation of compounds by chemical changes. If the activity is caused by a single compound, i.e. is not caused by two interacting compounds that are separated by the solvent/solvent fractionation, there should not be a reduction of antibacterial activity.

The solvent/solvent fractionation of the components in *C. microphyllum* into six fractions was a variation on the group separation procedure developed in the National Cancer Institute as described by Suffness and Douros [1979] [procedure as described in **Section** 2.7].

3.2.2 Quantities obtained and antibacterial activity of different fractions

The quantities present and the antibacterial activity of the different fractions were determined [**Table** 3.4]. The highest quantity, nearly 42 % [**Table** 3.5], was present in the hexane fraction. Nearly two-thirds of the total antibacterial activity was also present in the hexane fraction. This is different from other *Combretum* species investigated thus far, where most of the activity was in the carbon tetrachloride and/or chloroform fractions. If the more polar components, which may contain uninteresting polysaccharides and polyphenols/tannins are ignored,

the hexane fraction contained more than 88 % of the interesting non-polar antibacterial compounds. A comparison of the degree of purification can be obtained by dividing the % activity in each fraction with the % of the total mass [Table 3.5]. The procedure enriched the antibacterial activity of the polar compounds in the butanol and water fractions and the antibacterial activity of the non-polar fractions in the hexane fractions to a high degree. It had little effect on the methanol/water fraction and removed active compounds from the carbon tetrachloride and chloroform fractions.

Table 3.4 MIC values in mg/ml and total activity in ml of *C. microphyllum* leaves extract fractions obtained by solvent/solvent fractionation.

	H	CT	CF	B	MW	W
Total quantity obtained	1166	386	664	100	300	169
MIC						
E. faecalis	0.17	0.77	2.66	0.80	2.40	2.70
P. aeruginosa	0.33	0.77	2.66	0.05	0.15	0.08
E. coli	0.17	0.77	2.65	1.60	1.20	2.70
S. aureus	0.67	6.18	2.66	0.40	0.60	5.41
Total activity in ml						
E. faecalis	7000	500	250	125	125	62.5
P. aeruginosa	3500	500	250	2000	2000	2000
E. coli	7000	500	250	62.5	250	62.5
S. aureus	1750	62.5	250	250	500	31.3
Total for all organisms	19250	1563	1000	2438	2875	2156

Table 3.5 Distribution of mass and total antibacterial activity in different solvent/solvent fractions.

	H	CT	CF	B	MW	W
% of total mass	41.9	13.9	23.8	3.6	10.8	6.1
% of total activity	65.5	5.4	3.4	8.4	9.9	7.4
ratio activity/mass	1.6	0.4	0.1	2.3	0.9	1.2

The degree of enrichment is also apparent from the pie charts shown in Figure 3.8. Not all the bacteria had the same sensitivity to the compounds present in the different fractions. Especially the *P. aeruginosa* cultures were more sensitive to the polar fractions [Fig.3.9].

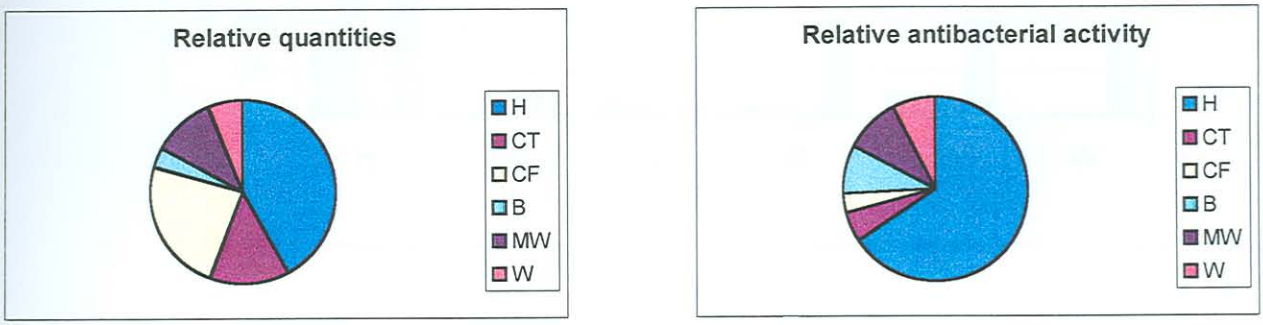

Figure 3.8 The relative quantities obtained by solvent/solvent fractionation and the relative antibacterial activities of the different fractions. H = hexane, CT = carbon tetrachloride, CF = chloroform, B = butanol, MW = 35 % methanol/water and W = water.

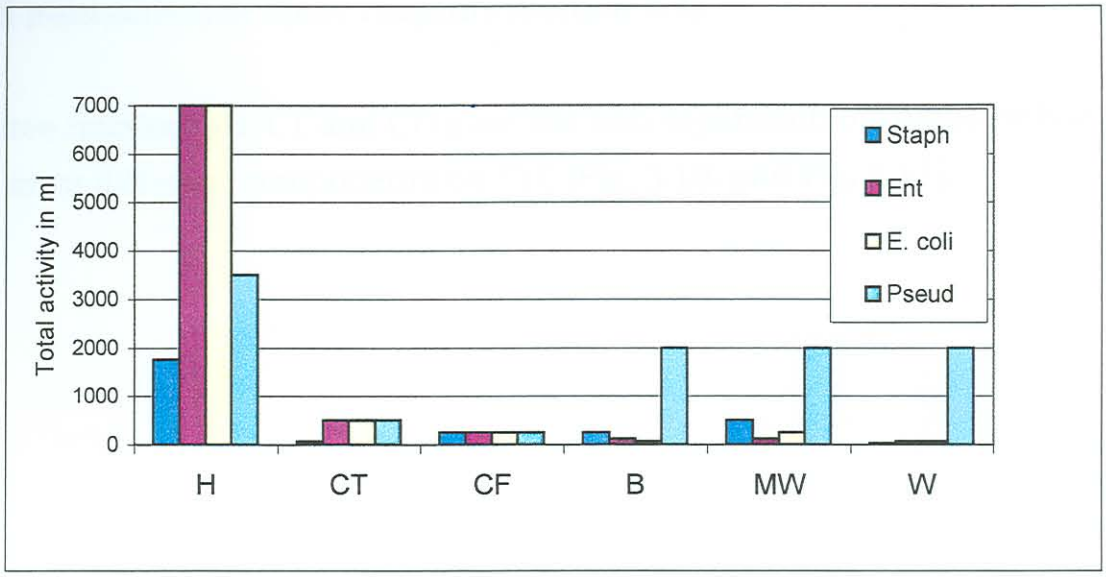

Figure 3.9 Total antibacterial activity to four test organisms of *C. microphyllum* leaves extracted with solvent/solvent fractions. (Fractions from left to right: H = hexane, CT = carbon tetrachloride, CF = chloroform, B = butanol, MW = 35 % methanol/water and W = water.)

Frequently Gram-negative bacteria are more resistant to plant extracts than Gram-positive bacteria, but this is not true for the compounds present in *C. microphyllum.*

3.2.3 Chemical composition of the six fractions obtained

Thin layer chromatography was on Merck TLC F_{254} plates. Initially 200 μg of the hexane fraction (H) was used. Due to the high concentration of the hexane fraction, the quantity of this fraction chromatographed was later reduced to 100 μg and gave better separation. Three different eluents were used: BEA, CEF and MA. Separated components were observed under visible and ultraviolet light (254

and 360 nm, Camac Universal UV lamp TL-600). Plates were sprayed with the vanillin and *p*-anisaldehyde spray reagents [**Section 2.4**].

The first three fractions (H, CT and CF) gave the best separation of compounds as well as the most different components on TLC [**Fig. 3.10. and Fig. 3.11**].

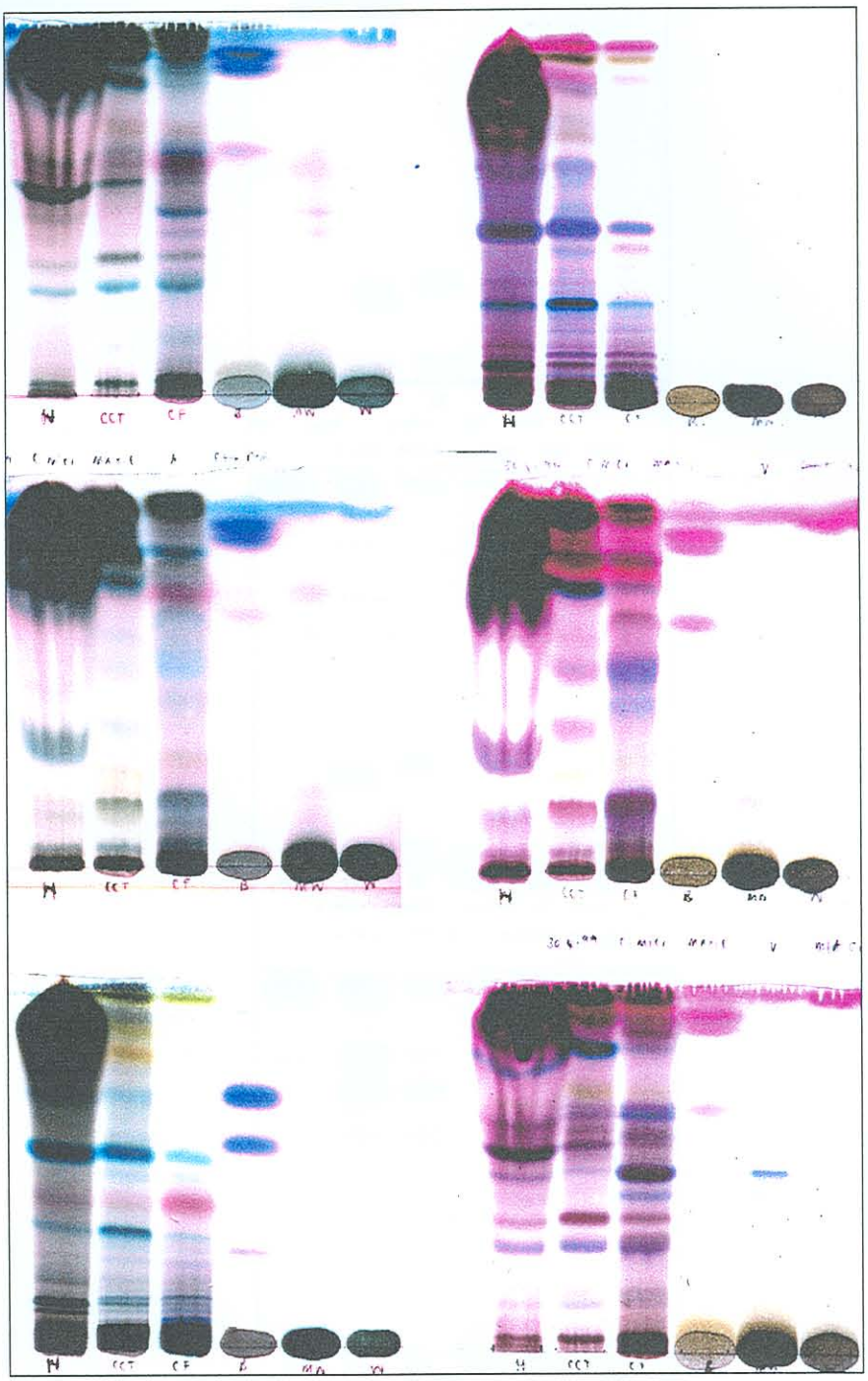

Figure 3.10 Chemical composition of solvent/solvent fractions [200 µg] separated by MDC/Acetone as eluent (top left and top right), BEA as eluent (middle left and right) and CEF as eluent (bottom left and right). Compounds visualized by *p*-anisaldehyde as spray reagent (top left, middle left and bottom left) and vanillin as spray reagent (top right, middle right and bottom right).

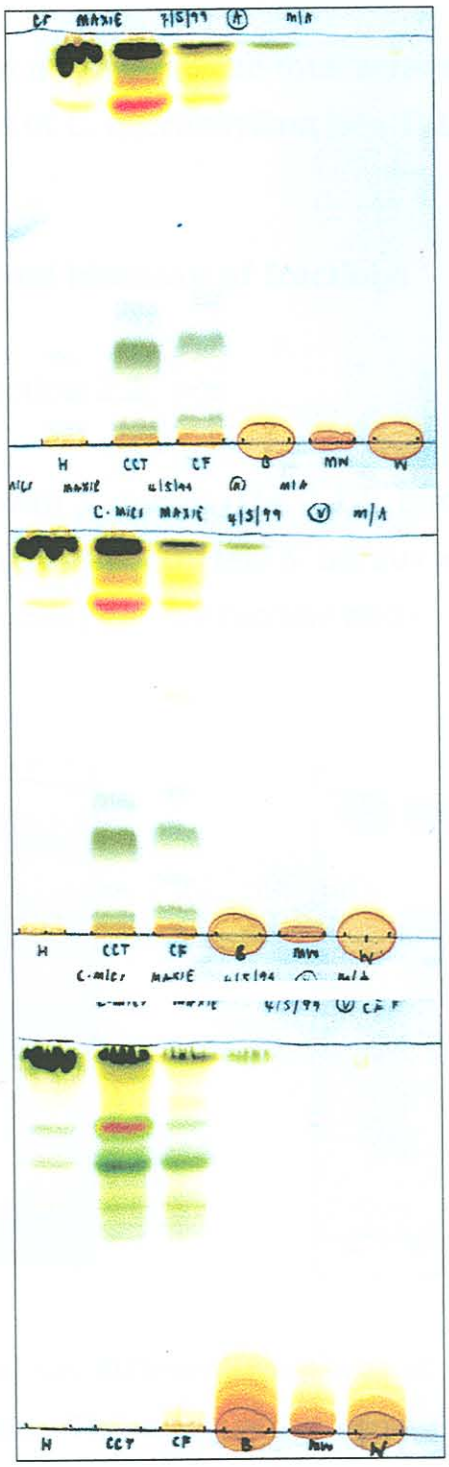

Figure 3.11 Chemical composition of solvent/solvent fractions [100 μg] separated by MA as eluent (top and middle) and CEF as eluent (bottom). Compounds visualized by *p*-anisaldehyde (top) and vanillin (middle and bottom) spray reagents.

MIC values were calculated in mg/ml and the total activity in ml was determined for the six different fractions of *C. microphyllum* [see **Table 3.4** above].

3.2.4 Bioautography and bioassay of fractions

Procedure as described in **Section 2.2.**

Bioautography worked well with *S. aureus* [**Fig. 3.12**], but was not as reproducible with the other test organisms. Unfortunately *S. aureus* was the least sensitive of the four test organisms, especially for the hexane and carbon tetrachloride fractions.

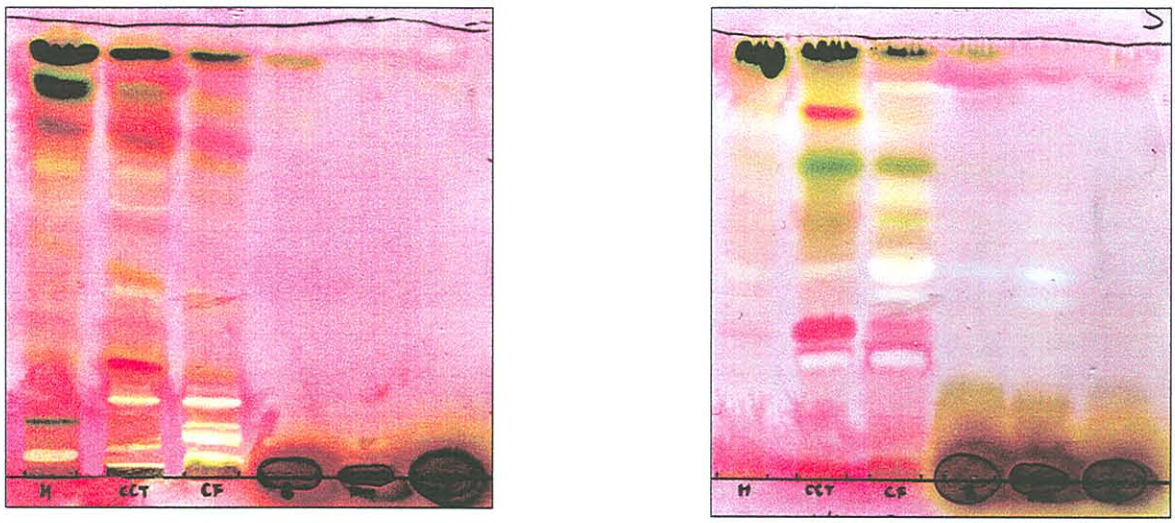

Figure 3.12 Bioautogram of six different fractions of *C. microphyllum* extract by solvent/solvent extraction. TLC using BEA [left] and CEF [right] as eluent, sprayed with *S. aureus* cell suspension, incubated and sprayed with INT. White areas indicate bacterial growth inhibition. (Fractions from left to right: hexane, carbon tetrachloride, chloroform, butanol, 35 % methanol/water and water.)

The hexane (H) fraction had the highest total antimicrobial activity by far for all four test organisms used [**Fig. 3.19**] and was chosen for the next steps in the isolation of bioactive compounds with column chromatography.

3.3 GROUP SEPARATION BY USING EXTRELUT AS PACKING MATERIAL

3.3.1 Introduction

Because the solvent/solvent fractionation is a tedious process, I decided to determine if Extrelut, sold by Merck, can be used instead of the solvent/solvent fractionation.

Extrelut is used as packing material for liquid-liquid extraction. It is used in analysing lipophillic substances in a complex matrix, e.g. body fluids where clean up is frequently necessary prior to the actual analysis. Conventional sample preparation, extraction using a separating funnel, is often associated with disadvantages: the formation of emulsion, poor phase separation, high solvent consumption, low degree of automation and high personnel costs. With Extrelut

- The eluate need not be dried prior to evaporation,
- Numerous published applications can be used for one's own laboratory problems,
- High rates of recovery, especially in the extraction of body fluids are obtained.

Liquid-liquid extraction becomes more efficient, easier and effective in routine laboratory work because:

- No emulsions are formed,
- Solvents, material and time are saved.

Extrelut consists of a specially processed wide-pore diatomaceous earth of high pore volume. The kieselguhr matrix of Extrelut is chemically inert and can be used within the pH-range 1 -13. Stringent initial control of raw material and equally stringent quality control of the final product guarantee uniform batch-to-

batch quality, although Extrelut is a naturally occurring product and therefore subject to certain fluctuations.

The aqueous sample is applied to the Extrelut sorbent. It distributes itself in the form of a thin film over the chemically inert matrix and thus acts as a stationary phase. Subsequently, elution takes place using organic solvents that are non-miscible with water, solvents like e.g. diethyl ether, ethyl acetate or halogenated hydrocarbons. All the lipophillic substances are extracted from the aqueous into the organic phase. During this process, the aqueous phase remains on the stationary phase. The eluate is free from emulsions and can be evaporated for further analysis.

Extrelut has been used for quite some time in clinical chemistry and toxicology for the sample preparation of urine, whole blood, plasma, serum, gastric juice, liquor, amniotic fluid, faeces, animal and plant tissue. Other applications are in the areas of environmental and residue analysis, e.g. the analysis of industrial, domestic and wastewater. The fractionated elution of acidic and basic substances like e.g. drugs and their metabolites from body fluids is also possible (Merck, Chrom Book 2 provided by Merck).

Initially Extrelut [Merck] was packed in short 10 ml polypropylene syringes as small columns. Because the instructions were not clear, two procedures were followed. One syringe was packed with Extrelut that was suspended in acetone (wet treatment) and another syringe was packed with dry Extrelut only (dry treatment).

3.3.2 Wet adsorbent

A 10 ml polypropylene syringe was packed with Extrelut (Merck) by suspending in acetone and pouring into the small column. Approximately 200 µl of the hexane extract was placed on the column. The first four fractions were eluted with

hexane (N1–N4) and collected by hand. This was followed by eluting with acetone (N5). The five collected fractions were dried in pre-weighed containers, dissolved in acetone and the components were separated by TLC [**Fig. 3.13**].

3.3.3 Dry adsorbent

The same procedure was followed, but in this case the adsorbent was not suspended in acetone before packing.

3.3.4 TLC of Extrelut fractions

Fractions N1–N5 (wet treatment), fractions D1–D5 (dry treatment) and a hexane fraction (c. 50 μg of each) were analyzed on TLC.

Very little fractionation took place with the dry column, but there was some fractionation of the more polar compounds by using Extrelut as packing material in the wet column [**Fig 3.13**]. The fractionation was not promising with this technique because it works best with aqueous extracts that are eluted with non-miscible organic solvents, and the column may have been overloaded.

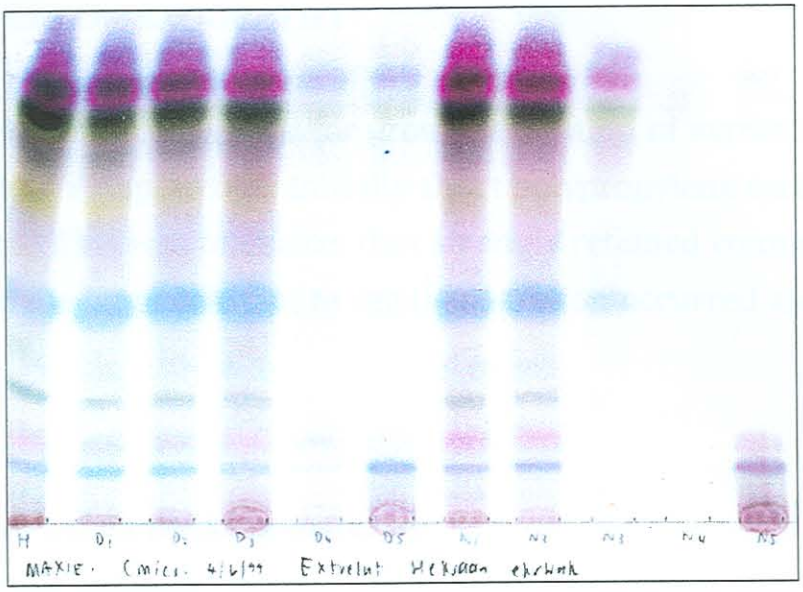

Figure 3.13 Separation of components in 50 μg of the different Extrelut fractions using the BEA eluent and the vanillin spray reagent. From left to right: H = hexane fraction, D1–D4 = dry adsorbent fractions eluted with hexane, D5 = dry adsorbent fraction eluted with acetone, N1–N5 = wet adsorbent fractions eluted with hexane and N5 = wet adsorbent fraction eluted with acetone.

3.4 COLUMN CHROMATOGRAPHY

Column chromatography was used for group separation of extracts and for isolating individual components. Initially short polypropylene syringes were used as small columns. This was to ensure that strongly retained components would not contaminate the large column, to see if separation occurred and to test stability.

3.4.1 Silica gel as packing material

Silica gel 60 [Merck] is a standardized silica gel widely used for preparative column chromatography that provides the user with unique method reliability. This is ensured by the fact that identical raw materials are used for all stationary phases, thus providing identical chromatographic selectivity. Practice has shown that for chromatographic separations in production, silica gel 60 types i.e. silica gels of a mean pore size of 60 A and in particle size ranges 40 - 63 μm and 63 - 200 μm are excellently suitable for the separation of complex mixtures (Merck Chrom Book 2 provided by Merck).

Subsequently polypropylene syringes (60 ml) were packed with silica gel 60 [Whatman 15- 40 μm]. Polypropylene was chosen because it has the best chemical resistance to the chemicals used and glass columns were not available at that stage. Glass wool was placed at the bottom of a 60 ml polypropylene syringe to prevent the silica gel from spilling.

Silica gel was suspended in a mixture of benzene : ethanol (9 : 1). A filter paper disk was placed on top of the silica gel after the silica gel suspension was poured into the small column to protect the top from disturbance.

Approximately half of the hexane extract (632.97 mg; 3.8 ml) was applied carefully in a narrow band to the top of the column with a pasteur pipette. It was

mixed with the benzene-mixture. As soon as the hexane extract was absorbed into the silica gel, the BEA eluent was added form a raised solvent flask to facilitate solvent flow and the hexane extract was carefully washed into the column without disturbing the top of the column. A small quantity was added to wash the extract into the column.

Gravitational force was used to elute the components through the column. The first 22 test tubes (A-V) were collected by hand. The rest of the test tubes (1-25) were collected by the fraction collector [Isco Foxy Jr] (600 drops per fraction, c. 2 ml/min)

Four solvents were used in the following order: BEA, CEF, acetone and methanol, based on the separation by TLC. I chose volatile solvents to make drying of extracts at room temperature under air stream easier:

Solvent 1: c. 150 - 200 ml BEA (fractions A-V)
Solvent 2: c. 140 ml CEF (fractions 1-7)
Solvent 3: c. 150 ml acetone (fractions 8-15)
Solvent 4: c. 150 ml methanol (fractions 16-25)

Individual fractions were separated by different TLC systems. Fractions A-V (non-polar) were analyzed by TLC using the BEA system (c. 3 µl) and fractions 1-25 (polar) were analyzed by TLC using the CEF system (c. 9 µl of each fraction) [**Fig. 3.14**].

Fractions A-F were very concentrated and gave the most different components on TLC. The same component occurred in fractions A-P.

Fractions 1-10 gave the same component and fractions 9-20 and 25 gave the same component on TLC.

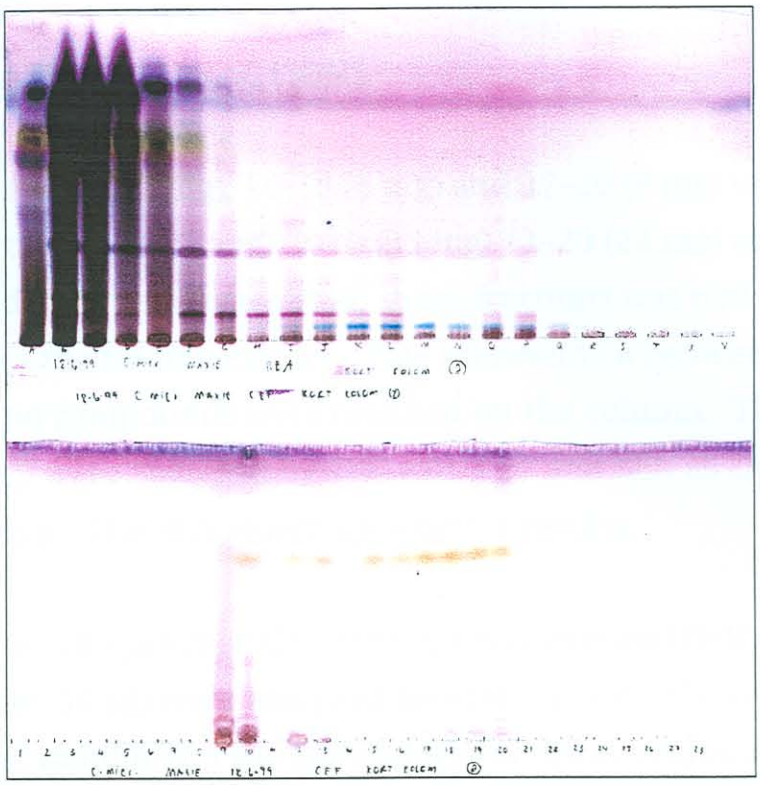

Figure 3.14 Top: Chromatogram of first highly non-polar fractions (A–V) using the BEA system as eluent and the vanillin spray reagent (c. 3 μl of each fraction placed on plates) and bottom: chromatogram of last polar fractions (1–25) using the CEF system as eluent and the vanillin spray reagent (c. 9 μl of each fraction placed on plates.)

3.4.2 COMBINATION OF FRACTIONS

Fractions 4-6 (4 mg), 11-13 (32 mg), 14-16 (4 mg) and 17-20 (8 mg) were combined. Later fractions 1-8 (15 mg), 9-10 (35 mg) 11-20 (22 mg) and 21-25 (17 mg) were re-combined. The total mass of all these fractions was 630 mg. I started with 633 mg of the hexane extract. This represents a recovery of 99.5 %, which indicates that no compounds were retained on the column. The combined fractions (c. 20 - 50 μg) were analyzed by TLC with CEF and BEA as eluents and vanillin as spray reagent. The BEA eluent gave better results.

Fractions ABC (431 mg), DEF (88 mg), GHI (9 mg), J-N (7 mg) and O-V (6 mg) were combined. Fractions (c. 50 μg) were analyzed several times by TLC using the BEA eluent system and the vanillin and the *p*-anisaldehyde spray reagents. Plates sprayed with the vanillin spray reagent, gave better separation of components [**Fig. 3.15**]. Fractions ABC and DEF gave the most definite bands and best separation on TLC.

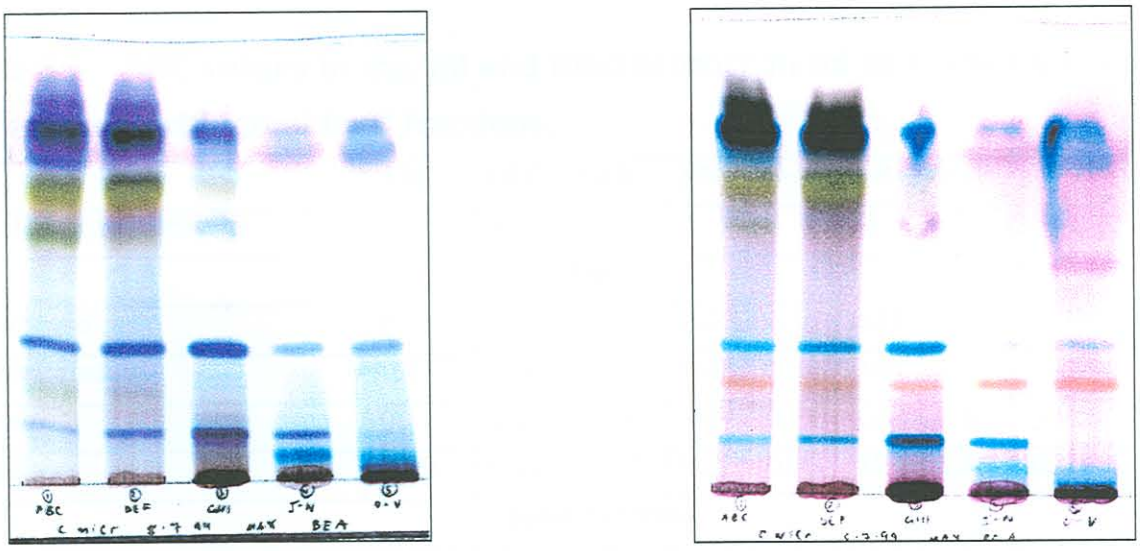

Figure 3.15 Separation of components present in 50 μg of fractions using the BEA eluent and the vanillin (left) and *p*-anisaldehyde (right) spray reagents. (From left to right: ABC, DEF, GHI, J-N and O-V combined fractions.)

The combined fractions were tested for antibacterial activity. By far the largest activity was in the first highly non-polar fractions [**Table 3.5**]. Lower activity was encountered with *S. aureus* [**Fig. 3.16**].

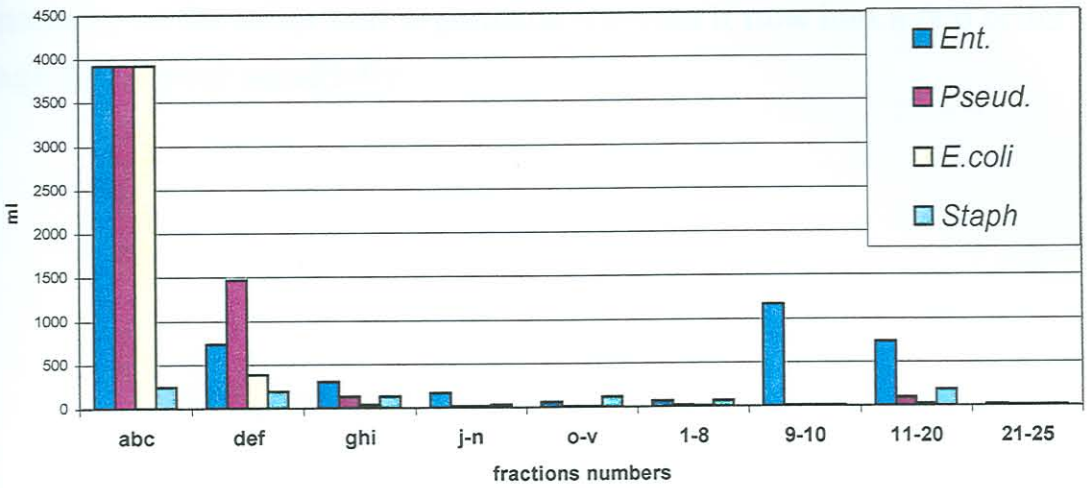

Figure 3.16 Total antibacterial activity in ml of *C. microphyllum* leaves extracted with combined fractions to four test organisms.

Table 3.5 MIC values in mg/ml and total activity in ml of *C. microphyllum* leaf extracts with combined fractions.

	ABC	DEF	GHI	J-N	O-V	1-8	9-10	11-20	21-25
Total quantity present	431	88	9	7	6	15	35	22	17
MIC									
E. faecalis	0.11	0.12	0.03	0.06	0.10	0.24	0.03	0.03	1.42
P. aeruginosa	0.11	0.06	0.07	0.44	1.50	0.94	3.50	0.23	2.83
E. coli	0.11	0.23	0.26	.044	0.75	1.88	3.50	0.92	2.83
S. aureus	1.79	0.47	0.07	0.22	0.05	0.24	3.50	0.12	2.83
Total Activity									
E. faecalis	3918	733	300	167	60	63	1167	733	12
P. aeruginosa	3918	1467	129	16	4	16	10	96	6
E. coli	3918	383	35	16	8	8	10	24	6
S. aureus	241	187	129	32	120	63	10	183	6
Total activity	11995	2770	593	231	192	150	1197	1036	30
Percentage of total	65.9	15.2	3.3	1.3	1.1	0.8	6.6	5.7	0.2

Results show that different fractions inhibited different organisms to varying degree, indicating that there is some specificity towards different test organisms. It is surprising that *S. aureus* was not nearly as sensitive as the other test organisms. In the original extract [**Table 3.3**] *S. aureus* had the same order of sensitivity as the other test organisms whereas it now had a two orders of magnitude lower sensitivity.

3.5 COMBINATION OF FRACTIONS AFTER TLC AND BIOASSAY

3.5.1 Developing a system to separate components by column chromatography

Attempts were made to develop a TLC system that would separate the components of fractions ABC and DEF well. This system would then be applied to column chromatography. Several combinations of volatile eluents were tested to develop an eluent system for column chromatography.

The best separation on column chromatography occurs if compound Rf is c. 0.5 on TLC. BEA as eluent has a relatively high solvent strength (c. 0.38). The higher the solvent strength, the more the components would occur at the top of the TLC plates. I was looking for a lower solvent strength. Toluene has a solvent strength of c. 0.29, but it is not volatile. Benzene has a solvent strength of 0.32, but is carcinogenic. Carbon tetrachloride may separate the components occurring at the top of the TLC plates.

Initially different combinations of carbon tetrachloride (CT) and methylene dichloride (MD) were tested as eluents in the following combinations:

CT : MD 1 : 2 (solvent strength c. 0.31)
CT : MD 3 : 2 (solvent strength c. 0.28)
CT : MD 2 : 1 (solvent strength c. 0.26)

Initially I used Seprachrom containers as eluent tanks. Fractions ABC and DEF were dissolved in acetone. (Initially fractions GHI were also tested). The volumes and concentrations of the combined fractions were determined. Approximately 50 μg of each fraction was applied to the plates. The combinations CT : MD 1 : 1,

CT alone and MD alone were also tested as eluents. (Plates were sprayed with the vanillin spray reagent).

Separated components were visualized under visible and ultraviolet light. The different bands on TLC were not as prominent and well separated as I had hoped for. The bands flowed together at the side ends of the plates. I did not use the Seprachrom containers any more. The best separation occurred with the combination CT : MD 1 : 1.

3.5.2 TLC in glass eluent tanks

The experiment was repeated in glass TLC tanks with the same combinations and better results were obtained.

New combinations for eluents were tested. Initially methanol (ME) and methylene dichloride (MD) were combined as eluents in a ratio of 1 : 1. Methanol (ME) as eluent pushed the components to the top of the TLC plates. The ME proportion was initially reduced to MD : ME 2 : 1 and MD : ME 3 : 1 to ensure better separation. Later MD : ME 8 : 1, 9 : 1 and 10 : 1 as eluent combinations were used with good results. MD : ME 15 : 1 and 20 : 1 were also tested.

The combination MD : ME 15 : 1 gave the best separation of components and the most definite bands [**Fig. 3.17**]. All the plates were sprayed with the vanillin spray reagent.

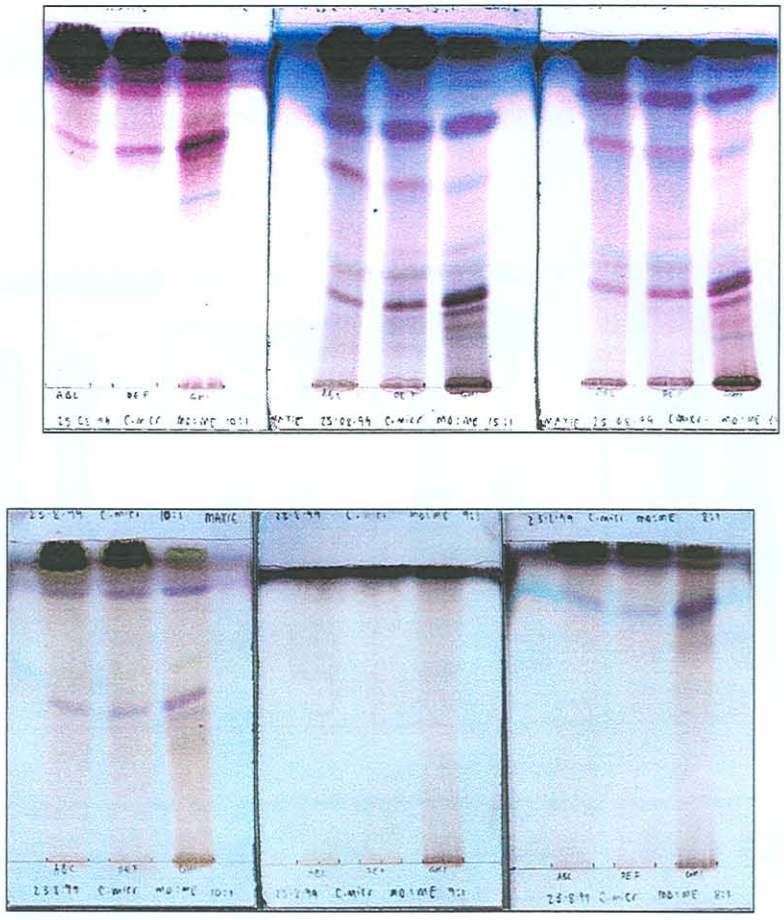

Figure 3.17 Different combinations of methylene dichloride (MD) and methanol (ME) as eluents for 50 μg of the ABC, DEF and GHI extracts. Plates were sprayed with the vanillin spray reagent. [MD : ME = 10 : 1 (top left), MD : ME = 15 : 1 (top middle), MD : ME = 20 : 1 (top right), MD : ME = 10 : 1 (bottom left), MD : ME = 9 : 1 (middle bottom) and MD : ME = 8 : 1 (bottom right)].

Later different combinations of acetone (A), hexane (H), methanol (ME), methylene dichloride (MD) and carbon tetrachloride (CT) were tested as eluents. Plates were sprayed with the vanillin and *p*-anisaldehyde spray reagents.

To determine the effect of heat on the components and the colour of the bands, some plates were put in the oven and the rest were dried with a hot air drier. The plates heated in the oven gave better results.

Good results were eventually obtained with a hexane-acetone mixture [**Fig. 3.18**]. The combination of hexane : acetone in a 2 : 1 ratio gave the best separation on TLC.

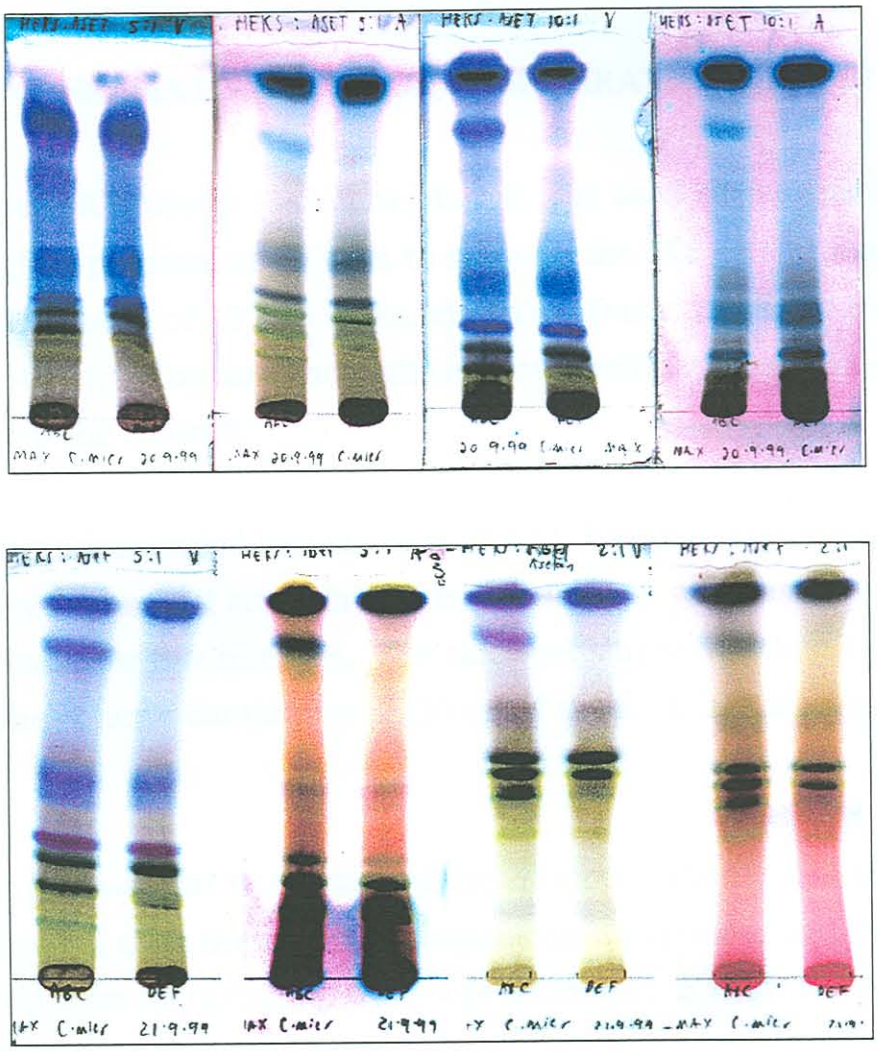

Figure 3.18 Different combinations of hexane (H) and acetone (A) as eluents for 50 μg of the ABC and DEF fractions. Plates sprayed with the vanillin spray reagent (top first, top third, bottom first and bottom third) and *p*-anisaldehyde spray reagent (top second, top fourth, bottom second and bottom fourth). [H : A = 2 : 1 (bottom third and bottom fourth), H : A = 5 : 1 (top first and second, bottom first and second) and H : A = 10 : 1 (top third and top fourth)].

This eluent combination (hexane : acetone 2 : 1) will be applied to the mixture in an attempt to isolate the bioactive compounds using a finer silica gel as packing material with column chromatography on a preparative column.

3.6 COLUMN CHROMATOGRAPHY ON A PREPARATIVE COLUMN

A finer silica gel, LiChroprep 15-25 μm [Merck], was used as packing material in a Michel-Miller glass preparative column to scale up the TLC separation. The column had a diameter of 25 mm and a length of 60 cm. I decided to continue with only the ABC fraction and chose the eluent combination of hexane : acetone (2 : 1) to develop the column.

The silica gel was suspended in a mixture of 2 : 1 hexane : acetone and was left to swell. I removed dissolved air in the gel by placing the suspension in a Bransonic 52 ultrasonic bath for five minutes. The silica gel slurry was then carefully poured into the column containing c. 150 ml of the 2 : 1 hexane : acetone mixture.

A filter paper disk was cut to the size of the column-diameter and was placed on top of the silica gel after the silica gel suspension was poured into the column to protect the top from disturbance. Care was taken not to allow the top of the column to dry out. A Michel-Miller adjustable bed height fitting was attached to the top of the column to minimize dead volume.

The ABC fraction was applied under pressure from a syringe to the top of the column through a Rheodyne 5020 low pressure injection valve injector in a closed column system.

Unfortunately I lost c. a quarter to a third of the ABC fraction, which was pushed back through the injector. This was probably caused by the volatile hexane which expanded in the injector and on the column due the higher temperature inside the

column. It was a very hot night and the hexane volatility caused cracks in the column when pressure was released.

Approximately another two litres of the hexane : acetone mixture was placed in a Bransonic 52 ultrasonic bath to remove gas, which could cause problems to the column. Pressure from a membrane pump was used to elute the components through the column. The column was left to run overnight, filling the test tubes with c. 15 ml every 3 minutes. Seventy test tubes were collected by the fraction collector [Isco Foxy Junior]. As soon as the separation was completed, the test tubes were placed under a stream of cold air, in order to concentrate the fractions for further analysis by TLC, bioautography and bioassay.

3.6.1 Chemical composition of different fractions

The fractions were analysed by TLC, using BEA and CEF as eluents and the vanillin spray reagent. Based on the TLC analysis and the quantity present in each fraction [**Fig. 3.19 and Fig. 3.21**], some fractions were pooled as follows: 7-12, 13-20, 21-30, 31-40, 41-50, 51-60 and 61-70. The quantity present in the first 6 and the rest of the pooled fractions is shown in **Figure 3.20**.

The separation was very disappointing. This was probably caused by a malfunction of the injection system. This caused the top of the column to dry out. To remove the air in the top, c. 4 cm of the top of the column had to be stirred with a glass rod. Consequently the extract was placed in a very broad band on the column and thus destroyed much of the potential separation.

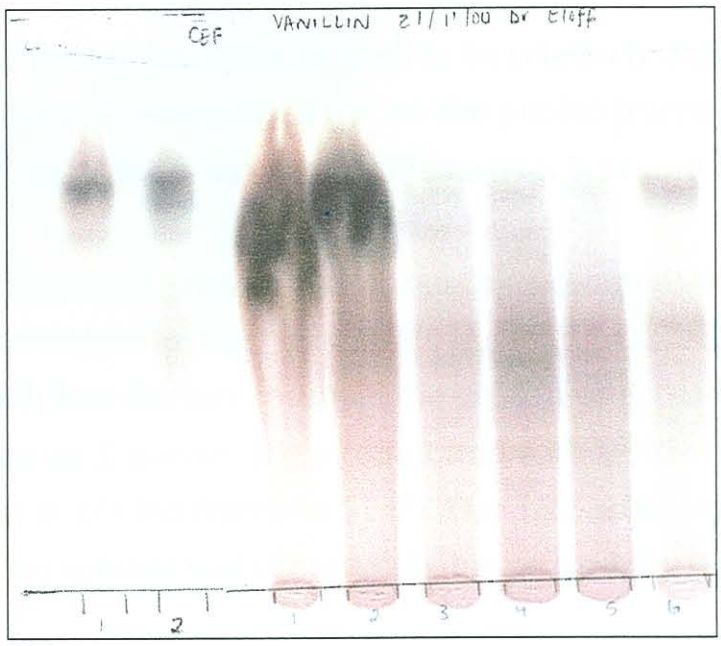

Figure 3.19 Separation of components present in 50 μg of fractions 1–6, using the CEF eluent and the vanillin spray reagent.

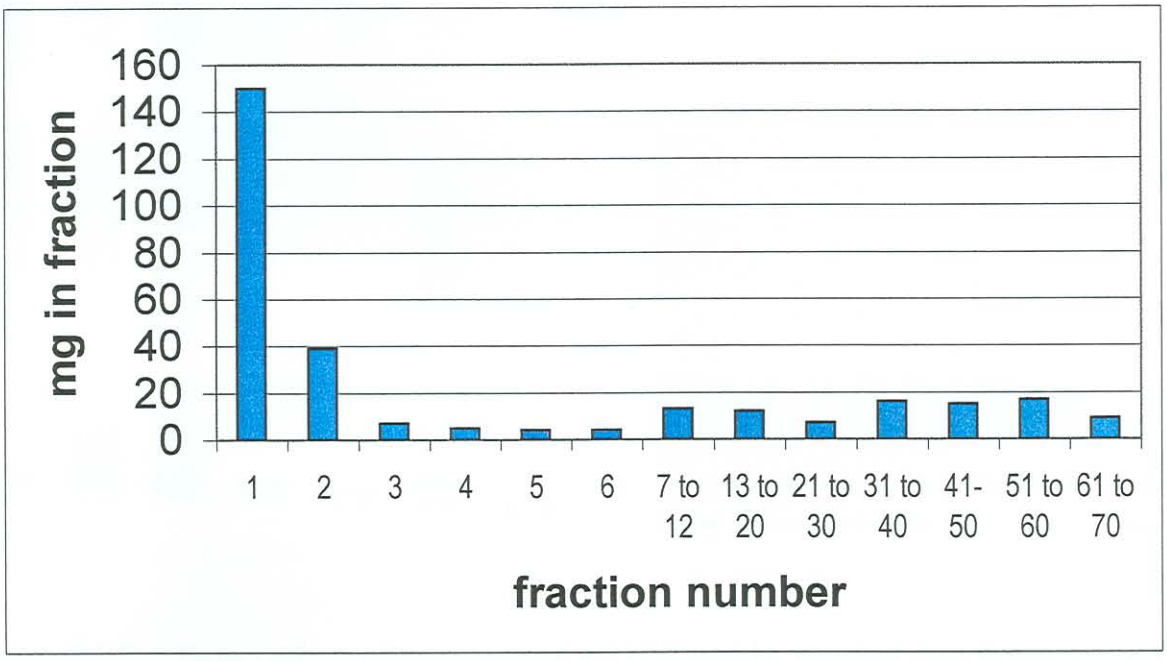

Figure 3.20 Separation of fractions from ABC by large scale column chromatography. (Fractions combined from fraction 7, based on quantity present in individual fractions.)

Because some of the pooled fractions seemed to be relatively pure based on the TLC results, bioautography was carried out on the pooled fractions using *S. aureus, E. coli* and *P. aeruginosa* as test organisms [**Fig. 3.21 and Fig. 3.22**].

There was clear inhibition of growth of *S. aureus* by a compound with an Rf-value of c. 0.74 with CEF as eluent by fractions 21-50. As found earlier, the other test organisms gave much less distinct results. The growth of *E. coli* was not inhibited by the same fractions as *S. aureus*, but a compound with an Rf-value of c. 0.84, gave clear inhibition in pooled fractions 51-60 [**Fig. 3.22 left**]. No clear inhibition of the growth of *P. aeruginosa* was observed by bioautography [**Fig. 3.22 right**].

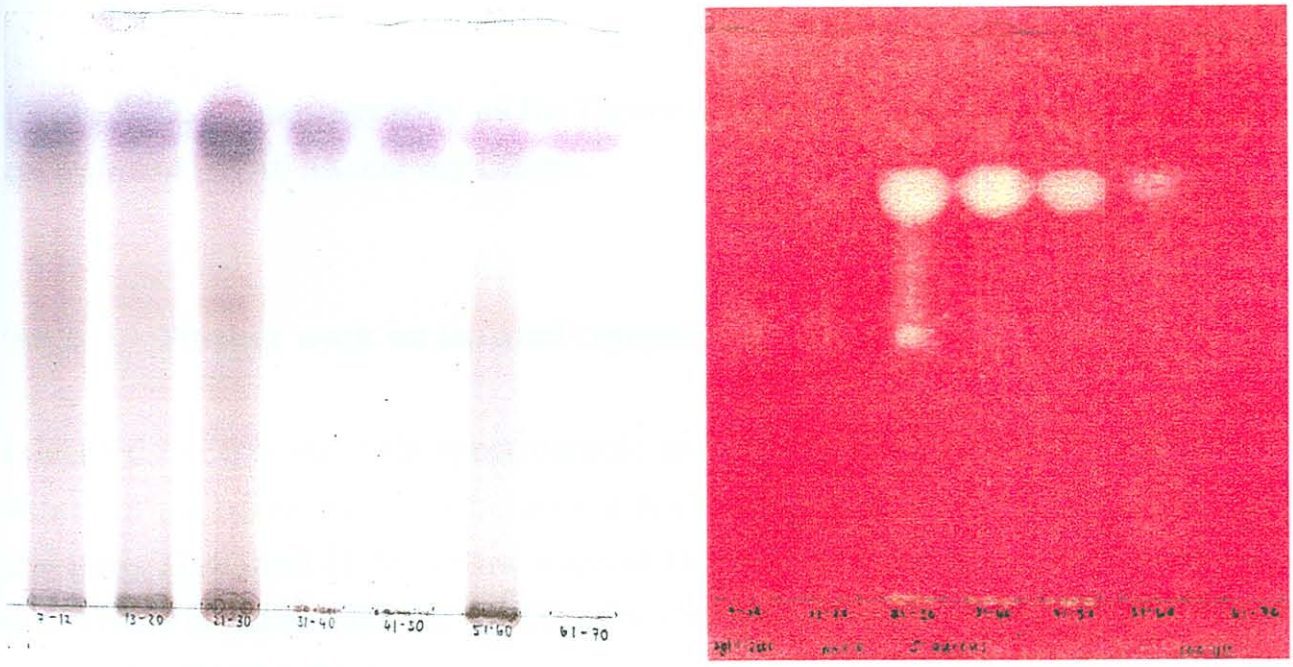

Figure 3.21 Separation of compounds [100 μg] in pooled fractions 7-20, 21-30, 31-40, 41-50 and 51-60 by TLC with CEF as eluent. Left sprayed with vanillin spray reagent and right bioautography with *S. aureus* as test organism.

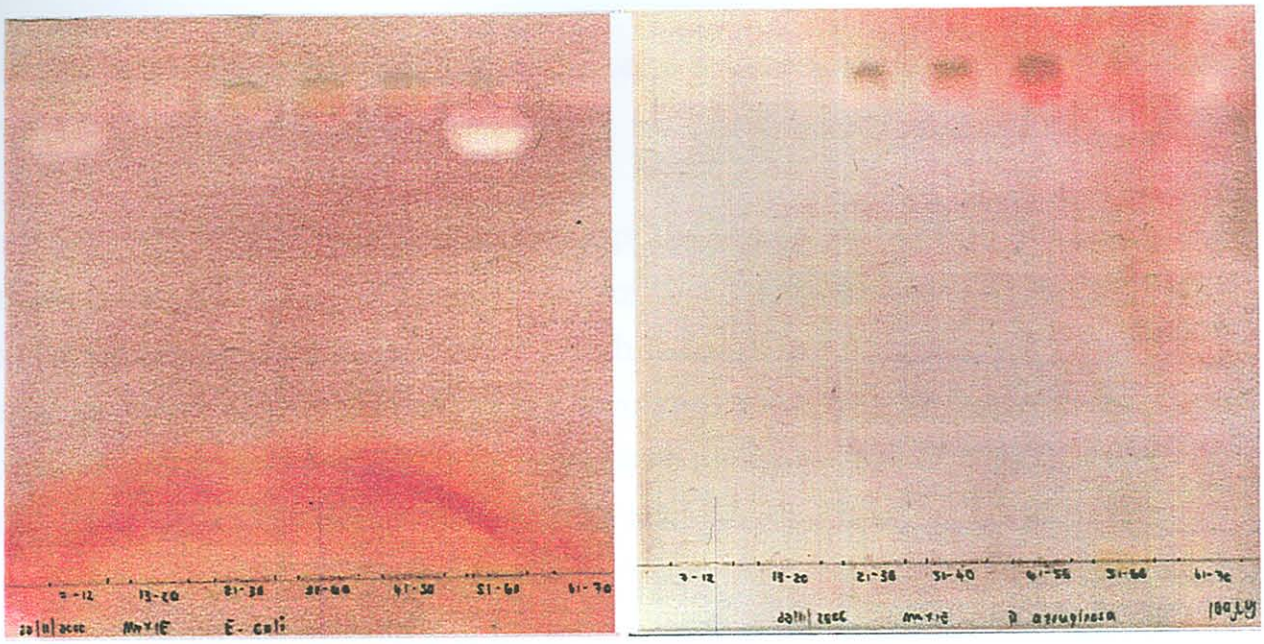

Figure 3.22 Bioautography as for Figure 3.21 with *E. coli* [left] and *P. aeruginosa* [right] as test organisms.

3.6.2 Further work on isolated compounds

I decided to carry out NMR spectroscopic analysis, before determining the MIC values of the active fractions because it is a non-destructive method. Pooled fractions 31-40 and 41-50, with a mass of 16 mg and 15 mg respectively, were apparently clean enough for NMR analysis based on the TLC results. These pooled fractions were dried over silica gel in a vacuum dessicator overnight to remove all traces of water that would give problems in the NMR analysis.

It was a big shock to find that only c. 3 mg of the material was left the next morning. Apparently the active compound was volatile enough to be removed by vacuumpump that was used for c. one hour before leaving the samples to dry overnight. Prof Fanie van Heerden, Chemistry Department, RAU was kind enough to carry out the NMR analysis on pooled fractions 31-40 and 41-50. She found that the remaining sample consisted mainly of phthalate, a common component

of plasticizers used in making plastics and also a common contaminant in certain solvents such as ethyl acetate.

To test whether I could have been chasing phthalic acid and to find out if this compound had antibacterial activity, I performed a number of tests. In the first place I evacuated phthalic acid under a high vacuum in a vacuum dessicator for an hour to determine if this compound is volatile enough to be removed by high vacuum. It did not seem likely because it has a melting point of 230 °C according to the Merck Index. No loss of the phthalic acid [Merck Chemicals] was found.

I then determined the Rf-values of phthalic acid and the active compound. Phthalic acid did not react with the vanillin spray reagent, but it gave very clear quenching in 254 nm UV light when TLC was performed on F_{254} TLC plates. The Rf-value was c. 0.33. **Figure 3.23** shows that phthalic acid could not be responsible for the antibacterial activity noticed in **Figure 3.21 [right].**

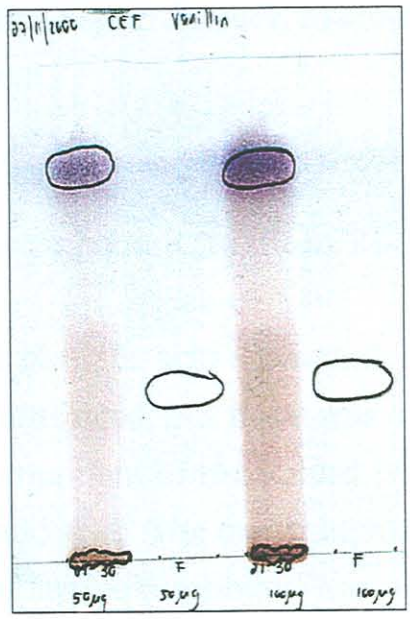

Figure 3.23 Separation of chemical components present in pooled fractions 21-30 [first, 50 μg and third, 100 μg] and phthalic acid [second, 50 μg and last, 100 μg] by TLC with CEF as eluent and visualizing components with vanillin spray reagent. Quenching of absorbance at 254 nm indicated by pencil circles.

To confirm the results, I also determined MIC values of phthalic acid and pooled fractions 21-30. In addition to this, bioautography with *S. aureus* was also carried out.

The MIC values for phthalic acid for the different test organisms were: *S. aureus* 2.5 mg/ml; *P. aeruginosa* 1.3 mg/ml; *E. faecalis* 0.6 mg/ml and *E. coli* 1.3 mg/ml. The MIC values for pooled fractions 21-30 were: *S. aureus* 0.9 mg/ml; *P. aeruginosa* 0.9 mg/ml: *E. faecalis* 1.8 mg/ml and *E. coli* 0.2 mg/ml.

Phthalic acid therefore does have a slight antimicrobial activity. Considering the structure [o-benzenedicarboxylic acid] this may even have been a localized pH inhibition effect at the high concentrations where inhibition took place.

The MIC values of all the fractions containing many non-active compounds, was usually below 0.5 mg/ml [**Table 3.5**]. It is therefore extremely unlikely that phthalic acid would represent a major portion of the antimicrobial activity measured. To investigate this aspect further, bioautography using *S. aureus* as test organism was carried out.

3.6.3 Bioautography with pooled fractions 21-30 and phthalic acid

The low quantity [50 μg] of phthalic acid separated, did not inhibit the growth of *S. aureus* under the conditions used, but there was a slight inhibition at 100 μg level. A minor bioactive component of the pooled fractions 21-30 had the same Rf-value of c. 0.33 as phthalic acid. The major bioactive compound was certainly not phthalic acid and the bioactive compound was also not the substance that quenched light at 254 nm indicated with the pencil lines. If the compound with an Rf-value of c. 0.33 is indeed phthalic acid, it means that the main bioactive compound must be present in a very low concentration and consequently must have a very high bioactivity because a relatively large quantity of phthalic acid is required for the growth inhibition noted [**Fig. 3.24**].

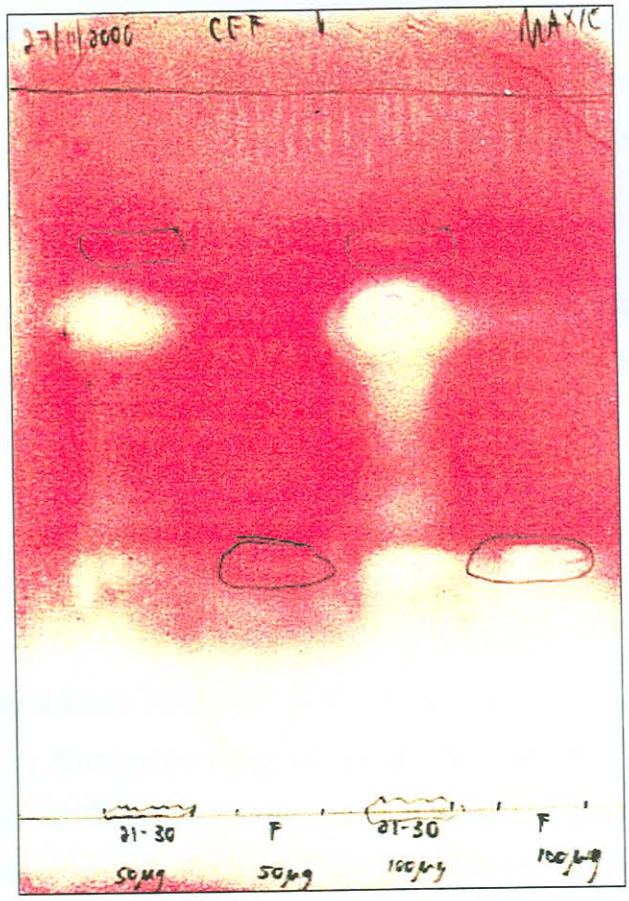

Figure 3.24 Bioautogram of fractions 21-30 [50 μg first and 100 μg third] and phthalic acid [50 μg second and 100 μg last]. TLC using CEF as eluent, sprayed with *S. aureus* cell suspension, incubated and sprayed with INT. White areas indicate bacterial growth inhibition.

3.6.4 Quantitative evaluation of results

Original 90 g of plant material was extracted with acetone. The acetone extract weighed 8.4 g. Solvent fractionation led to nearly 80 % of the non-aqueous activity being concentrated in the hexane fraction. The first column chromatography lead to 65.9 % of the total activity going into fractions ABC. The final chromatography did not yield good separation of the highly non-polar components, but it did yield an antibacterial compound in a relatively pure state.

If I estimate that this compound was 90 % pure, c. 30 mg would have been obtained from 90 g of the original plant material. With an estimated loss of 30 % in the final column chromatography, this compound was probably present at a quantity in the order of 40 mg. This means that it was present at a level of c. 0.04 % in the original material.

It is a pity that MIC values of these pooled fractions were not determined before it was lost, otherwise it would have been possible to calculate what percentage of the total activity is presented by this compound. It is in any case a minor bioactive component because, the majority of the activity resided in the first two fractions. The only reason for investigating the minor compound first was because it seemed to be relatively pure according to the TLC data.

The experience gained here stresses the fact that not all compounds separated by TLC are visualized by the spray reagent used, that one has to be careful to use high quality solvents in the latter stage of the isolation procedure and that there is a danger that volatile compounds can be lost by vacuum drying.

Unfortunately due to personal circumstances it was not possible to follow up on the results obtained thus far. A major avenue for further work exists in the concentrated fractions 1-3 of the final separation. There is also the possibility to capitalize on the suspected volatility of the bioactive fractions by separating these compounds from other compounds through a cold finger vacuum distillation process.