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CHAPTER 4

EXTRACTION AND ISOLATION OF ANTIBACTERIAL COMPOUNDS

4.1. Extraction efficiency of ten extractants

To determine the best extractant for isolating antibacterial compounds, ten different solvents were used to extract 500 mg of a finely ground leaf material of *C. apiculatum*. The extraction was done three times with 5 ml of each solvent on the same plant material. The extracting efficiency for antibacterial compounds of each solvent was determined. This was measured
in terms of the percentage yield of the extracts from the original 500 mg leaf material, the chemical complexity of the extract and lastly the antibacterial activity of the components extracted by a particular extractant. The same plant material was extracted twice more to ensure maximal extraction. The individual extracts were placed in pre-weighed containers and dried in an air stream at room temperature.

Tetrahydrofuran and acetone were the best extractants by extracting 14.4% and 13.2% respectively [Table 4.1]. Water extracted only 0.8% of the original powder mass of the leaf. This indicates that *C. apiculatum* leaves contain a low concentration of very polar components because water is highly polar and hydrophilic therefore its extracting spectrum was limited to very polar and water-soluble compounds only.

The rate of extraction is calculated as the mass of the first extract divided by the total mass extracted by three successive extractions and multiplied by 100. The rate of extraction varied from 50% using water, hexane 60%, methanol 63%, methylene dichloride 72%, ethyl ether 78%, isopropyl ether 80%, acetone 85%, ethanol 86%, ethyl acetate 88% and tetrahydrofuran 89% [Fig. 4.1]. TH and AC extracted the largest quantities of 72 mg and 66 mg respectively compared to the other extractants. This indicates that *C. apiculatum* leaves contain a high concentration of intermediate polarity compounds. According to fig 4.2 the extractants with intermediate polarity were by far the most efficient in extraction. They extracted metabolites over a wide range of polarities.

Table 4.1. Extraction of 500 mg leaf material of *C. apiculatum* by three successive extractions with 5 ml of different extracts. Rate of extraction (percentage) = first extract / total extracted. Hexane (HE), Isopropyl ether (IE), Ethyl ether (EE), Methylene dichloride (MD), Ethyl acetate (EA), Tetrahydrofuran (TH), Acetone (AC), Methanol (ME), Ethanol (ET) and Water (WA).

Solvents	**HE**	**IE**	**EE**	**MD**	**EA**	**TH**	**AC**	**ET**	**ME**	**WA**
1st	6	12	14	23	21	64	56	19	10	2
2nd	3	2	2	5	2	7	9	2	3	1
3rd	1	1	2	4	1	1	1	1	3	1
Total (mg)	*10*	*15*	*18*	*32*	*24*	*72*	*66*	*22*	*16*	*4*
% Extracted	2	3	3.6	6.4	4.8	14.4	13.2	4.4	3.2	0.8
Rate Extraction	60%	80%	78%	72%	88%	89%	85%	86%	63%	50%

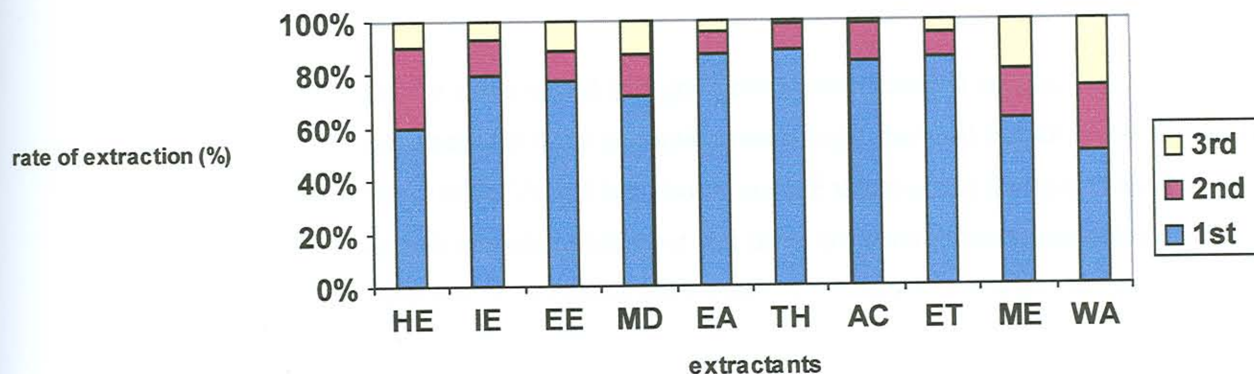

Fig.4.1 Quantities extracted as percentage of the total quantity extracted in three rounds. (Extractants arranged from non-polar to polar)

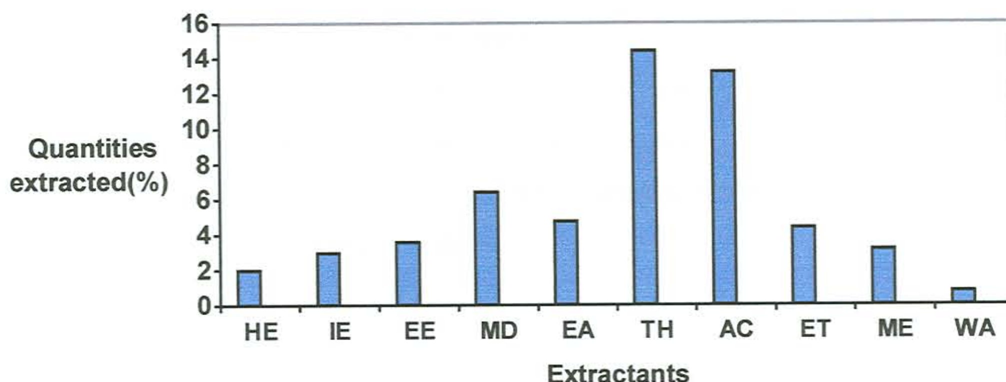

Fig.4.2. Quantities extracted by the ten different extractants in percentage of dry mass.

One would have thought that there would be a good correlation between the rate of extraction and the total quantity extracted because if the extraction rate is high, the total extraction should also be high. In some cases notably with EA, ET but also IE and EE this was not the case. This may mean that whatever EA extracts, it extracts quickly but that there are several components that are not extracted and that the chemical composition must differ. The complexity of the different extracts were therefore examined next.

4.1.1. Complexity of extracts

The different extracts were separated by developing TLC plates in EMW, CEF and BEA (section 3.3). The plates were dried and sprayed with vanillin SR and anisaldehyde SR to detect separated compounds. The components were separated well by CEF and EMW solvent systems. The following colours of components were common to all of the extractants: green, red, yellow and purple. The yellow compound appeared in all the extracts except with water. The red compound was present in all extractants except hexane, when the components were separated by BEA and CEF. Purple and green compounds appeared sparingly under the CEF and BEA on IE, EE, ET, TH and MD extractants. The BEA solvent system yielded the same colours on the constituents but the separation was not good. The water extractant showed only the red colour on all the TLC plates under the three solvent systems. Therefore, the red colour could indicate a polar constituent.

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The purpose was to determine the complexity of compounds present in the extract. The larger the variety of compounds extracted by the different extractants, the better the chance that biologically active components are present in screening studies. On the other hand, the extractants that extracted the bioactive compounds preferably to non-active compounds would be the best in isolation studies.

The BEA did not give good separation of compounds in these extracts; hence it was not used as extensively as the other solventsfor further work in this study. Of the spray reagents used, anisaldehyde SR led to better detection of colours of the constituents on the chromatogram than vanillin SR and was used for further work.

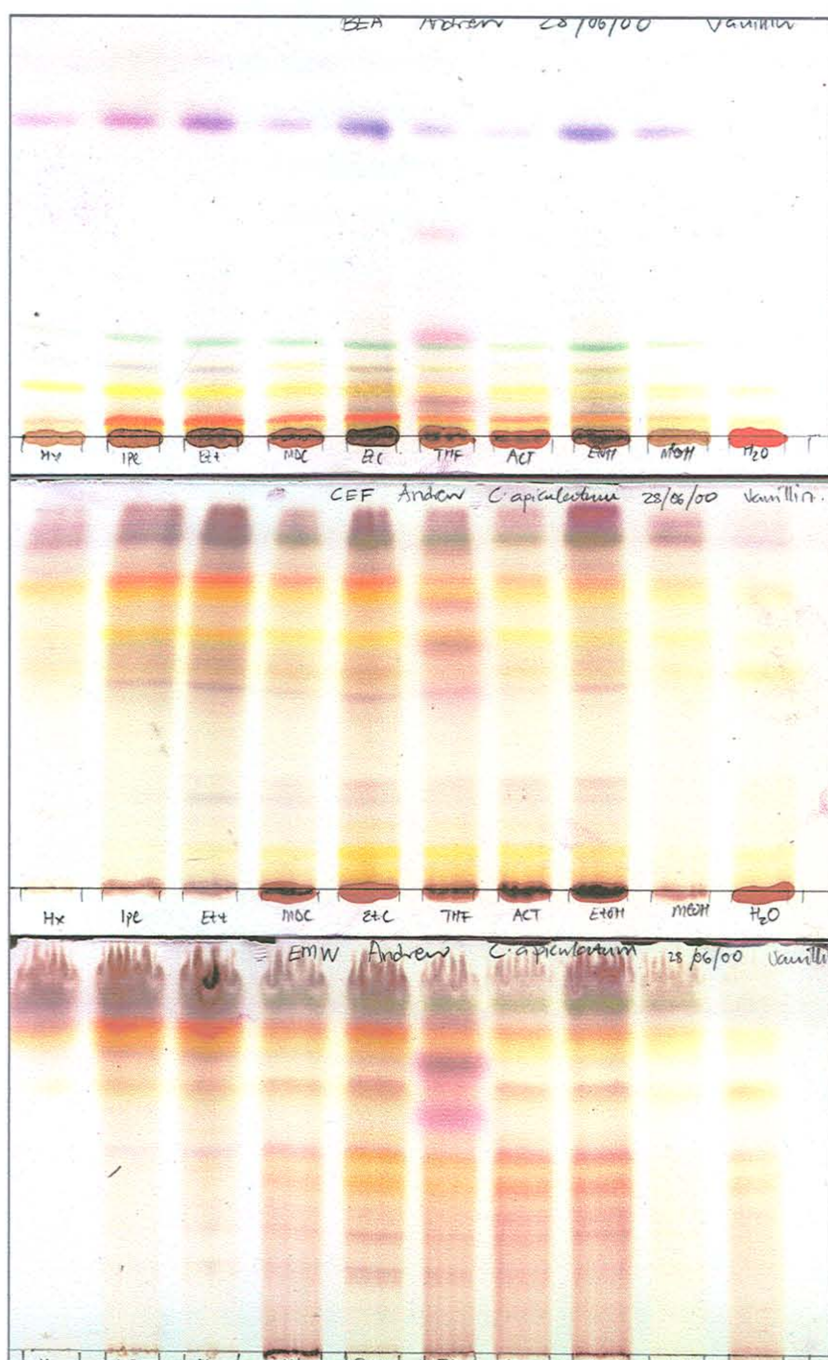

Fig.4.3. Separation of components present in 100 µg of 10 different extracts with BEA (top), EMW (bottom) and CEF (middle) as eluents using vanillin SR as spray reagent. Extractants from left: (HE), (IE), (EE), (MD), (EA), (TH), AC), (ME), (ET) and (WA).

Since different solvents are able to extract certain classes of phytochemicals based on similar polarity, one can argue that the *C. apiculatum* powdered leaves contain less fixed oils, waxes and

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fats, since only solvents of low polarity, e.g. hexane can extract this. Hexane (HE) and methylene dichloride (MD) extracted fewer compounds from the extraction mixture. In addition the non-polar eluent, BEA did not separate any visible compounds after spraying with anisaldehyde SR. According to table 4.2. the spectrum of compounds present in large quantities in *C. apiculatum* may possibly be narrowed to aglycones, alkaloids, chalcones, flavonoids, glycosides and other related phytochemicals, (Houghton and Rama, 1998).

Table 4.2. Types of phytochemicals extracted by different extractants
(Houghton and Rama, 1998)

Polarity	Solvent	Types of chemicals extracted
Low	Hexane	waxes, fats, fixed oils
Moderate	Dichloromethane	alkaloids, aglycones, volatile oils
	Diethyl ether	alkaloids, aglycones
	Ethyl acetate	alkaloids, aglycones, glycosides
	Acetone	alkaloids, aglycones, glycosides
	Ethanol	glycosides
	Methanol	sugars, amino acids, glycosides
High	Water	sugars, amino acids, glycosides

4.2. Bioassay of extracts using different extractants

The ten extracts were tested for antimicrobial activity. Cultures of the four bacteria described in section 3.2 were used. Minimum inhibitory concentration and total activity were determined for all extractants on the test microorganism, [Table 4.3 and 4.4] and [Figure 4.9 and 4.10]. The water and hexane extracts only had slight inhibition to the four microorganisms hence were left out on Figure 4.9 and 4.10 below.

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4.2.1. Bioautography

The procedure followed is explained in section 3.5 of chapter 3. The bioautographic technique worked better with the *S. aureus* and *E. coli* than with the other two test organisms. These two were more sensitive. Most of the activity on the test organisms was exhibited by components in IE and EA extracts. There was little or no activity on extracts of very high or very low polarity. Only those with intermediate polarity showed good activity. Bioautography plates developed in EMW showed one compound, which inhibited the growth of *S. aureus*. This was found with the following extracts: IE, EE, MD, EA and ME. The same activity was exhibited by IE, EE, MD and EA using CEF. The BEA bioautogram had no clear zones of inhibition. The inhibition of the growth of *E. coli* was more distinct than that of *S. aureus* in all the solvent systems. All the extractants under EMW and CEF inhibited the growth of *E. coli* by one compound. The zones of inhibition in CEF were clearer than those in EMW. Only IE, EE, MD and EA inhibited the growth of *E. coli* with one compound.

P. aeruginosa was more resistant to all extracts. It is a Gram-negative microorganism with known ability to resist antimicrobial regimens due to the nature of its cell wall (Nakae et al, 1986 in Goodman and Gilman, 1996). Polar and non-polar compounds contain yellow or brown colours and some of these compounds may mask the inhibition of the growth of the test organism (Martini and Eloff, 1998).

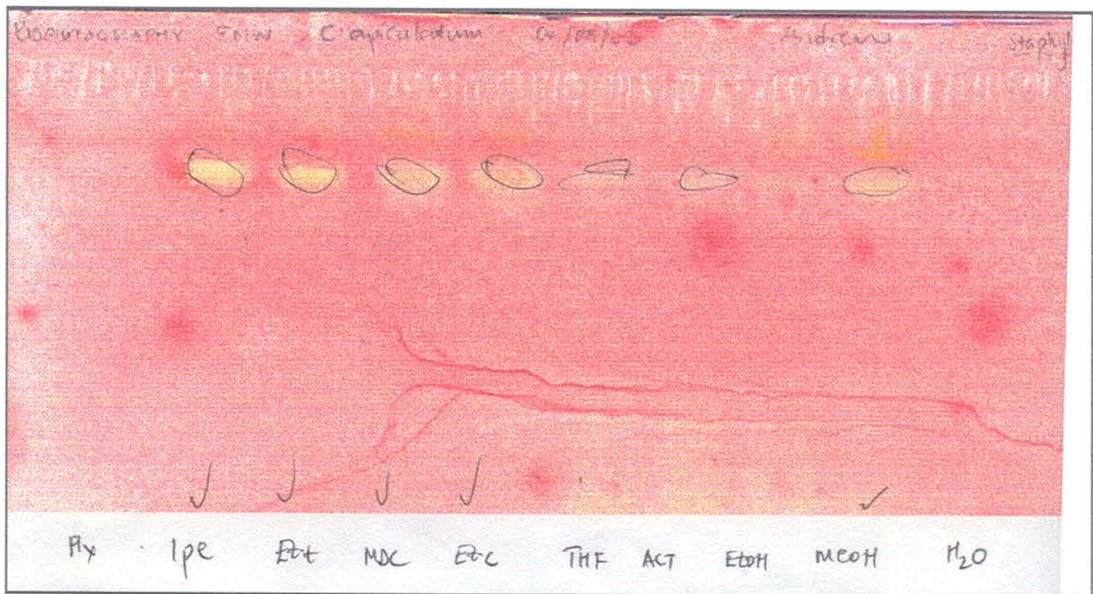

Fig. 4.4 Bioautogram of *C. apiculatum* leaves extracted by 10 different extractants. TLC developed in EMW and sprayed with *S. aureus* culture, incubated overnight then sprayed with INT solution.

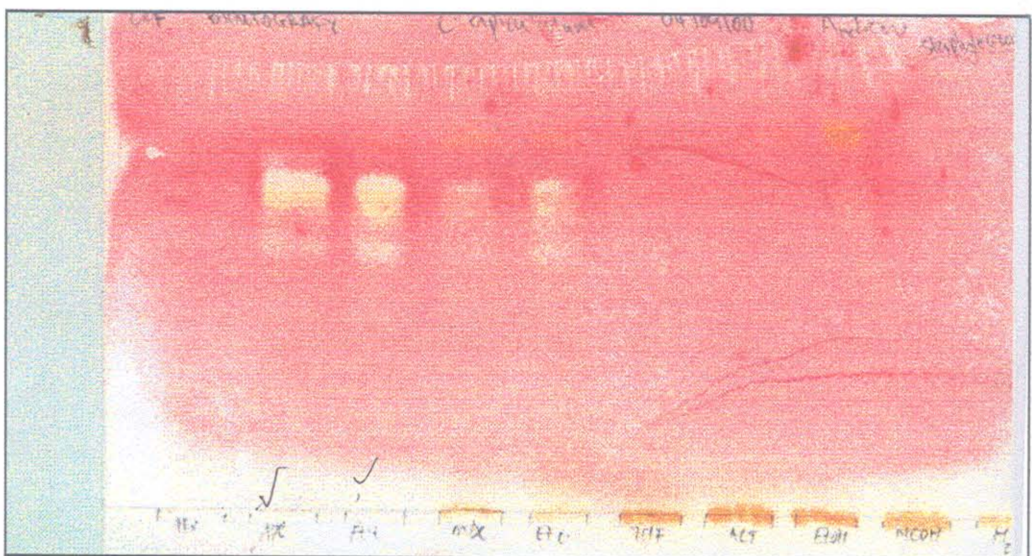

Fig. 4.5 Bioautogram of *C. apiculatum* leaves extracted by 10 different extractants. TLC developed in CEF and sprayed with *S. aureus* culture, incubated overnight then sprayed with INT solution.

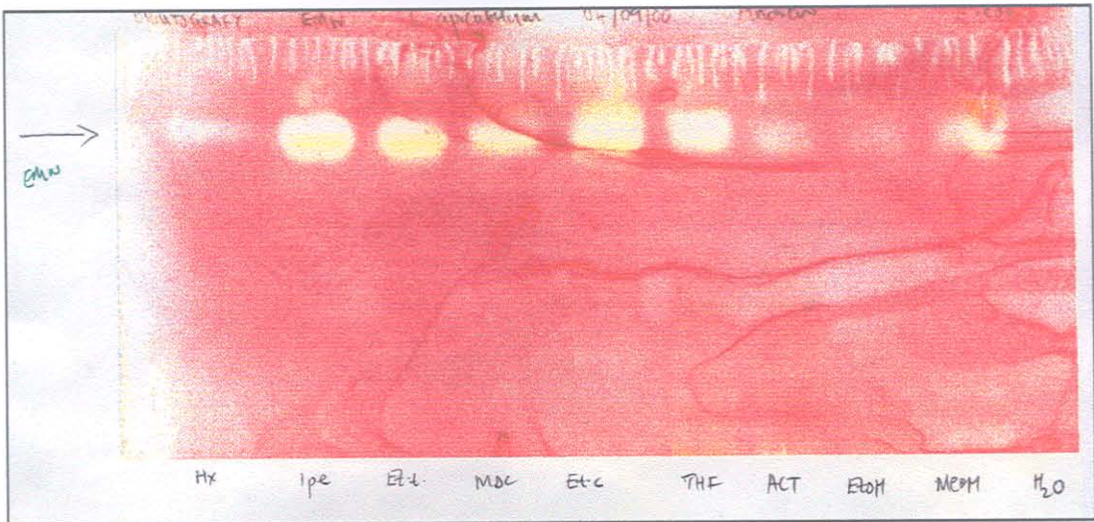

Fig. 4.6 Bioautogram of *C. apiculatum* leaves extracted by 10 different extractants. TLC developed in EMW and sprayed with *E. coli* culture, incubated overnight then sprayed with INT solution.

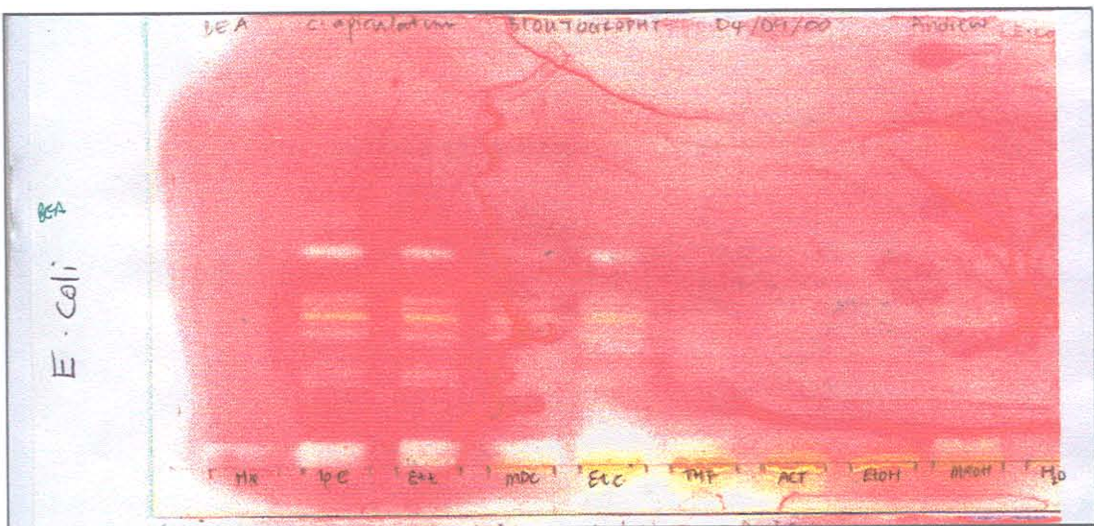

Fig. 4.7 Bioautogram of *C. apiculatum* leaves extracted by 10 different extractants. TLC developed in BEA and sprayed with *E. coli* culture, incubated overnight then sprayed with INT solution.

Fig. 4.8 Bioautogram of *C. apiculatum* leaves extracted by 10 different extractants. TLC developed in CEF and sprayed with *E. coli* culture, incubated overnight then sprayed with INT solution.

4.2.2. Minimum inhibitory concentration (MIC)

The MIC was determined as explained in section 3.4 above. Extracts were dried and reconsituted in acetone in all cases. Acetone control did not inhibit the bacterial growth rate. All the extractants except water and hexane inhibited the growth of *E. coli* at relatively low concentrations of 0.1 mg /ml. *E. faecalis* and *S. aureus* followed with an average MIC value of 0.2 mg/ml. The MIC values of on all the extractants ranged from 0.04 mg /ml to 2.5 mg/ml. EE and EA had the lowest MIC values of 0.04 mg /ml on the activity of *E. faecalis*. This however differs with the results obtained in the bioautography on the same microorganism. Water on the contrary had the highest value of 2.5 mg/ml. The average MIC value of 0.11 mg /ml was exhibited by EA. *P. aeruginosa* was resistant to most of the extracts.

Table 4.3. MIC values in mg/ml of *C. apiculatum* leaves, extracted with ten different extractants (HE), (IE), (EE), (MD), (EA), (TH), (AC), (ME), (ET) and (WA).

Solvents	**IE**	**EE**	**MD**	**EA**	**TH**	**AC**	**ET**	**ME**	**HE**	**WA**
MIC values (mg/ml)										
E. coli	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.6	0.6
P.aeruginosa	0.2	0.2	0.2	0.1	0.6	0.6	0.2	0.1	0.3	2.5
E. faecalis	0.1	0.04	0.2	0.04	0.2	0.2	0.3	0.3	0.3	0.5
S. aureus	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.6	1.3
Average	0.15	0.4	0.18	0.11	0.3	0.3	0.2	0.15	0.45	1.23

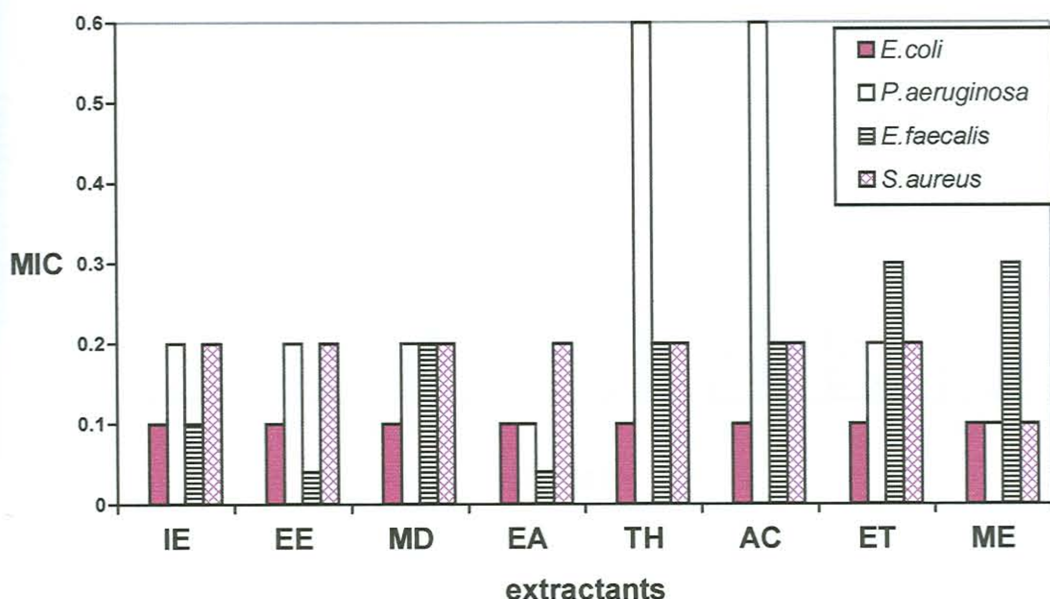

Figure 4.9. Minimum inhibitory concentrations (mg/ml) of the different extracts on the test organisms: *S. aureus, E. coli, P. aeruginosa and E. faecalis.*

Total activity is defined as the total volume (ml) to which the bioactive compounds present in 1 g of dried leaf material or extract can be diluted and still inhibit the growth of the test microorganism. Total activity = Quantity extracted ($mg.g^{-1}$) / MIC ($mg.ml^{-1}$) (Eloff, 2000).

Table 4.4. Total activity in ml/g of *C. apiculatum* leaves, extracted with ten different extractants. (HE), (IE), (EE), (MD), (EA), (TH), (AC), (ME), (ET) and (WA).

Solvents	IE	EE	MD	EA	TH	AC	ET	ME	HE	WA
Total activity (ml)										
E. coli	307	358	588	537	163	1433	486	256	19	6
P.aeruginosa	153	179	294	537	204	179	243	255	38	2
E. faecalis	307	716	294	1075	819	717	121	64	38	1
S. aureus	153	179	294	268	820	717	243	256	19	3
Average	230	358	368	604	502	762	273	208	29	3

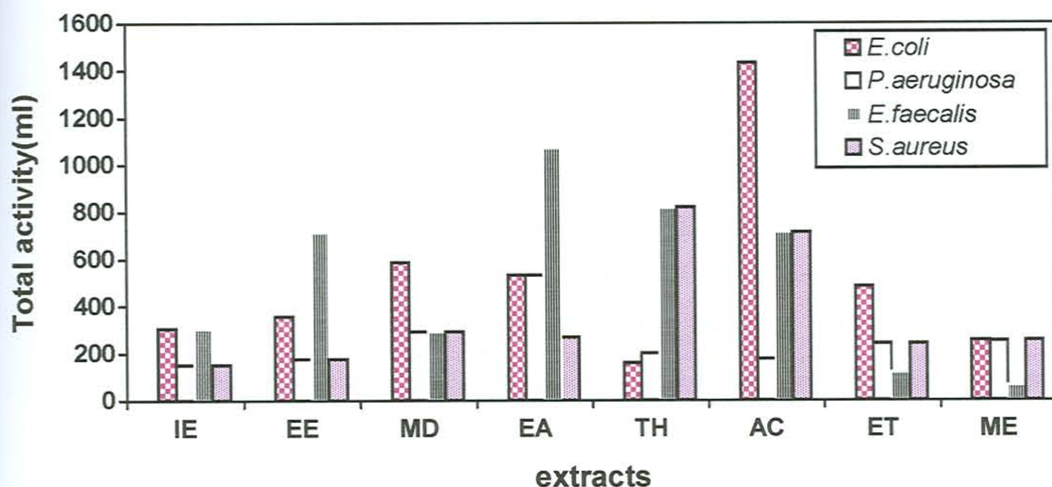

Figure 4.10. Total activity in ml/g of the different extractants on the test organisms: *S. aureus, E. coli, P. aeruginosa and E. faecalis.*

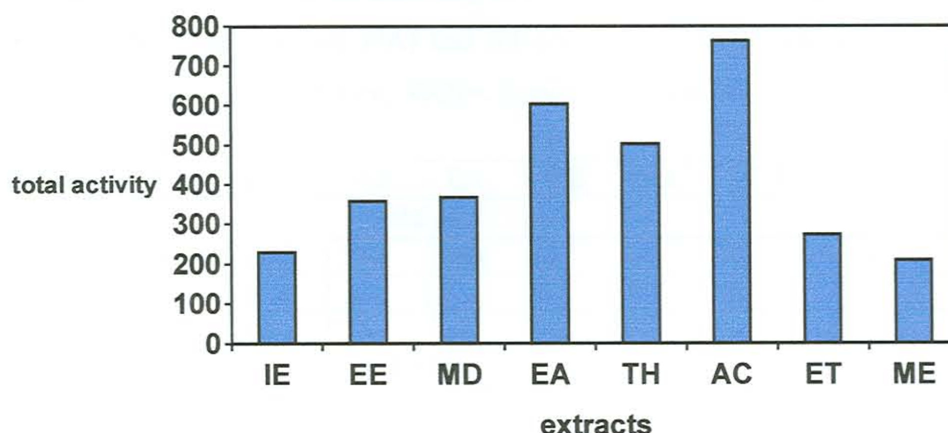

Figure 4.11. Average total activity of the different extracts on the test organisms: *S. aureus, E. coli, P. aeruginosa* and *E. faecalis.*

Even though AC did not show high activity against *P. aeruginosa,* it had low MIC values on the other entire test organism, hence high activity. AC and EA had high average total activity of 762 and 604 respectively. Consequently AC was used in the large-scale extraction to isolate bioactive compounds. EA would also have been a good candidate as extractant especially if compounds active against *E. faecalis* were to be isolated. The lower volatility and non-water miscibility of EA, however decreases its usefulness.

It appears that the active principles shown by the chromatograms could be acidic compounds because the R_f value in the relatively non-polar acidic CEF was nearly as high as in the much polar neutral EMW. In acidic medium compounds with an ionizable hydroxyl group will be in the unionized (non-polar) form whereas in a neutral solvent system the compounds would be ionized (polar).

The compounds that were active against *E. coli* had R_f values ranging from 0.4 to 0.8; against *S. aureus.* R_f values from 0.5 to 0.7 and against *E. faecalis* R_f values from 0.1 to 0.8. This was using the three solvent systems, BEA, EMW and CEF. The compound that exhibited activity against both *E. coli* and *S. aureus* had the same R_f value of 0.8. This compound showed this activity using EMW and CEF. Compounds active against *E. coli* only had R_f values of 0.1 and 0.5 with BEA as solvent system.

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Table 4.5. R_f values of compounds inhibiting *E. coli* growth using different extractants (HE, IE, EE, MD, EA, TH, AC, ME, ET and WA) and solvent systems. (CEF, BEA and EMW). X=. Slight inhibition, XX=. Moderate inhibition, XXX=. Strong inhibition.

	HE	IE	EE	MD	EA	TH	AC	ET	ME	WA
BEA										
0.1	x	xx	xx	xxx	xxx	x			x	
0.3			x	x						
0.5		x	xx							
EMW										
0.8	xx	xx	xx	xx	xx	xx	x	x	x	x
CEF										
0.4						x				
0.7						x				
0.8	xx	xx	xx	x	xx	x	x	x		

Acetone, water and ethanol extracts did not show activity against *E. coli* in the non-polar BEA, solvent system. Slight activity was exhibited on the plates developed in EMW and CEF. This shows that the active components appear to be non-polar because they could only be separated by non-polar solvent systems. This is in contrast to other extracts, which showed a slight inhibition.

Table 4.6. R_f values of compounds inhibiting *S. aureus* growth using different extractants (HE, IE, EE, MD, EA, TH, AC, ME, ET and WA) and solvent systems. (CEF, BEA and EMW). X=. Slight inhibition, XX=. Moderate inhibition,
XXX=. Strong inhibition.

	HE	IE	EE	MD	EA	TH	AC	ET	ME	WA
CEF										
0.7		xx	xx	x	x					
0.4		x	x							
EMW										
0.8		x	x	x	x				x	

Inhibition of growth could only be seen under EMW and CEF. The BEA chromatogram did not show any clear zone that could be associated with inhibition. The active extracts in this regard were isopropyl ether (IE), ethyl ether (EE), methylene dichloride (MD) and ethyl acetate (EA).

Table 4.7. R_f values of compounds inhibiting *E. faecalis* growth using different extractants (HE, IE, EE, MD, EA, TH, AC, ME, ET and WA) and solvent systems. (CEF, BEA and EMW). X=. Slight inhibition, XX=. Moderate inhibition, XXX=. Strong inhibition.

	HE	IE	EE	MD	EA	TH	AC	ET	ME	WA
BEA										
0.1		x	x		x					
EMW										
0.8		x	x	x	x				x	

E. faecalis was only slightly inhibited by a compound with a R_f value of 0.1 in BEA and 0.8 in EMW. No inhibition was found when CEF was used as solvent system.

4.2.3. Conclusion

The most effective extractants are TH and AC according to total quantity extracted and AC and EA according to the total antibacterial activity. Although the bioautography results with acetone were not as good as with other extractants, acetone was used for the large-scale extraction for the following reasons (Eloff, 1998):

- Acetone dissolves both hydrophilic and lipophilic compounds.
- It is miscible with water
- It has low toxicity to the test microorganisms in the bioassay
- It is easy to remove by evaporation
- It is non-toxic to humans
- Other students in our group have used it and it makes comparisons with their results easier

4.3. Large-scale extraction and group separation of extracts

4.3.1. Fractionation of extracts

The purpose was to extract and separate the extracts of the *C. apiculatum* leaf into groups of varying polarities by solvent/solvent fractionation. By simplifying the extracts, it may be easier to isolate the antibacterial compounds, if it were present in only one or two fractions, containing fewer non-active compounds.

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4.3.2. Extracted quantities

The finely ground *C. apiculatum* leaf powder (100 g) was extracted with 1000 ml of acetone three times. The extract was dried under vacuum in a Buchi; PE 120 rotary evaporator to yield 16.484 g. The dried extract was dissolved with equal volumes of chloroform and water in a separating funnel. The procedure was followed as discussed in section 3.6. The exact quantities of fractions are shown in Fig.4.12 and Table 4.8 below.

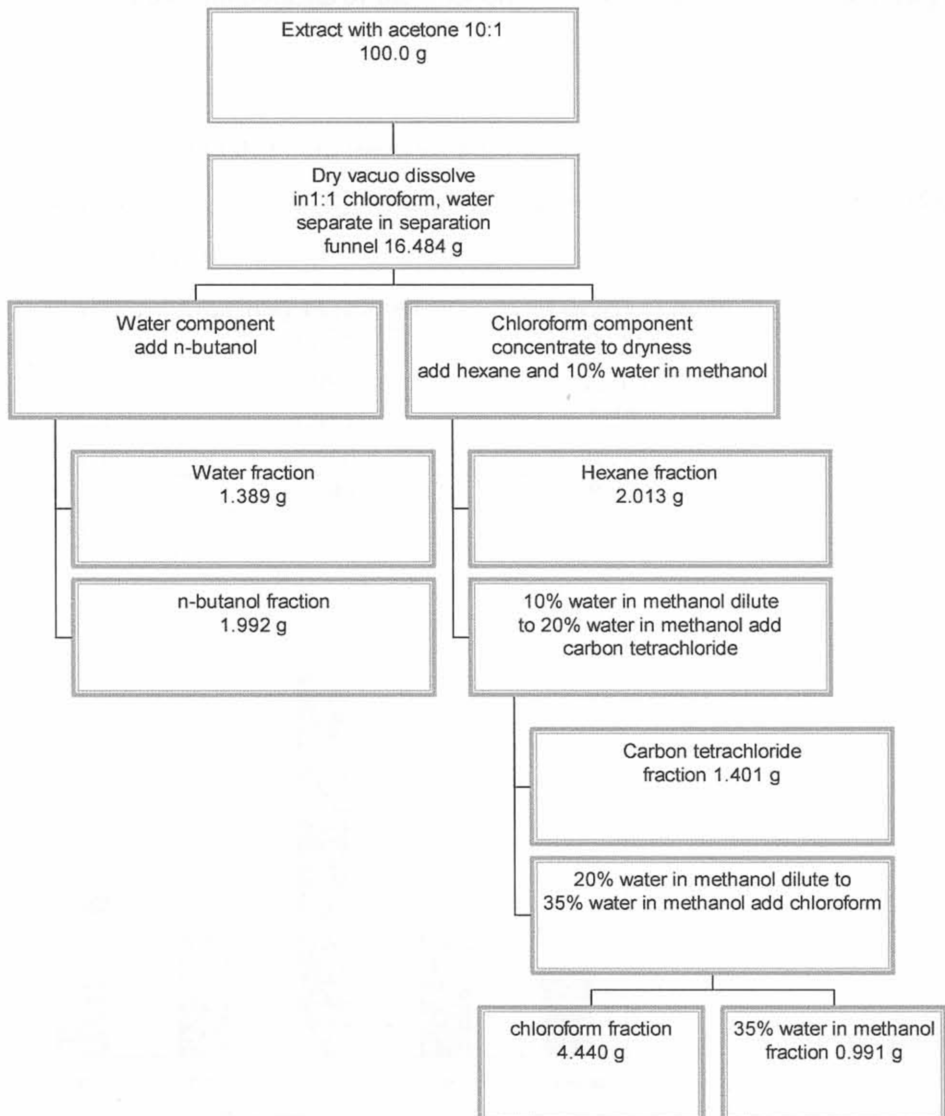

Figure 4.12. The procedure and quantities obtained during the solvent/solvent fractionation of the components in the *C. apiculatum* leaf extract.

The different fractions were dried in a pre-weighed round bottom flask. The following quantities of extracts were collected, CF (4.440 g), HE (2.013 g), CT (1.4001 g), B (1.992 g), W (1.389 g) and W/M (0.991 g) [table 4.8]. The fractionation started with 16.484 g crude extract of acetone. Total quantity extracted by different extracts was 12.226 g. The highest percentage of acetone extract was in chloroform (26.9%), the lowest was 6.0% extracted by 35% W/M. The quantity of the extract lost during fractionation was approximately 4.258 g. It is not easy to explain how nearly 25% of the extract was lost. The pellicle formed in some cases could not represent such a large loss. Possibly some of the components of the fractions were volatile and were lost during drying.

Table 4.8. Quantities (mg) present in different fractions after solvent/solvent fractionation of *C. apiculatum* leaf extract, water (W), butanol (B), hexane (H), carbon tetrachloride (CT), chloroform (CF) and 35% water in methanol (W/M)

Fraction	Quantity extracted (g)	% of original mass
HE	2.013	12.21
B	1.992	12.10
CT	1.401	8.50
CF	4.440	26.93
W	1.389	8.42
W/M	0.991	6.01

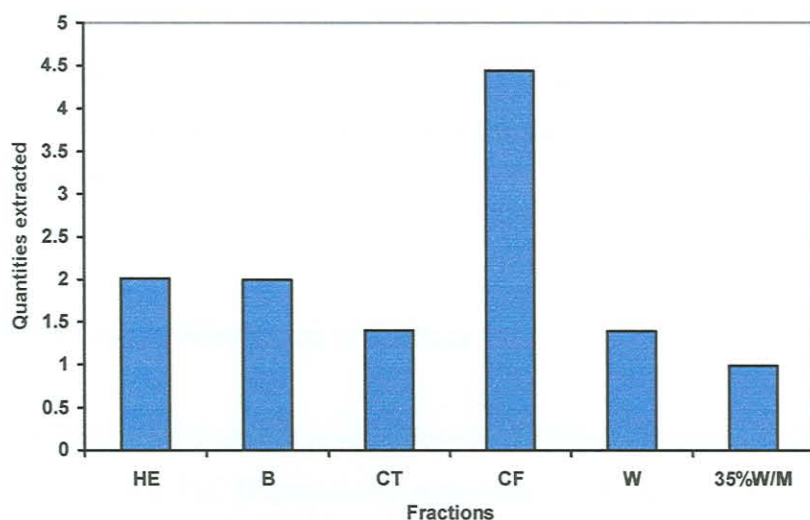

Figure 4.13. Quantities (mg) separated into different fractions by the solvent/solvent fractionation of *Combretum apiculatum* leaf extract.

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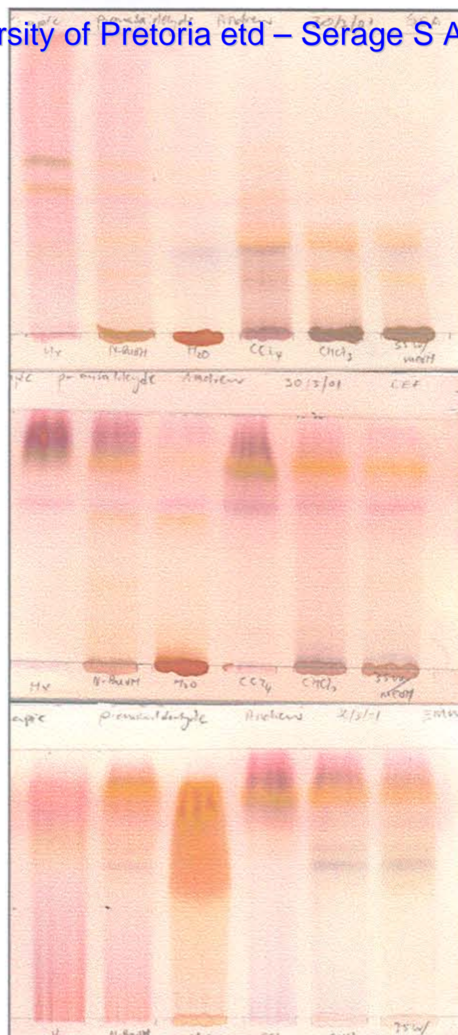

Fig.4.14. Chemical composition of solvent/solvent fractions separated by BEA (top), CEF (middle) and EMW (bottom) as eluants. Compounds were visualized using anisaldehyde SR. Fractions from left Hexane (H), butanol (B), water (W), carbon tetrachloride (CT), chloroform (CF) and 35% water in methanol, (W/M)

4.3.3. Chemical composition of fractions

To determine the chemical complexity of the different fractions, 100 µg of each fraction was separated using TLC [Figure 4.14]. Anisaldehyde SR was used to spray the TLC plates. The EMW and CEF separated the components better than BEA did. The CF, CT and M/W fractions contained more components than the other fractions. As observed with the plates from the preliminary extraction, the most dominating constituents were those with a red, purple and yellow colours. The three colours were distinct on the CF and CT fractions. The water fraction was slightly separated in

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the BEA and CEF solvent systems. However, all the fractions were separated well with EMW and CEF as solvent systems. Consequently the major constituents present in the extracts were moderately polar to polar.

4.3.4. Bioassay of fractions

The different fractions were tested for antimicrobial activity using the test organisms discussed above, section 3.2. The compounds, which inhibited *S. aureus*, had the same R_f values as obtained under extraction. *E. coli* was inhibited by compounds with R_f values of 0.1, 0.3, 0.4 and 0.8, table 4.9. According to chromatograms the acetone extract contains more than one compound that inhibit *S. aureus* and *E. coli*. The compounds with R_f values of 0.8, 0.4 and 0.5 correspond to the yellow and purple compounds obtained under extraction. Constituents in the CF extract showed good inhibition. Inhibition of *E. coli* was clearer than that on *S. aureus*.

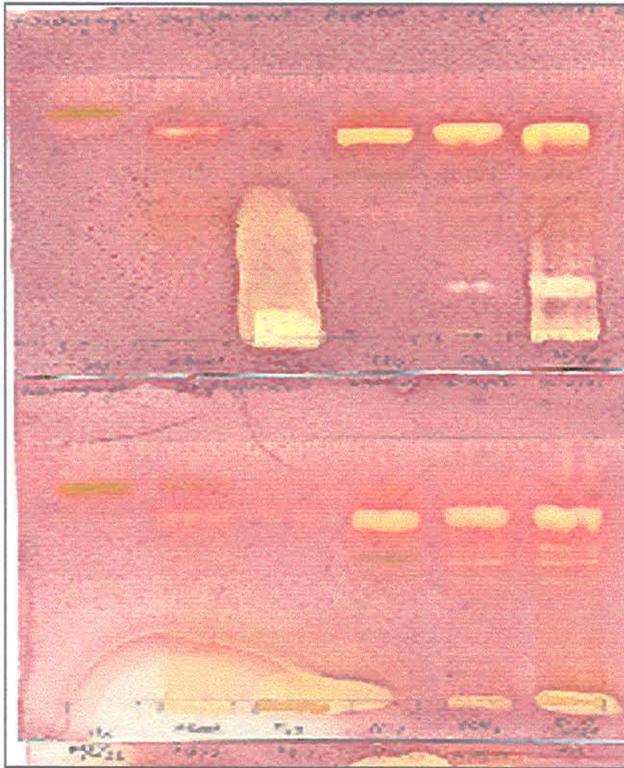

Fig.4.15. Bioautogram of six different fractions of *C. apiculatum* extract separated by solvent/solvent extraction. TLC plates were sprayed with *S. aureus*. Solvent systems used EMW (top) and CEF (bottom). Fractions from left hexane (H), butanol (B), water (W), carbon tetrachloride (CT), chloroform (CF) and 35% water in methanol, (W/M).

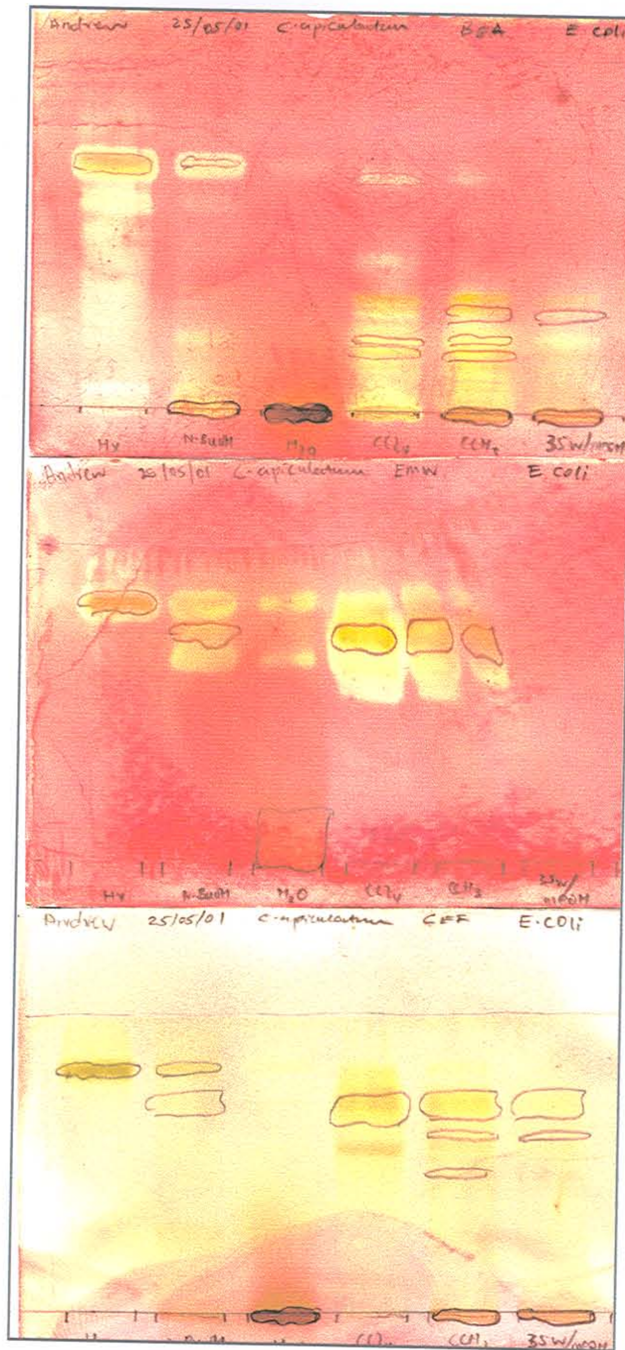

Fig.4.16. Bioautogram of six different fractions of *C. apiculatum* extract separated by solvent/solvent extraction. TLC plates were sprayed with *E. coli.* Solvent systems used BEA (top), EMW (middle) and CEF (bottom). Fractions from left hexane (H), butanol (B), water (W), carbon tetrachloride (CT), chloroform (CF) and 35% water in methanol, (W/M).

Table 4.9. R_f values of antibacterial compounds from CF, CT and 35%W/M fractions using *E. coli* as test organism, and BEA as solvent system. (X = slight inhibition, XX= moderate inhibition, XXX= strong inhibition)

R_f values	CF	CT	W/M
0.8		x	
0.4	x		x
0.3	x	x	
0.1	x	x	

Table 4.10. R_f values of antibacterial compounds from CF, CT and 35%W/M fractions using *S. aureus* as test organism and EMW as solvent system. (X= slight inhibition, XX= moderate inhibition, XXX= strong inhibition)

R_f values	CF	CT	35%W/M
0.8	xx	xx	xx
0.2	x		xx

4.3.5 Selecting best fraction

Further work was done on the chloroform extract because it displayed good activity against the test organisms. In comparing the bioautograms it must be kept in mind that the same quantity was separated from each fraction. The selection of the fraction to be investigated further, total quantity was considered. The CF fraction contained nearly the same quantity as the B, CT and W fractions combined. Based on the antibacterial activity and mass of each fraction, the CF fraction was the most promising and this was used for further work.

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4.4. Isolation by bioassay guided fractionation

4.4.1. Introduction

Further work using column chromatography to isolate bioactive constituents was carried out on the chloroform (CF) fraction obtained from the group separation of extracts (section 3.6). The procedure for isolating the compounds is summarized in section 3.7 and shown schematically by figure 4.18 below.

The 100 x 2.5 cm glass column was packed with freshly prepared silica gel slurry. About 4.44 g of CF extract was loaded onto the column. The column was developed with hexane, chloroform and methanol. Fractions were collected into test tubes in volumes of approximately 50 ml. A total of 248 test tubes were collected at the end of the run. These were combined into 29 fractions based on similar chemical complexity after analyzing the fractions by TLC.
Fractions 1-18 and 25-29 were relatively simple and were dried in a stream of air in a fume cupboard and crystals formed, which were recrystalized from methanol, chloroform and hexane to yield relatively pure compounds. The complexity of fractions 19 to 24 necessitated the setting up of another column for further separation.

The second column of 1 x 50 cm long was set up using silica gel slurry made in hexane. Chloroform and methanol mixtures were used as mobile phases. Many fractions were collected and after TLC were pooled into fractions which were combined with fractions containing the same compounds. Bioautography was carried out on the same 29 fractions. Ten fractions, which had biological activity from both the 1st and 2nd columns, were selected. The components were separated by TLC and antibacterial activity determined by bioautography on these selected fractions fig 4.21

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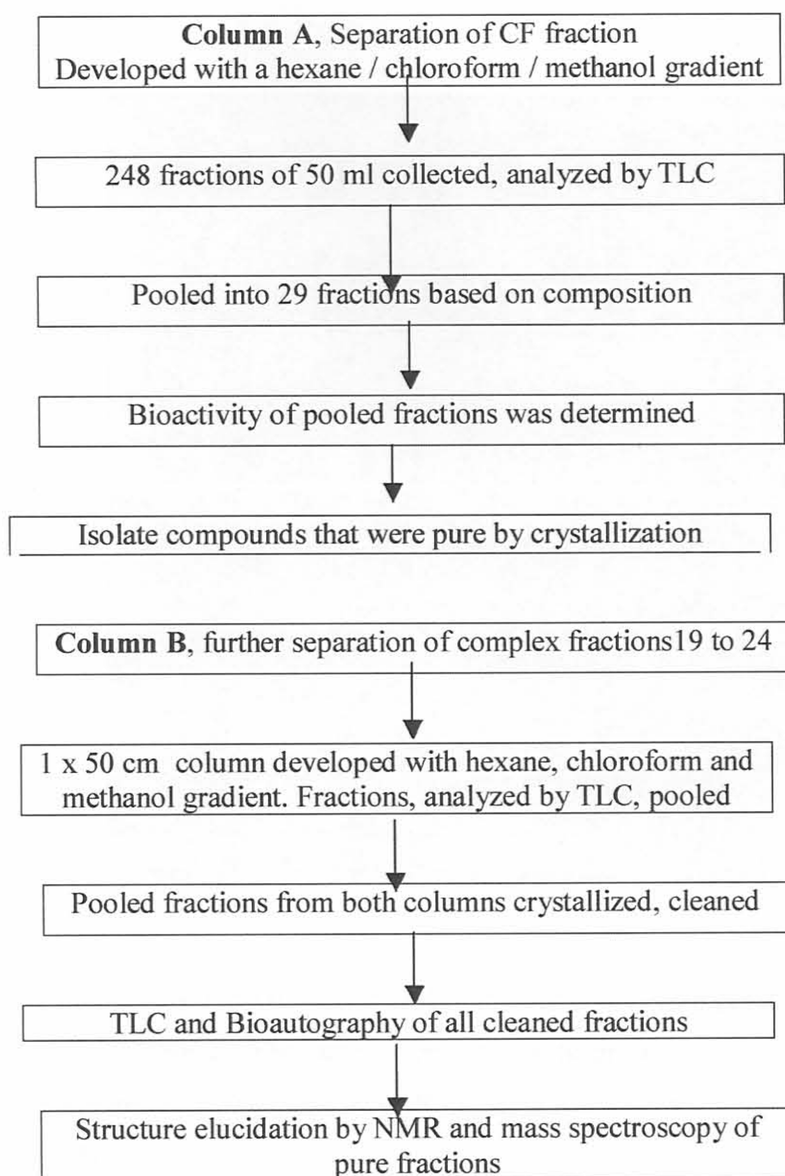

Figure 4.17. Steps followed in the extraction and characterization of active compounds

4.4.2. Antibacterial activity of fractions

Bioautography was carried out on the 29 pooled fractions to establish which of the fractions had bioactive compounds. Test microorganisms, *E. coli* and *S. aureus* were used for the bioassay, because these microorganisms were more sensitive to the chloroform extract (Fig 4.15 and 4.16). An aliquot from the fractions was spotted onto the TLC plates and the procedure followed as described in section 3.3 and 3.5. This revealed the presence of compounds of interest. Figure 4.21.

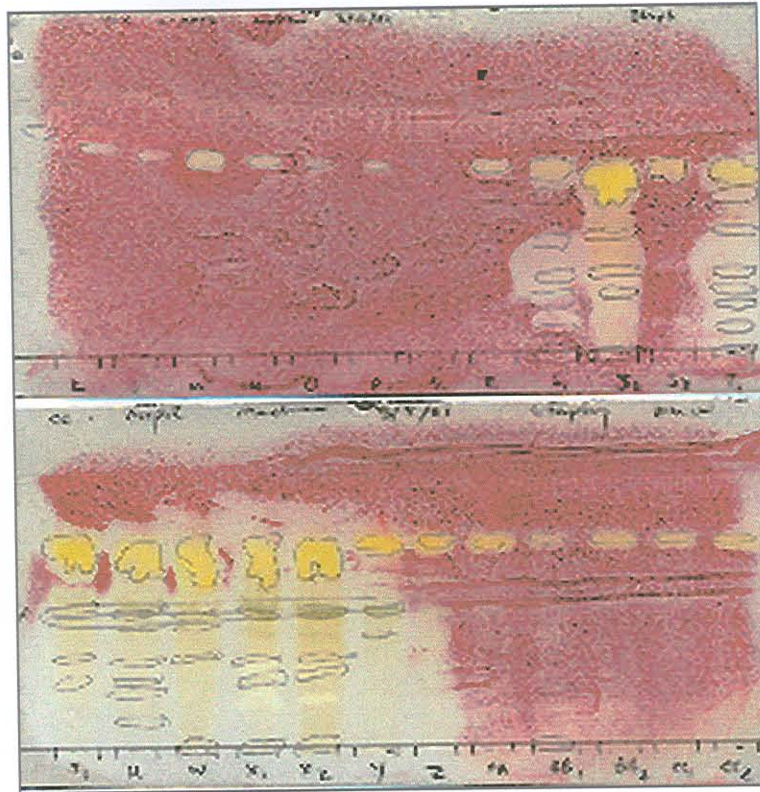

Fig.4.18. Bioautogram of pooled fractions of *C.apiculatum* obtained from column chromatography, EMW as an eluent using *S. aureus*. The clear zones indicate the growth inhibition of *S. aureus* by the fractions. Top fractions: K, L, M, N, O, P, Q, R, S_1, S_2 and bottom fractions: T, U, W, $X._1$, X_2, Y, Z, AA, BB, CC, DD.

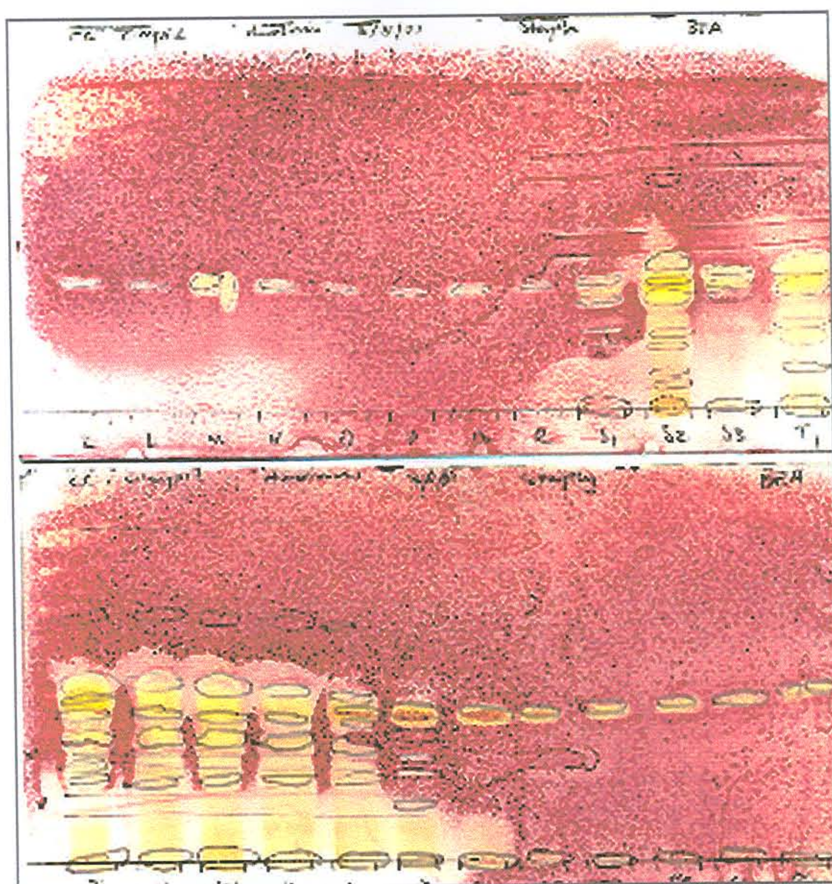

Fig 4.19. Bioautogram of pooled fractions of *C. apiculatum* obtained from column chromatography, BEA as an eluent using *S. aureus.* The clear zones indicate the growth inhibition of *S. aureus* by the fractions. Top fractions: K, L, M, N, O, P, Q, R, S_1, S_2 and bottom fractions: T, U, W, $X_{\cdot 1}$, X_2, Y, Z, AA, BB, CC, DD.

The region of activity had the similar R_f values as those found on one of the CF compounds in solvent/solvent fractionation. It was however observed that fractions 19 to 24 had a high complexity of different components, which appeared red, yellow and brownish upon spraying the TLC plates with vanillin SR. Therefore, this necessitated the setting up of another column to further separate fractions, 19 – 24. The separated fractions from 19 to 24 had the same biological activity as those of the first column. Ten fractions, which had biological activity from both the 1st and 2nd columns, were selected. TLC was run on all the selected fractions [figure 4.20].

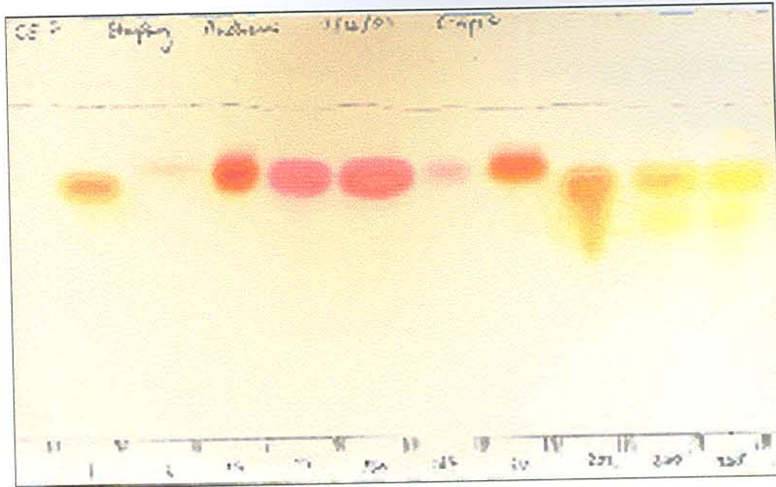

Fig.4.20. TLC of the 10 active samples from column chromatography using CEF as an eluent. Fractions from left, 1, 13, 19, 75, 130, 164, 34, 202, 207, and 238.

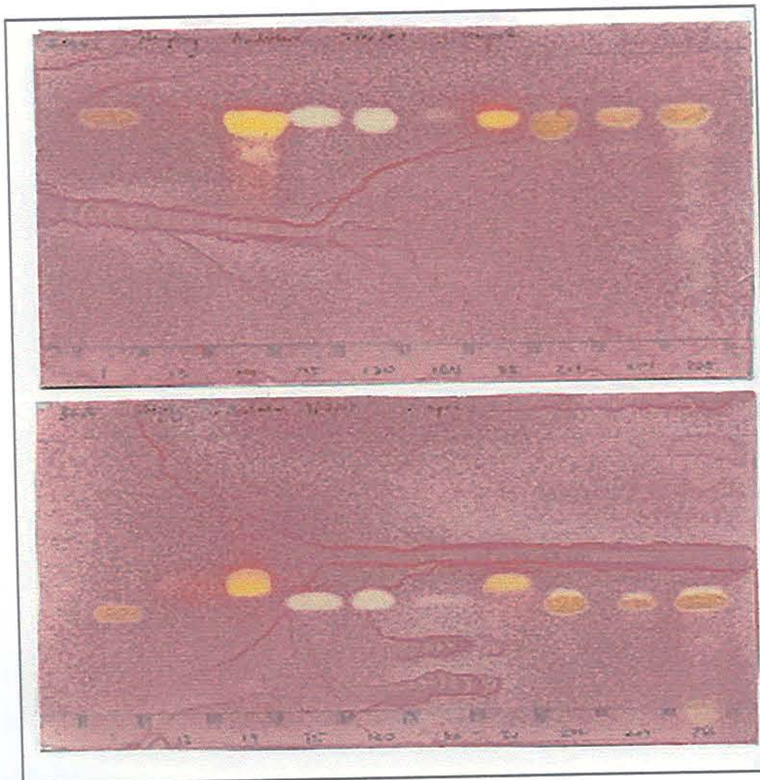

Fig.4.21. Bioautogram of the 10 active samples obtained from column chromatography, using EMW (top) and BEA (bottom) as eluants. The plates were sprayed with *S. aureus*. Fraction numbers from left, 1, 13, 19, 75, 130, 164, 34, 202, 207, and 238

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Bioautography was carried out with the ten active fractions obtained from column chromatography. Fractions and their quantities were [1 (37 mg), 13 (40 mg), 19 (35 mg), 34 (40 mg), 75 (17 mg), 130 (24 mg), 164 (20 mg), 202 (68 mg), 207 (47 mg), and 238 (20 mg)]. Some fractions showed good activity on *S. aureus* and *E. faecalis*, but *P. aeruginosa* and *E. coli* appeared to be resistant. The active fractions were analyzed using NMR on a Varian 300 MHz machine. Four were sufficiently pure for structure elucidation. The spectra showed them to be flavonoids.

4.4.3. R_f values of isolated compounds

It is important to record the R_f values of isolated compounds to make it easier to identify bioactive compounds present in other Combretaceae extracts. It also makes it possible to dereplicate data, i.e. to ensure that known compounds are not isolated again. Based on the behaviour during column chromatography the compounds isolated were probably different, but the similar R_f values after TLC using EMW indicates a possible degree of similarity or that the solvent system is not useful in separating these compounds.

Table 4.11. R_f values of the bioactive compounds obtained from column chromatography

Samples	**AS-1**	**AS-13b**	**AS-164**	**AS-130**
BEA	0.36	0.4	0.38	0.4
EMW	0.8	0.8	0.8	0.79

4.4.4. MIC values of isolated compounds

MIC values were determined on the isolated compounds as described in section 3.4.
Good inhibition was shown by samples AS-1, AS-13b and AS-130 on *E. faecalis* and *S. aureus* with MIC value of 40 μg/ml. AS-13b inhibited both *E. faecalis* at the same MIC value of 40 μg/ml. AS-164 inhibited *E. faecalis* and *S. aureus* at 80 μg/ml. If the MIC values of the isolated compounds are compared to that of the crude extract (Table 4.3) in most cases the MIC values are two to five times lower with the exception of *E. coli* results. With *E. coli* the isolated compounds are up to 5.4 times less active that the crude extract. This may be due to not isolating the important

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bioactive compounds present in the crude extract, but the R_f values of the isolated compounds were similar to that of the crude extract according to bioautography. A more likely explanation is that in the crude extract there are synergistic effects, i.e. two or more compounds may inhibit *E. coli* substantially more than the single isolated compounds. This would also explain the difference obtained between bioautography where compounds are isolated and MIC of crude extracts where several compounds are present.

Table 4.12. MIC values (µg/ml) of the four active samples on four test organisms: *E. faecalis, S. aureus. E. coli* and *P. aeruginosa*.

Samples	**AS-1**	**AS-13b**	**AS-130**	**AS-164**
P. aeruginosa	300	130	300	300
E. faecalis	400	40	40	80
E. coli	600	250	130	130
S. aureus	40	40	80	80
Average MIC's	**335**	**106**	**138**	**148**

AS-13b was the least active against *P. aeruginosa* and *E. coli.* Based on the bioassay results below, using the four test organisms, AS-1, AS-13b, AS-130 and AS-164 can be regarded as having substantial antibacterial activity against some of the pathogens. The values of AS-130 and AS-164 were very similar.

One difficulty with the presentation in Table 4.12 is that the higher activity is, the lower the value is. To compensate for this the total activity per mg of compound can be calculated. This has been done to compare the plant extracts (Eloff, 2000). In this case total activity per mg can be defined as 1/MIC in mg/ml. This value represents the volume in ml into which the one mg of the active compound can be diluted and still inhibit the growth of bacteria. One mg of AS-13b can therefore be diluted to 25 ml and still inhibit the growth of *S. aureus*.

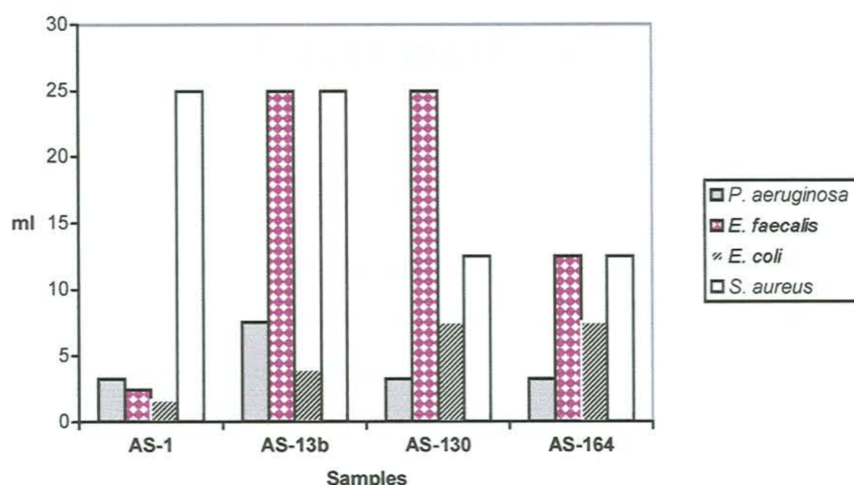

Figure 4.22. Total activity in ml per mg of the four bioactive samples

It is strange that the MIC value of the EE and EA extracts had a MIC value of 40 µg/ml, the same value as the isolated compounds. This may indicate that the compounds isolated may have a minor antibacterial activity. In the crude extracts, R_f values of the bioactive compounds, according to the bioautography results, were 0.4 and 0.8 the same as the isolated compounds. This indicates that the compounds isolated were the major bioactive compounds present in the original extract and not minor bioactive compounds. The only logical explanation at t his stage is that there may have been synergistic effects. Unfortunately there was not enough material available to test this hypothesis and elucidate the chemical structure.