CHAPTER 5

5. RESULTS AND DISCUSSION

5.1 EXTRACTION

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

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

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

† Methanol/chloroform/water (12:5:3)

‡ Methylene dichloride

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

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

5.2 SOLVENT / SOLVENT EXTRACTION

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

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

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

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

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

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

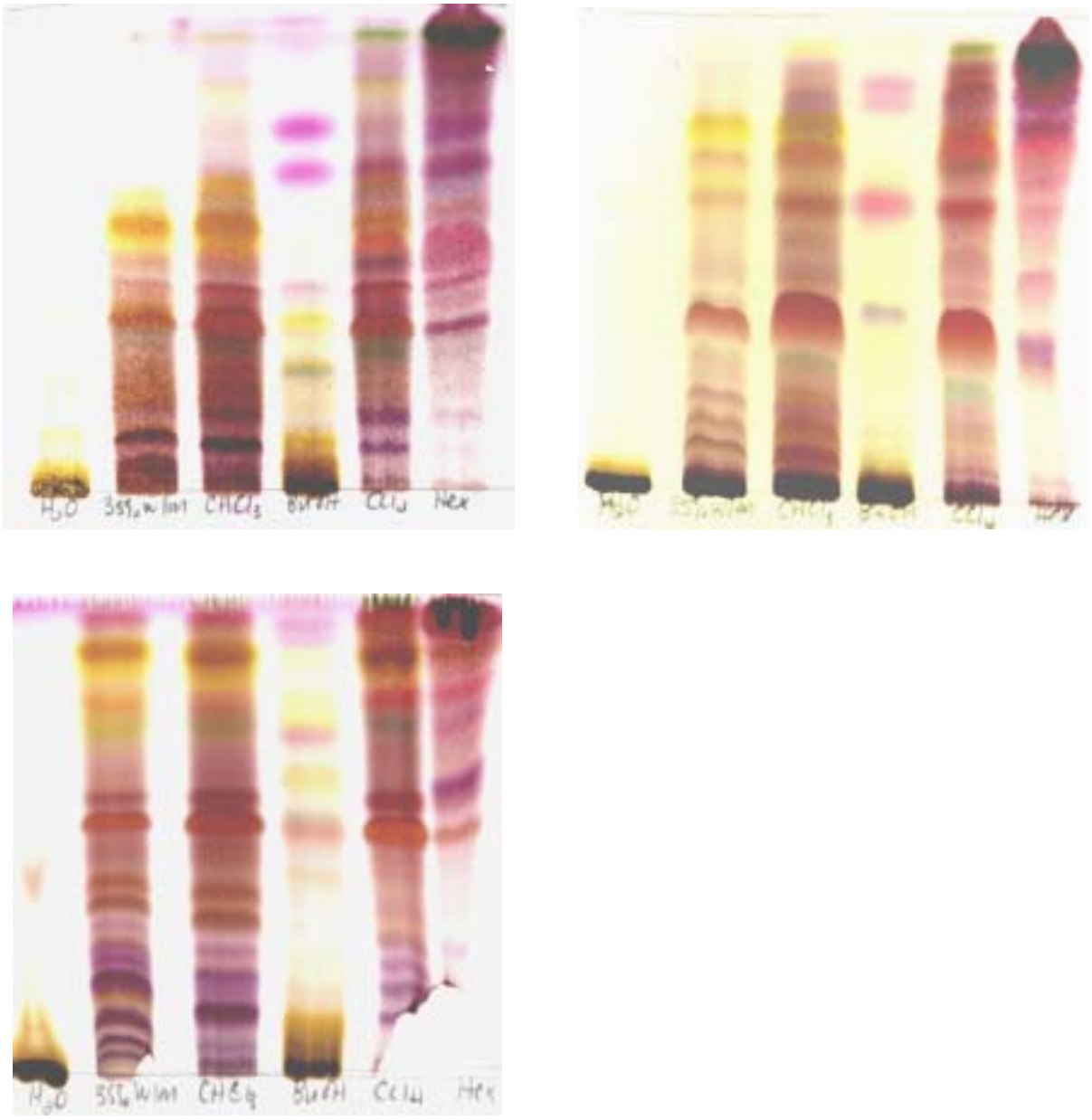


Figure 5.1: Solvent/solvent fractions depicting from left to right: water, 35% water/methanol, chloroform, butanol, carbon tetrachloride and hexane. Clockwise from the top are the fractions run in BEA, 2 acetone:3 MDC and CEF

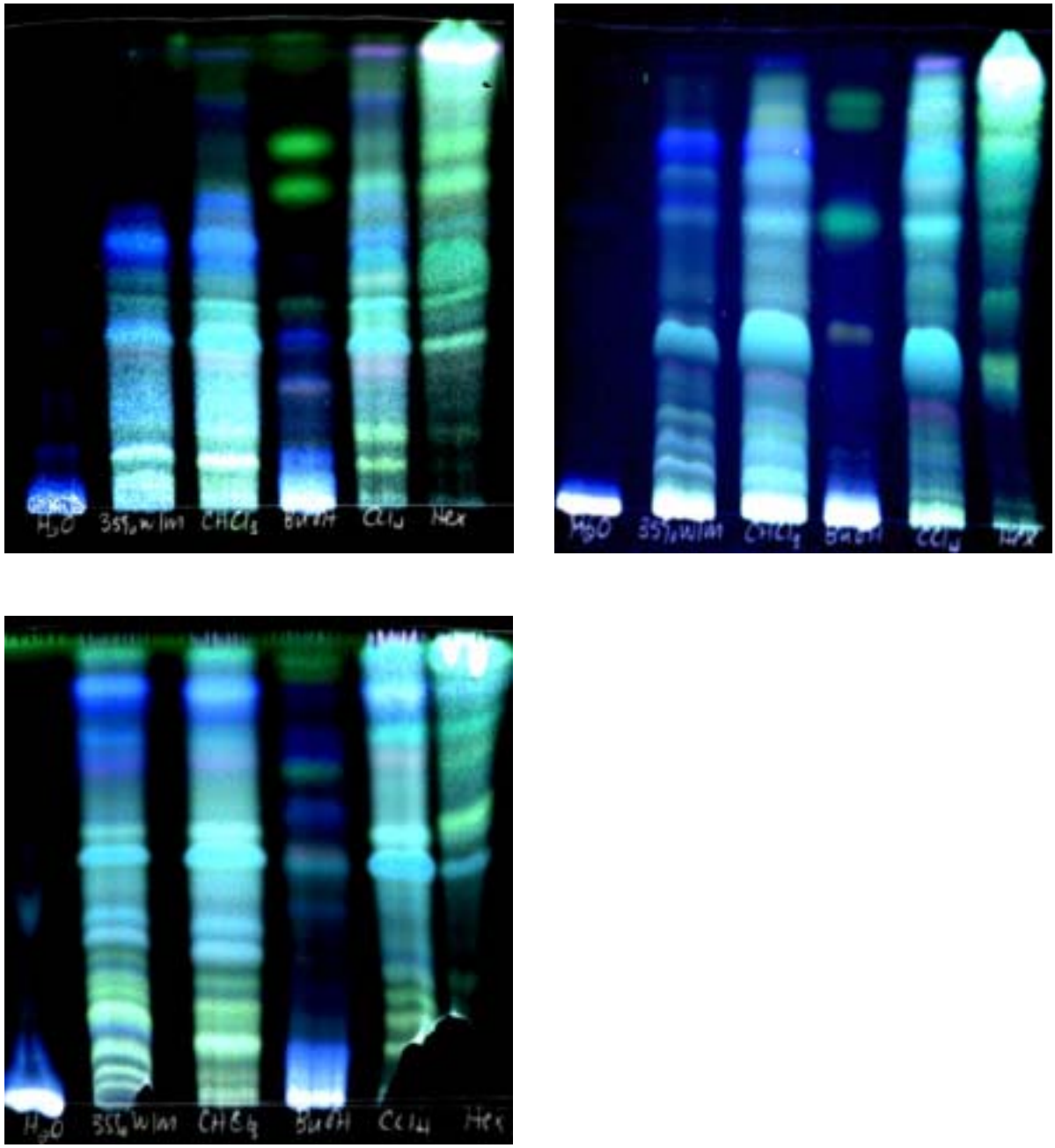


Figure 5.2: Negative image of solvent/solvent extraction depicting from left to right: water, 35% water in methanol, chloroform, butanol, carbon tetrachloride and hexane. Clockwise from the top are the mobile phases BEA, 2A:3MDC and CEF

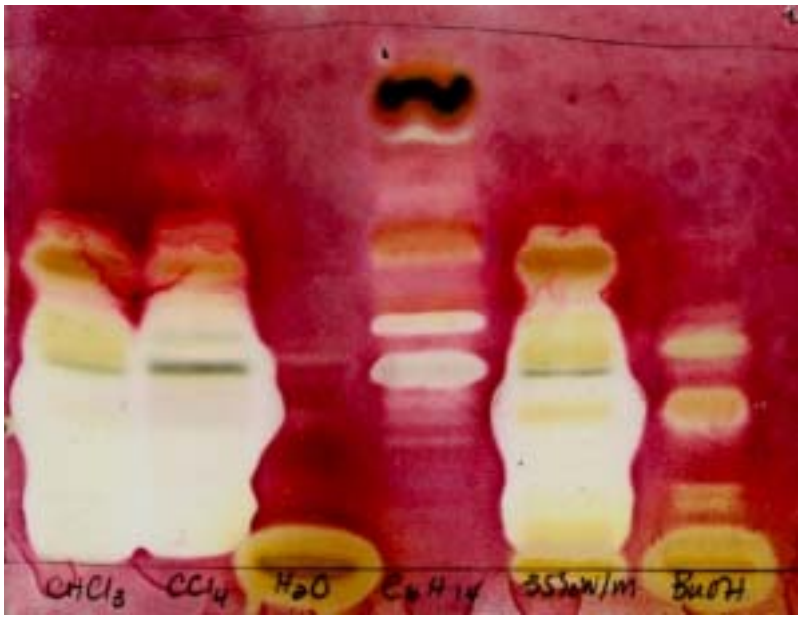


Figure 5.3: Bioautography of the solvent/solvent fractions using BEA as mobile phase. From left to right are chloroform, carbon tetrachloride, water, hexane, 35% water in methanol and butanol.

5.3 COLUMN CHROMATOGRAPHY

5.3.1 Hexane fraction

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

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

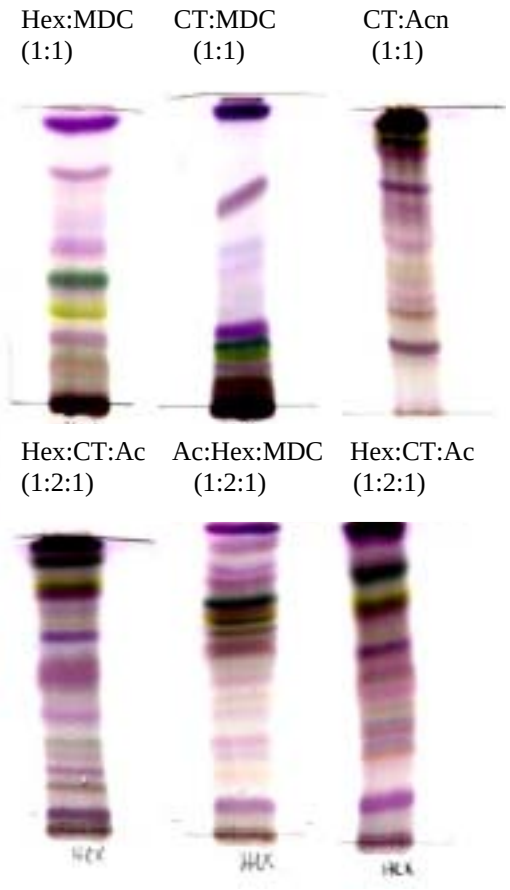


Figure 5.4: TLC plates showing the separation obtained by various mobile phases using the hexane fraction. Hexane: methylene dichloride (1:1), shown top left, separated the non-polar fractions and acetone: methylene dichloride (1:1) (not shown) separated the polar fractions the best.

5.3.1.1 TLC analysis and combination of fractions

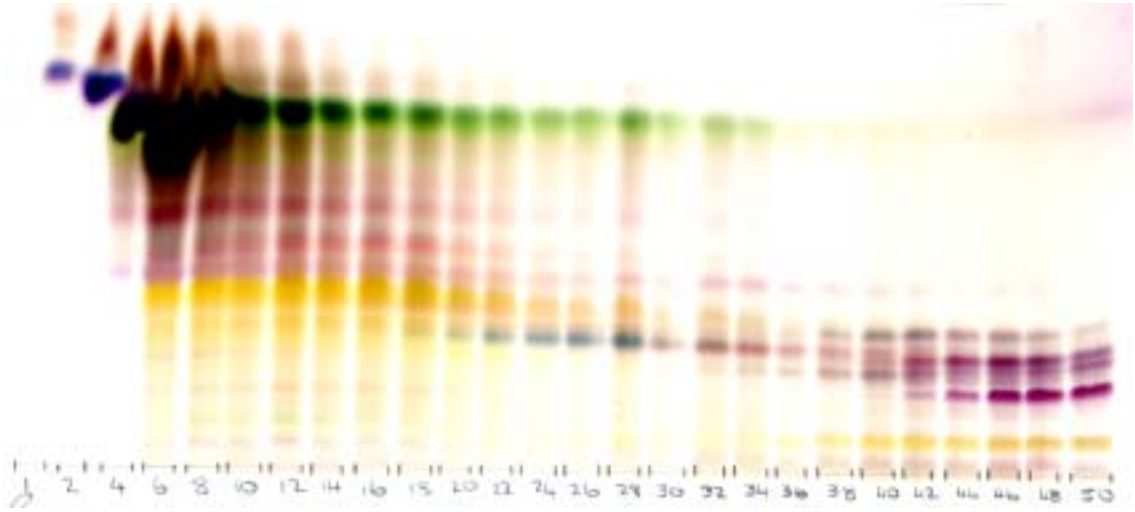
BEA was used for TLC analysis for the first 50 non-polar fractions and CEF for the remaining polar fractions (Figure 5.5), which enabled us to combine fractions with similar TLC profiles. Combination of fractions resulted in 16 fractions (A-P), which were then further analysed by TLC and bioautography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

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

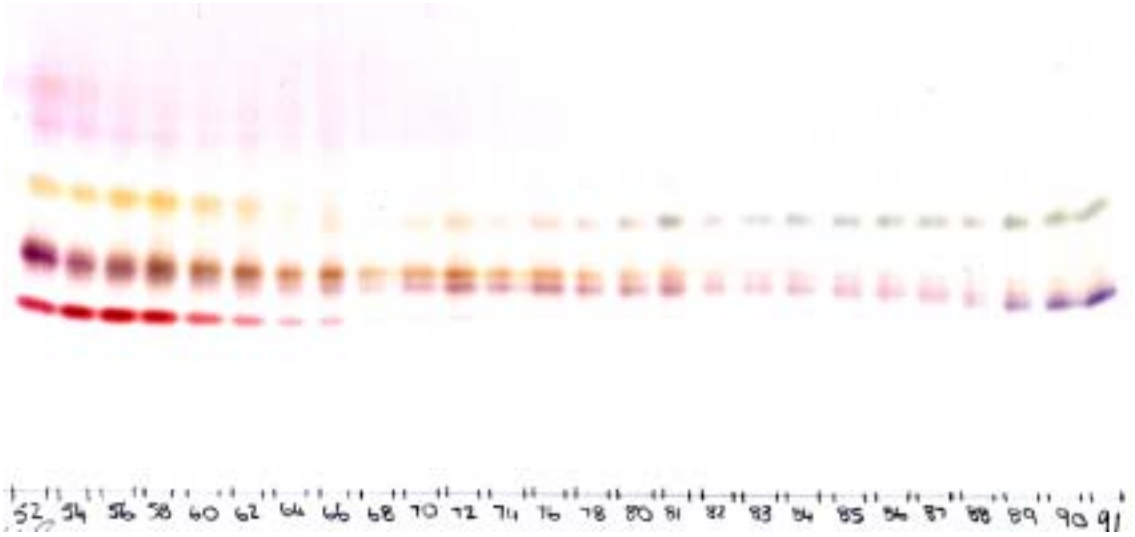
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
Combined	1-3	4-8	9 19	20 29	30 39	40 50	51 68	69 88	89 108	109 117	118 121	122 127	128 138	139 153	154 167	168 180
Complex	2	3	3	4	4	5	5	5	5	5	5	5	3	3	2	2
Mass (mg)	460	1166	5375		708	492	455	366	250	160	78	225	96	92	220	202

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

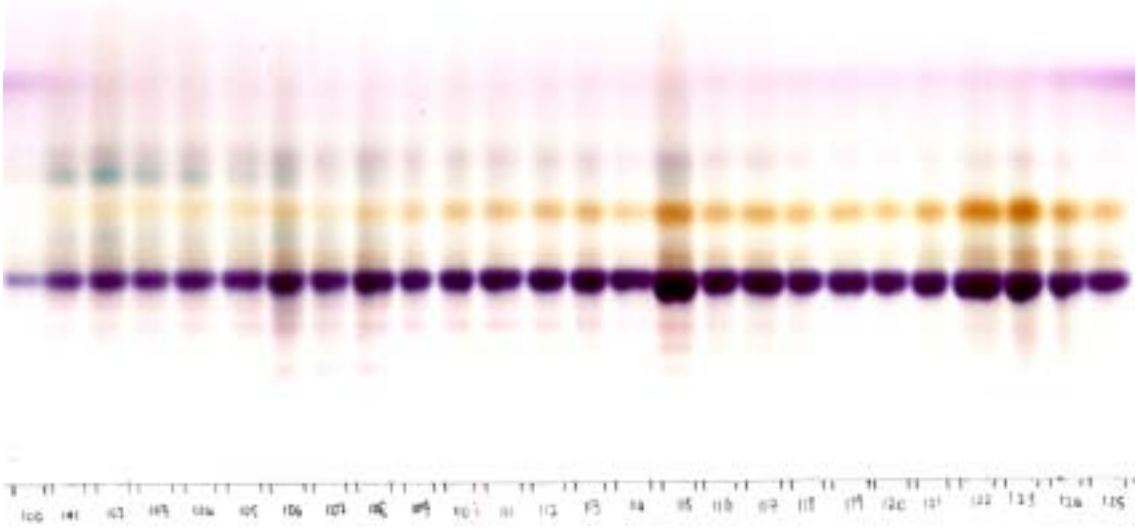
A



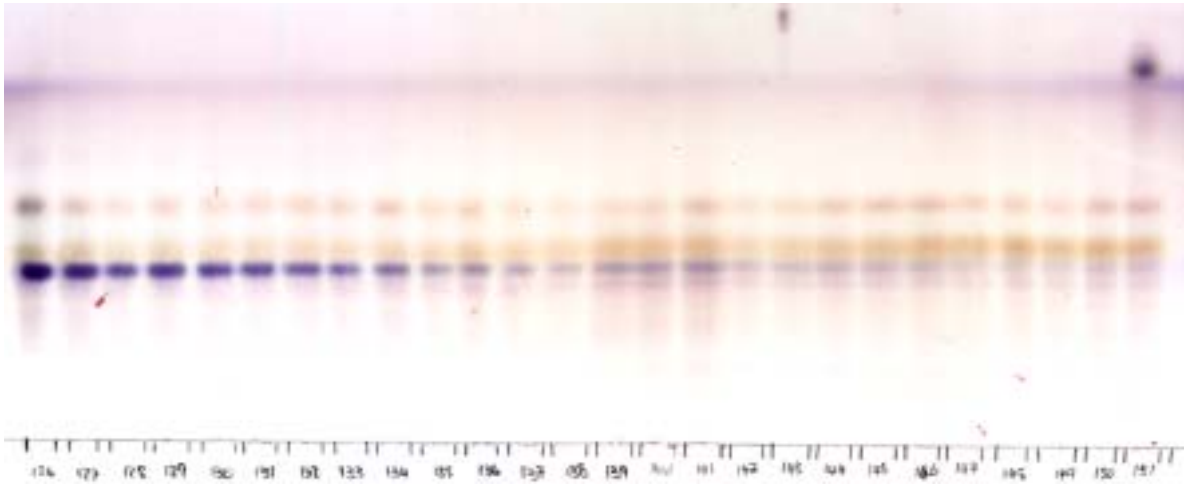
B



C



D



E

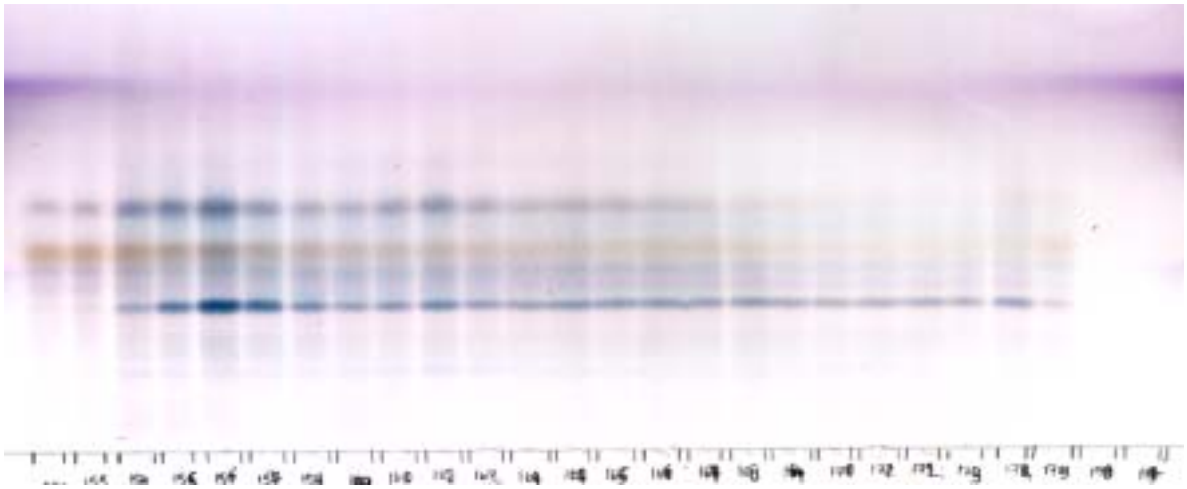


Figure 5.5: TLC of hexane fractions collected after passing through a silica gel column. A depicts the first 50 samples eluted with BEA, while B (52-91), C (100-125), D (126-151) and E (152-178) are eluted with CEF. All plates were sprayed with vanillin.

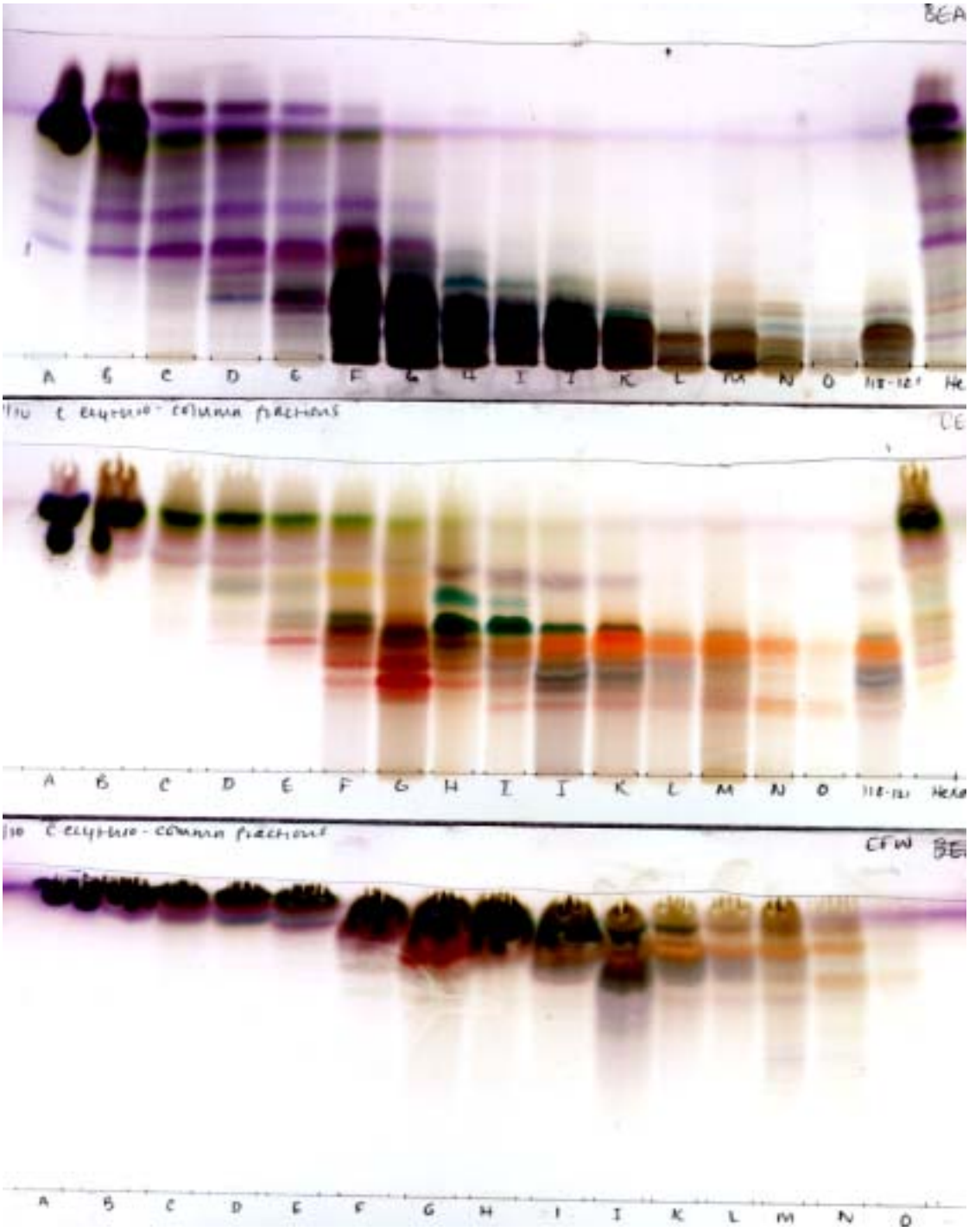


Figure 5.6: TLC of the fractions, which were combined according to the TLC results depicted in Figure 5.5. From the top are the fractions eluted with BEA, CEF and EMW and sprayed with vanillin.

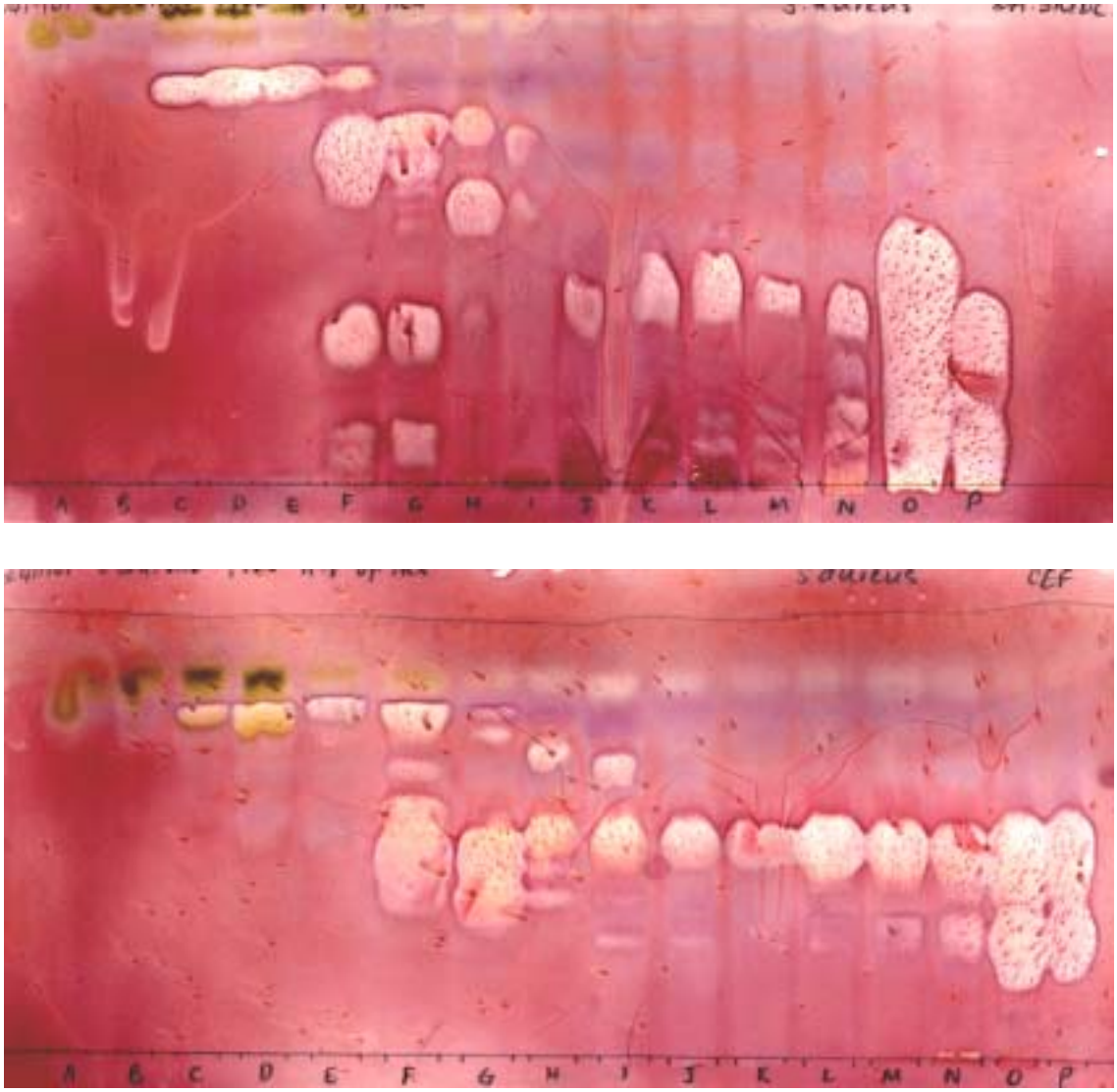


Figure 5.7: Bioautography of fractions A-P using *S. aureus* as test organism and two different mobile phases, i.e. 2A:3MDC (top) and CEF (bottom).

5.3.1.2 Separation of fraction C

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

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

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

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

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

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

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

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

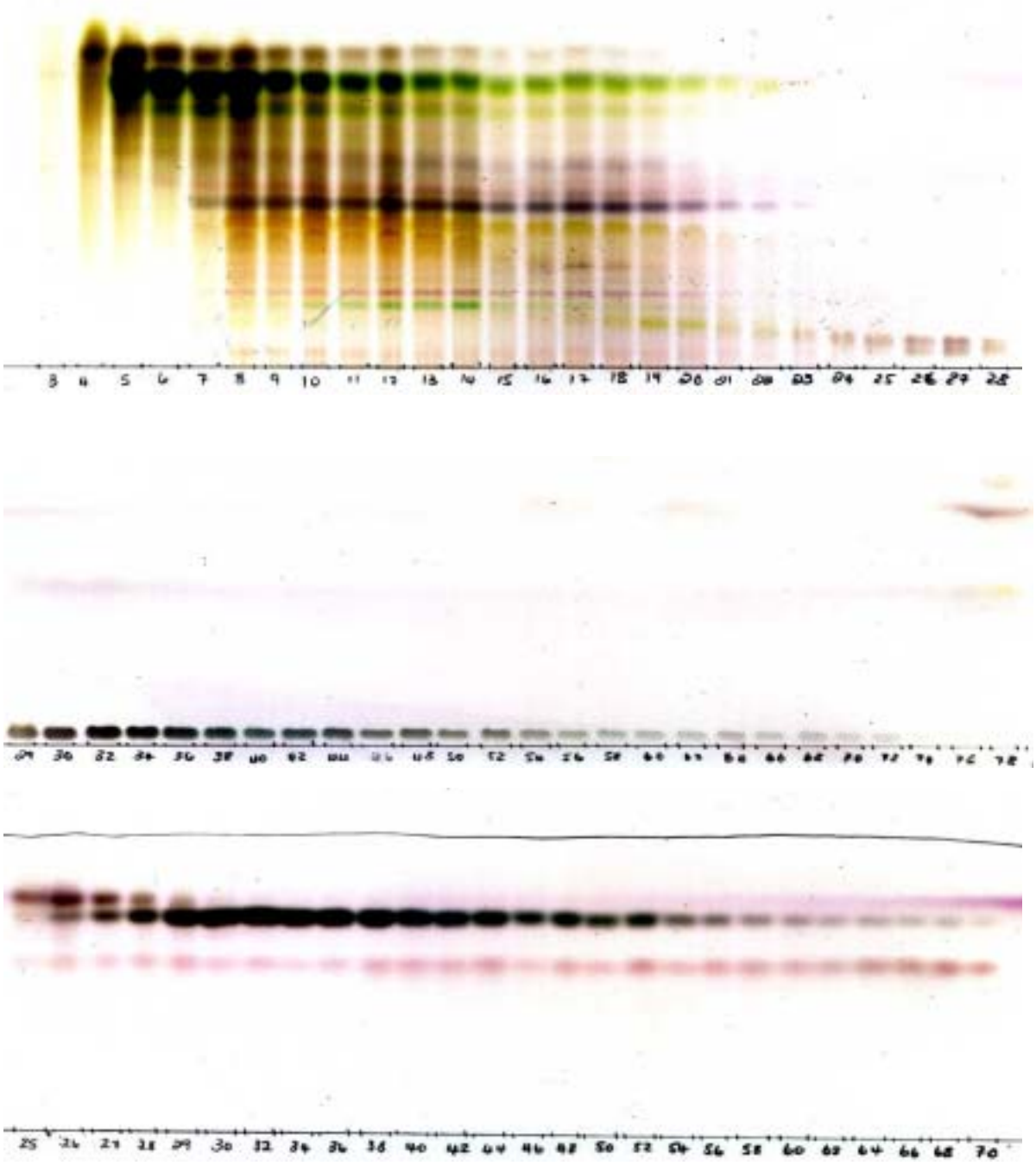


Figure 5.8: TLC of fraction collected from column chromatography of sample C. First 28 samples were eluted using BEA (top) and the rest with both BEA and CEF (middle and bottom) and sprayed with vanillin.

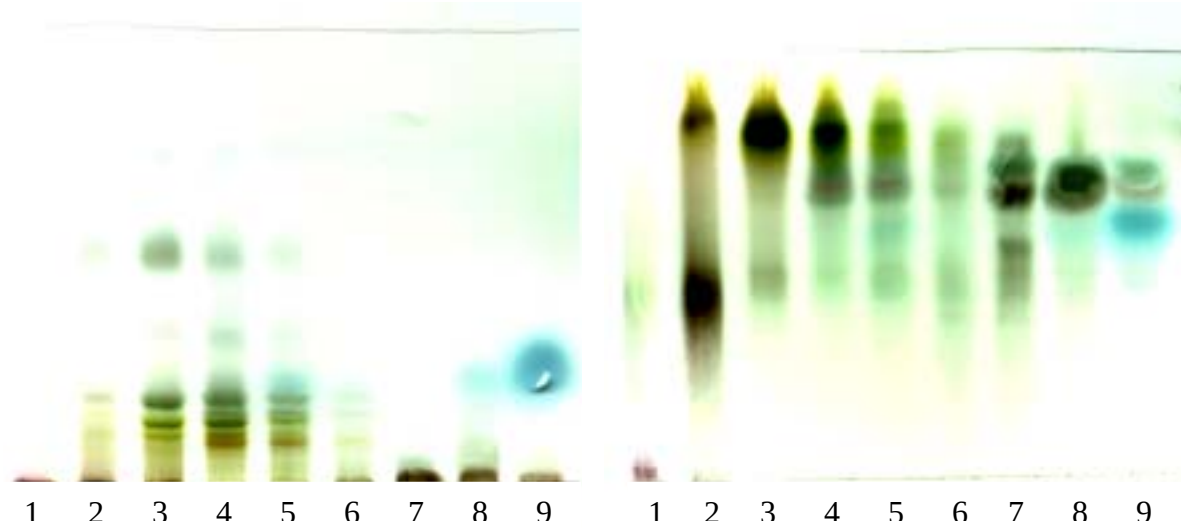


Figure 5.9: Combination of the fractions shown in figure 5.8 into 9 fractions. The TLCs show the fractions eluted with BEA (left) and CEF (right) and sprayed with vanillin.

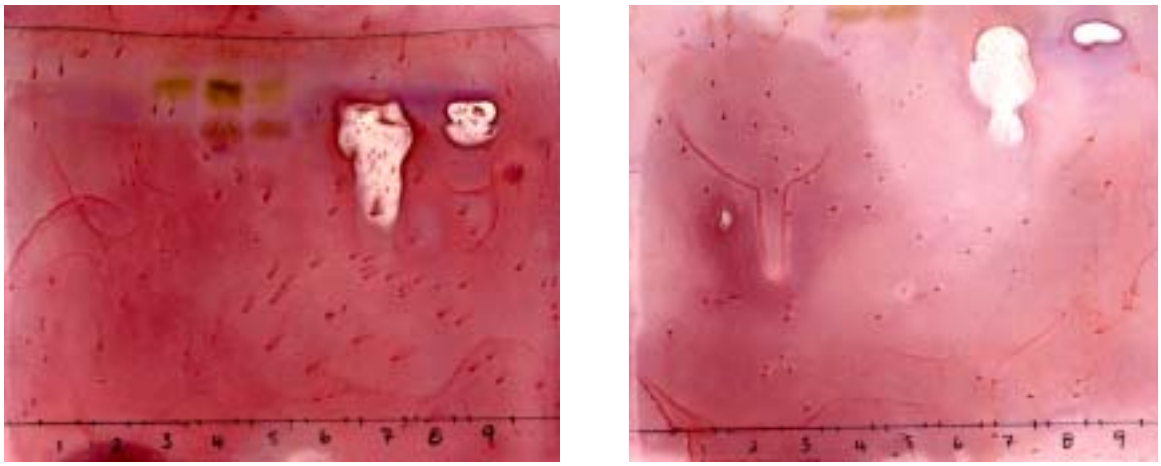


Figure 5.10: Bioautography showing inhibitory compounds of fractions 1-9 eluted with CEF (left) and 2A:3MDC (right). Although depicted on the plate, fractions 2 and 8 were not included as they had already been used for further analysis.

5.3.1.3 Preparative TLC of fraction 2C

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

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

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

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

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

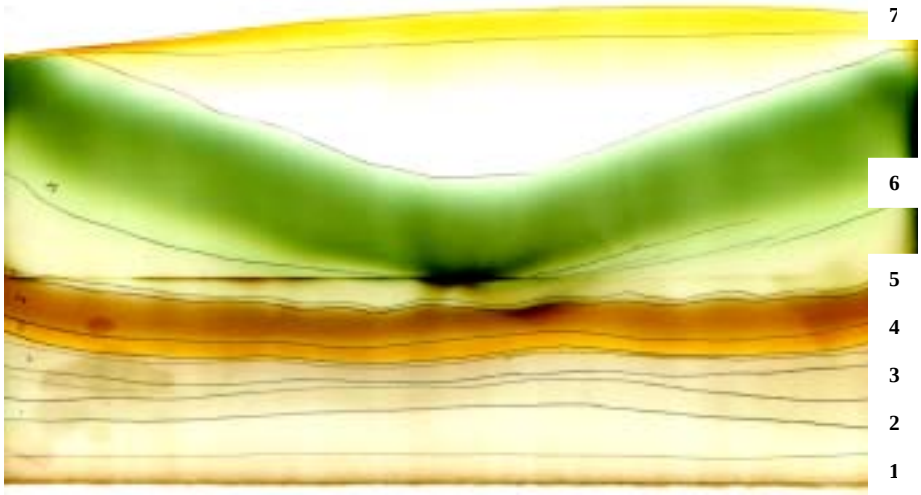


Figure 5.11: Preparative TLC of fraction 2C eluted with CEF. Compounds marked off were seen under both UV and visible light.

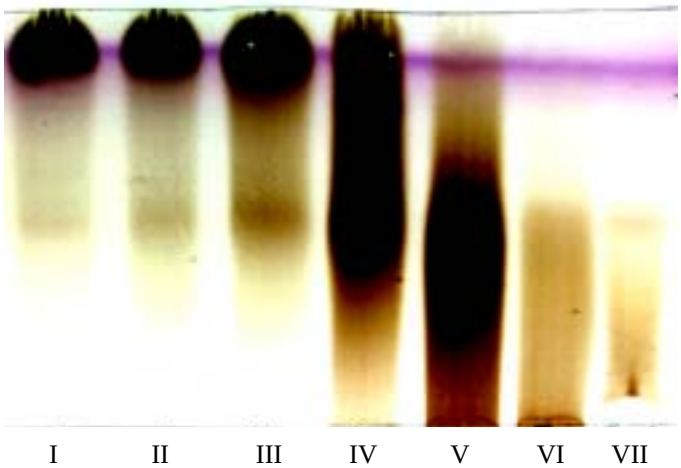


Figure 5.12: TLC of fractions I – VII after PTLC, eluted with CEF and sprayed with vanillin

5.3.1.4 Analysis of fraction 8C by GC-MS

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

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

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

5.3.1.5 Toyopearl separation

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

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

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

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

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

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

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

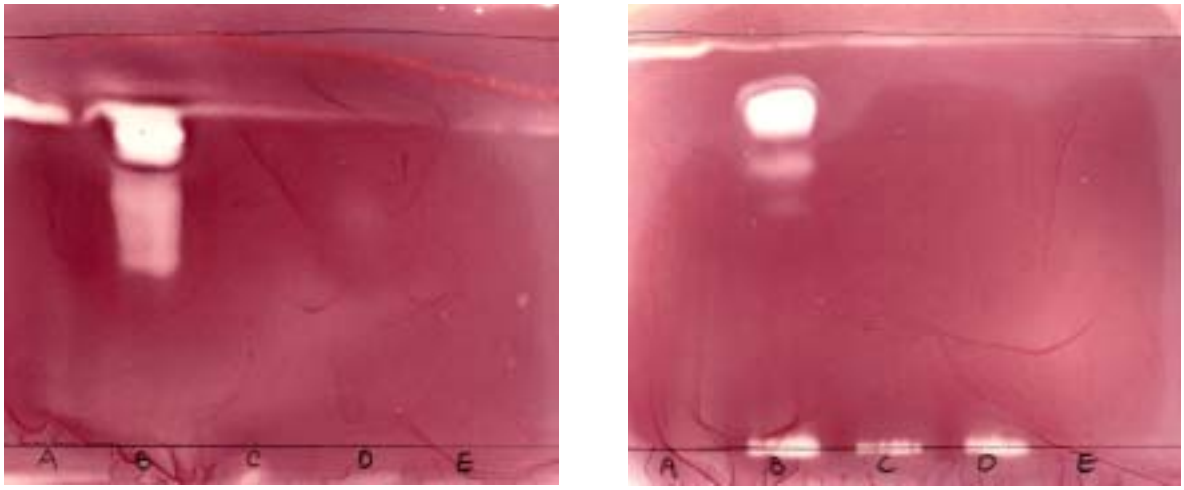


Figure 5.13: Bioautography of samples A1-E5 using *S. aureus* as test organism and eluting samples with CEF (left) and 2A:3MDC (right)

5.3.1.6 HPLC and preparative HPLC of fraction B2

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

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

5.3.1.7 Preparative TLC of fraction C3

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

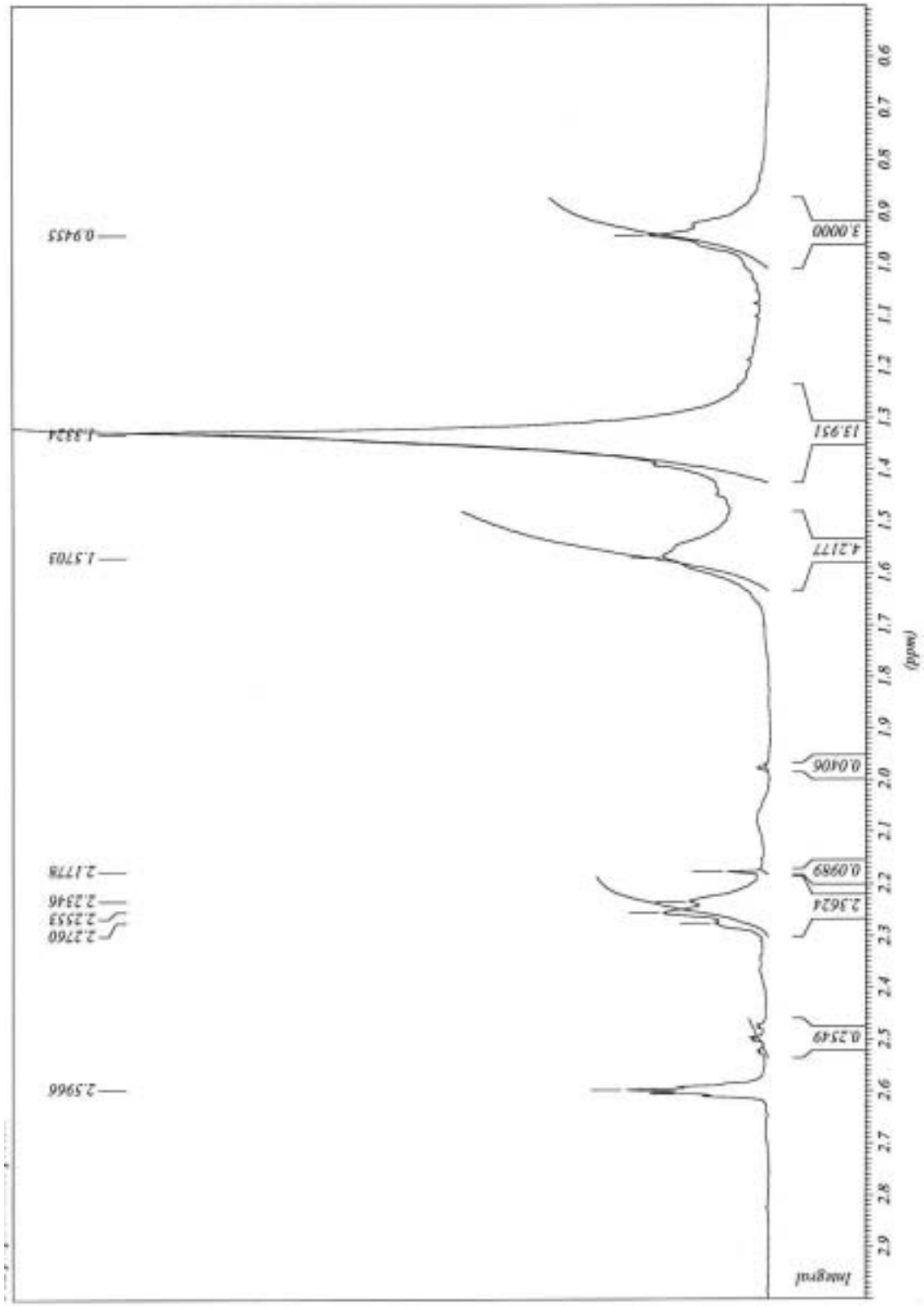
5.3.1.8 NMR of fraction B2 and C3

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

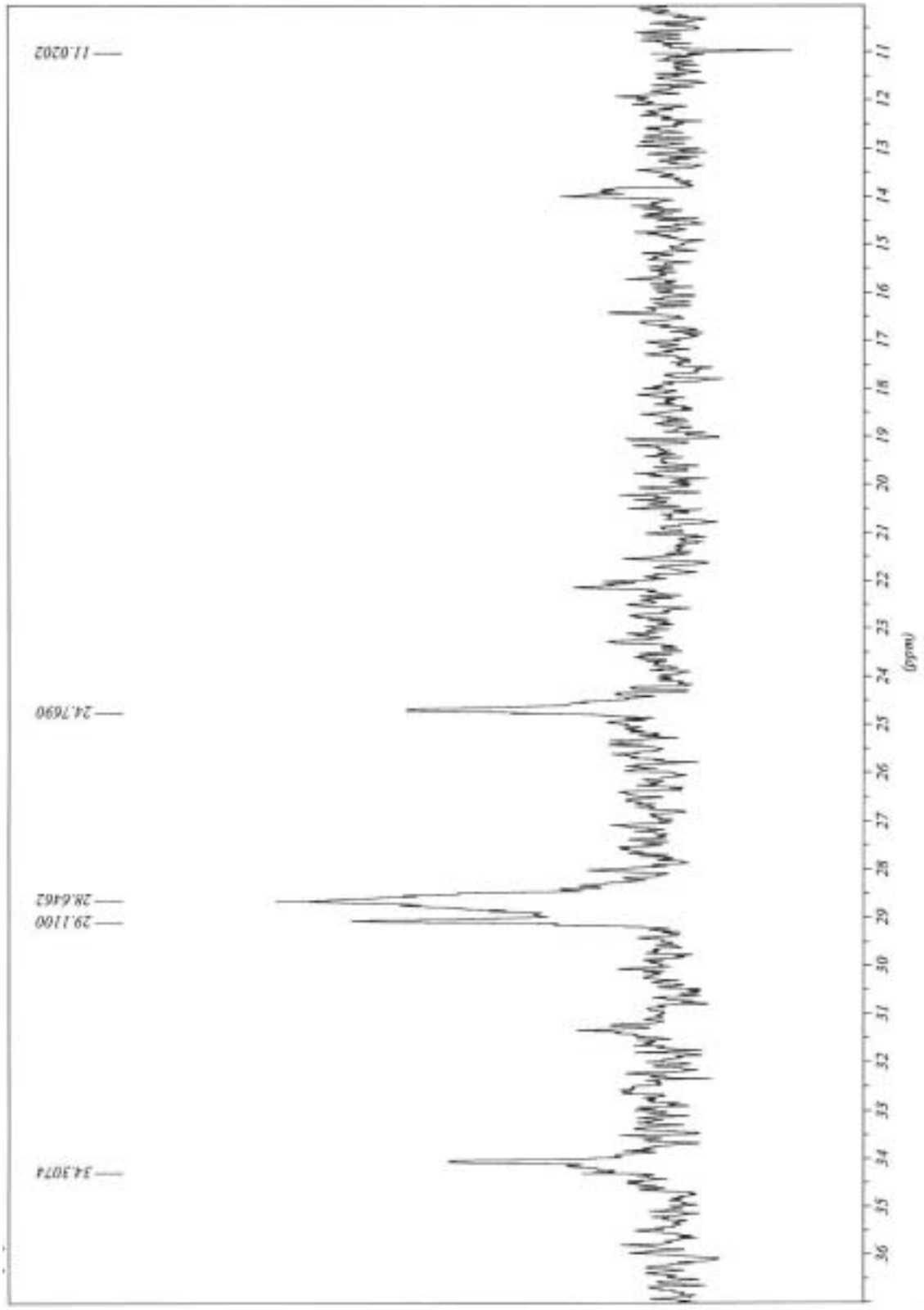
Fraction C3 was dissolved in deuterated DMSO and ran at 300 MHz but once again the ^{1}H NMR exhibited many peaks of which none could identify the compound. All that could be deduced from this spectrum was that this was an alkane (Spectrum 5.1). ^{13}C NMR was very noisy and could not help in the identification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

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

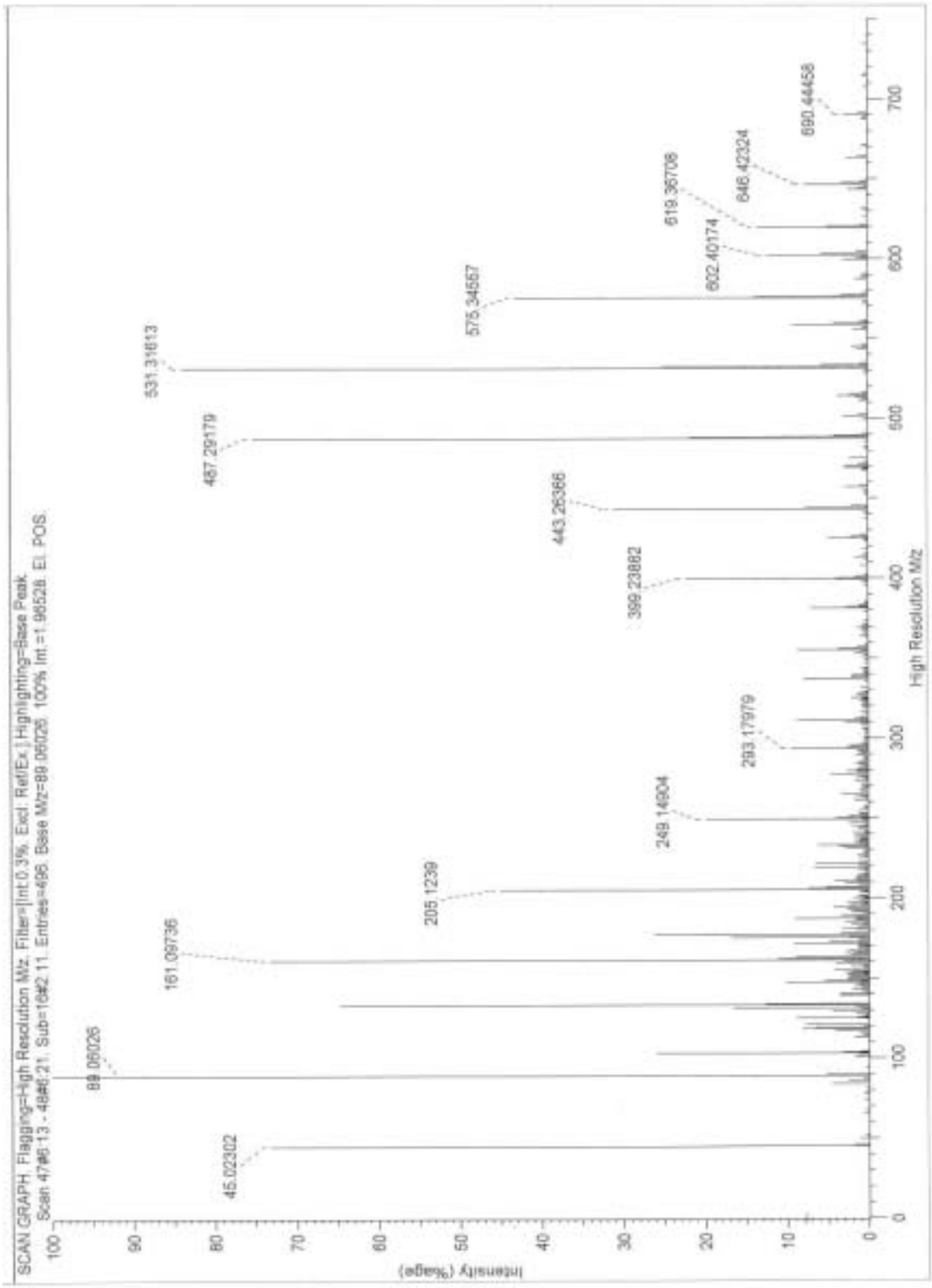
Other fractions that were collected from the hexane column chromatography, were sent for NMR analysis. They were all suggestive of long chain aliphatic compounds. No follow up work was done due to lack of time. Selected spectra are shown in Appendix C.



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



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



Spectrum 5.3: HREIMS of C3

5.3.2 Chloroform fraction

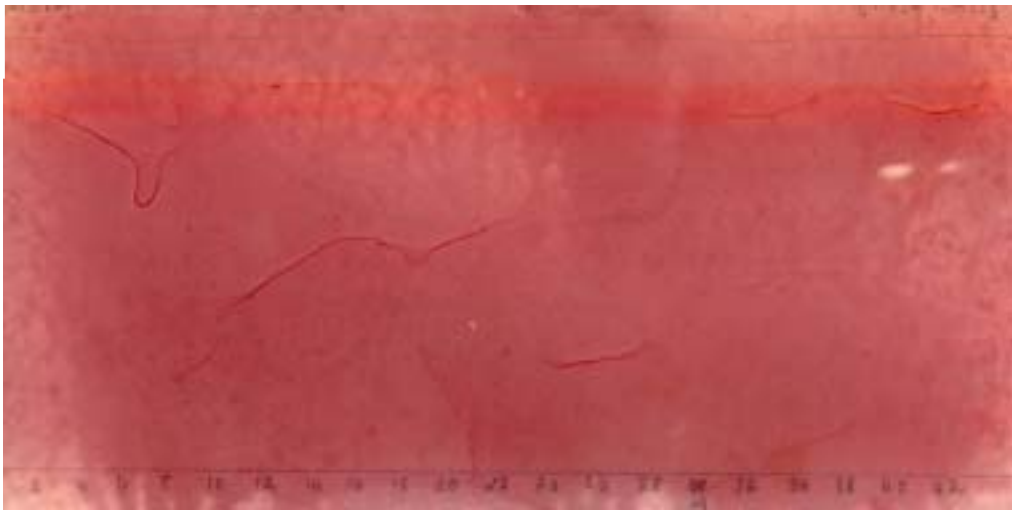
5.3.2.1 Column I (CE)

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

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

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

A



B

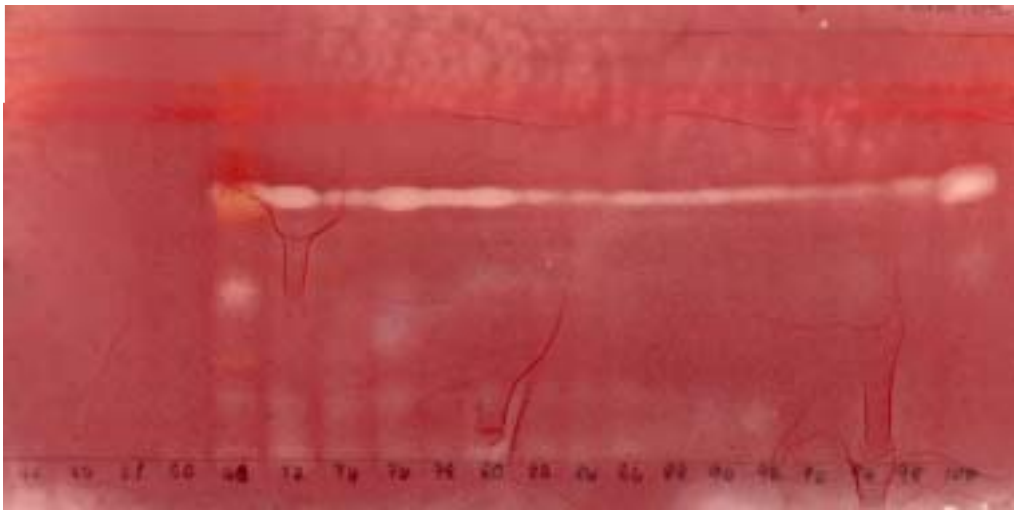
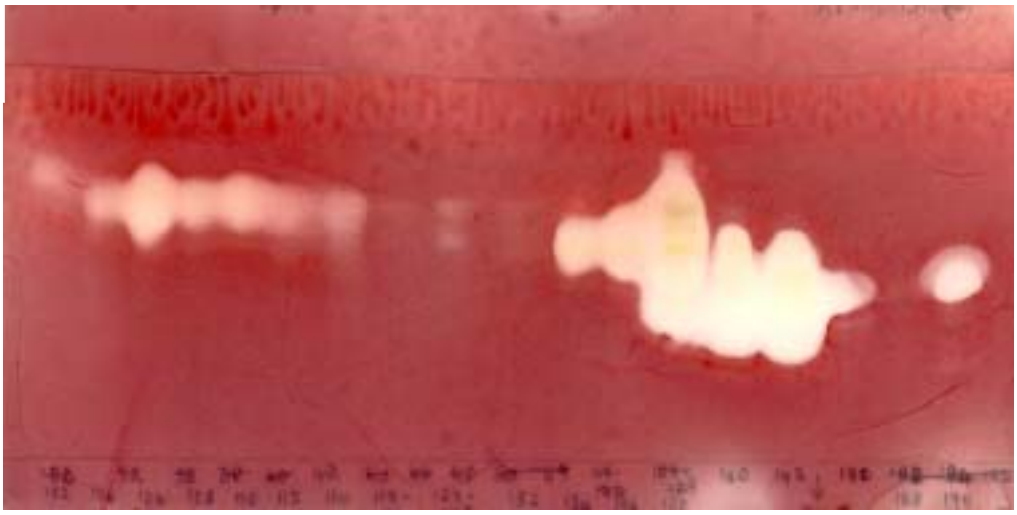


Figure 5.14a: Bioautography of fractions A (2-42) and B (44-100) of Column I eluted with 1% methanol/chloroform and sprayed with *S. aureus*

C



D

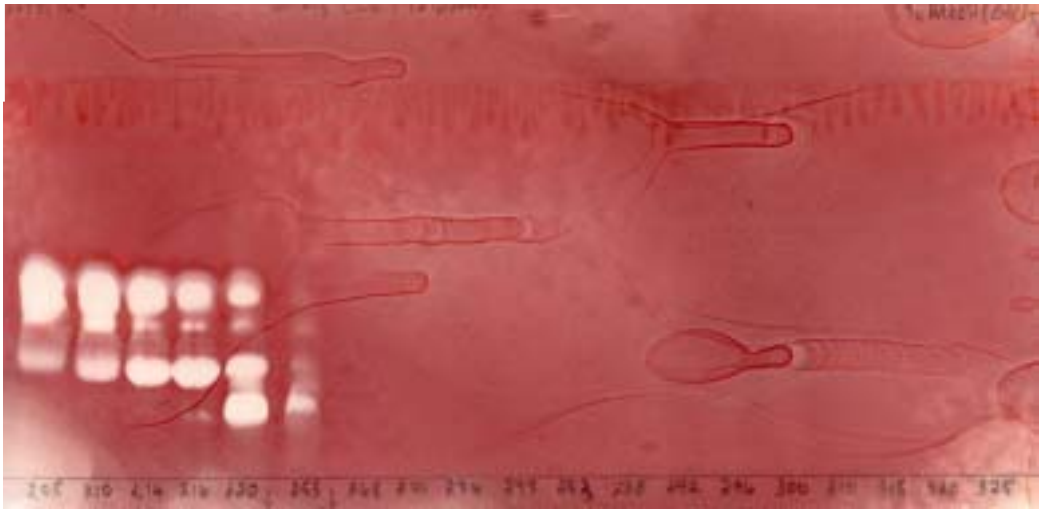


Figure 5.14b: Bioautography of fractions C (102-190) and D (205-325). And eluted with 10% methanol/chloroform and 15% methanol/chloroform respectively.

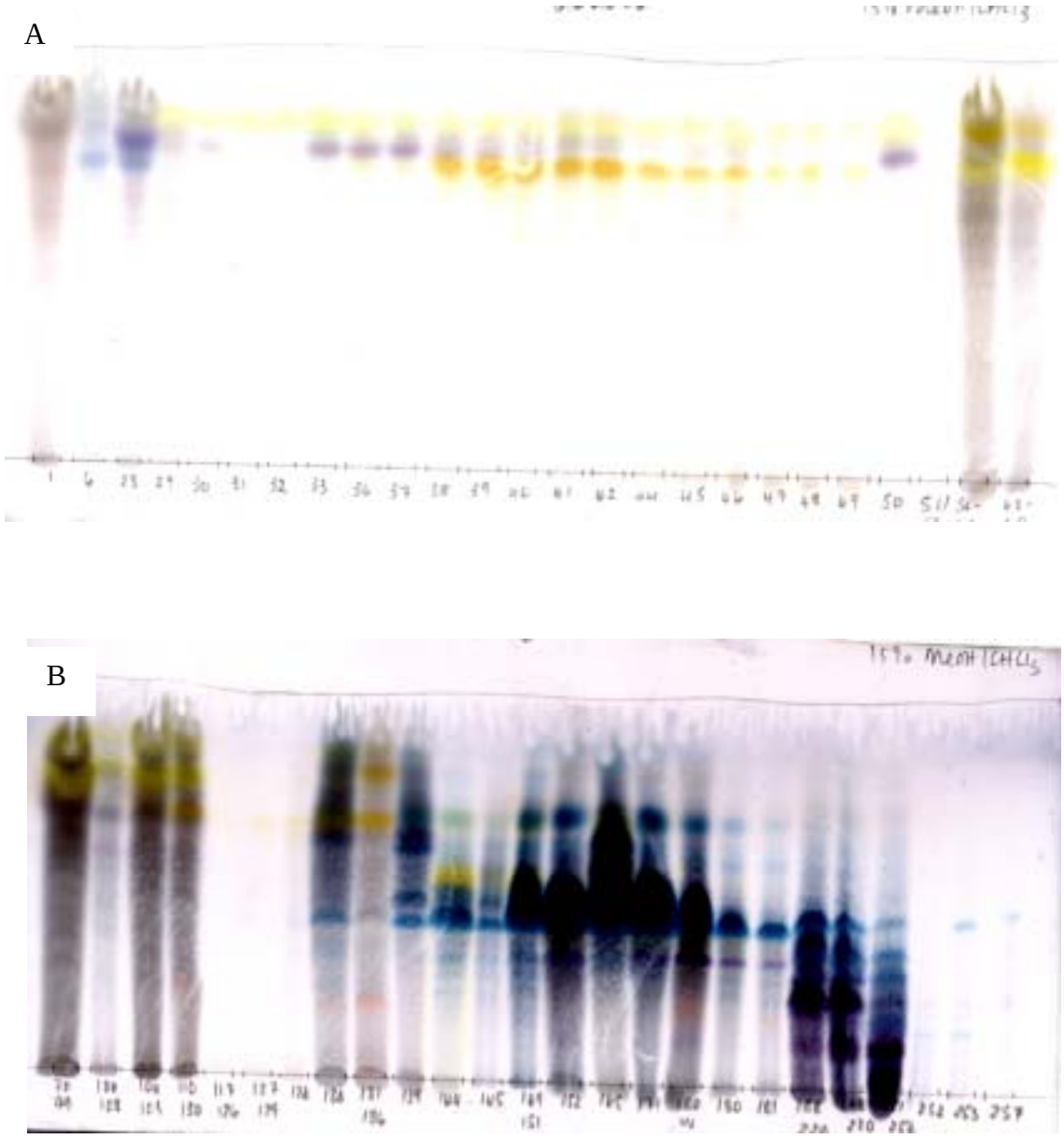


Figure 5.15: TLC of Column I of chloroform fraction. A are the fractions 1-69 and B are fractions 70-259 both eluted with 15% methanol in chloroform.

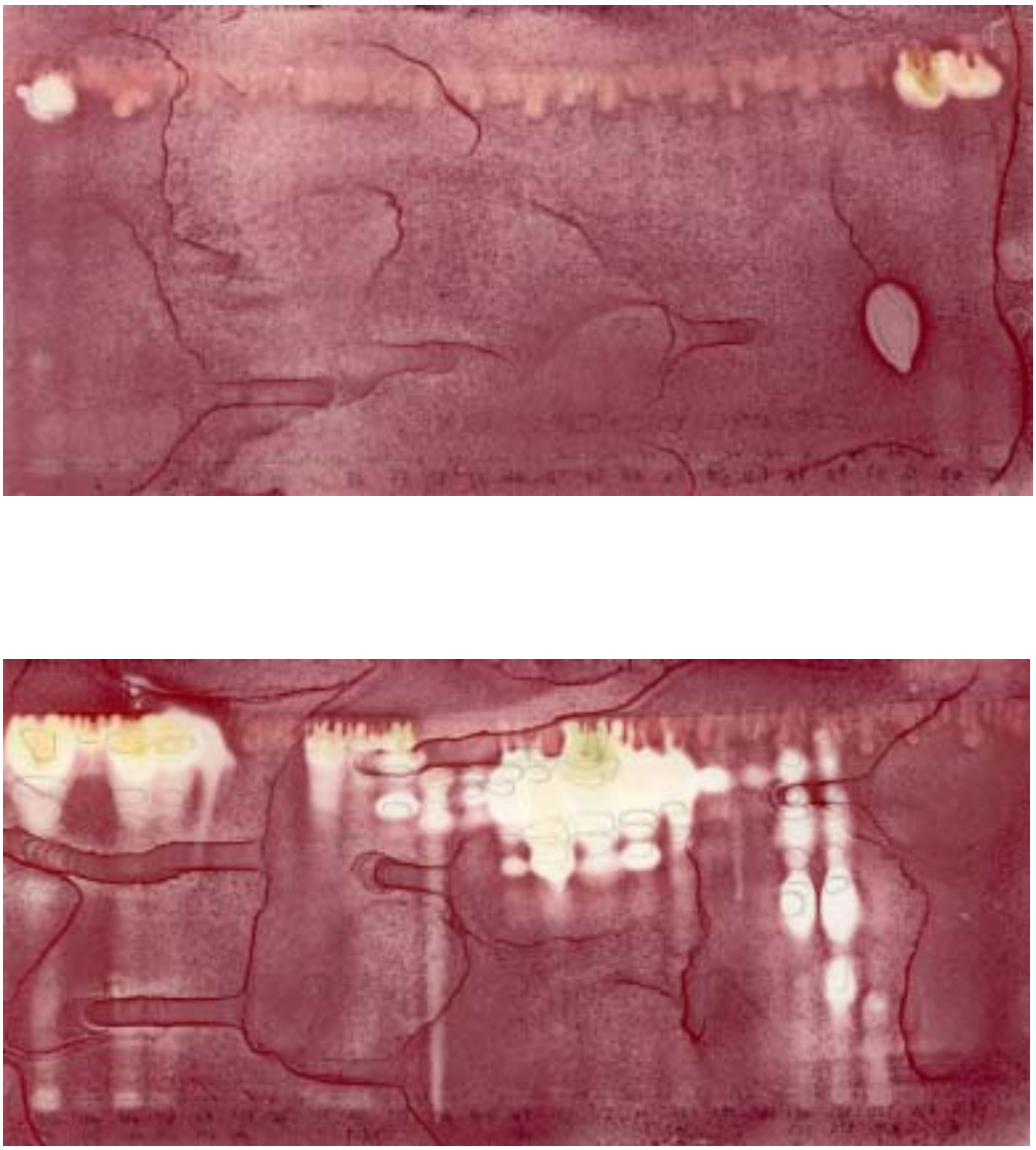


Figure 5.16: Bioautography of the combined fractions of the chloroform column fractionation (fig. 5.15) using 15% methanol/chloroform as eluant.

5.3.2.2 Column II / Sephadex I

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

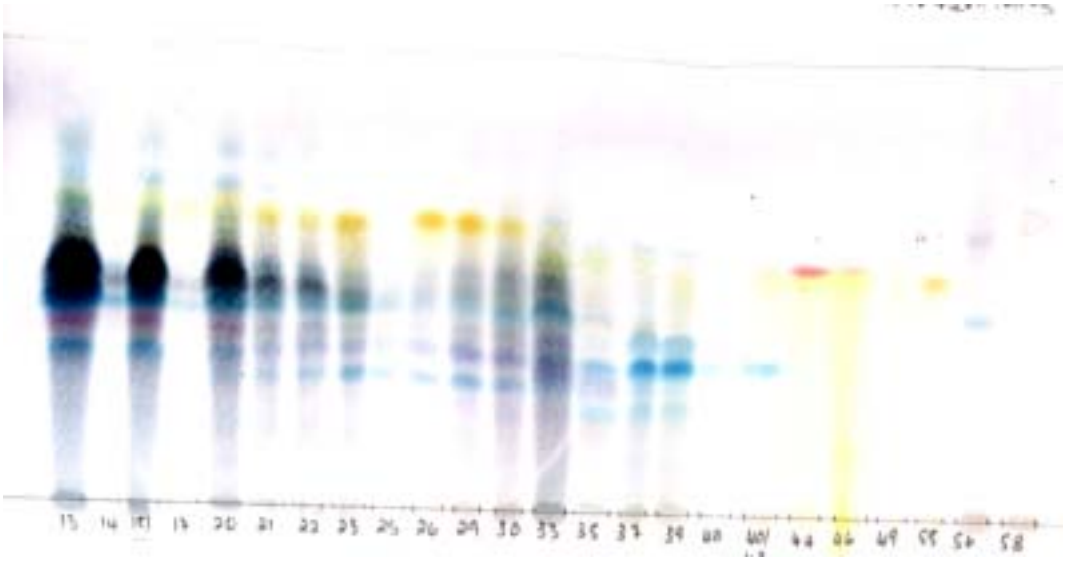


Figure 5.17: TLC of combined fractions of column II using 15% methanol in chloroform as eluant.

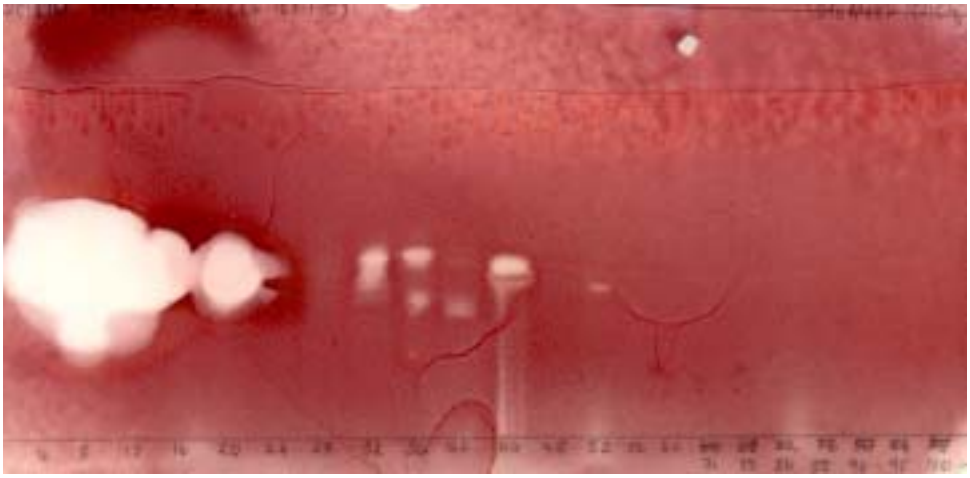


Figure 5.18: Bioautography of random fractions from Column II before combination. Elution was performed using 10% methanol in chloroform.

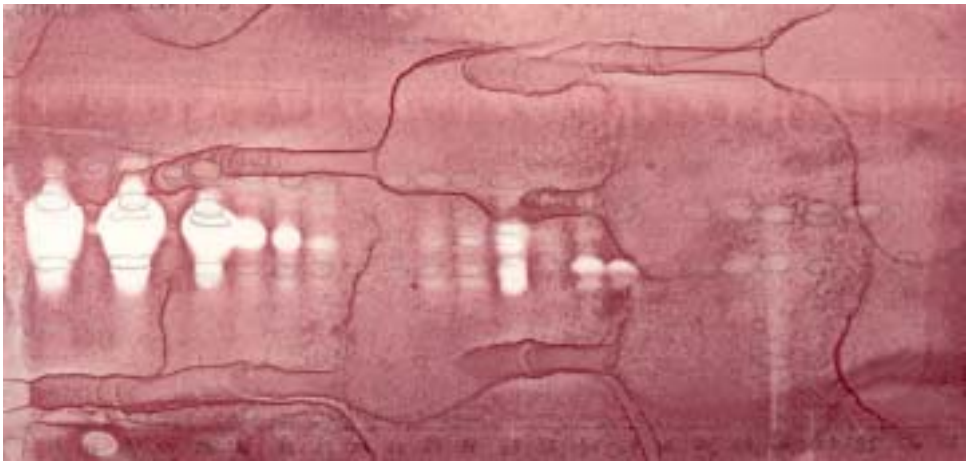


Figure 5.19: Bioautography of combined fractions from Column II coinciding with those of Fig. 5.17. Elution was performed using 15% methanol in chloroform.

5.3.2.3 Column III / Sephadex II

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

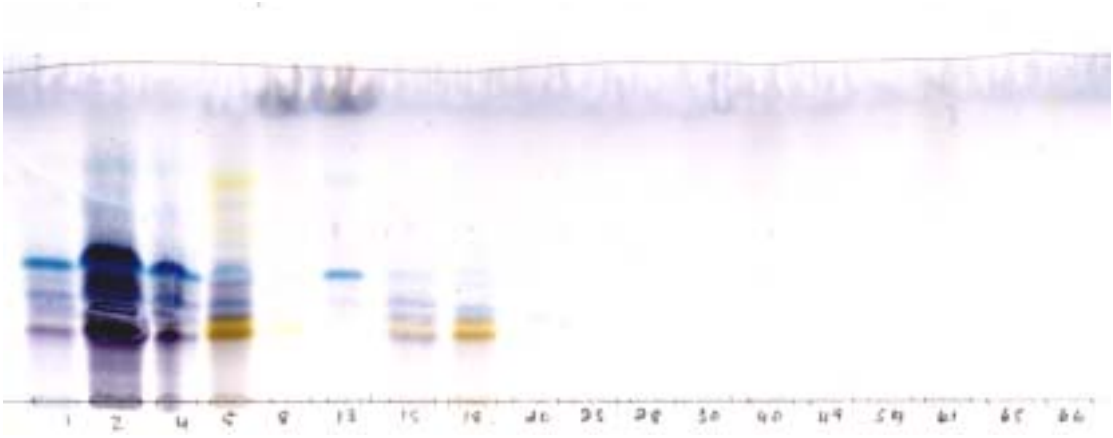


Figure 5.20: TLC of combined Column III fractions eluting with 15% methanol in chloroform.

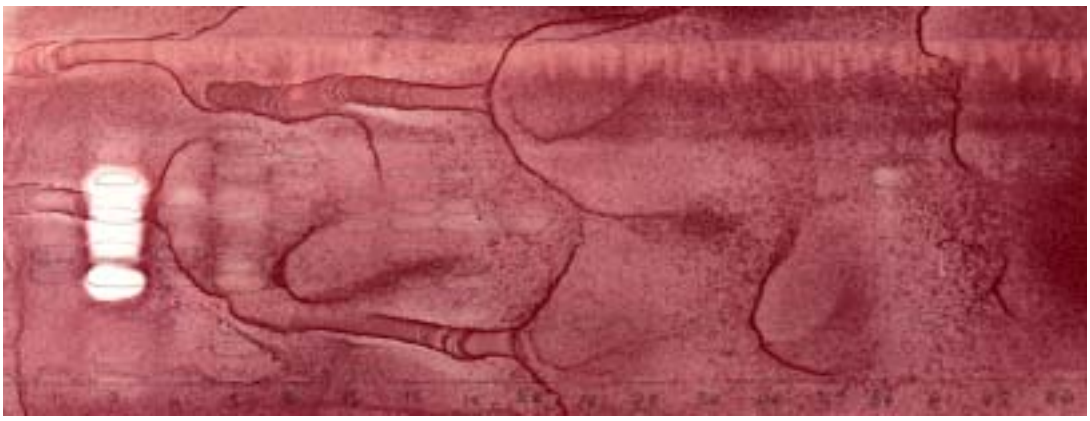


Figure 5.21: Bioautography of the same fractions applied to TLC in Figure 5.21 and eluted with 15% methanol in chloroform.

5.3.2.4 Column IIIa

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

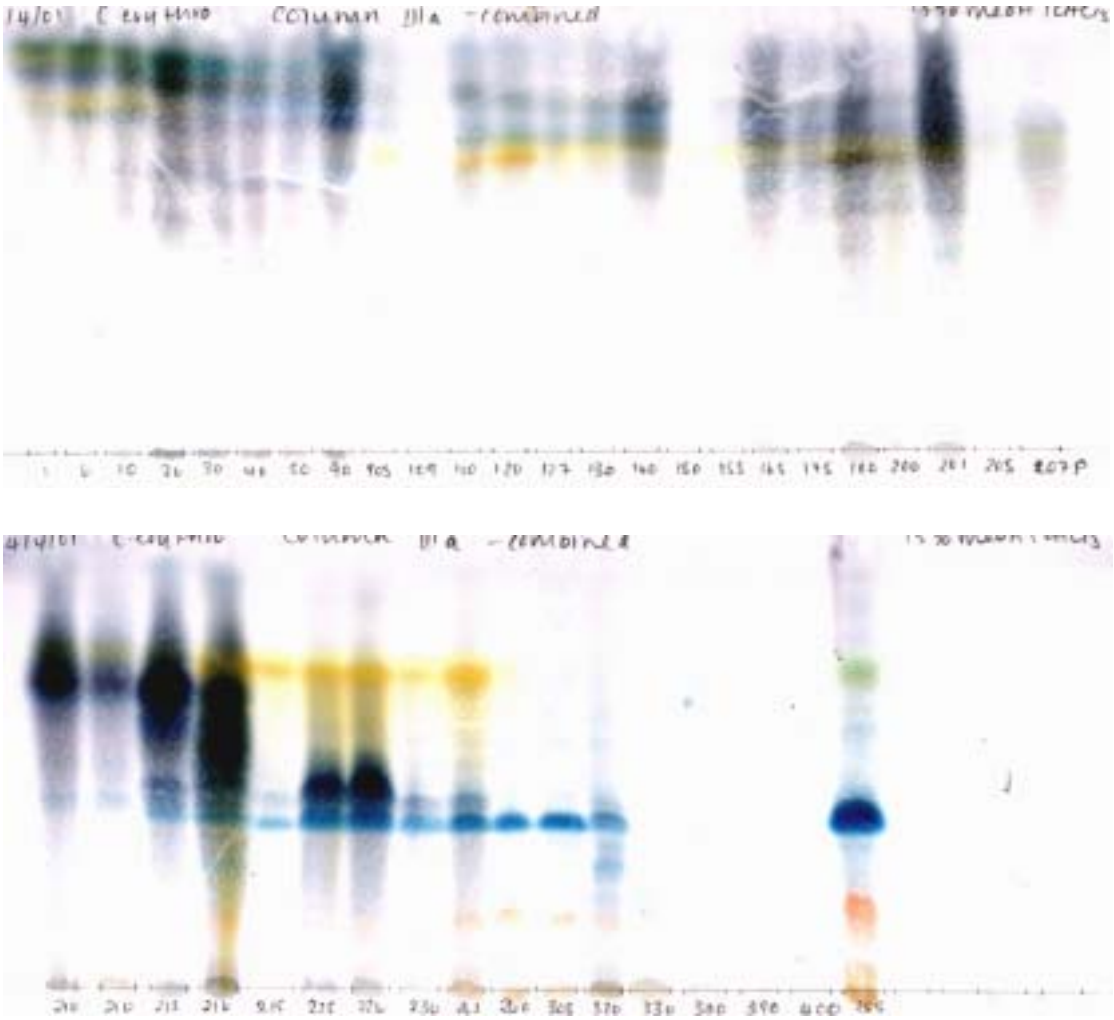


Figure 5.22: TLC of combined fractions of Column IIIa eluted with 15% methanol in chloroform.

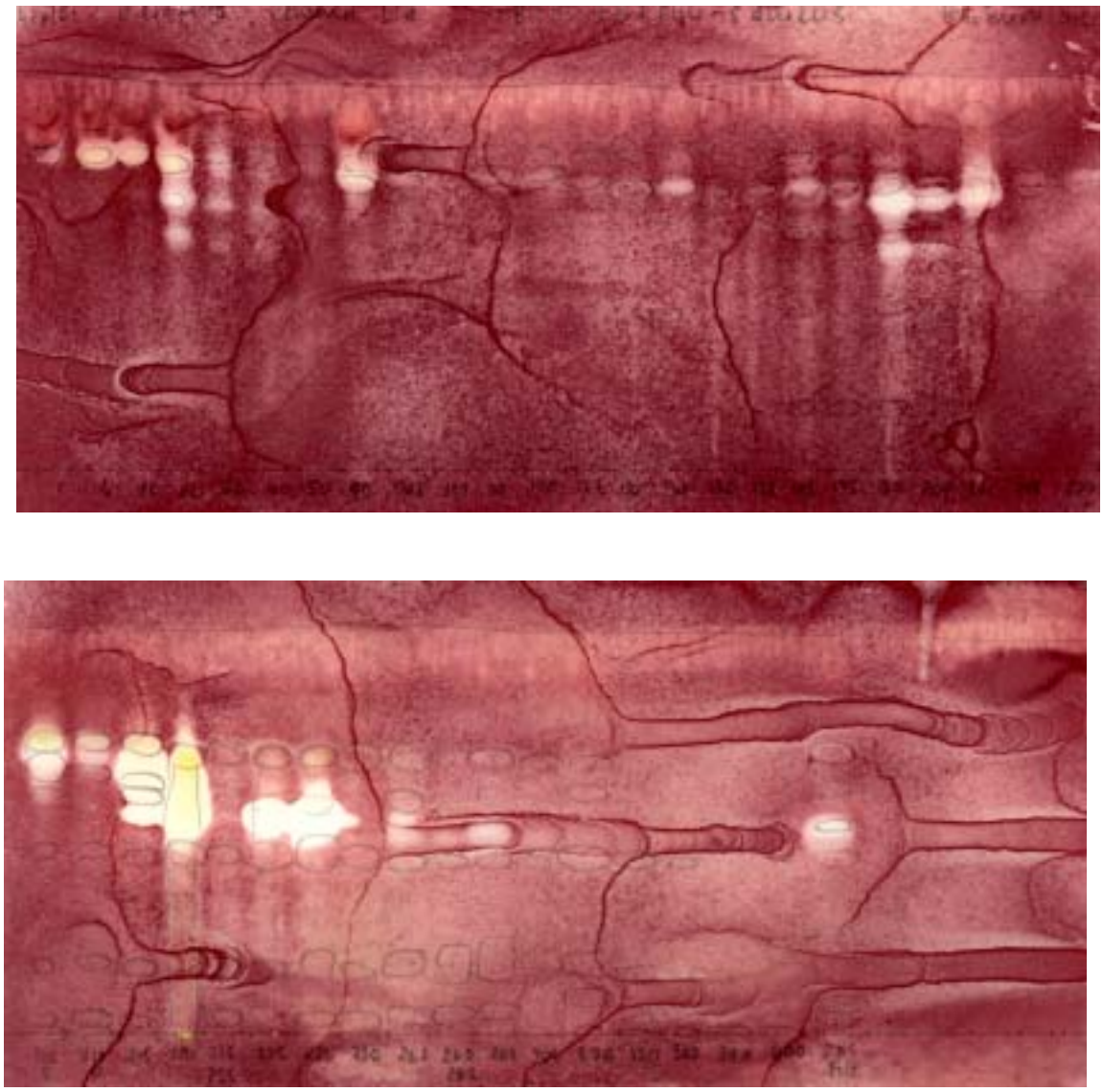


Figure 5.23: Bioautography of combined fractions of Column IIIa corresponding to those shown in Figure 5.22. Elution was with 15% methanol in chloroform.

5.3.2.5 Column IV

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

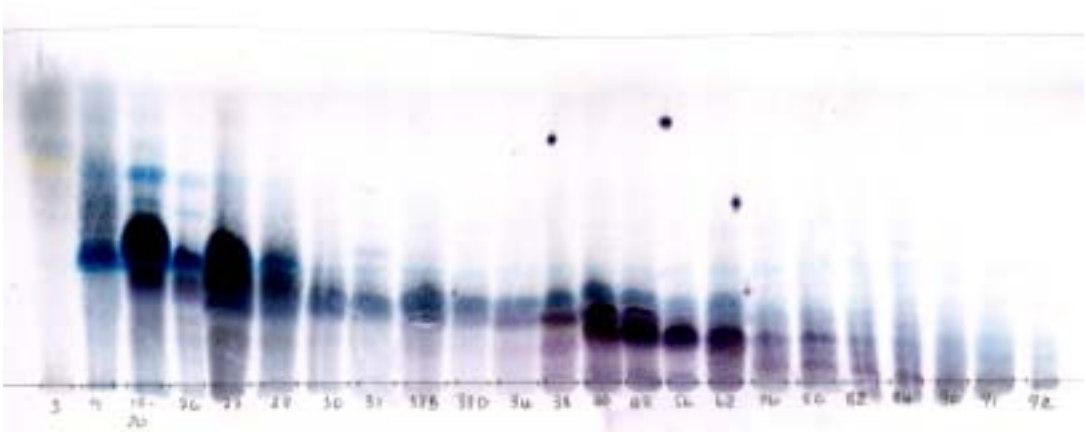


Figure 5.24: TLC of combined fractions of Column IV eluted with 15% methanol in chloroform.

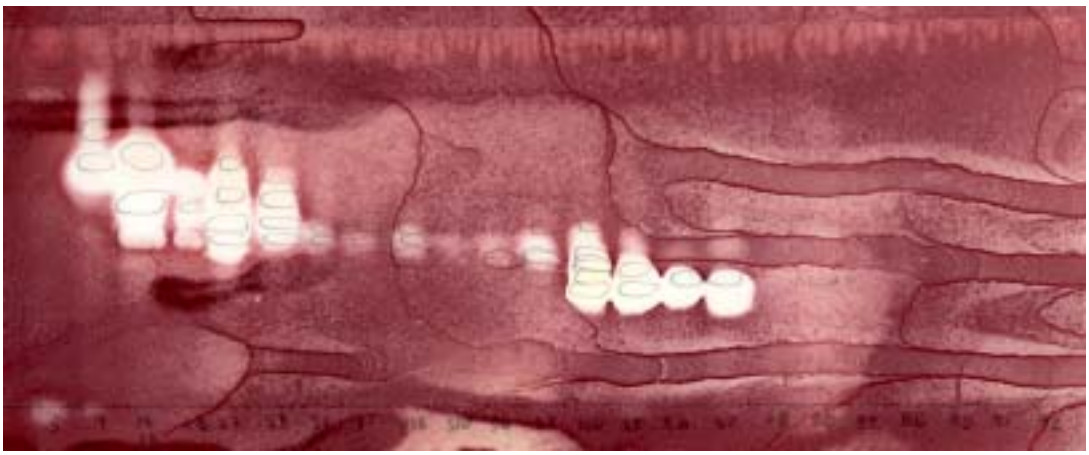


Figure 5.25: Bioautography of combined Column IV fractions corresponding to those shown in Figure 5.24.

5.3.2.6 RP-207S

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

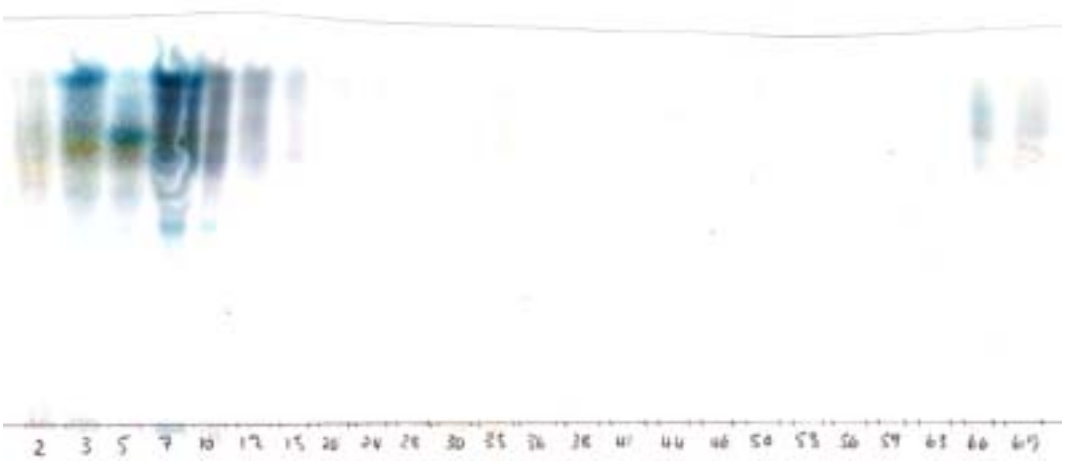


Figure 5.26: TLC of the first few fractions of the RP207S column. Compounds were eluted using 15% methanol in chloroform on normal phase TLC plates.

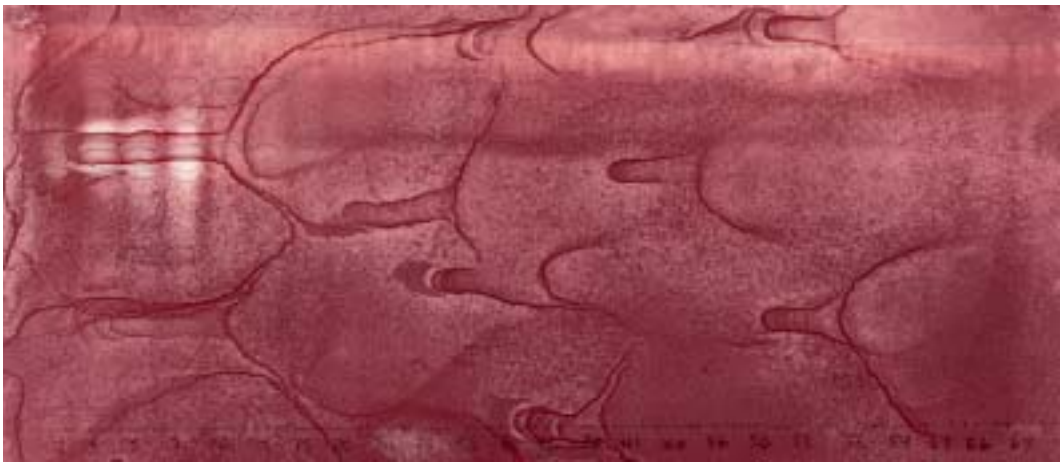


Figure 5.27: Bioautography corresponding to the samples show in Figure 5.26 elutes with 15% methanol in chloroform on normal phase TLC plates.

5.3.2.7 Column 221

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

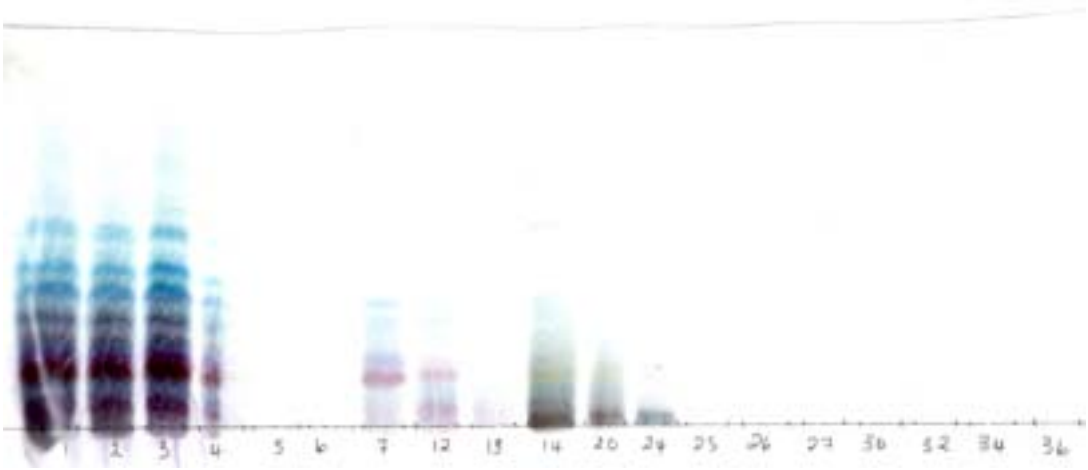


Figure 5.28: TLC of fractions collected from Column 221 and eluted with 15% methanol in chloroform.

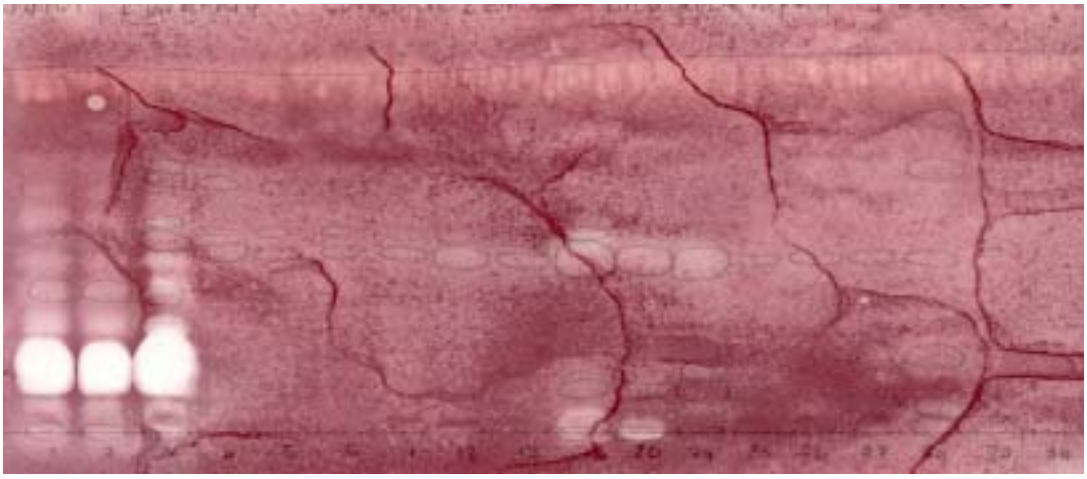


Figure 5.29: Bioautography results corresponding to fractions shown in Figure 5.28 eluted with 15% methanol in chloroform.

5.4 VARIATION IN BIOLOGICAL ACTIVITY

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

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

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

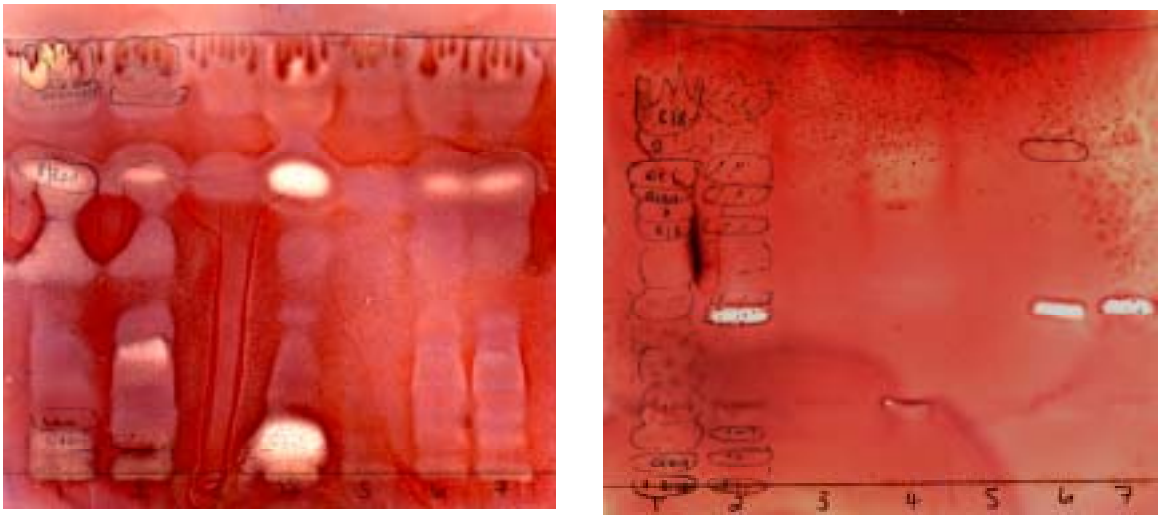


Figure 5.30: Bioautography of *C. erythrophyllum* leaves collected from 7 different plants around Pretoria, eluted with 2A:3MDC (left) and CEF (right)