CHAPTER 4

RESULTS AND DISCUSSION ON ISOLATION OF COMPOUND

4.1 **Isolation of active compound**

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

4.1.1 Method development

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

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

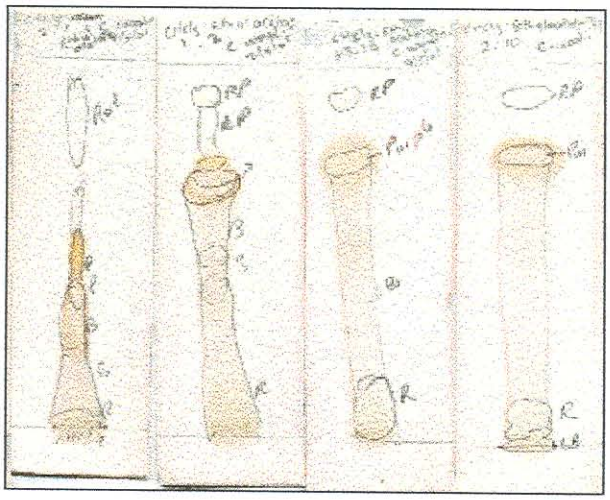

Fig. 33 TLC of chloroform fraction using different ratios of chloroform and ethyl acetate combination as solvent systems. From left to right, ratios 2:1, 1:2, 1:3, 1:5. Lines indicate fluorescing compounds.

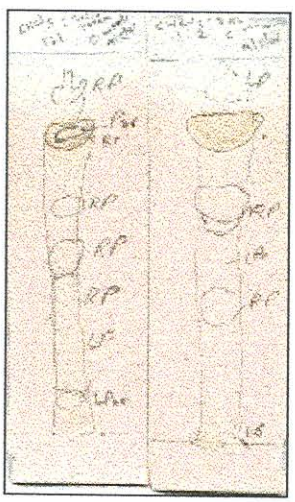

Fig. 34 TLC of chloroform fraction using different ratios of chloroform and tetrahydrofuran combination as solvent systems. From left to right: ratios 1:1, 1:2. Lines represent fluorescing compounds.

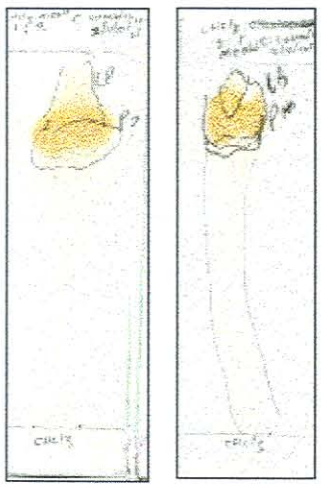

Fig. 35 TLC of chloroform fraction using different ratios of chloroform and methanol combination as solvent systems. From left to right: ratios 1:2, 2:1. Lines represent fluorescing compounds.

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

4.1.2 Column chromatography of chosen fraction

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

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

4.1.3 Analysis of collected fractions by TLC

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

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

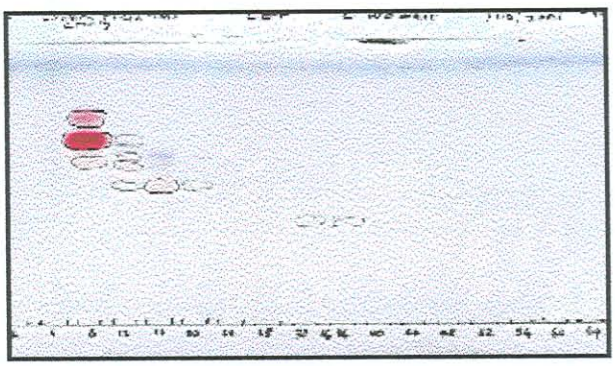

Fig. 36 TLC of every fourth collected fraction separated by CEF and sprayed with vanillin-sulphuric acid reagent. Lanes from left to right: fractions 4, 8,…, 60, 64.

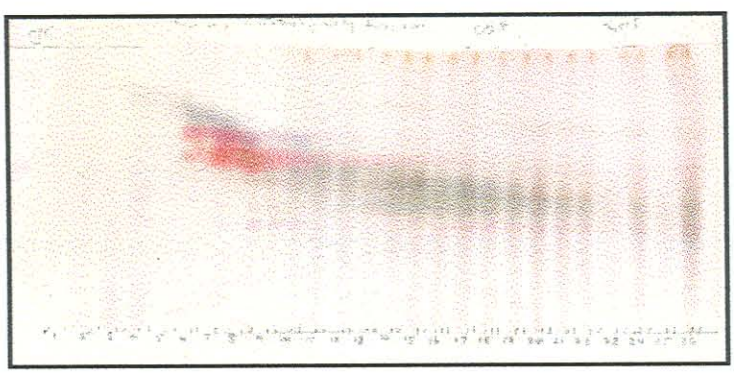

Fig. 37 TLC of collected fractions separated by CEF and sprayed with vanillin-sulphuric acid reagent. Lanes from left to right: fractions 1, 2, 3, …, 24, 25.

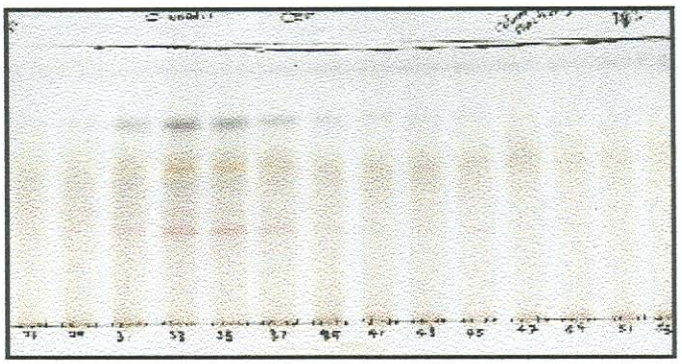

Fig. 38 TLC of collected fractions separated by CEF and sprayed with vanillin-sulphuric acid reagent. Lanes from left to right: fractions 26, 27, 28, …., 53, 54.

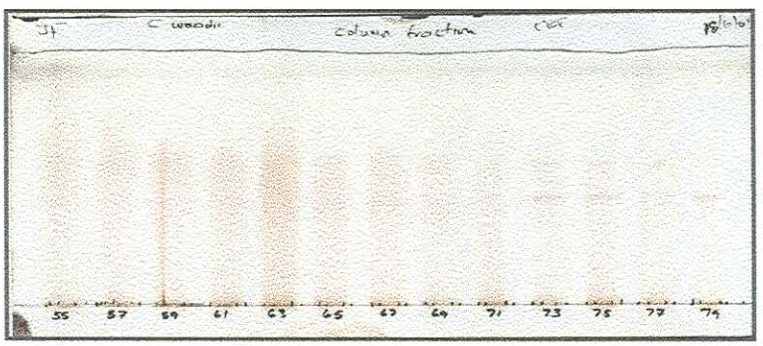

Fig. 39 TLC of column chromatography fractions separated by CEF and sprayed with vanillin-sulphuric acid reagent. Lanes from left to right: fractions from 55, 56, …, 78, 79.

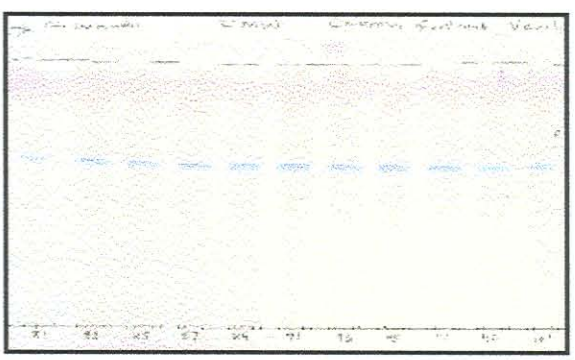

Fig. 40 TLC of collected fractions separated by EMW and sprayed with vanillin-sulphuric acid reagent. Lanes from left to right: fractions 81, 82,…, 100, 101.

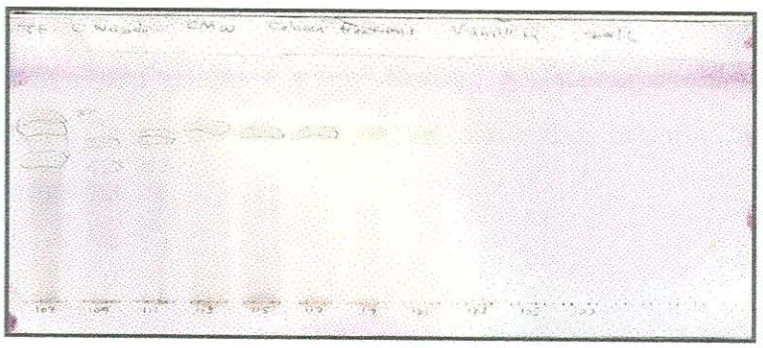

Fig. 41 TLC of collected fractions separated by EMW and sprayed with vanillin-sulphuric acid reagent. Lanes from left to right: fractions 107, 108,..., 122, 123.

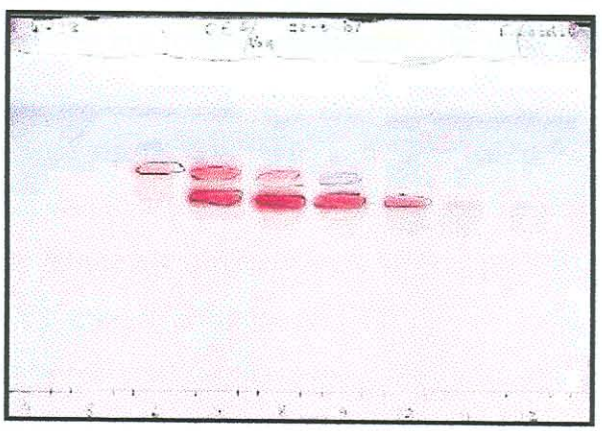

Fig. 42 TLC of collected fractions separated by CEF and sprayed with a vanillin-sulphuric acid reagent. Lanes from left to right: fractions 4, 5, ..., 11, 12.

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

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

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

4.1.4 Combination of collected fractions

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

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

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

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

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

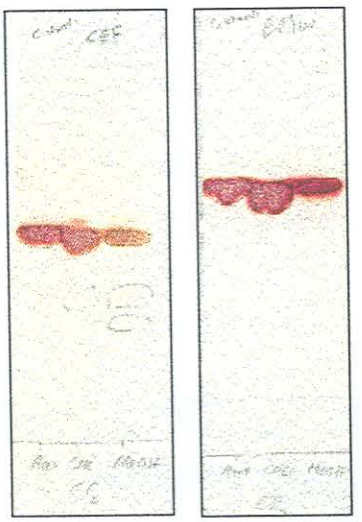

Fig. 43 TLC of some of CF2 that dissolved in three different solvents using CEF (left) and EMW (right) as the solvent systems. From left to right: acetone (ACN), chloroform (CHC), and methanol (MEOH) components. Lines indicate fluorescing compounds.

4.1.5. Analysis of CF1 by TLC and Column chromatography

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

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

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

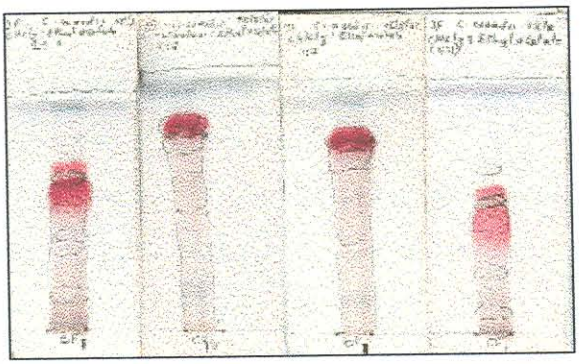

Fig. 44 Separation of CF1 by different solvent systems using vanillin-sulphuric acid as the spray reagent. The TLC plates from left to right were in developed in different chloroform-ethyl acetate combinations (2:1, 1:5, 1:3, 4:1).

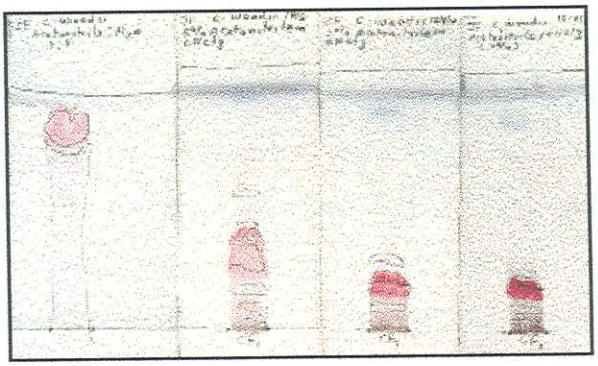

Fig. 45 Separation of CF1 by different solvent systems using vanillin-sulphuric acid as the spray reagent. The TLC plates from left to right were developed in acetonitrile:water (1:1), 5% acetonitrile in water, 2% acetonitrile in water, and 1% acetonitrile in water. Lines represent fluorescing compounds.

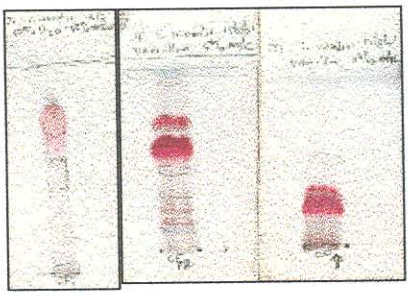

Fig. 46 Separation of CF1 by different solvent systems using vanillin-sulphuric acid as the spray reagent. The TLC plates from left to right were developed in methanol: water. (1:1), 5% methanol in chloroform, and 1% methanol in water.

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

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

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

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

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

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

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

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

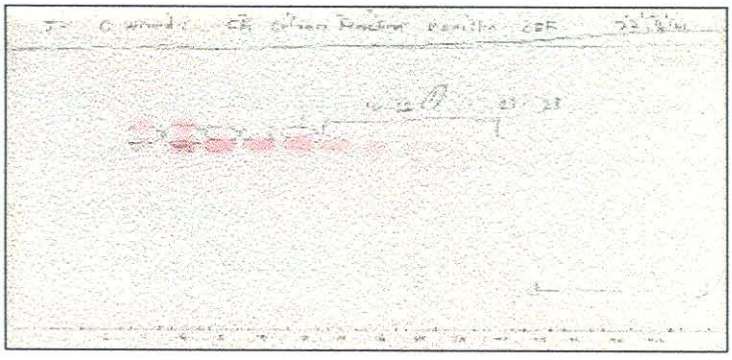

Fig. 47 TLC separation of fractions obtained from column chromatography of CF1 by CEF and using vanillin-sulphuric acid spray reagent. From left to right: fractions 1, 2, …, 29, 30.

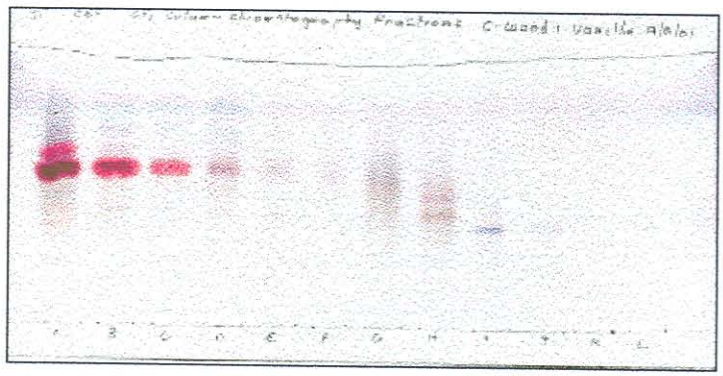

Fig. 48 TLC separation of grouped fractions from column chromatography of CF1 by CEF using vanillin-sulphuric acid spray reagent. From left to right, A, B, …, K, L.

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

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

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

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

4.1.6 Minimum inhibitory concentration of isolated active compound

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

The results are as shown below [Table 15]

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

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

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

4.1.6 Bioautography of isolated compound

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

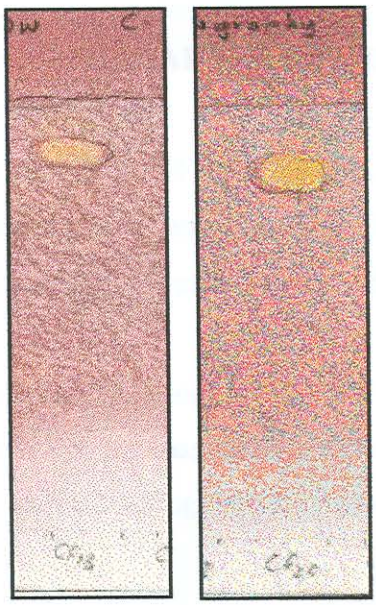

Fig. 49 Bioautograms of CF1b (left) and CF2C (right). TLC developed in EMW and sprayed with actively growing *S. aureus* culture and later with INT. Brownish-yellow colour indicated growth inhibition zone.

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

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

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