

CHAPTER 4

ISOLATION OF ANTIFUNGAL COMPOUNDS FROM *C. dentata* (Burm.f) C.A. Sm.

4.1. INTRODUCTION

4.1.1. Compounds isolated from Cornaceae family

Reports concerning isolation of compounds from *Curtisia dentata* are scanty, if not unpublished. From other members of the family Cornaceae several phenolic compounds, flavonoids and terpenoids have been isolated (Jang *et al.*, 1998; Nakaoki & Moira, 1958; Lee *et al.*, 2000). The genus *Cornus* is well known to contain large amounts of polyphenols such as tannins (Hatano, 1989). Several iridoid compounds were isolated from the roots of *Cornus capitata* (Tanaka *et al.*, 2001). Lee *et al.* (2000) reported the isolation of two phenolic compounds, namely, (-)-2,3-digalloyl-4-(*E*)-caffeoyl-L-threonic acid and (-)-2-galloyl-4-(*E*)-caffeoyl-L-threonic acid from fresh leaves of *Cornus controversa* Hemsl (Cornaceae). Several anthocyanins are also present in *Cornus florida*, *C. controversa*, *C. kousa* and *C. florida* (Vareed *et al.*, 2006).

The broad aim of this study was to isolate compounds active against *Candida albicans* from extracts of *Curtisia dentata*.

The objectives of this section were to:

- 1) Compare the activity the extracts of the leaves and stem bark of *C. dentata* in order to select the plant part to isolate compounds from.
- 2) Isolate active compounds from the leaves through bioassay-guided fractionation.
- 3) Identify the isolated compounds using Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectrometry (MS).

4.2. MATERIALS AND METHODS

4.2.1. General methods

4.2.1.1. Plant part collection

Leaves and stem bark of *C. dentata* were harvested from the University of Pretoria's Botanical Gardens in March 2005, dried at room temperature and ground to powder using a Macsalab mill (Model 200 Lab). The powders were stored in sealed glass containers at room temperature, in the dark until needed.

4.2.1.2. Serial exhaustive fractionation

The leaves and stem bark extracts of *C. dentata* were compared to select the best plant part for isolation of compounds. The leaves contained more active compounds and resulted in better MIC values than the stem bark extracts. For isolation of active compounds, serial exhaustive extraction of the leaves of *C. dentata* leaves (830 g) was performed as outlined in Chapter 2.

4.2.2. Overview of approach followed

The procedure used to isolate active compounds from the leaves of *Curtisia dentata* is presented schematically in Figure 4.1.

4.2.2.1. Selection of stationary phase

Column chromatography using silica gel as the stationary phase was chosen for separation of compounds for the following reasons: 1) silica gel was previously used in the Phytomedicine laboratory for isolation of compounds with a high degree of success (Martini and Eloff, 1998), 2) separation on silica gel represents one of the cheapest methods for isolation of compounds, 3) silica gel is readily accessible and preparation of separating systems is simple and quick, and 4) eluent system polarity can be varied to adjust elution of active compounds.

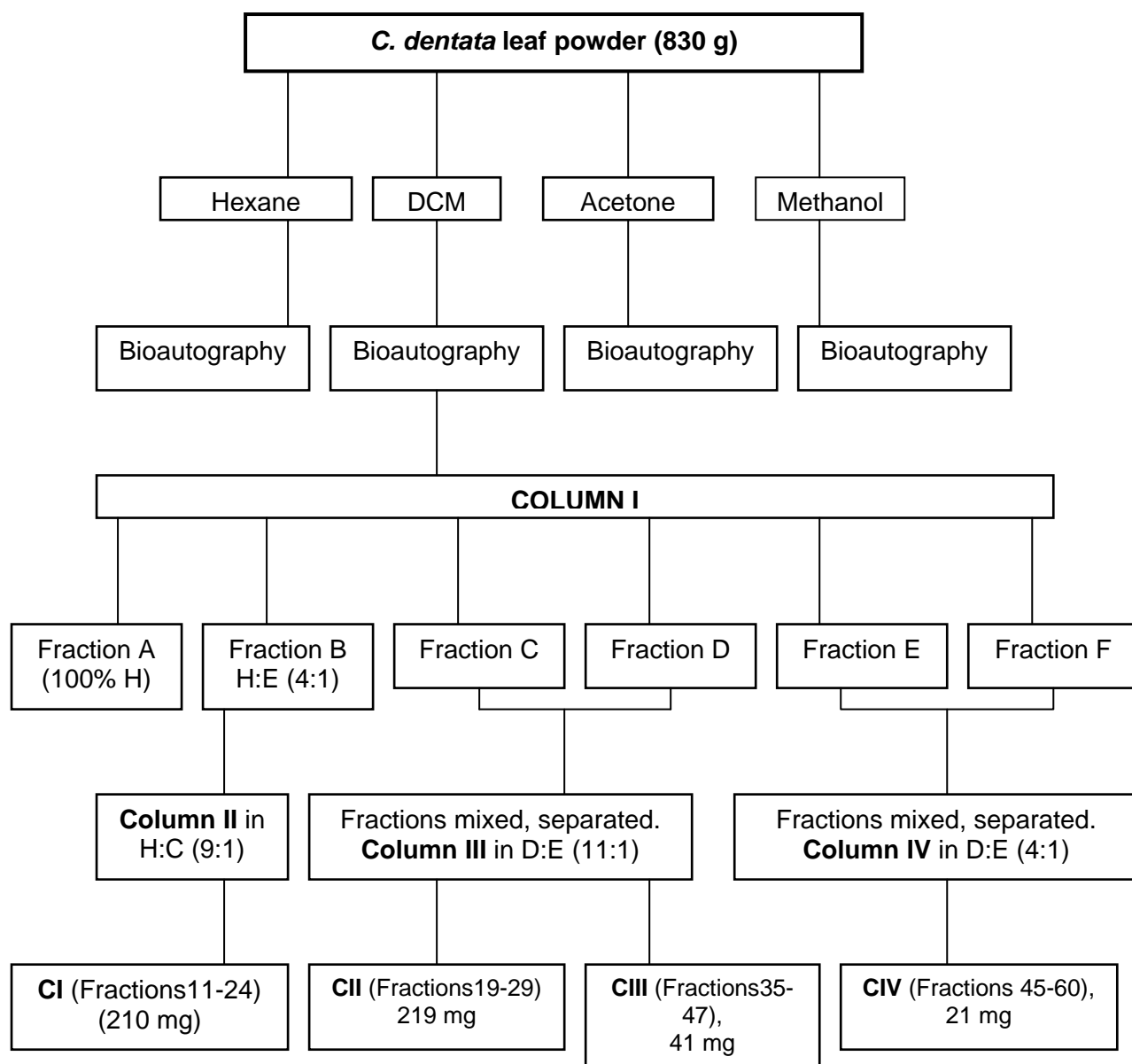


Figure. 4.1. Schematic diagram showing the isolation of active compounds from *Curtisia dentata* leaves using column chromatography with silica gel 60 as the stationary phase.

4.2.2.2. Group separation (Column I)

Silica gel (800 g) was mixed with hexane to form a homogenous suspension/slurry and stirred using a stirring rod to remove bubbles. The silica gel slurry was poured into a glass column (10 cm diameter and 50 cm length) whose outlet was plugged with cotton wool to retain the gel in the column. The solvent was allowed to flow out of the column opening to allow the gel to settle.

The plant extract sample was prepared by dissolving 15 g of the DCM extract in 100 ml of ethyl acetate. To the solution 30 g of silica was added and mixed by stirring with a glass rod. The mixture was allowed to dry at room temperature under a stream of air for approximately 5 hours. The dried silica gel-extract mixture was carefully layered on the column gel bed. For elution hexane was used as the mobile phase with the polarity increasing by 10% increments of ethyl acetate. For each eluent mixture 1.5 L volumes were used and 500 ml fractions collected in glass beakers. Collected fractions were concentrated using a Büchi R-114 Rotavapor. TLC was used to analyze fractions, and those with similar chemical components were combined.

Thirty three (33) fractions were collected and concentrated using a Rotavapor. These fractions were again analyzed by TLC, and those containing similar components were mixed, resulting in 6 major fractions (A – F). These major fractions were dried in pre-weighed beakers and their masses determined.

4.2.2.3. Combination of fractions from column I (DCM extract)

Fractions 1-3 were combined to form major fraction A, as they contained similar components when analyzed using TLC. Fractions 4 & 5 were combined to result in major fraction B. Fractions 6 & 7 were also combined to yield major fraction C. Fraction 8 was not combined with any fraction, and it was designated as fraction D. Fractions 9-14 were combined and the resultant mixture was called fraction E. Fractions 15-33 were combined and to result in fraction F. The antimicrobial activities of the fractions were assayed as described in chapter 2, using bioautography as the method of choice with *Candida albicans* as the test fungal organism. In most instances active compounds were found in more than one fraction. These fractions were combined to maximize the level of active compounds in order to obtain a high yield of the compounds.

4.2.2.4. Isolation of compound CI (Column II)

A glass column with a length of 50 cm and diameter of 2 cm was used for fractionation of fraction B. Silica gel 60 (150 g) was mixed with hexane by stirring and the slurry poured into the column whose bottom opening was plugged with cotton wool. The solvent was allowed to drip out for the silica gel to settle and establish a column bed. The sample was prepared by mixing 2 g of fraction B with 4 g silica gel

in 60 ml of ethyl acetate. The mixture was stirred vigorously and dried under a stream of air. The dried sample was loaded onto the gel in the column. After a series of separations of the fraction on TLC plates in different solvent mixtures to obtain a system resulting in good resolution, hexane: chloroform (HC) (9:1) was selected as the solvent system. The loaded column was eluted using HC (9:1) mixture with 50 ml fraction volumes collected at 5 ml/min. Fractions 11-24 contained pure compound **C1**.

4.2.2.5. Isolation of compound CII and CIII (column III)

Fractions C and D were mixed and from this mixture compounds CII and CIII were isolated. A glass column with a length of 40 cm and diameter of 4 cm was used for fractionation of the mixture of fractions C and D. Silica gel (200 g) was mixed with hexane by stirring and the slurry poured into the column whose bottom opening was plugged with cotton wool. The solvent was allowed to drip out to settle the gel and establish a column bed. The sample was prepared by mixing 3 g of fractions C and D mixture with 6 g silica gel in 90 ml ethyl acetate. The mixture was stirred vigorously and dried under a stream of air. The dried sample was then loaded onto the gel bed and eluted with dichloromethane:ethyl acetate (11:1) mixture at 5 ml/min. Fifty ml fractions were collected. Fractions 19-29 contained pure compound **CII** and fractions 35-47 contained pure compound **CIII**.

4.2.2.6. Isolation of compound CIV (column IV)

Fraction E – F were combined and the mixture used for isolation of compound CIV. A glass column with a length of 40 cm and diameter of 4 cm was used for fractionation of the mixture. The column was prepared as in **section 4.2.2.5**. The sample was prepared by mixing 3 g of fractions E and F mixture with 6 g silica gel in 90 ml ethyl acetate. The mixture was stirred vigorously and dried under a stream of air. The dried sample was then loaded onto the gel bed and eluted with dichloromethane:ethyl acetate (4:1) mixture with polarity increased by 10% increments of ethyl acetate. Fifty ml fractions were collected at 5 ml/min.

4.2.2.7. Isolation of compound HI (Column V)

The hexane fraction (2 g) was subjected to column chromatography using toluene as a solvent (mobile phase). A glass column with a length of 40 cm and diameter of 4 cm was used for fractionation of a mixture. About 200 g of silica gel was mixed with

toluene and slurry poured into the column whose bottom end opening was plugged with cotton wool. The sample was prepared by dissolving 2 g of hexane extract in 10 ml hexane and mixed with 4 g silica gel. The mixture was dried under a stream of air at room temperature. The dry mixture was carefully layered onto the column and eluted with toluene. Fifty-milliliter (50 ml) fractions were collected in glass test tubes. Similar fractions were combined. Fractions 14-19 contained pure compound **HI**.

4.3. RESULTS

4.3.1. Serial exhaustive fractionation

The serial exhaustive extraction of *C. dentata* leaves is described in section 2.2.3. The same plant material was extracted with different solvents, starting with the least polar, hexane (H), with methanol (M) completing the extraction. The masses of extracts obtained with each solvent are displayed in Fig. 4.1. Acetone and methanol resulted in the largest mass (24 and 21 g, respectively) of extracted chemical components and the least amount (13 g) was obtained with hexane.

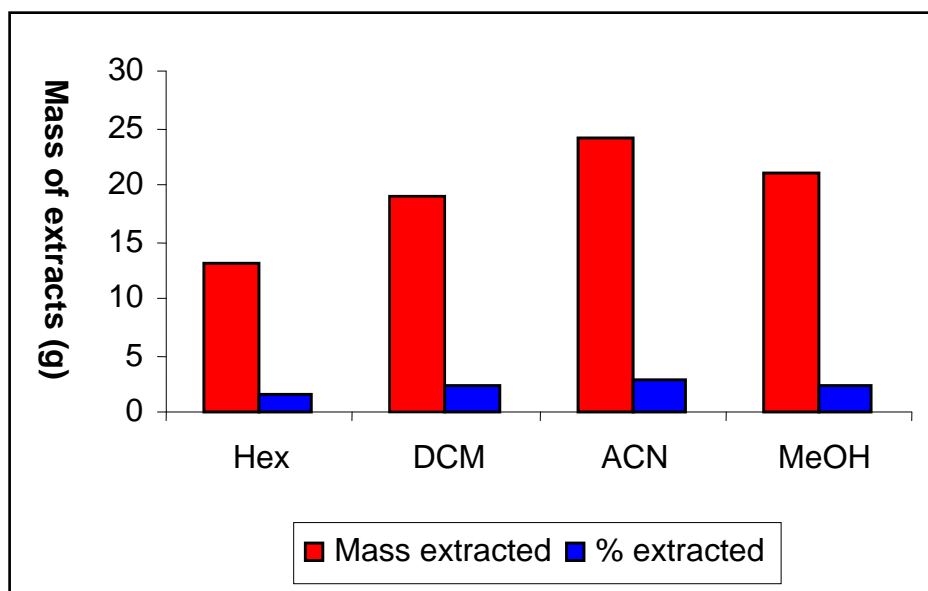


Figure 4.1. Amount extracted from *Curtisia dentata* leaves (837 g) using solvents of varying polarities. The dried leaf material was extracted serially with hexane (HEX), dichloromethane (DCM), acetone (ACN) and methanol (MeOH) in the order listed.

4.3.2. Activity of serial exhaustive extraction samples

4.3.2.1. Anti-*Candida* activity

Extracts acquired from serial exhaustive extraction of *Curtisia dentata* leaves were investigated to determine the presence of active compounds using bioautography and serial microplate dilution. Investigation of activity against *C. albicans* revealed that the acetone and dichloromethane extracts of the leaves of *Curtisia dentata* contained the highest number of active compounds (Fig. 4.2). The active compound in the hexane fraction was also observed in the dichloromethane extract. The methanol extract was not active against *Candida albicans* in bioautography.

The lowest MIC value recorded (0.107 mg/ml) against *C. albicans* was observed with the dichloromethane extract of *C. dentata* leaves (Table 4.1). The hexane extract did not show any observable activity against *C. albicans*, with an MIC value of over 2.5 mg/ml. Acetone and methanol extracts resulted in MIC values of 0.207 and 0.50 mg/ml, respectively. The highest total activity (4240 ml) recorded per bulk fraction resulted from the dichloromethane fraction, whilst the hexane extract resulted in the least total activity (79.3 ml) (Table 4.1). The methanol and the acetone fractions resulted in total activity values of 1050 and 3312 ml, respectively.

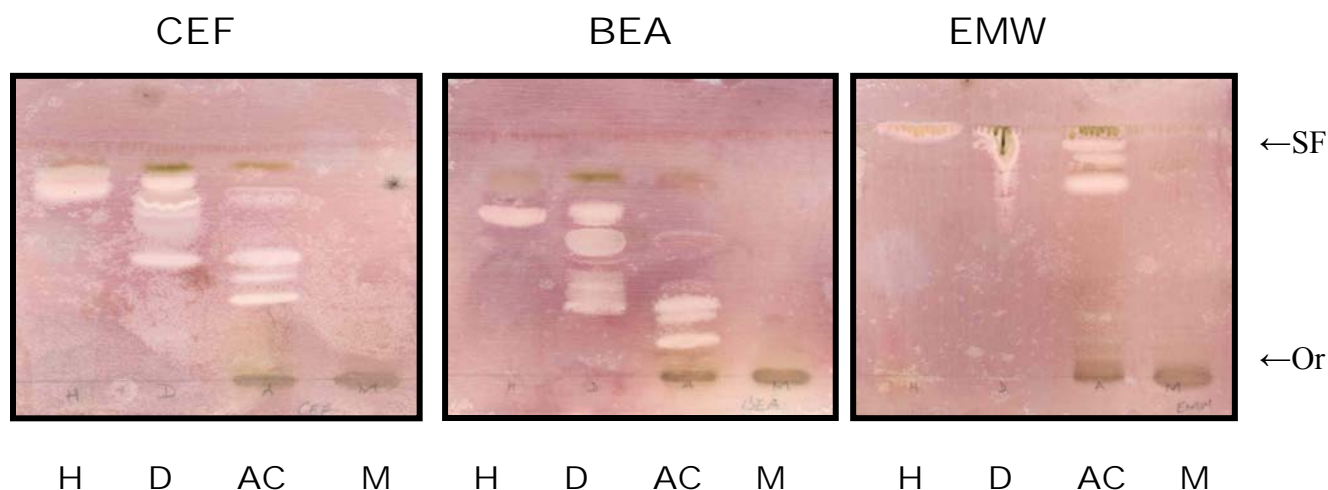


Figure 4.2. TLC plates showing growth inhibiting activity of *Curtisia dentata* leaf extracts against *Candida albicans*. Plant material was extracted with hexane (H), dichloromethane (D), acetone (AC) and methanol (M). SF, solvent front; Or, origin

Table 4.1. Minimal inhibitory concentrations and total activity of various bulk fractions of *C. dentata* against *C. albicans*

Fraction	MIC (mg/ml)	Total Activity (ml)/fraction
Hexane	>2.5	79.3
Dichloromethane	0.107	4240
Acetone	0.207	3312
Methanol	0.50	1050

4.3.2.2. Antibacterial activity

Bioautography is generally much more difficult with fungi than with bacteria. To facilitate the bioassay guided fractionation, bioautography was carried out with four important nosocomial bacteria to determine if the same compounds are active against *C. albicans*. If this is the case, it is easier to use bacterial cultures in the isolation work.

The serial exhaustive extracts of *Curtisia dentata* leaves were also tested for activity against bacterial test organisms using bioautography resulting from serial exhaustive extraction. Dichloromethane and acetone extracted more chemical components active against all test bacterial organisms. The methanol extract had the lowest number of antibacterial compounds against the four bacterial organisms under investigation (Fig. 4.3). Based on these findings, and that the dichloromethane extract contained high numbers of active compounds, the dichloromethane extract was chosen for further fractionation to isolate active compounds.

In the previous Chapter it was shown that the same compounds that were active against *C. albicans* on bioautograms had activity against bacterial species, hence in bioassay-guided fractionation bacteria may be used to follow the active compounds. The TLC plates of the extracts sprayed with vanillin-sulphuric acid are shown on Figure 4.4, revealing the different compounds extracted with each of the four extractants.

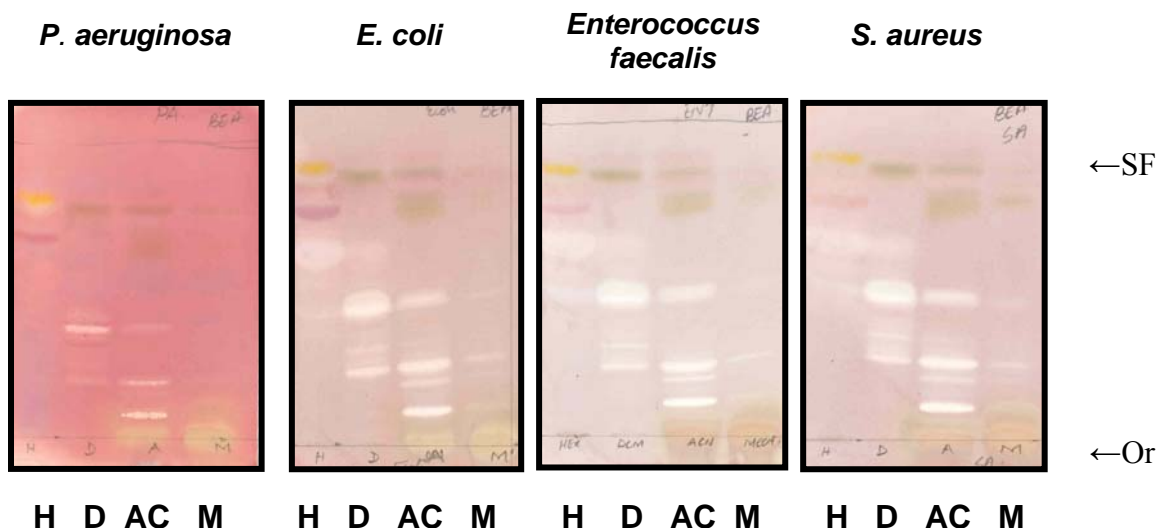


Figure 4.3. TLC bioautograms (BEA as eluent) showing activity of hexane (H), dichloromethane (D), acetone (AC) and methanol (M) extracts of *Curtisia dentata* against *P. aeruginosa*, *E. coli*, *Enterococcus faecalis* and *S. aureus*. SF, solvent front; Or, origin

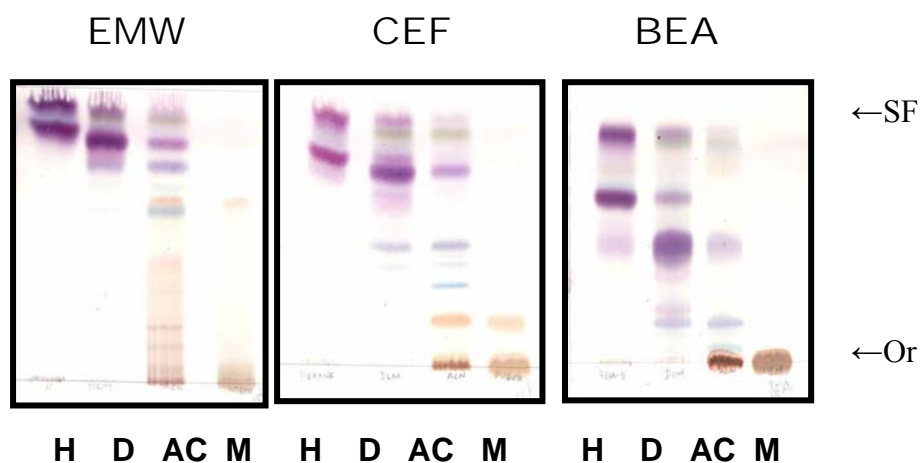


Figure 4.4. TLC chromatograms of serially extracted *C. dentata* leaves developed in EMW, CEF and BEA. Plant material was extracted with hexane (H), dichloromethane (D), acetone (AC) and methanol (M). SF, solvent front; Or, origin.

4.3.3. Isolation of compounds

From column I several fractions were collected and analyzed on TLC plates (Figs. 4.5 & 4.6). Similar fractions were combined into six major fractions A-F. The masses

of the different major fractions are displayed in Table 4.2. These fractions were tested for antifungal activity (Fig. 4.7 and Table 4.3). Active fractions were identified for isolation of anti-*Candida* compounds. Fractions C-F were active against *Candida albicans* on bioautograms (Fig. 4.7).

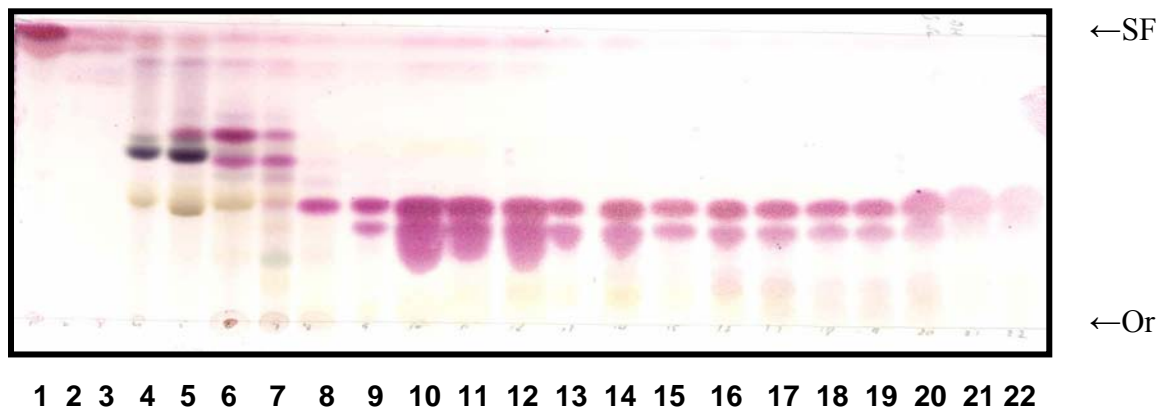


Figure 4.5. Fractions collected from separation of dichloromethane extract of *Curtisia dentata* leaves (fraction 1-22). The sample was first eluted with hexane and polarity was increased with 20% increments of ethyl acetate and then methanol. TLC plates were developed in hexane : ethyl acetate (7:3) and visualized using vanillin-sulphuric acid spray. SF, solvent front; Or, origin.

Fraction 11-24 from column II was a yellow solution, and a white precipitate formed. The precipitate was collected and washed several times with hexane. The dry compound **CI**, white in colour had a mass of 210 mg. The TLC chromatogram of the isolated compound CI is displayed in Fig. 4.8.

Fractions 19 – 29 from column III contained a single spot on TLC plates sprayed with vanillin-sulphuric acid and were pooled together and dried using a Rotavapor (fig. 4.9). The mass of the compound **CII** was recorded as 219 mg. Fractions 35 – 47 contained a single compound, which stained purple-red on TLC plates sprayed with vanillin-sulphuric acid. These fractions were combined and dried using a Rotavapor. The resultant compound **CIII**, creamy-white in colour had a mass of 41 mg (Fig. 4.9).

Fractions 45 – 60 from column IV contained a compound with some minor impurities on TLC chromatograms. These fractions were combined and solvent evaporated using a Rotavapor. The white powder was washed several times with hexane and then dried. The recorded mass of the compound **CIV** was 21 mg.

From column V, 36 fractions were collected. Fractions 14 -19 contained a single compound after separation on TLC plates (Fig. 4.10). These fractions were combined and dried using evaporation under a stream of air. The resultant compound was washed several times in methanol, dried and weighed. The resulting compound **HI**, 86 mg, was a white powder that stained purple upon spraying with vanillin-sulphuric acid.

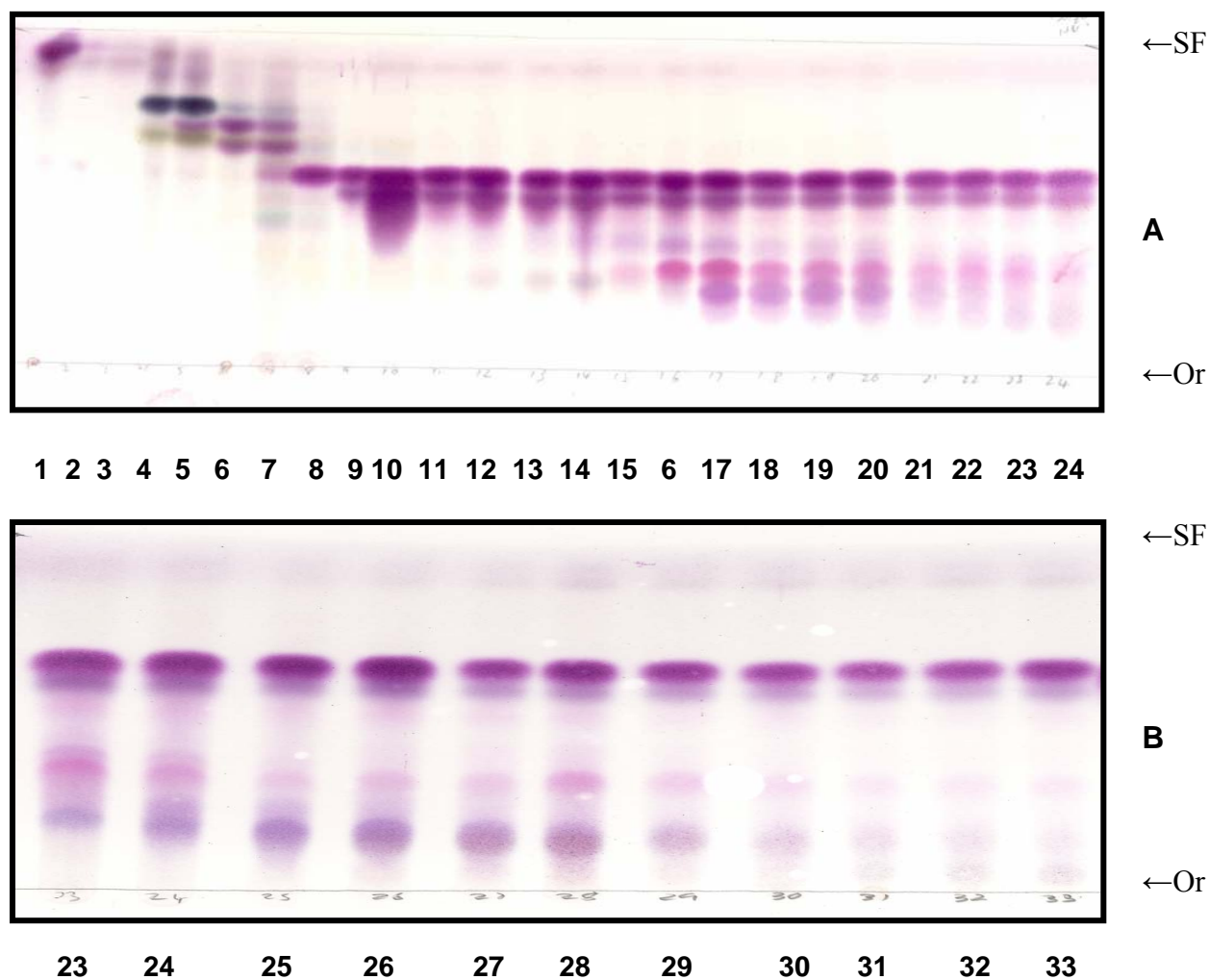


Figure 4.6. Fractions collected from separation of dichloromethane extract of *C. dentata* leaves (fraction 1-24 [A] and 23-33 [B]). TLC plates were developed in hexane: ethyl acetate (1:1) and visualized using vanillin-sulphuric acid. SF, solvent front; Or, origin.

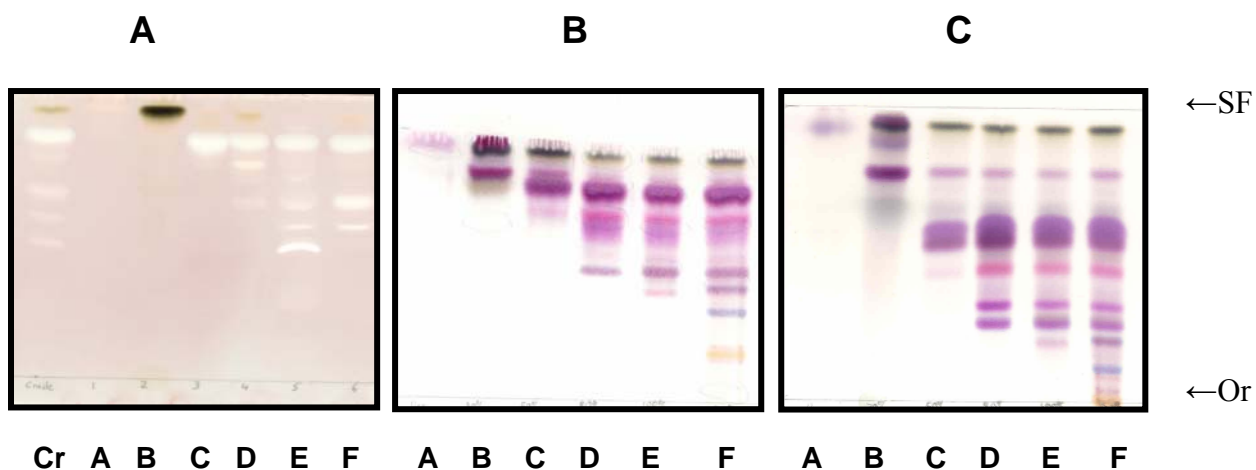


Figure 4.7. TLC bioautograms (A) (CEF) showing activity of fractions A-F against *Candida albicans*, and vanillin-sprayed TLC plates developed in CEF (B) and BEA (C). The dichloromethane crude extract (Cr) was tested alongside various fractions using the TLC bioautography method. SF, solvent front; Or, origin.

Table 4.2. Mass (g) of the major fractions from column I

Column I Fraction	No. of active compounds	Mass (g)
A	0	1.45
B	1	2.15
C	1	2.50
D	4	1.68
E	5	2.09
F	5	2.22

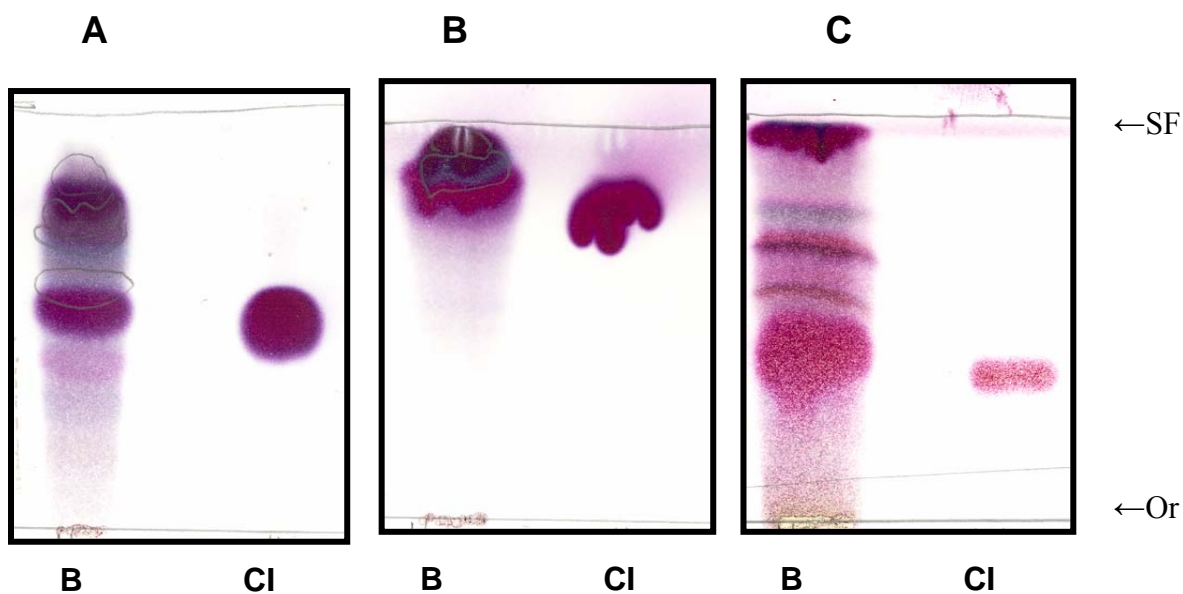


Figure 4.8. TLC chromatograms showing compound CI analysed side-by-side with fraction B from column I. The TLC plates were developed in BEA (A), CEF (B) and dichloromethane (C), and sprayed with reagent vanillin-sulphuric acid reagent. SF, solvent front; Or, origin.

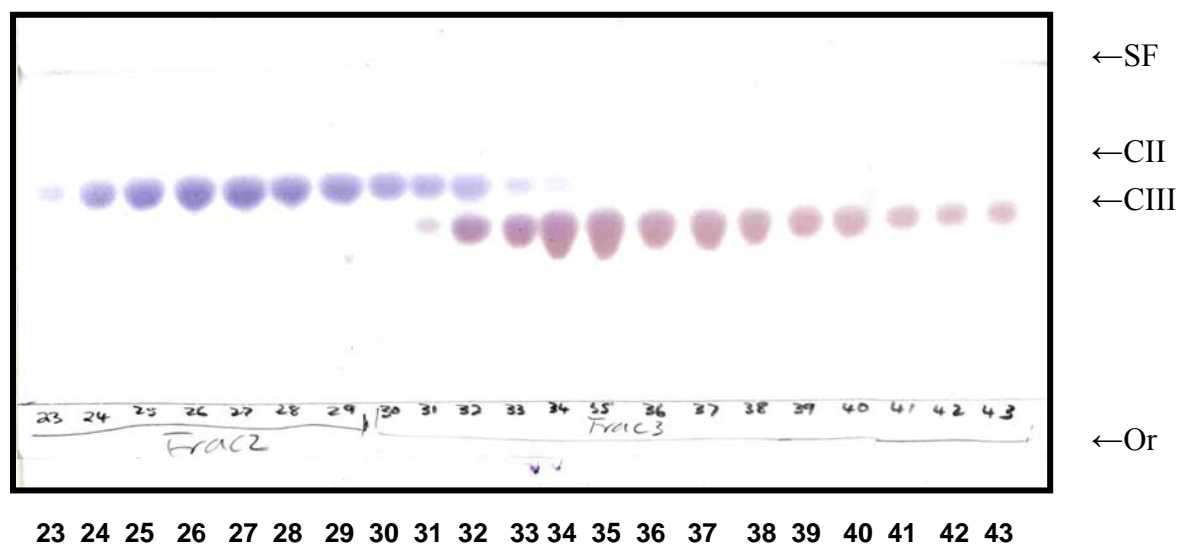


Figure 4.9: TLC chromatogram showing separation of CIII from CII in column III. The TLC plates were developed in HE (1:1) and then sprayed with vanillin sulphuric acid. Only fractions 23 – 43 are shown on the chromatogram. SF, solvent front; Or, origin.

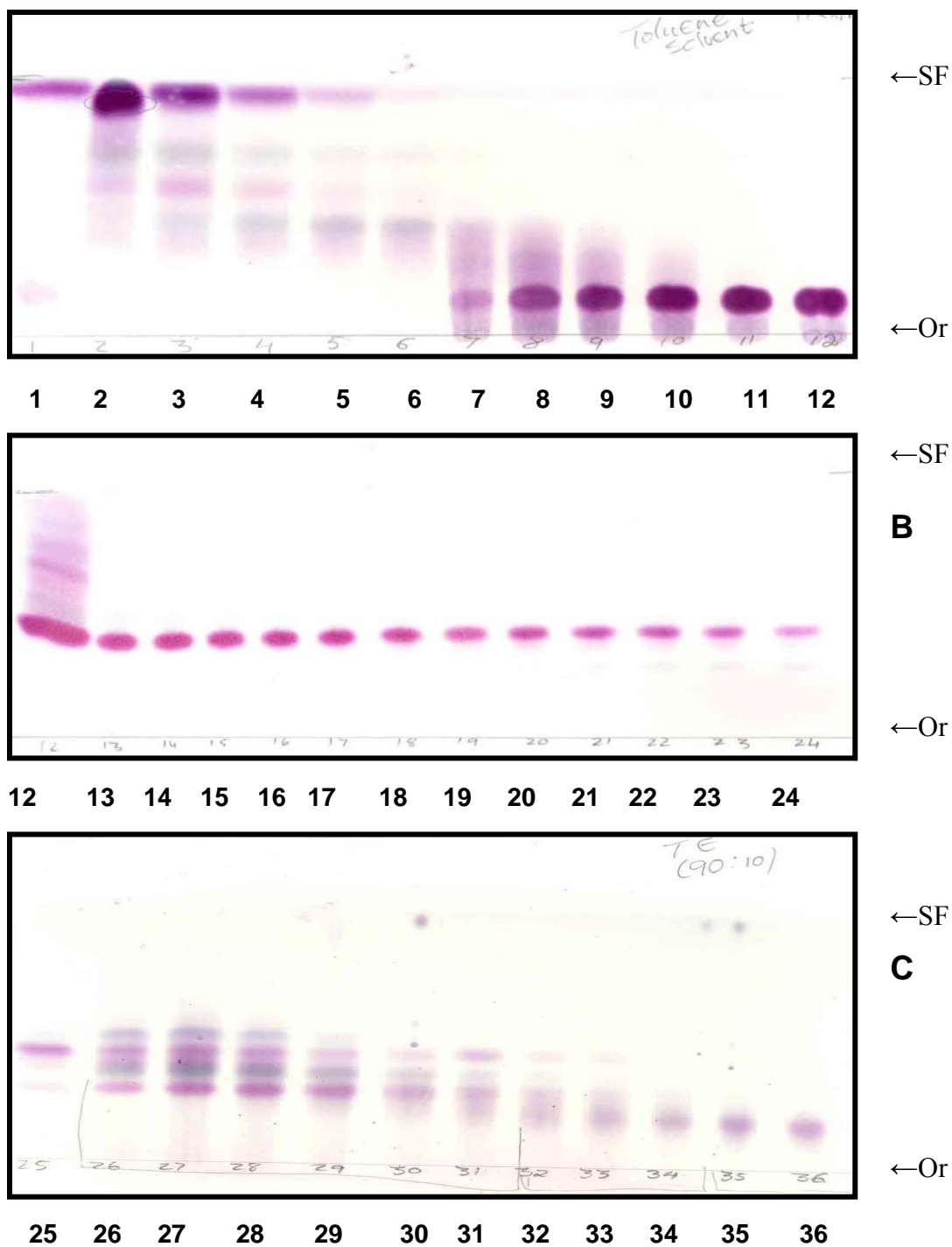


Figure 4.10. Fractions 1 – 36 collected from separation of hexane extract of *Curtisia dentata*. The sample was first eluted with toluene. TLC plates were developed in toluene (A) and toluene: ethyl acetate (9:1) mixture (B, C) and visualized using vanillin-sulphuric acid. SF, solvent front; Or, origin.

4.4. DISCUSSION

The plant species used, *Curtisia dentata*, is utilized for treatment of different health problems by traditional cultures in South Africa. The stem bark is the preferred plant part for preparation of medicines.

4.4.1. Serial exhaustive extraction of *Curtisia dentata* leaves

A higher number of active compounds was present in the acetone extracts of the leaves of *Curtisia dentata* compared to that the bark. The same powdered leaf material was serially extracted with solvents of varying polarities, starting with the least polar (hexane) and ending with methanol. The extracted material was subjected to bioautography experiments with bacterial and fungal species as test organisms. Bioautography results revealed that the highest number of active compounds was present in the dichloromethane and acetone extracts. The bulk methanol fraction showed no visible presence of antimicrobial compounds on bioautograms against *Candida albicans* and bacterial test organisms. The absence of active compounds in the methanol extract is not clear, though it may seem that active compounds were removed by acetone before methanol could be used as the extractant since serial exhaustive extraction was used.

Though the presence of active compounds was observed in the hexane extract against the five test organisms in bioautography, the MIC value recorded against *Candida albicans* was above 2.5 mg/ml. The antifungal compounds extracted by hexane may have been volatile and were lost during evaporation of the TLC solvents from the chromatograms. The lowest MIC values (0.11 and 0.21 mg/ml) resulted from the bulk DCM and acetone fractions against *Candida albicans*, further correlating the MIC data with bioautography results. Furthermore, the bulk DCM fraction resulted in the highest total activity calculated as 4240 ml/fraction. The total activity values for the bulk methanol, hexane and acetone fractions were 1050, 79.3 and 3312, respectively. This finding further emphasized that the hexane fraction was the least active while the acetone and the DCM fractions, with good MIC and total activity values were the most active fractions.

The methanol extract failed to reveal active compounds on bioautograms but was active in the serial microplate method with a MIC value of 0.5 mg/ml. The reasons for the discrepancy are unclear. Synergistic effects may be necessary for the activity of

some of the compounds, and thus serial exhaustive extraction may separate compounds that act together synergistically. Furthermore, some compounds may be volatile, resulting in their loss from TLC plates during drying for bioautography assay.

4.4.2. Isolation of compounds

Four compounds were isolated using column chromatography with silica gel as the stationary phase. The isolated compounds may belong to the same family of compounds as they stained similarly when sprayed with vanillin-sulphuric acid (purple-red colour). Compound **CI**, whose mobility on TLC was closer to the solvent front was the most non-polar whilst compound **CIV** was the most polar of the isolated compounds judging by its mobility in all the mobile phases used. This is the first report of isolation and identification of compounds with antifungal and antibacterial activity from *Curtisia dentata* leaves. Compound **HI** was later found to be the same as compound **CI**, as revealed by analysis on TLC plates.

4.5. CONCLUSIONS

Curtisia dentata has been used for many decades to treat different ailments. The findings of this study reveal that there are several antimicrobial compounds in the leaves of this plant species. Four compounds were isolated using silica gel as the stationary phase in column chromatography, following the principles of bioassay-guided fractionation. This is the first report of isolation of compounds from *Curtisia dentata*.