CHAPTER 2

MATERIALS AND METHODS

2.1 COLLECTION OF PLANT MATERIAL

Leaves of *C. microphyllum*, a creeper, were collected in June 1998 from a plant growing in the Lowveld National Botanical Garden, Nelspruit (Eloff, 1999). The reference number of the plant is 151/72 indicating that it was collected in 1972 and was the 151st collection of that year. The origin of the plant is recorded in the database of the botanical garden and a voucher specimen from the plant is deposited in the garden's herbarium.

2.2 PREPARATION OF LEAF MATERIAL

Leaves were dried at room temperature (c. 20 °C) by spreading out on a table in the laboratory. All the old, brown and diseased leaves were removed. The leaves were ground to a fine powder in a Jankel and Künkel Model A10 mill, which ensured homogenized tissue. The ground leaf material was stored at room temperature in the dark in tightly closed glass containers.

Intact dried leaves were used for the initial extraction with acetone and sodium bicarbonate.

2.3 EXTRACTION

Initially bicarbonate extraction was attempted. In a preliminary experiment, 3 g intact, dried leaves were extracted with 100 ml 1 % aqueous sodium bicarbonate ($NaHCO_3$) (Rogers, 1998) by shaking for 1 hour on an ultrasonic sound bath (Bransonic 52 ultrasonic bath). After filtration through Whatman # 1 filter paper, the extract was acidified by adding up to 4 ml concentrated HCl, to precipitate the extracted acidic compounds. The solution was left in a cold room.

The same quantity of leaves was also extracted with acetone under the same conditions without acidifying. The acetone extract was dried under reduced pressure and the precipitate was dissolved in 1 ml acetone. The process was repeated three times and the extracts were decanted and combined.

The procedure subsequently used was extracting 500 mg of finely ground, dried leaf material with 5 ml of the extractant. The following extractants were used (polarity and selectivity group, according to Snyder and Kirkland, 1979, given in brackets): hexane [0.1, 0], carbon tetrachloride [1.6, 0], isopropyl ether [2.4, I], diethyl ether [2.8, I], methylene dichloride [3.1, V], tetrahydrofurane [4.0, III], acetone [5.1, VIa], ethanol [4.3, II], methanol [5.1, II] and water [10.2, VIII]. Later ethyl acetate [4.4, VIa] was also used. Extractants used were of technical grade.

The material was extracted in polyethylene centrifuge tubes while shaking vigorously in a Vortex model K-500-4 test tube mixer for 5 minutes. After balancing and centrifuging at 5300 x g for 5 minutes, the extract was decanted in a pre-weighed container. The process was repeated three times on the same plant material. (Filtration was used with carbon tetrachloride because it could not be separated from the solid materials by centrifugation, due to its high density.) The combined extracts were dried in a stream of air at room temperature.

Due to problems with drying the water extract in a stream of air, water was removed by vacuum distillation in a Büchi rotary evaporator and placed in a dessicator overnight to determine yield. The dried extracts were dissolved in

acetone to yield c. 25 mg/ml of the extract and stored at c. 7 °C in sealed, labeled containers.

2.4 TLC ANALYSIS OF EXTRACT

Thin layer chromatography (usually 5 - 10 µl of a 50 - 100 mg extract/ml solution) was on Merck TLC F_{254} plates with one or more of the following eluents:

- benzene/ethanol/ammonium hydroxide (90/9/1, v/v/v) [BEA],
- chloroform/ethyl acetate/formic acid (5/4/1, v/v/v) [CEF],
- methylene dichloride/acetone (3/2, v/v) [MA].

Extracts were applied to the plates 1 cm from the bottom and allowed to develop by ascending chromatography for c. 9 cm. Different volumes of the same concentration of each set of samples were applied with a micropipette. Development of chromatograms took place in a saturated, closed TLC tank. The atmosphere was saturated by placing filter paper, wetted with the eluent, against the walls of the tank. Separated components were investigated under visible and ultraviolet light (245 and 360 nm, Camac Universal UV lamp TL-600). TLC plates were sprayed with one of the following spray reagents (Stahl, 1969):

- 0,59 g vanillin dissolved in 100 ml sulphuric acid : ethanol (4 : 1) [vanillin spray reagent],
- 20 % aqueous perchloric acid [perchloric spray reagent],
- 15 ml 85 % phosphoric acid diluted to 100 ml with methanol [phosphoric acid spray reagent],
- 5 ml *p*-anisaldehyde dissolved in 90 ml ethanol and 5 ml concentrated sulphuric acid (Carr and Rogers, 1986) [*p*-anisaldehyde spray reagent].

The plates were heated at 105 °C until the development of colour bands was complete.

2.5 MINIMUM INHIBITORY CONCENTRATION BY INT MICROPLATE BIOASSAY

Minimum inhibitory concentration (MIC) was determined with a microplate serial dilution method (Eloff, 1998c). The following test organisms were used: *Staphylococcus aureus* (American Type Culture Collection number 29213), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922) and *Enterococcus faecalis* (ATCC 21212). The National Committee also recommends these bacterial strains for Clinical Laboratory Standards. These bacteria are also responsible for most nosocomial diseases in hospitals. These cultures were grown at 37 °C and regularly subcultured (1 % inoculum). Approximately every 6 months, fresh cultures were obtained from Dr. F. Huygens, Department Medical Microbiology, University of Pretoria to ensure that no contamination of cultures influenced our results.

2.5.1 Dilution of extract

The 96-well microplate was labeled with sample codes after deciding on two-fold serial dilutions of 8 times (landscape mode) or 12 times (portrait mode). Distilled water (100 µl) was placed in each of the wells with a Socorex multichannel micropipette and 100 µl of extract was added with a single channel micropipette to the first well in the relevant series producing 50 % of the original concentration. The pipette was rinsed between applications with a small quantity of acetone.

After all extracts have been diluted in the first well, the multichannel pipette was used to remove 100 µl from the first well and was placed into the next well. The plunger was pushed up and down three to four times to ensure that the content of the well was properly mixed. (This produced 50 % of the original extract concentration; the next well 25 %, etc at this stage.) The process was repeated all the way to the bottom of the plate. The first 100 µl from the last row of wells was discarded to ensure that all the wells contained 100 µl of the extract. The first

column had a series of two-fold dilutions of extract number 1, the second of extract number 2, etc.

2.5.2 Addition of bacteria

The growth of bacteria was arranged in advance to ensure having an active culture of the bacteria required. The active culture used, was never older than 10 days (Eloff, 1998c). To ensure that the active culture was not contaminated, activities were completed within a laminar flow cabinet. After 100 µl of the relevant bacterial culture was placed into each of the wells, it was mixed thoroughly. This yielded 25 % of the original extract concentration in the first row, 12.5 % in the second row, etc. Either ampicillin (4 mg/ml) or gentamicin (1 mg/ml) solutions (100 µl) was added to well A12 to serve as a positive control of inhibitory activity of the extract. The microplate was sealed in a plastic bag and incubated overnight at 37 °C at 100 % relative humidity in an incubator.

The following morning 40 µl of a 0,2 mg/ml solution of INT (*p*-iodonitrotetrazolium violet) was added to each row with a multichannel micropipette. The plate was returned to the incubator for at least 30 minutes to ensure adequate colour development and was then examined. The microplates were again examined for colour development after 60 minutes and after 120 minutes. (*E. faecalis* took much longer to react - up to 16 hours).

A clear solution or a definite decrease in colour reaction compared to the next two-fold dilution, indicated inhibition of growth. The minimum inhibitory concentration (MIC) of the extract was calculated from the original concentration of the extracts.

2.6 BIOAUTOGRAPHY

The bioautography procedure described by Begue and Kline (1972) was used. The developed thin layer chromatogram plates were dried overnight in an air stream to remove any remaining eluent. The TLC plates were sprayed with a concentrated suspension of actively growing *S. aureus* cells, *P. aeruginosa* cells, *E. coli* cells and *E. faecalis* cells. The plates were incubated overnight at 38 °C in a chamber at 100 % relative humidity. Plates were sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet [Sigma Chemicals] in water.

Inhibition of growth was indicated by clear zones on the chromatogram after incubating for 30 - 90 minutes. Toxicity of solvents of the different test organisms used was determined by serial dilution as medium to levels where growth was not inhibited.

2.7 COMPARISON OF DIFFERENT SOLVENTS

The different solvents used were methanol, ethanol, acetone, water, methylene dichloride and methanol : chloroform : water (12 : 5 : 3). The total quality extracted, rate of extraction, total number of components extracted, number of inhibitory components extracted and the toxicity to the micro-organisms were determined.

Plant material (0,5 g) was weighed into each of six centrifuge tubes and 5 ml of each solvent was added to the respective tubes, to test the rate of extraction and the quantity extracted [**Section 2.3** for procedure]. The process was repeated two more times. After each extraction the extracts were placed in pre-weighed glass vials for separate analysis. Solvents were removed at room temperature for quantitative analysis. The vials with the dry material were weighed and the quantity extracted was calculated. The quantity obtained after each extraction was divided by the total amount extracted, and the rate of extraction was calculated.

2.7.1 Solvent/solvent fractionation

The solvent/solvent group separation procedure used by the USA National Cancer Institute as described by Suffness and Dourus (1979) was applied with a slight variation.

Fine, dry plant material (90 g) was extracted with 900 ml acetone by vigorously shaking for 30 minutes and repeated three times. The acetone extract was taken to dryness in a pre-weighed round-bottomed flask in a Büchi PE120 rotary evaporator under reduced pressure, rotating at c. 100 r.p.m. and the water bath temperature at c. 50 °C. When the extracts were nearly dry, the round-bottomed flask was lifted from the water and taken to dryness at ambient room temperature to prevent possible heat inactivation or change.

The acetone extract was dissolved in 100 ml water and 100 ml chloroform and the two layers were separated in a separatory funnel. The lighter fraction (water) was extracted with an equal volume of *n*-butanol in a separatory funnel to yield the water (W) and the butanol (B) fractions. The heavier fraction (chloroform) was taken to dryness in a rotary evaporator (in a pre-weighed round-bottomed flask) under reduced pressure and extracted with a 1 : 1 mixture of hexane and 10 % water in methanol.

The hexane (H) fraction was recovered with a separatory funnel. The 10 % water in methanol extract was diluted to 20 % water in methanol by adding water and extracted with carbon tetrachloride (CT) fraction. The 20 % water in the methanol extract was diluted to 35 % methanol in water and extracted with chloroform to yield the chloroform (CF) fraction and the 35 % water in methanol (MW) fraction. Equal volumes of the different solvents were used each time.

In all cases, the upper phase was re-extracted with the lower phase two or three times with c. 10 % of the original lower phase volume to ensure adequate separation. A khaki colour appeared when water was added to the 10 % and 20 % water in methanol solutions. The mass of each round-bottomed flask used on the

rotary evaporator was determined before and after with the dry fractions to determine the difference. The extracts were redissolved in acetone or acetone : water (1 : 1) in pre-weighed amber flasks with tight sealing lids and the concentration of each fraction was determined. The process is schematically represented in **Figure 2.1**.

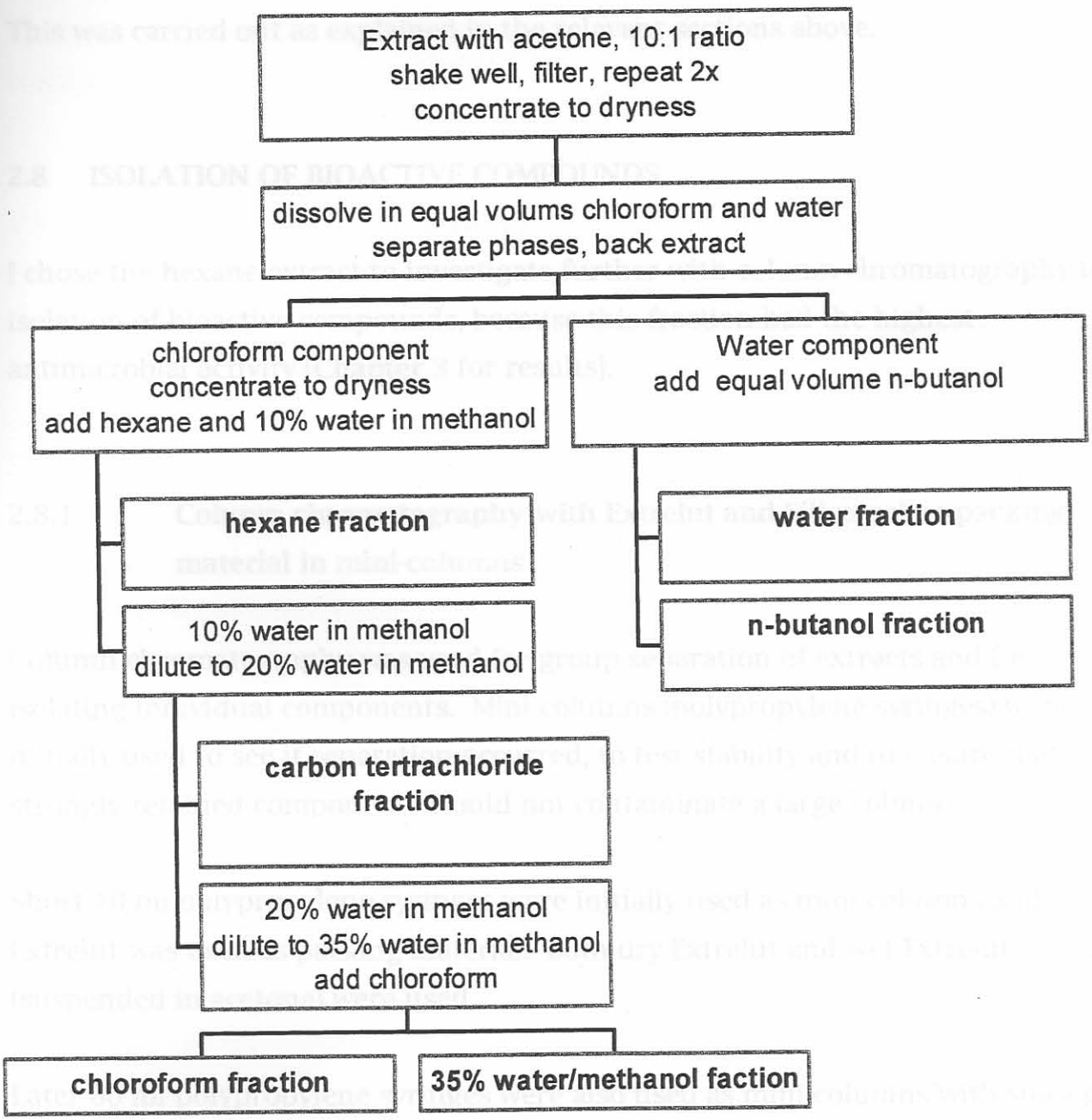

Figure 2.1 The procedure used for solvent/solvent extraction and fractionation of the components in *C. microphyllum* into six fractions.

2.7.2 Thin layer chromatography, bioautography and bioassay of fractions

This was carried out as explained in the relevant sections above.

2.8 ISOLATION OF BIOACTIVE COMPOUNDS

I chose the hexane extract to investigate further with column chromatography for isolation of bioactive compounds, because this fraction had the highest antimicrobial activity [**Chapter 3** for results].

2.8.1 Column chromatography with Extrelut and Silica gel as packing material in mini-columns

Column chromatography was used for group separation of extracts and for isolating individual components. Mini columns (polypropylene syringes) were initially used to see if separation occurred, to test stability and to ensure that strongly retained components would not contaminate a large column.

Short 10 ml polypropylene syringes were initially used as mini-columns and Extrelut was used as packing material. Both dry Extrelut and wet Extrelut (suspended in acetone) were used.

Later 60 ml polypropylene syringes were also used as mini-columns with silica gel as packing material.

Gravitational force was used to elute the components through the column, in some cases and air pressure from a membrane pump in other cases.

2.8.2 Combination of fractions after TLC and bioassay

An aliquot of the different fractions were separated by TLC using one of the three eluent systems and the results were used to pool fractions with similar composition.

2.9 TLC OF COMBINED FRACTIONS

2.9.1 TLC in Seprachrom containers

Seprachrom consists of plastic 5 cm x 10 cm containers, which can be closed. Several combinations of volatile eluents were tested to develop an eluent system for column chromatography. The best separation on column chromatography is if the wanted compound had an Rf-value of c. 0.5 on TLC. Later only glass TLC-tanks were used due to the unsatisfactory results in the Seprachrom containers.

2.9.2 TLC in small glass tanks

Attempts were made to develop a TLC system that would separate the components of fractions well so that this system could be used in subsequent column chromatography. Initially different combinations of carbon tetrachloride and methylene dichloride were tested as eluents.

Good results were eventually obtained with a hexane-acetone combination. The combination of hexane : acetone in a 2 : 1 ratio, gave the best separation on TLC.

2.9.3 Column chromatography on preparative Silica gel columns

A Michel-Miller glass preparative column, specially designed for high performance low pressure liquid chromatography, was used (length: 60 cm and diameter: 25 mm) to scale up TLC separation. These columns have Teflon couplings, fittings and adapters which resolves problems of plastic incompatibility with halogenated aliphatic compounds and ketones.

A finer silica gel, LiChroprep 15-25 μm [Merck] was used as packing material. The silica gel was suspended in the eluent mixture and was left to swell. Air was removed by placing the suspension in a Bransonic 52 ultrasonic bath for five minutes. The silica gel slurry was carefully poured into the column containing the eluent mixture.

In order to protect the top of the column from disturbance, a filter paper disk was cut to fit the column-diameter and was placed on top of the column. To minimise dead volume, a Michel-Miller adjustable bed height fitting was attached to the top of the column and adjusted to remove all the free space on the top of the column.

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

Pressure from a membrane pump was used to elute components through the column and the column was developed at a rate of c. 3 ml/min. Fractions were collected by an Isco Foxy Junior fraction collector. A gradient was formed by joining two Schott bottles at the bottom and taking eluents off to the column from the near side bottle while adding a more polar eluent to the far side.

2.9.4 Combination of collected fractions

Some of the collected fractions were combined, based on the analysis by TLC and the quantity present in each fraction. Bioautography was carried out on the combined fractions, using *S. aureus*, *E. coli* and *P. aeruginosa* as test organisms.

NMR spectroscopic analysis was carried out on combined fractions based on the TLC results.