CHAPTER 2

MATERIALS AND METHODS

2.1 Plant material

Leaf material was collected from two trees growing in the Lowveld National Botanical Garden, Nelspruit [Fig. 6]. The origin of each tree is recorded in the database of the botanical garden and voucher material from each tree was deposited in the garden's herbarium (Eloff, 1999).

2.2 Preparation and Extraction.

Leaves were dried under shade at room temperature (20 ^{0}C) and milled into a fine powder with a Junkel and Kunkel model A10 mill. The powder was stored at room temperature in the dark in tightly closed glass containers.

In a preliminary extraction, 0.5 g each of finely ground plant material was extracted in 5 ml in each of 10 solvents of varying polarity: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethyl acetate, tetrahydrofuran, acetone, methanol and water with vigorous shaking. The extracts were decanted after centrifuging at 5300 x g for 5 minutes.

The process was repeated twice more. The extractants used were of technical grade (Merck). The solvents were removed under a cold air stream at 7 ^{0}C. The extracts were weighed and re-dissolved in acetone to yield 10 mg/ml solutions.

2.3. TLC analysis of extracts

Thin layer chromatography was used to determine the composition of extracts. A quantity of 50 µg of extract was separated by TLC (Merck, Kieselgel 60 F_{254}) using the following solvent systems developed in our laboratory:

Benzene:ethanol:ammonium hydroxide (BEA) (36:4:0.4)

Ethylacetate:methanol:water (EMW) (40:5.4:4)

Chloroform:ethylacetate:formic acid (CEF) (20:16:4)

A 5 µl of the extract solution was applied by micropipette 1 cm from the bottom of the TLC plates and allowed to develop in solvent in the solvent systems. The development of the chromatograms was carried out in a closed, saturated TLC tank. Separated components were visualized under visible and ultraviolet light (254 nm and 360 nm, Camac Universal UV lamp TL-600). TLC plates were sprayed with one of the following reagents (Stahl, 1969):

- *p*-anisaldehyde-sulphuric acid (a freshly prepared mixture of 1 ml *p*-anisaldehyde,

18 ml ethanol, 1 ml sulphuric acid) for detection of sugars, steroids, terpenes, etc (Smith 1937 as referred to by Stahl, 1969)

- vanillin-sulphuric acid (0.1 g vanillin, 28 ml methanol, 1 ml sulphuric acid) for detection of higher alcohols, phenols, steroids and essential oils (Stahl, 1969).

The plates were heated at 105 ^{0}C until the development of colour was complete.

2.4 Bioautographic assay

The bioautography procedure described by Begue and Kline (1972) was used.

2.4.1 Preparation of TLC plates for bioautography

The TLC plates were prepared in duplicate (3 x 2) and developed in different mobile systems above. Chromatography plates were first dried overnight under a stream of air to remove the remaining TLC solvents that might kill the bacteria before spraying the plates. Duplicate plates were sprayed with the vanillin spray reagent or with bacteria.

2.4.2 Preparation of bacteria

A 10 ml of highly dense fresh bacteria culture was added into two centrifuge tubes and centrifuged at 5300 x g for 20 minutes to concentrate the bacteria. The supernatant was discarded and the pellet at the bottom of the tube was visible. The pellet was resuspended in 2 – 4 ml of fresh Mueller Hilton broth.

2.4.3 Bioassay method

The dried chromatographic plates were sprayed with a concentrated suspension of actively growing cells of *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 25922), *Escherichia coli* (ATCC 27853) or *Enterococcus faecalis* (ATCC21212).
The selection of test organisms was based on recommendation of the National committee for clinical laboratory standard (NCCLS) that these are bacteria strains for clinical Laboratory Standards. (Waitz et al., 1992) These species of bacteria are also the major cause of nosocomial infections in hospitals (Sacho et. al., 1993). The plates were sprayed until they were wet and opaque, before being incubated overnight at 38 ^{0}C in a clean chamber at 100 % relative humidity. After overnight incubation, plates were sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (Sigma Chemicals). Inhibition of growth was indicated by clear zones on chromatogram after incubating for about one hour (Begue and Kline, 1972).

2.5 Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) values were determined by INT microplate bioassay (Eloff, 1998). The test organisms used are the same as those used above for bioautography. The work was done within the Laminar flow cabinet to prevent the contamination of the working culture.

2.5.1. Dilution of extract

The 96-well microplate was labeled with sample code after deciding on two-fold serial dilution of eight times (landscape model) or 12 times (portrait model). Distilled water (100 µl) was placed in each of the wells with a Socorex multichannel micropipette to the first well in the relevant series. This reduced the concentration in this well to 50% of the original concentration. The pipette tip was rinsed between applications with a small quantity of water. After all extracts have been diluted in the first well, the multiple channel pipette was used to remove 100 µl from the first well and place the content into next well. The plunger was pushed up and down three to four times to ensure that the contents of the well were properly mixed. The concentration in this well was 25% of the original extract concentration, the next well 12.5%, etc.

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 contain 100 µl of the extract. The first column will then have a series of two-fold dilutions of extract number one.

2.5.2 Addition of bacteria

The working culture of the bacteria was prepared ahead to get active bacteria culture required. The working culture was grown for 4 – 6 hours at 37 ^{0}C and then stored in the fridge for up to10 days before using (Eloff, 1998c). The 100 µl of the relevant bacteria culture was placed in each of the wells and mixed by squirting the bacteria into wells. This resulted in 25% of the original extract concentration in the first row, 12.5% in the second row, etc.

The microplates were incubated overnight in the incubator at 37 ^{0}C. Then, 40 µl of 0.2 mg/ml *p*-iodonitrotetrazolium (INT) solution was added to each row with a multichannel micropipette. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis* cultures were used in the microplates. The microplates were examined for colour changes after 30, 60 and 120 minutes of incubation. The *E. faecalis* cultures took much longer to react, up to 16 hours.

The lowest concentration where growth is inhibited was recorded. The minimum inhibitory concentration (MIC) of the extract was calculated from the original concentration of the extracts.

2.6 Solvent-Solvent fractionation

This method was used to simplify the extract into different fractions. The solvent-solvent group separation procedure used by the USA National Cancer Institute as described by Suffness and Douros (1979) was adopted with minor modifications. The acetone extract of *C. woodii* leaves was fractionated into solvent of different polarities. The protocol is depicted diagrammatically below [Fig. 8].

2.6.1 Analysis and bioautographic assay of fractions

The thin layer chromatography and the bioautography assay of fractions obtained from solvent-solvent extraction were carried out as explained in the section 2.5 above. The MIC values of different fractions obtained were similarly determined.

2.7 Isolation of bioactive compounds

The best fraction from solvent-solvent extraction was subjected further to silica gel column chromatography.

2.7.1 TLC analysis

The TLC analysis of chosen fraction was used to select the best solvent system that could be employed as mobile phase for column chromatography. Thin layer chromatography was also carried out on the chosen fraction in different solvent systems [Section 4.1.1].

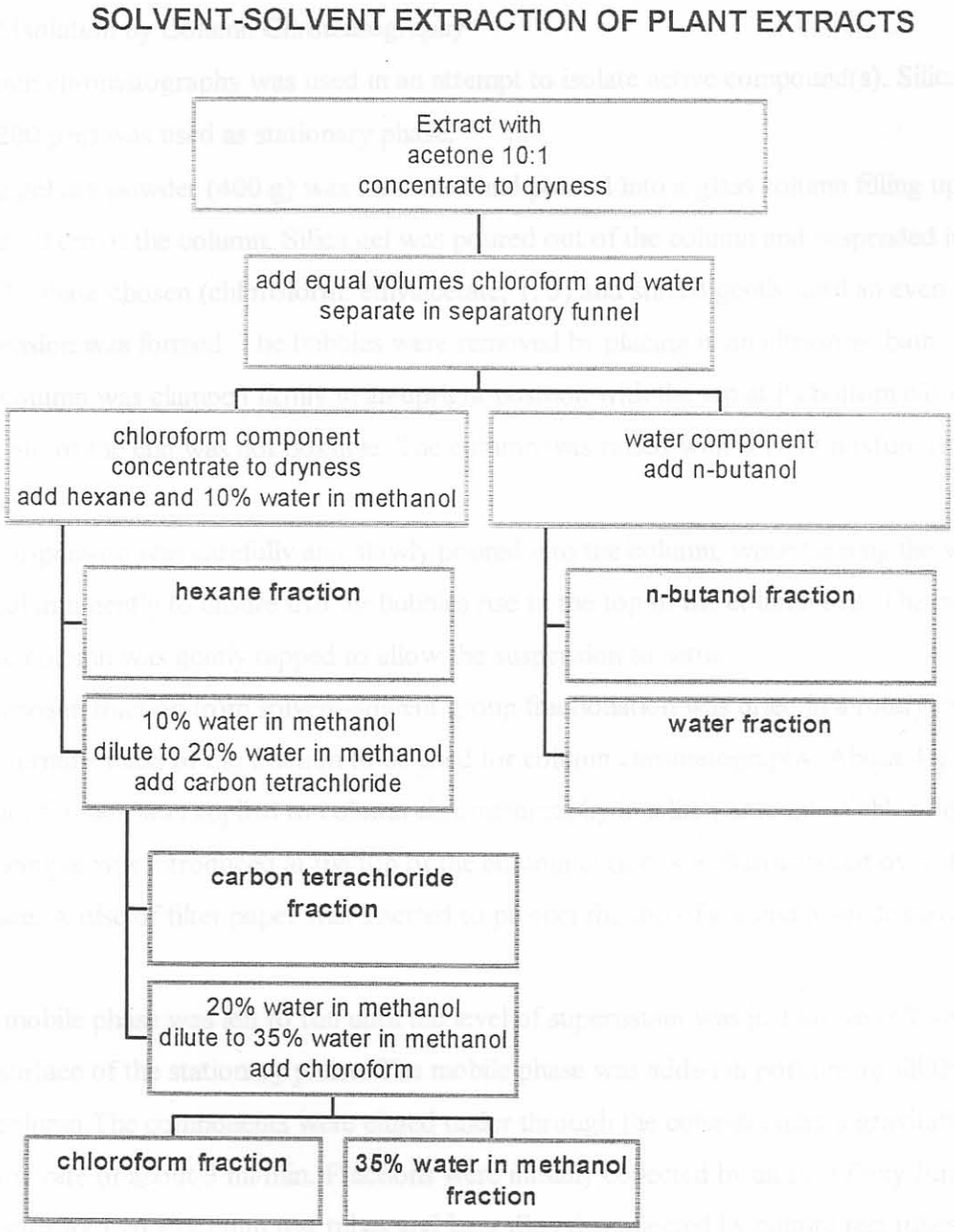

Fig. 8 The procedure used for the solvent-solvent fractionation of the components in the *C. woodii* leaves extracts.

2.7.2 Isolation by Column Chromatography

Column chromatography was used in an attempt to isolate active compound(s). Silica gel 60 (63-200 μm) was used as stationary phase.

Silica gel dry powder (400 g) was measured and poured into a glass column filling up to about 70 cm of the column. Silica gel was poured out of the column and suspended in the mobile phase chosen (chloroform: ethylacetate, 1: 5) and stirred gently until an even suspension was formed. The bubbles were removed by placing in an ultrasonic bath.

The column was clamped firmly in an upright position with the tap at its bottom closed so that flow out of the end was not possible. The column was rinsed with solvent mixture (mobile phase).

The suspension was carefully and slowly poured into the column, while tapping the wall of the column gently to ensure that air bubbles rise to the top of the column bed. The bottom end of the column was gently tapped to allow the suspension to settle.

The chosen fraction from solvent-solvent group fractionation was dried in a rotary evaporator to determine mass of the fraction to be used for column chromatography. About 4 g of the extract fraction was applied to column chromatography in a little amount of chloroform

The sample was introduced at the top of the column until it is well distributed over the whole surface. A disc of filter paper was inserted to protect the top of column from disturbance.

The mobile phase was left to run until the level of supernatant was just above (<3 mm) at the top surface of the stationary phase. The mobile phase was added in portions to fill the top of the column.The components were eluted under through the column under a gravitational force at flow rate of about 3 ml/min. Fractions were initially collected by an Isco Foxy Junior collector into 16 x125 mm test tubes and later directly collected by culture test tubes (25 x 150 mm). About 500 ml of 10% methanol in acetone was gradually introduced into the column after about 1500 ml to elute components which could not moved by chloroform : ethylacetate mixture.

As the separation was completed, the test tubes were placed in the fume cupboard under a stream of air to concentrate the fractions for bioassay and further analysis by TLC.

Approximately 160 test tubes were collected in separating the chloroform fraction. Various TLC analyses were done on the fractions to isolate the active compound.

The isolated compounds were purified by recrystallization in different solvents of varying polarities.

2.7.3 Bioassay work on isolated compounds

Bioautography was carried out on the purified compounds. MIC values were determined using all the four tested organisms in order to compare the strength of inhibition in comparison to standard antibiotics (ampicillin and chloramphenicol) used as controls.

2. 8 Spectroscopic analysis of isolated compounds

The samples were weighed and dissolved in deuterated chloroform for NMR analysis. Both ^{1}H- and ^{13}C-NMR experiments were carried out at Medical University of Southern Africa on a 300 MHz Varian NMR machine (Oxford instruments).

The isolated compounds were also sent to Cape Technikon for mass spectrometric analysis on a VG70-SEQ instrument.