CHAPTER 3

MATERIALS AND METHODS

3.1. Sample Preparation and selection of best extractant

C. apiculatum leaves were collected from a tree in the Lowveld National Botanical Gardens, Nelspruit. The plant label and a site map identified the tree. A voucher specimen is kept in the Garden Herbarium. The leaves were collected in April 2001, allowed to dry for 2 months in the shade at room temperature. Stems and thick veins were removed. The leaves were ground to a fine powder in a Junkel and Kunkel Model A10 mill.

The powder was initially extracted on a shaking machine with a 10:1 solvent to dry weight ratio for 4 hours, 6 hours and 24 hours. Later 0,5 g of powder was extracted with 5 ml of the extractant in a centrifuge tube by vigorous shaking and the extract was decanted after centrifuging at 3000-x g for 5 minutes. The solvents used were of technical grade (Merck). For quantitative determination, solvent from extracts placed in pre-weighed glass vials was removed by a stream of air at room temperature. The ten different solvents used were Hexane (HE), Isopropyl ether (IE), Diethyl ether (EE), Methylene dichloride (MD), Ethyl acetate (EA), Tetrahydrofuran (TH), Acetone (AC), Methanol (ME), Ethanol (ET) and Water (WA). To afford quantitative extraction; the procedure was repeated twice. The purpose of extracting the leaf material with the ten different solvents was to establish which one would extract most antibacterial components in the least chemically complex fraction.

3.2. Microorganisms used in Bioassay

The following served as test organisms in this study.

Staphylococcus aureus *(ATCC 292163),* Gram-positive

Enterococcus faecalis (ATCC 29212), Gram-positive
Escherichia coli (ATCC 27853), Gram-negative
Pseudomonas aeruginosa *(ATCC 25922),* Gram-negative

These microorganisms were chosen on the basis that they are the common cause of nosocomial infections in hospitals, (Sacho et al 1993). They are the reference strains recommended by the National Committee for Clinical Laboratory Standards, Villanova, Pennsylvania. (NCCLS, 1990). Bacteria were grown at 37°C in Mueller-Hinton broth for 4 to 6 hours, then stored at 8°C. Every 6 months a new liquid culture was established from agar cultures.

3.3. Thin layer chromatography (TLC)

Thin layer chromatography was carried out using the TLC F254 MERCK plates with the following solvent systems developed in our laboratory:

- Chloroform/ethyl acetate/formic acid, (CEF) [5:4:1],
- Benzene/ethanol/ammonia (BEA), [36:4:0:4],
- Ethyl acetate/methanol/water (EMW), [40: 5.4: 4.0].

The solvent systems separate components with a wide range of polarities and different R_f values.

In this study; 20 x 20 cm aluminium plates were cut in half and prepared in duplicate for each of the solvent systems described above. To each plate, 5 µl of a 20 mg extract/ml solution (ie: 100 µg) different extracts was applied in a line of about 1 cm length. The plates were then put into the TLC tanks to develop for 10-20 minutes. The tanks were saturated before development by lining with filter paper moistened with the eluent. The separated components were visualized under UV light at 240 nm and 360 nm. They were also sprayed with spray reagents and heated at 100°C. Spray reagents (SR) used were:

- Vanillin SR, was prepared as follows: 0.3 g of vanillin powder dissolved in 84 ml of methanol to which 3 ml of sulphuric acid was added carefully.

- Anisaldehyde SR, was prepared as follows: 1 ml of *para*-anisaldehyde was added to 18 ml of ethanol and 1 ml of sulphuric acid was added carefully.

3.4. Microplate Bioassay

In the assay of extracts for antimicrobial activity, some scientists have been using an agar plate diffusion technique. An extract is placed in a hole punched in a nutrient agar plate seeded with the test organism. A zone of growth inhibition indicates bioactivity. The pitfalls with this agar diffusion assay are that, agar is hydrophilic and the components of a complex extract may diffuse at different rates. The correlation between zone of inhibition and MIC values is very low (Eloff, unpublished data).

In this study, a serial dilution microplate technique was used to determine the minimum inhibitory concentration (MIC) values of the extracts to the microorganisms (Eloff, 2000). In brief, 100 µl of water was added to all the wells of the microplate in relevant order using multichannel micropipette. To the 100 µl of water in each of the first wells, 100 µl of different extracts at 10 mg/ml concentrations was added. This reduced the concentration of each well by 50% of the original concentration. When all extracts have been diluted in this manner, multichannel micropipette was used to remove 100 µl from the first wells into the wells of the next row. This was done all the way down to the last well. The concentration was therefore reduced from 100% down to 0.39% in the wells of the eight row. Caution was taken not to contaminate extracts between the wells by rinsing the pipette thoroughly with small quantity of water between each application. To each of the diluted wells, 100 µl of actively growing culture of bacteria was added, see section 3.2 above. The microplate was covered and incubated overnight at 37°C, after which 40 µl of a 0.2 mg/ml *para*-iodonitrotetrazolium (INT) (Sigma) solution was added to each well. The microplates were examined for colour changes after 30, 60, and 120 minutes of incubation at 37 °C. The lowest concentration at which a decrease in red colour is apparent compared to the next dilution is taken as the MIC value. Bacterial growth is indicated by the red color of the INT reduced to formazan, (Eloff, 2000).

3.5. Bioautographic assay

Bioautography is used to determine the number of active compounds present. The bioautographic procedure described by Begue and Kline (1972) was used. The TLC plates were spotted with the extracts, and developed using the eluants described in section 3.3. The plates were dried overnight in a stream of air to remove all traces of the eluents. The following day, the plates were sprayed with a concentrated suspension of an actively growing culture of the four test organisms discussed in section 3.2. The plates were then incubated overnight at 37°C and 100% relative humidity. This was followed by the spraying of the plates with a solution of INT to detect biological activity on the chromatograms. Clear zones on the chromatograms indicated inhibition of growth.

3.6. Solvent/solvent fractionation

Solvent/solvent fractionation was used as a preliminary separation to simplify the complex extracts. The USA National Cancer Institute has used this method (Suffness and Douros, 1979). The *C. apiculatum* leaf powder (mg) was extracted on a shaking machine with a 10:1(mg:ml) ratio of acetone to powder. This was repeated three times. The extracts were decanted and filtered, combined and dried under reduced pressure using a rotary evaporator. The dried acetone extract was dissolved in a 1:1 mixture of chloroform and water. The separated water fraction was extracted with an equal volume of n-butanol in a separatory funnel to yield the water (W) and the butanol (B) fractions. The chloroform fraction was taken to dryness in a rotary evaporator under reduced pressure and extracted with a 1:1 mixture of hexane (HE) and 10% water in methanol (10%W/M). The hexane fraction was recovered in a separatory funnel while the 10% W/M was diluted to 20% water in methanol and extracted with carbon tetrachloride to yield the carbon tetrachloride (CT) fraction. The 20% water in methanol was further diluted to a 35% water in methanol and extracted with chloroform to yield the 35% water in methanol (W/M) and the chloroform (CF) extracts. In all cases, equal volumes of the solvents were used and the extraction process repeated with a small volume approximately two or more times. TLC and bioautographic assay of the six fractions obtained was carried out as

described in sections 3.3 and 3.5 respectively. R_f values were also determined from the bioautographic plates.

3.7. Column chromatography

Column chromatography is used in the isolation of different components from crude extract. The column is prepared by packing a solid stationery phase onto which the sample mixture is applied. A mobile phase is then allowed to move down the column by gravity. The constituents of the mixture elute at different rates through the column as bands.

In this study, a one meter by 25 mm diameter glass column was packed with silica gel slurry in hexane. Caution was taken to prevent the formation of bubbles within the column. The glass column was then clamped in an upright position with its tap carefully controlled to regulate the flow. The CF fraction of about 4.44 g was applied onto the top of the column and the mobile phase allowed to move down the column through gravity. The mobile phase was added in portions to fill the top of the column.

Elution was started with 100% hexane and followed with a mixture of increasing proportions of dichloromethane in hexane until 100% of dichloromethane, then a mixture of dichloromethane and methanol until 100% methanol is reached. Fractions of 10 ml were collected each time. The collected fractions were concentrated in a stream of air at room temperature and analyzed by TLC.
Fractions were then pooled based on similar chemical composition. Fractions 19 to 24 appeared to have complex chemical composition. These fractions were combined and separated in the second run of the column. The column was prepared in exactly the same manner as the first one, except that this one 50 cm long with 1 cm diameter.
Bioautography using *S. aureus* and *E. coli* was carried out on the fractions. Active compounds were isolated by crystallization and subsequent washing with different solvents to remove contaminants.

3.8. Spectroscopic analysis

Nuclear magnetic resonance (NMR) is used to determine the structure of organic compounds by measuring the magnetic moments of their hydrogen and carbon atoms. In most compounds H-atoms are attached to different functional groups such as –CHO, -CH_2, -CH_3, -NH_2, -CHOH etc and the NMR spectrum provides a record of the number of H-atoms in the different positions. At least 5 – 10 mg of a sample is needed for NMR analysis. However, the advantage of using NMR over the other methods like mass spectroscopy is that the sample can be recovered and used for other tests. In practice, a sample is placed in a solution of an inert deuterated solvent, between the poles of a powerful electromagnet. The protons or carbon atoms undergo chemical shifts according to their molecular environment within the bulk molecule, upon being subjected to the radio waves. The complexity of the spectrum is related to the number of different protons and functional groups. Interpretation of the spectrum leads to the determination of the chemical structure. The spectra in this study were obtained on a 300 MHz Varian (Oxford instruments) machine at Medunsa.

Mass spectroscopy depends on the production of ions in a gaseous phase from the sample. These ions then separate according to mass to charge ratio (*m/e*). The principle of mass spectroscopy is that when an organic molecule is bombarded with electrons of sufficient energy, it loses an electron and so becomes a positive ion. The imparted excess energy accumulates in a particular bond which then cleaves. Different bonds require different energies to break. Each molecule will give rise to unique fragments. The mass spectrometer separates and records positive ions according to their mass to charge ratio (*m/e*).