CHAPTER 4

CONCLUSIONS

4.1 INTRODUCTION

The aim of the study was to investigate whether *Combretum microphyllum* contains antibacterial compounds. I also attempted to isolate one or more of these antibacterial compounds.

I worked on *C. microphyllum*, which forms part of another section (*Conniventia*) of the subgenus *Cacoucia*. There has not been any publication on the antibacterial activity of *C. microphyllum* that I could find. *C. microphyllum* possesses different chemical and antibacterial compounds from other *Combretum* species as well as a reasonably high antibacterial activity. The chemical composition and Rf-values of antibacterial compounds present in extracts also differ from that of other sections of the genus according to preliminary studies.

Other students in our laboratory are investigating species from other sections in an effort to provide chemotaxonomic evidence for the sectional classification and to confirm the occurrence of different antibacterial compounds in the different species. Car and Rogers (1986) identified certain differences in the chemical composition of the *Combretum* species in a preliminary study. They did not however investigate *C. microphyllum*.

4.2 EXTRACTION

The extracts were very complex and I investigated whether extraction with different solvents could yield less complex extracts with high antibacterial activity. In order to find an extractant that would be optimally useful both in screening and isolation of antimicrobial components from plants, I decided to compare a number of extractants of varying polarity.

In a preliminary experiment, I compared acetone with aqueous sodium bicarbonate, as extractants using intact dried leaves of *C. microphyllum* as proposed by Carr and Rogers (1986).

Different spray reagents were examined. The vanillin spray reagent gave the best results and I decided to use it in future work.

Acetone as extractant gave more promising results than sodium bicarbonate. The acetone extract yielded the highest concentration, as well as the most different compounds on TLC. The acetone extract inhibited growth most in all four cultures. The sodium bicarbonate extract inhibited Gram-positive bacteria to a larger extent than Gram-negative bacteria, but *E. coli* was inhibited much more than *E. faecalis* by the acetone extract. This indicated that there may be some selectivity.

The total quantity extracted by acetone from intact leaves was substantially lower than values from Eloff (1998a) using finely ground leaf material and the MIC values for the extracts of intact leaves were much lower (thus more active). Consequently I decided to continue working on finely ground leaf material.

I also experienced difficulties in the acid precipitation of the bicarbonate extracts and decided not to continue with sodium bicarbonate as extractant.

Because extracts of *Combretum* spp are complex, I decided to test more extractants with varying polarity to determine if I could simplify extracts and/or extract more antibacterial activity with different extractants.

Eleven different extractants were subsequently tested on finely ground, dried leaf material for the extraction of antibacterial compounds from *C. microphyllum*. (Extractants used were hexane, carbon tetrachloride, isopropyl ether, diethyl ether, methylene dichloride, tetrahydrofurane, ethyl acetate, acetone, ethanol, methanol and water.)

There were large differences in the compounds extracted. Methylene dichloride and ethanol extracted the most different compounds that could be separated by TLC at the quantity analyzed. With hexane, carbon tetrachloride, acetone and water, no coloured compounds were visible before spraying with the 50 μg applied to the plates.

The separation of the more non-polar compounds, using BEA as eluent yielded many compounds. Surprisingly, a relatively polar extractant as ethanol, extracted similar concentrations of non-polar compounds (high Rf-values in normal phase chromatography). TLC with the intermediate polarity eluent CEF indicated that hexane did not extract polar compounds, that the carbon tetrachloride and tetrahydrofurane extracts had serious streaking and that acetone had the highest concentration and most diverse number of compounds that reacted with the vanillin spray reagent.

MIC values were determined. Although methanol and methylene dichloride extracted more activity, I still decided to continue with acetone as extractant due to its relatively low toxicity to test organisms and good TLC separation of compounds obtained with acetone as extractant and ease of removal from extracts.

4.3 PRELIMINARY SEPARATION OF EXTRACTS

4.3.1 Group separation of extracts by solvent/solvent fractionation

I investigated to what extent solvent/solvent extraction could simplify extracts without reducing antibacterial activity. This fractionation procedure separated the components of *C. microphyllum* into six fractions: hexane, carbon tetrachloride, chloroform, butanol, 35 % methanol/water and water soluble.

The first three fractions (hexane, carbon tetrachloride and chloroform) gave the best separation of compounds as well as the most different components on TLC.

The quantities present and the antibacterial activity of the different fractions were determined. The highest quantity, nearly 42 %, was present in the hexane fraction. Nearly two-thirds of the total antibacterial activity was also present in the hexane fraction. This was different from other *Combretum* species investigated thus far where most of the activity was in the carbon tetrachloride and/or chloroform fractions. If the more polar components (which may contain

uninteresting polysaccharides and polyphenols/tannins) are ignored, the hexane fraction contained more than 88 % of the interesting non-polar antibacterial compounds.

This procedure enriched the antibacterial activity of the polar compounds in the butanol and water fractions and the antibacterial activity of the non-polar fractions in the hexane fraction to a high degree. It had little enrichment effect on the methanol/water fraction and removed bioactive compounds from the carbon tetrachloride and water fractions. Not all the bacteria had the same sensitivity to the compounds present in the different fractions. Especially *P. aeruginosa* cultures were more sensitive to the non-polar fractions.

Frequently Gram-negative bacteria are more resistant to plant extracts than Gram-positive bacteria, but this is not true for the compounds present in *C. microphyllum.*

Bioautography worked well with *S. aureus*, but was not as reproducible with the other test organisms. Unfortunately *S. aureus* was the least sensitive of the four test organisms, especially for the hexane and carbon tetrachloride fractions.

Because the hexane fraction had the highest antimicrobial activity by far for all four test organisms used, I chose this fraction for the future steps in the isolation of bioactive compounds with column chromatography.

4.3.2 Group separation by using Extrelut as packing material

Because the solvent/solvent fractionation is a tedious process, I decided to determine if Extrelut, a diatomaceous sold by Merck, can be used instead of the solvent/solvent fractionation.

Small columns were initially used to see if separation occurred, to test stability and to ensure that strongly retained components would not contaminate a large column. Both wet and dry adsorbents of Extrelut were used.

The Extrelut fractions were analyzed on TLC. Very little fractionation took place with the dry column, but there was some fractionation of the more polar compounds in the wet column. The fractionation was not promising with this technique because it works best with aqueous extracts that are eluted with non-miscible organic solvents, and the column may have been overloaded.

4.4 COLUMN CHROMATOGRAPHY

4.4.1 Initial column chromatography with silica gel [15–40 μm] as packing material on a small column

A 60 ml Polypropylene syringe was packed with a slurry of silica gel 60 [Whatman 15 - 40 μm] in a mixture of benzene : ethanol (9 : 1). Four solvents were used in the following order: BEA, CEF, acetone and methanol, based on the separation by TLC. I chose volatile solvents to make drying of extracts at room temperature under air stream easier.

The first 22 test tubes were collected by hand and the rest were collected by the fraction collector.

Individual fractions were separated by different TLC systems based on the expected polarity. The first non-polar fractions were analyzed using the BEA system and the more polar fractions with the CEF system. Fractions A-F were very concentrated and gave the most different components on TLC.

Fractions were analyzed by TLC and combined. Fraction ABC gave the most definite bands and best separation on TLC. The BEA system and the vanillin spray reagent gave the best results.

The combined fractions were tested for antibacterial activity. By far the largest activity was in the first highly non-polar fractions (ABC and DEF). *S. aureus* was not nearly as sensitive as the other test organisms. In the original extract, *S. aureus* had the same order of sensitivity as the other test organisms whereas it now had a two orders of magnitude lower sensitivity. It is not clear whether this change was due to experimental error or a change in activity due to the procedure followed.

The results showed that different fractions inhibited different organisms to varying degree, which indicated that there was some specificity towards different test organisms.

4.4.2 Developing a system to separate components by column chromatography on a large column

Attempts were made to develop a TLC system using volatile eluents that would separate the components of fractions ABC and DEF well, with the purpose of applying this system later in column chromatography.

Initially different combinations of carbon tetrachloride and methylene dichloride were tested as eluents. Later different combinations of acetone, hexane, methanol, methylene dichloride and carbon tetrachloride were also tested as eluents.

Good results were eventually obtained with a hexane-acetone mixture with a 2:1 ratio.

4.4.3 Final column chromatography on a preparative column

A finer silica gel, LiChroprep 15–25 μm, was used as packing material on a glass preparative column to scale up the TLC separation.

I decided to continue with only the ABC fraction and chose the eluent combination of hexane : acetone 2:1 in an attempt to isolate the bioactive compounds using this finer silica gel as packing material with column chromatography on a preparative column.

A syringe was used to apply the ABC fraction under pressure to the top of the column. Unfortunately I lost c. a quarter to a third of this fraction, which was pushed back through the injector. The reason for this could be due to the volatility of hexane which could have expanded in the

injector and on the column due to the higher temperature inside the column.

4.5 ISOLATION OF BIOACTIVE FRACTION

The fractions were combined based on the TLC analysis and the quantity present in each fraction. Some of the combined fractions seemed to be relatively pure based on the TLC results and I decided to carry out bioautography on the combined fractions using *S. aureus*, *E. coli* and *P. aeruginosa* as test organisms.

The growth of *S. aureus* was inhibited by a compound with a Rf-value of c. 0.74 with CEF as eluent by fractions 21-50. As found earlier, bioautography with the other test organisms gave much less distinct results. The growth of *E. coli* was not inhibited by the same fractions as *S. aureus*, but a compound with an Rf-value of c. 0.84, gave clear inhibition in pooled fractions 51-60. This pool did not inhibit the growth of *S. aureus* indicating that there is some selectivity of the different bioactive compounds.

NMR spectroscopic analysis was carried out before determining the MIC values of the active fractions because it is a non-destructive method. Combined fractions 31-40 and 41-50, with a mass of 16 mg and 15 mg respectively, were apparently clean enough for NMR analysis based on the TLC results. These combined fractions were dried in a vacuum dessicator overnight after evacuating for one hour under high vacuum. Unfortunately only c. 3 mg of the material was left the next morning. The active compound was apparently volatile enough to be removed by vacuumpump.

NMR analysis showed that the remaining sample consisted mainly of phthalate, a common component of plasticizers used in making plastics and also a common contaminant in certain solvents such as ethyl acetate.

Phthalic acid was evacuated under vacuum to determine if this compound is volatile enough to be removed by high vacuum, but no loss of the phthalic acid was found. I determined the Rf-values of phthalic acid and the active compound. Phthalic acid did not react with the vanillin spray reagent, but it gave very clear quenching in UV light when TLC was performed. The Rf-value with CEF was c. 0.33 and phthalic acid could not be responsible for the antibacterial activity.

MIC of the pooled fractions 21-30 and phthalic acid was carried out as well as bioautography with *S. aureus*. Phthalic had a slight antimicrobial activity with an MIC in the order of 1 - 2 mg/ml. This could be an indirect pH related effect. The MIC values of all the fractions containing many non-active compounds were usually below 0.5 mg/ml. It is therefore unlikely that phthalic acid would represent a significant portion of the antimicrobial activity measured. A quantity of 50 µg pure phthalic acid did not exhibit any bacterial growth inhibition whereas 50 µg of an acetone leaf extract showed several antibacterial zones at Rf-values different from phthalic acid.

The main bioactive compound must have been present in a very low concentration and consequently must have a very high bioactivity, because a relative large quantity of phthalic acid would have been required for the growth inhibition noted.

4.6 RECOMMENDATIONS ON FUTURE WORK

There are still many aspects left to explore. The next logical step is that the non-polar fractions from the final column chromatographic separation should be investigated further.

There is also the possibility to capitalize on the suspected volatility of the bioactive fractions by separating these compounds from other compounds through a cold finger vacuum distillation process.

It should not be difficult to remove any contaminating phthalic acid from bioactive fractions by anion exchange chromatography.

At an international medicinal plant conference held in Zürich in September 2000, it was reported that *Combretum paniculatum* was the most active of all plants tested against the HIV virus replication (Anti-HIV-1 and -HIV-2 Activity of Ethnobotanically Selected Ethiopian Medicinal Plants. K. Asres, F. Bucar, T. Kartnig, M. Witvrouw, C. Pannecouque and E. De Clercq). Most taxonomists consider *C. paniculatum* to be synonymous with *C. microphyllum*. We found that the chemical fingerprints are similar. This result increases the importance of continuing the work initiated in this first modest attempt to isolate bioactive compounds from *C. microphyllum*.