Chapter 3

Materials and Methods

3.1 Plant collection

Plant material (leaf, stem and bark) of *Combretum paniculatum* was collected from the garden of Prof J.N. Eloff (Murrayfield, Pretoria) and identified by Prof Eloff. Collection was done in April 2004 when leaves were still green. A voucher specimen is deposited in the medicinal plant herbarium of the Pharmacology and Toxicology Section of the Department of Paraclinical Sciences at the University of Pretoria.

3.2 Preparation and extraction of plant material

The leaves were carefully examined and old, insect-damaged or fungus-infected leaves, twigs and flowers removed. Healthy leaves were spread out and dried in the laboratory at room temperature for about ten days. The plant material was ground to a fine powder using a Jankel and Kunkel model A 10 mill.

Small scale extraction was done using several solvents, from non-polar to polar. Extraction was done in small quantities (3 g to 30 ml solvent) in order to determine the solvent that extracted the most active components. Separate aliquots of plant material were used for each solvent. The following solvents were used: hexane, carbon tetrachloride, ethanol, acetone, dichloromethane, tetrahydrofuran, methanol and water. Extracts were reconstituted to 10 mg/ml for biological assays.

3.3 Analysis by thin layer chromatography (TLC)

In TLC, 100 µg of the plant extract (10 µl of 10 mg/ml) was separated on Merck TLC F_{254} analytical plates using as eluents solvent systems of different polarities, namely BEA (benzene/ethanol/ammonium hydroxide (90:10:1), CEF (chloroform/ethyl acetate/formic acid (5:4:1) and EMW (ethyl acetate/methanol/water (40:5.4:4). Separated components were visualized under visible and UV light at wavelengths of 254 nm and 365 nm (Camac Universal UV lamp TL-600). Plates were afterwards sprayed with *p*-anisaldehyde or vanillin sulphuric acid spray reagents and heated for about

five minutes at 100°C for development of colour (Wagner and Bladt, 1996). The two spray reagents were respectively prepared thus: 1 ml of *p*-anisaldehyde mixed in 18 ml of ethanol and 1 ml of concentrated sulphuric acid. For vanillin, 0.1 g of vanillin was dissolved in 28 ml methanol and 1 ml of sulphuric acid was added (Wagner and Bladt, 1996).

3.4 Bioassay-guided isolation

A bioassay-guided isolation method was used with the objective of isolating the antibacterially active compounds. Leaves of the plant were extracted and fractionated with the activity of the fractions identified by bioautography. The extracts were spotted on thin layer chromatography (TLC) plates and developed in the solvent system that separated the compounds most effectively. This was done initially using BEA, CEF and EMW. Duplicate chromatograms were dried overnight and sprayed with a concentrated suspension in Müller-Hinton (MH) broth of actively growing cells of Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis)* or Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa)* bacteria. The plates were incubated at 37°C overnight in a chamber at 100% relative humidity. Plates were sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium (INT) salt. Clear zones on the chromatogram indicated inhibition of growth after incubating for one hour (Begue and Kline, 1972). The INT is metabolized to a coloured formazan product by actively growing cells.

3.5 Solvent/Solvent fractionation

This procedure simplifies extracts by fractionating the components based on polarity. The solvent/solvent group procedure used by the US National Cancer Institute as described by Suffness and Douros (1979) was applied.

3.6 Chromatography

Column chromatography is the most widely used technique to isolate the components of complex mixtures. Various stationary phases were used to separate compounds either according to polarity or size of the compounds (normal and reverse silica gel, Sephadex and Amberlite XAD-16).

3.6.1 Amberlite XAD-16

Amberlite XAD-16 is a polymeric adsorbent supplied as insoluble white beads. It is a non-ionic, hydrophobic, cross-linked polymer which derives its adsorptive properties from its macroreticular structure (containing both a continuous polymer phase and a continuous pore phase), high surface area, and the aromatic nature of its surface.

3.6.2 Chromatotron

The chromatotron is a preparative, centrifugally accelerated, radial, thin-layer chromatograph designed by the authors of Compendium of Organic Synthetic Methods. It replaces preparative TLC plates, small columns and HPLC. The sample to be separated is applied as a solution using a dropper or syringe near the center of a spinning disk coated with a thin layer of sorbent. Elution by a solvent forms circular bands of the separated components which are spun off from the edge of the rotor together with the solvent. A novel collection system brings the eluate to a single output tube. The capacity that can be introduced to the plate should not be above 1 g.

Special advantages of the chromatotron include:

- No spotting of samples or scraping of bands.
- Separations are completed rapidly, typically within 20 minutes.
- A UV transparent lid allows direct observation of UV absorbing or coloured compounds during the separation.
- The pump recycles the output to the input for increased resolving power.
- Layer thickness of 1, 2 or 4 mm of sorbent on the inner plate gives high capacity. The sorbent layer is easily regenerated *in situ* for re-use.
- Solvents are used sparingly. Gradient elution is easy. A nitrogen atmosphere prevents oxidation of samples.
- Compact (easily moved from lab to lab), few controls, no high pressures.
- Low price: half a dozen chromatotrons cost less than a single preparative HPLC (high performance liquid chromatograph).

UV transparent compounds may be detected in the eluted fractions by conventional TLC.

3.7 High pressure liquid chromatography (HPLC)

Chemical separations can be accomplished using high pressure (or performance) liquid chromatography (HPLC) utilizing the fact that certain compounds have different migration rates given a particular column and mobile phase. The chromatographer can separate compounds using HPLC, and

the extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase.

In this research HPLC was also used in the isolation process. There is the option of using different types of columns and solvent systems for the separation of compounds as well as a variety of detectors to interface with the HPLC in order to achieve optimal analysis of the compound.

Detection of compounds by HPLC is a crucial part of any HPLC assay. In order to detect any compound by HPLC, a detector must first be selected. Once the detector is selected and is set to optimal detection settings, a separate assay must be developed. The parameters of this assay should be such that a clean peak of the known sample is observed on the chromatograph. The identifying peak should ideally have a reasonable retention time and should be well separated from extraneous peaks at the detection levels which the assay will be performed. To alter the retention time of a compound, several parameters can be manipulated. One is the choice of mobile phase, and another is the choice of column.

Selection of the separation system by HPLC is accomplished by researching the literature and by trial and error. A sample of a known compound must be utilized in order to assure identification of the unknown compound. Identification of compounds can be confirmed by combining two or more detection methods.

3.8 Analysis and concentration of fractions

When column chromatography was completed, the fractions collected in test-tubes were placed under a stream of air to facilitate concentration of the fractions for TLC analysis and bioassay. After concentration and reconstitution to 10 mg/ml, the contents of the test tubes were spotted on TLC plates, developed in a suitable solvent system, viewed under UV, sprayed wit a chromogenic spray reagent and heated for about 5 minutes. Those fractions with similar TLC profiles were combined. Plates were also developed for bioautography except that they were not sprayed with a spray reagent. Minimum inhibitory concentrations (MIC) of the fractions were also determined.

3.9 Antiviral activity

The crude extracts of *C. paniculatum* were tested for antiviral efficacy at the University of Pretoria. The active extracts were taken to the Hans-Knöll Institute (HKI) in Jena, Germany, where compounds were isolated from the plant material. These compounds were then tested for antiviral activity at the HKI.

3.9.1 University of Pretoria method

3.9.1.1 Cell culture

Crandell feline kidney cells (CRFK) were obtained from the Department of Veterinary Tropical Diseases, University of Pretoria. Cultures were grown in Eagle's Minimum Essential Medium (MEM) containing 10% foetal calf serum (FCS) and 0.05 mg/ml gentamicin. Confluent cell cultures were maintained at 37°C.

3.9.1.2 Virus

The virus used in this assay was feline herpesvirus type 1 (FHV-1), an enveloped virus. Enveloped viruses are highly sensitive to environmental influences. The virus used in the assay was cultured in 75 cm^2 flasks of confluent CRFK cells. Flasks were inoculated with virus stock and then incubated until approximately 90% of the monolayer showed cytopathic effect (CPE). The monolayer was then trypsinised and the resulting cell suspension centrifuged at 1000 X g for 10 minutes and the supernatant stored at -70°C.

3.9.1.3 Determination of the antiviral efficacy of the extract

The extract (see section 3.2) was diluted in sterile de-ionized water to a final dilution of 1:50. Virus stock (0.5 ml) was then mixed with 0.5 ml of extract (contact time 20 minutes). A serial 10-fold dilution was performed by taking 0.5 ml from the mixture of virus and extract and placing in tubes containing 4.5 ml MEM. The above-mentioned 10-fold dilutions were performed in 8 different tubes to give a dilution range of 1:10 to 1:1280. Two hundred µl of each dilution was placed in wells of a 96-well flat bottom microtitre plate. Eighty µl of CRFK cells (480,000 cells/ml) were added to each well in 5 replicates. Each extract test included a virus control and a toxicity test. The plates were incubated for 5 days at 37°C in a 5% CO_2 atmosphere. The CPE was observed by the use of an inverted light microscope. One hundred

percent cell damage was scored with a 4 while 75% cell damage was scored a 3 and so on. A zero indicated that the cells were unaffected. The tissue culture infectious dose 50 ($TCID_{50}$) was calculated using the Karber method (Karber, 1931).

3.9.1.4 Determination of the cytotoxicity of the extracts (MTT assay)

Viable cell growth after incubation with the test compound was determined using the tetrazolium based colorimetric assay described by Mosmann (1983). Cells (CRFK) of a sub-confluent culture were harvested and centrifuged at 200 x g for 5 min, and resuspended in growth medium to 4.8 x 10^5 cells/ml. The growth medium used was MEM supplemented with 0.1% gentamicin and 5 % foetal calf serum. A total of 200 µl of the cell suspension was pipetted into each well of a 96-well microtitre plate. The plates were incubated for 24 h at 37°C in a 5% CO_2 incubator, until the cells were in the exponential phase of growth. The MEM was aspirated from the cells using a fine tube attached to a hypodermic needle, and replaced with 200 µl of test compound at differing concentrations (serial dilution prepared in growth medium). The cells were disturbed as little as possible during the aspiration of medium and addition of test compound. Each dilution was tested in 5 replicates. The microtitre plates were incubated at 37°C in a 5% CO_2 incubator for 5 days. Untreated cells and positive control (berberine chloride, Sigma) were included.

After incubation, 30 µl MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, Sigma, stock solution of 5 mg/ml in PBS) was added to each well and the plates incubated for a further 4 h at 37°C. After incubation with MTT the plates were centrifuged for 10 min at 1500 rpm. The medium in each well was carefully removed, without disturbing the MTT crystals in the wells, before adding 150 µl fresh PBS to each well. The microtitre plates were again centrifuged for 10 min at 1500 rpm and the PBS removed from the wells. The MTT formazan crystals were dissolved by adding 50 µl DMSO to each well. The plates were shaken gently until the MTT solution was dissolved. The amount of MTT reduction was measured immediately by detecting absorbance in a microplate reader (Titertek Multiscan MCC/340) at a test wavelength of 540 nm and a reference wavelength of 690 nm. Wells containing medium and MTT but no cells, were used to blank the plate reader. The lethal concentration 50 (LC_{50}) value was calculated as the concentration of test compound resulting in a 50% reduction of absorbance compared to untreated cells.

3.9.2 Hans-Knöll Institute (HKI) method

3.9.2.1 Cytotoxicity test to determine the maximum tolerated dose (CC_{10}) of the test compound in HeLa, MDCK and Vero cell monolayers

MDCK (Madin-Darby Canine Kidney) and Vero cells were seeded in microtitre plates and incubated for 48 hours at 37°C in 95% humidity and in the presence of 5% CO_2 to form a monolayer. The medium was then discarded and the compounds added at various concentrations (100 µl per well; 2-fold dilutions). To determine the control value (6 untreated cell controls) 100 µl medium was used in each control well respectively. Cells were stained with crystal violet/methanol 72 hours after the compounds had been added and incubation had taken place. Following elution of the stain, the optical density of the individual wells was measured with a Dynatech 570/630 plate photometer and compared with the mean values of the controls. The CC_{10} values (µg/ml) were estimated with the aid of the foregoing data provided that more than 90% of the control cells were viable.

3.9.2.2 Determination of the antiviral efficacy of the test compounds by means of inhibition of the cytopathic effect (CPE)

The test system consisted of:

1. HeLa cells infected with coxsackievirus strain B3 Nancy
2. MDCK cells infected with influenzavirus type A strain Hong Kong
3. Vero cells infected with herpes simplex virus type 1 strain K1

The replication of the viruses used in the test results in pronounced CPE leading to complete destruction of the cells. The virus-induced CPE can be inhibited by adding antiviral substances in volumes of 100 µl per well and diluted by means of 2-fold dilutions. In the test, treated and untreated cell layers were infected with a dose of virus that after 24 hours (B3 and Hong Kong) and 48 hours (K1) resulted in complete CPE in the untreated virus controls. At this stage cells that were still adhering were fixed and stained with crystal violet/formalin solution. The inhibition of the virus-induced CPE was quantified photometrically using a Dynatech plate reader following elution of the stain. The antiviral efficacy was determined by comparing the optical densities of treated and untreated virus-infected cells with the average optical densities of the controls that was set as 100%. As internal test control, a known virustatic compound was included simultaneously in each microtitre plate.

3.10 Antibacterial activity

3.10.1 Microdilution assay for MIC determination

The MIC for each plant extract against a range of bacteria was determined by serial two-fold dilution of extracts beyond the level where no inhibition of growth of test organism was observed (Eloff, 1998b). The test organisms were the Gram-positive *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213, and the Gram-negative *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. The assay was performed in microtitre plates by adding 100 µl of sterile water to all wells. In row A, 100 µl of extract was added with a micropipette. From row A, 100 µl was transferred to row B after using a micropipette to take up and release the liquid three times to ensure adequate mixing. The process was repeated until all the rows down the column were completed and 100 µl from row H was discarded. Two wells were used as a sterility control containing only water, whilst the growth control contained both water and test organism. Gentamicin was used as a positive control antibiotic.

After adding 100 µl of the bacterial suspension to each row (except for the sterility control), the microplate was sealed and incubated at 37^0C at 100% relative humidity overnight. The following morning 40 µl of a 0.2 mg/ml solution of INT (iodonitrotetrazolium chloride) was added to each row and the plate was returned to the incubator for at least half an hour to ensure adequate colour development. INT is a dehydrogenase activity detecting reagent, which is converted into an intensely coloured red-purple formazan by metabolically active micro-organisms.

Inhibition of growth was indicated by a clear solution or a definite decrease in colour reaction. This value was taken as the MIC of the extract. Extracts used for MIC determination were either dissolved in acetone, water or DMSO.

3.10.2 Total activity

Total activity is a measure of the amount of material extracted from a plant in relation to the MIC of the extract, fraction or isolated compound. Reasons for screening studies abound: to find new lead biologically active compounds, and to confirm the ethnomedicinal use of plants to develop phytomedicines for use as herbal medicine. In many screening studies, activities are reported non-quantitatively. Even if extract data are expressed in quantitative terms such as MIC, it is usually not

possible to compare different plants or different fractions with the results presented. To compare activity of plants or fractions, however, the quantity extracted from the plant or present in the fraction should be included in the equation (Eloff, 2004). In mathematical terms it can be expressed as:

$$\text{Total activity (ml/g)} = \frac{\text{Amount extracted from 1 gram (mg) or amount present in fraction (mg)}}{\text{MIC (mg/ml)}}$$

The units are expressed in ml/g and indicate the degree to which the active extracts, fractions or compounds present in one gram can be diluted and still inhibit the growth of the test organisms (Eloff, 2004).

3.10.3 Bioautography

Extracts were reconstituted to a concentration of 10 mg/ml in acetone and 10 µl (100 µg) of each extract were applied as narrow lines on TLC plates. Extracts of the leaves and root bark were used for bioautography. The organic solvent was evaporated by a stream of air and plates were eluted by a solvent system that would provide efficient separation. The solvent was once more evaporated by a stream of air overnight. In the case of TLC plates run in CEF, plates were dried much longer to ensure adequate removal of formic acid. The plates were then sprayed with a suspension of actively growing bacterial cells and incubated overnight at 37^0C in a chamber at 100% relative humidity. The next morning, the plates were sprayed with a 2 mg/ml solution of INT. Inhibition of growth was indicated by clear zones on the chromatogram (Begue and Kline, 1972). This method was chosen for its simplicity, low cost, accuracy and fast results.

3.11 Antifungal activity of extracts

A serial microdilution assay with INT added as growth indicator (Masoko *et al.,* 2005) was used to determine the minimum inhibitory concentration (MIC) values for plant extracts against fungi. This method had previously been used successfully for antibacterial activities (Eloff, 1998b and McGaw *et al*., 2001). By applying the tetrazolium assay for measuring antifungal activities, a slight modification was made to suit fungal growth conditions. Extracts were resuspended to 10 mg/ml in acetone. The plant extracts (100 µl) were serially diluted in water in 96-well microtitre plates (Eloff, 1998b). Using a sterile swab, fungal cultures were transferred from Sabouraud agar plates into fresh Sabouraud dextrose broth, and 100 µl of this suspension was added to each well. Amphotericin B was used as the reference antibiotic and positive control, and appropriate solvent blanks were included. As an indicator

of growth, 40 µl of 0.2 mg/ml *p*-iodonitrotetrazolium violet (INT) dissolved in water was added to each of the microplate wells. The microplates were incubated at 37°C for 2-3 days. The MIC was recorded as the lowest concentration of the extract that inhibited antifungal growth after 24 and 48 h.

3.12 Agar diffusion method for antibacterial and antifungal activity

Nine ml of Müller-Hinton agar for bacteria and Sabouraud Dextrose Agar for fungi were poured into Petri dishes (9 cm in diameter) and inoculated with the respective test organisms to form a lawn. Wells (4 mm) were punched out of the solid agar using pipette tips, and 1 ml of 50 µg/ml test compounds and control antibiotics were placed into each well. The Petri dishes were incubated at 37°C for 20 h and the average diameter of the inhibition zone surrounding the wells was measured.

3.13 Antioxidant activity

Metabolism, like other aspects of life, involves trade-offs. Oxidant by-products of normal metabolism cause extensive damage to DNA, proteins, and lipids. It is argued that this damage (the same as that produced by radiation) is a major contributor to ageing and to degenerative diseases of ageing such as cancer, cardiovascular disease, immune-system decline, brain dysfunction, and cataracts. Antioxidant defenses against this damage include ascorbate, tocopherol, and carotenoids. Dietary fruits and vegetables are the principal source of ascorbate and carotenoids and are one source of tocopherol. Low dietary intake of fruits and vegetables doubles the risk of most types of cancer as compared to high intake and also markedly increases the risk of heart disease and cataracts. Since only 9% of Americans eat the recommended five servings of fruits and vegetables per day, the opportunity for improving health by improving diet is great (Ames *et al.*,1993).

Plant extracts may protect against infection by stimulating or protecting the immune system of the user. Immune system stimulation is associated with antioxidant activity. The most widely used methods for measuring antioxidant activity are those that involve the generation of a radical species and measurement of a range of end points as a fixed time. Two types of approaches may be followed, namely inhibition assays where the extent of the scavenging by hydrogen or electron donation of a preformed free radical is the marker of antioxidant activity as well as assays involving the presence of antioxidant systems during the generation of the radical. This study made use of the DPPH free radical assay.

DPPH (1, 1 diphenyl-2-picryl hydrazyl) is a stable purple-coloured free radical which does not dimerize and can hence be prepared in crystalline form. The DPPH method measures hydrogen atom or electron donating activity and hence provides an evaluation of antioxidant activity due to free radical scavenging. Reaction of the DPPH with the antioxidant results in a decolourisation of the free radical, which can be followed spectrophotometrically. DPPH may also be used as a TLC spray reagent in a quick screening procedure which detects the presence of antioxidants when the initial purple background on the TLC plate turns yellow in the presence of an antioxidant (Brand-Williams *et al*., 1995). In this way, the qualitative antioxidant activity can be determined easily by spraying a chromatogram with 0.2% DPPH. The yellow zones on the purple background represent compounds with antioxidant activity.

3.14 Anti-inflammatory activity

The NAD(P)-linked enzyme, 3α-hydroxysteroid dehydrogenase, has been purified to homogeneity from rat liver cytosol (Penning, 1985). This enzyme is known to reduce a variety of 3-ketosteroids, e.g. 5α-dihydrotestosterone (5α-androstan-17β-o-one), 5β-dihydrocortisone (5β-pregnan-17α, 21-diol-3, 11, 20-trione), to the corresponding 3α-hydroxysteroids, and therefore plays an important role in cortisone metabolism (Penning, 1985). A surprising property of the purified enzyme is that it is potentially inhibited by the major classes of non-steroidal and steroidal anti-inflammatory drugs in rank order of their therapeutic potency (Penning, 1985). A high correlation exists between the logarithms of the concentration of drug required to produce 50% inhibition of the purified 3α-hydroxysteroid dehydrogenase (log IC_{50} value) with the dose required to produce an anti-inflammatory response in man. These observations led to the suggestion that the extent of inhibition of 3α-hydroxysteroid dehydrogenase could be used to predict anti-inflammatory drug potency (Penning, 1985).

3.14.1 Enzyme assay

The reduction of 5β-dihydrocortisone was monitored by measuring the changes in the absorbance of the pyridine nucleotide at 340 nm. Each assay (1.0 ml) contained the following: 0.840 ml of H_2O, 0.100 ml of 1 M potassium phosphate buffer (pH 6.0), 20 µl of 9 M NADPH, 10 µl of 5 mM 5β-dihydrocortisone, and 30 µl of acetonitrile. The reactions were initiated by the addition of enzyme (30-50 µg of cytosolic protein or 0.6 µg of purified enzyme), and optical density change was observed over a period of 5 minutes. Control incubation experiments by addition of cytosol in which either the 5β-

dihydrocortisone or NADPH was absent, indicated that the presence of both substances was required before the cytosol would promote a change in absorbance at 340 nm.

The percentage inhibition of isolated compounds was detected at three concentrations (30 µg/ml, 3 µg/ml and 0.3 µg/ml). Increasing amounts of the isolated compound were added to the standard assay and the concentration of the compound required to reduce the rate of 5β-dihydroxycortisone by 50% inhibitory concentration (IC_{50}) was computed from the resulting dose-response curves.

Chapter 4

Selection of the best extractant for the plant material

4.1 Introduction

In phytochemical analysis, the botanical identity of the plant under study must be authenticated by an acknowledged authority and a voucher specimen stored so that results can be reproduced on the correct species by other scientists. Also, if taxonomy changes, the identity of the species investigated can be verified.

There are different methods available for extracting compounds from plant material. One method for obtaining organic constituents from dried plant tissue is to use a Soxhlet apparatus with a sequential range of solvents, starting in turn with ether, petroleum and chloroform (to extract lipids and terpenoids) and then using alcohol and ethyl acetate (for more polar compounds). This method is useful when working on the gram scale. The extract obtained is clarified by filtration and concentrated in a rotary evaporator. This procedure leads to exhaustive extraction but labile compounds will be destroyed by the high temperature used.

A direct cold extraction procedure of finely ground material was developed for use in the Phytomedicine Programme (Eloff, 1998a). This chapter will focus on how to select the best extractant, quantity and number of compounds separated, and whether there is a difference between compounds extracted from *C. paniculatum* and *C. microphyllum.* The aim of this chapter is to determine the best extractant to use in the isolation of the bioactive compounds and also to compare data for the closely related *C. paniculatum* and *C. microphyllum.* Germishuizen and Meyer (2003) consider *C. paniculatum* and *C. microphyllum* the same species while Carr (1988) considers them to be different species.

4.2 Extraction

Extraction was carried out on the powdered leaf material of *C. paniculatum*. This was performed on a Labotec Model 20.2 shaking apparatus with a 1:10 dry weight plant sample (g) to solvent (ml) ratio. This extraction was carried out in parallel using eight solvents ranging from non-polar to polar (hexane, carbon tetrachloride, dichloromethane, tetrahydrofuran, ethanol, acetone, methanol and water). One

gram of plant material (separate aliquots for each solvent) was placed in a polyethylene centrifuge tube and 10 ml of the extracting solvent placed into the polyethylene tube. The sample and solvent were shaken for 10 minutes on the shaker and centrifuged for 10 minutes at 1500 x g before filtering using Whatman No. 1 filter paper. The solvent was evaporated and the residue resuspended to 10 mg/ml. The plates were developed in three solvent systems, namely BEA, EMW and CEF, and the fingerprint profile observed. The composition of the solvent systems is described in section 3.3.

4.3 Results

Water extracted the largest quantity (246 mg) with a 24.6% yield followed by methanol (194 mg and 19.4% yield). The lowest quantity extracted was for dichloromethane (DCM) (15 mg) and 1.5% of material was extracted (Table 4.1). Water and methanol extracted the highest quantity of material from *C. paniculatum*, and this implies that there are many polar compounds in the sample since water and methanol are the most polar solvents among the solvents used. The values for *C. microphyllum* that were obtained from the work of Kotze and Eloff (2002) are also included in Table 4.1 for comparative purposes. In general, the results for the two plants were dissimilar.

Table 4.1. Quantity of material extracted (mg/g) from *C. paniculatum* and *C. microphyllum* (Kotze and Eloff, 2002)

Plant species	Hexane	CCl_4	DCM	THF	EtOH	Acetone	MeOH	Water
C. paniculatum	23	27	15	47	43	29	194	246
C. microphyllum	36	48	106	64	36	32	174	48

Figure 4.1 depicts TLC separations of the leaf extracts developed in EMW and sprayed with vanillin sulphuric acid spray reagent.

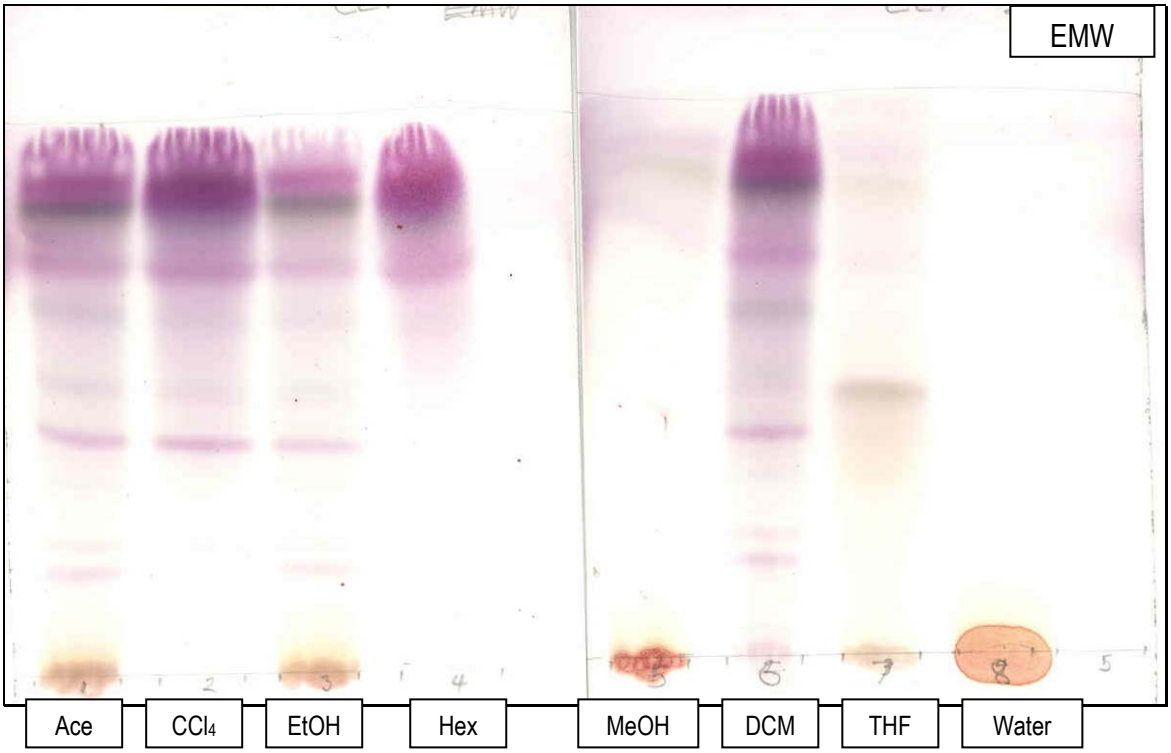

Fig 4.1. TLC plate showing separation of components present in 8 different solvent extracts of *C. paniculatum* leaf material using EMW as eluent and vanillin spray reagent

With the spray reagent used, about 11 compounds were detected by TLC in the EMW solvent system. No components visualized by the spray reagent separated in the methanol and water extract using this eluent. The retention factors of the components of the leaf extract are shown in Table 4.2.

Table 4.2. Retention factors (R_f) of compounds present in leaf extracts developed in EMW and sprayed with vanillin/sulphuric acid spray

Solvents	Retention factor (R_f)						
Hexane					0.77	0.88	
CCl_4		0.46	0.56		0.77	0.89	
DCM	0.25	0.46	0.65		0.74	0.88	
THF			0.52				
EtOH		0.46			0.77	0.87	0.91
Acetone	0.24	0.46	0.56	0.68	0.77	0.85	0.89
MeOH	0						
H_2O	0						

Acetone extracted the highest number of compounds (7), followed by dichloromethane (5). Water and methanol extracts did not move from the origin so the retention values are zero in the EMW solvent system. The TLC chemical profile of the acetone extract indicated that a wide range of compounds was extracted by acetone. The retention factors had a wider range for acetone and the major compounds isolated by the other solvents are also found in the acetone extracts. Extracts of water and methanol did not separate even with the polar EMW solvent system, meaning that the compounds isolated were highly polar and stayed at the bottom of the plate.

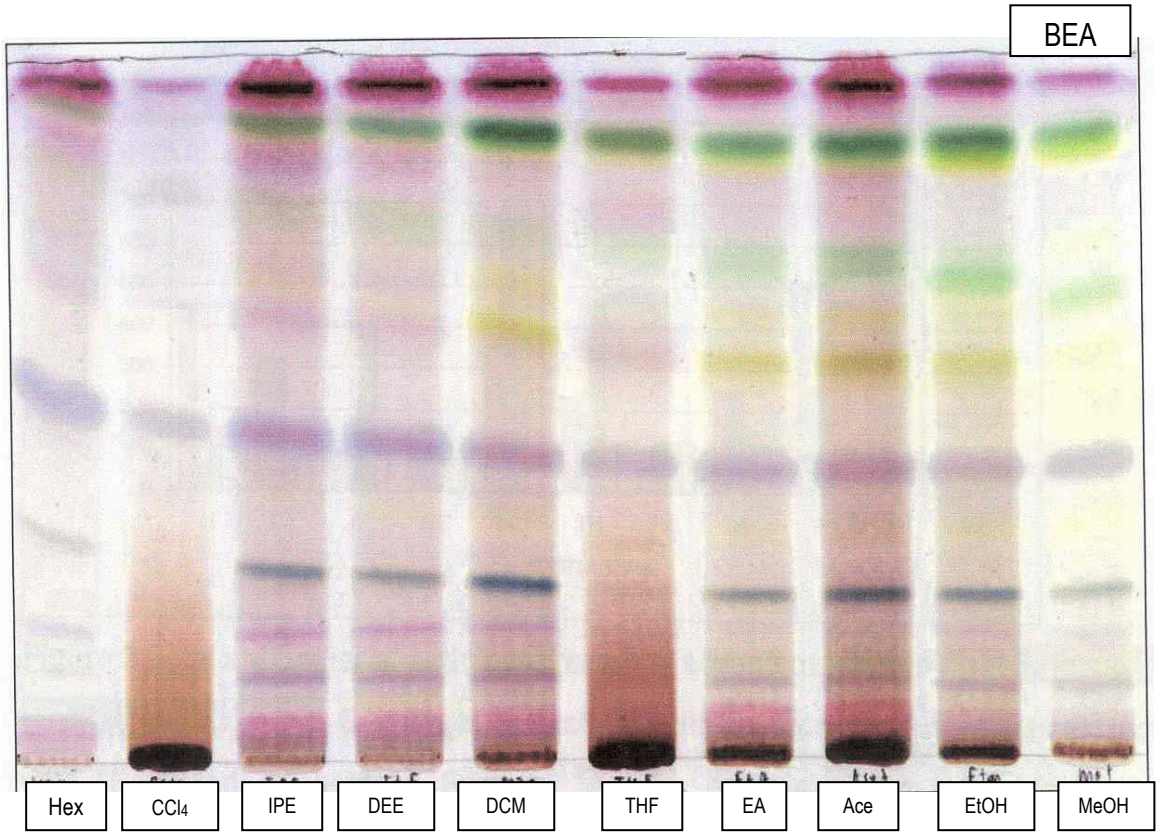

Fig 4.2. Separation of components present in 100 µg of extracts of *C. microphyllum* leaves, developed in BEA and sprayed with vanillin/sulphuric acid spray reagent (from Kotze and Eloff, 2002)

The solvents used from left to right are: hexane, carbon tetrachloride, isopropyl ether, diethyl ether, dichloromethane, tetrahydrofuran, ethyl acetate, acetone, ethanol and methanol.

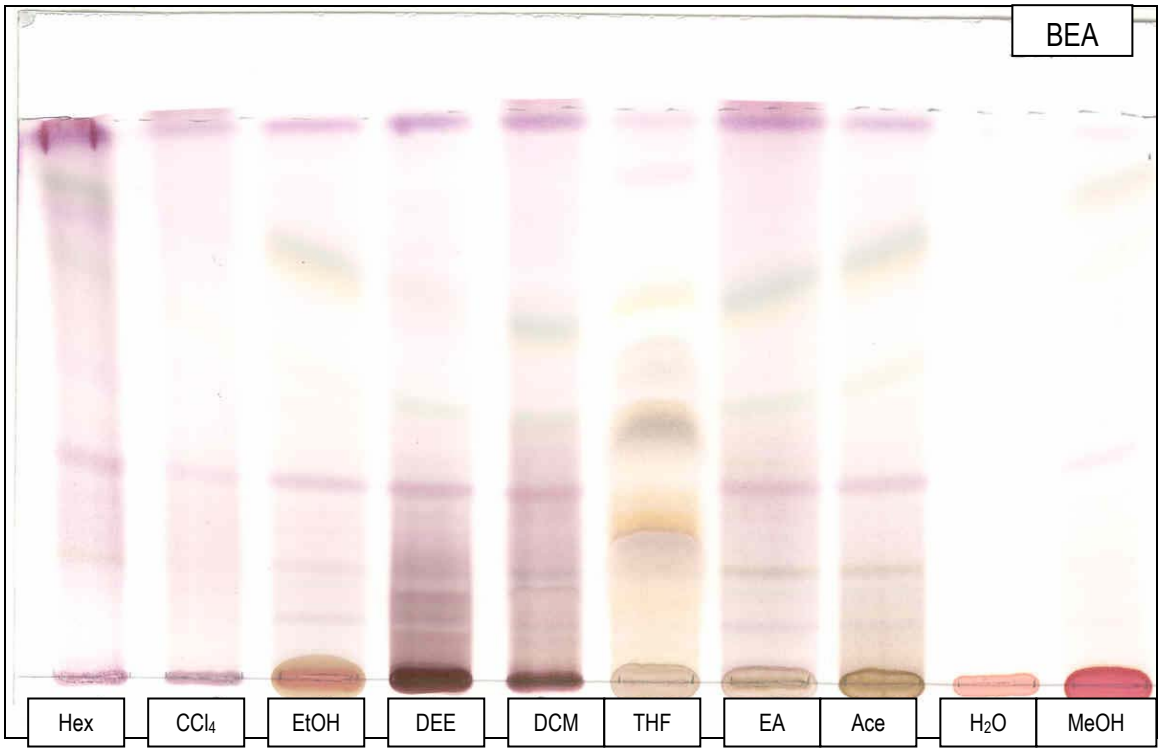

Fig 4.3. Separation of components present in 100 µg of extracts of *C. paniculatum* leaves developed in BEA and sprayed with vanillin/sulphuric acid spray reagent

Lanes from left to right represent compounds extracted by hexane, carbon tetrachloride, ethanol, diethyl ether, dichloromethane, tetrahydrofuran, ethyl acetate, acetone, water and methanol. No components separated in the water and methanol extract.

4.4 Discussion

The amount of material extracted per gram of powdered *C. paniculatum* leaves ranged from 15 mg to 246 mg (Table 4.1). These values are different from those obtained by Kotze and Eloff (2002) working on the closely related *C. microphyllum* (26 to 174 mg). The amounts of material extracted by water and DCM for the two species were notably different but the amounts for the other solvents were closer. This may be related to the season of collection of plant material, when sugars (polar and soluble in water) may have been converted to non-polar metabolites (soluble in DCM).

For *C. paniculatum,* water, methanol and THF extracted the most components while for *C. microphyllum*, methanol, DCM and THF extracted the most compounds. *C. microphyllum* extracts were separated into many more compounds using TLC analysis than *C. paniculatum* extracts. The

differences shown in the fingerprints and the amounts of material extracted from the two plants supports the view of Carr (1988) that the plants represent different species. The differences in amounts of material extracted from *C. microphyllum* and *C. paniculatum* and in TLC analysis of fingerprint profiles can be seen in Table 4.1, Fig 4.2 and Fig 4.3 respectively.

TLC was used to determine the chemical composition of the extracts. The solvent systems used were BEA, CEF and EMW. The acetone extract had as many as seven compounds on the TLC fingerprint profile compared to the other solvents used for extraction. Dichloromethane, carbon tetrachloride, hexane and tetrahydrofuran extracts were separated into 5, 4, 2, and 1 compounds respectively by TLC in the EMW solvent system. Water and methanol extracts did not separate into compounds visible with the spray reagent.

As far as the selection of the extractant of choice is concerned, Eloff (1998a) found that acetone was the best extractant, especially for the Combretaceae family. This conclusion was confirmed in work done on several *Combretum* and *Terminalia* species investigated in the Phytomedicine Programme. Based on the presence of compounds visualized with the vanillin spray reagent, acetone extracted the largest number of different compounds from *C. paniculatum* leaves in this study. The ease of evaporation and the fact that it is not toxic to bacteria at the concentrations used in the bioassays in this study makes acetone a good solvent to work with. Asres *et al.* (2001) also used acetone as extractant for the leaves of *C. paniculatum*, and the extract exhibited antiviral activity against HIV-2 with an EC_{50} of 3.0 µg/ml and a selectivity index of 32. Acetone was therefore selected as the initial extractant in further work.