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CHAPTER 4

4. MATERIALS AND METHODS

4.1 PLANT MATERIAL

Fresh or dried plant material can be used as a source for secondary plant components. Most scientists have opted to use dry material for several reasons: (i) traditional healers frequently use dried plant material, (ii) the time delay between collecting plant material and processing it makes it difficult to work with fresh material and (iii) there are fewer problems associated with the large scale extraction of dried plant material. Freshly harvested and dried materials are more commonly used since old dried material may undergo some quantitative losses and qualitative changes. In the present study dried material was used.

Combretum erythrophyllum [Burch] Sond. leaves were collected from a tree in the Pretoria National Botanic Gardens. The tree was identified by the plant label and the identity was confirmed by Mrs C.L. Bredenkamp, the curator of Combretaceae at the National Botanical Institute. A voucher specimen was deposited in the National Herbarium (C.L. Bredenkamp 1542 PRE). With later studies leaves were collected from various sites around Pretoria and voucher specimens were deposited in the National Herbarium.

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4.2 PREPARATION OF LEAF MATERIAL AND EXTRACTION

Farnsworth [1994] states that the biggest problem in drug development from plants is choosing the appropriate extracting solvent.
Soxhlet extraction of dried plant material is widely used employing solvents of increasing polarity, e.g. ether, petroleum ether, chloroform, ethyl acetate and ethanol. This method only works for compounds that can withstand the high temperatures of the boiling solvent but cannot be used for thermolabile compounds. This problem can be overcome by boiling at reduced pressure but this can be difficult and was therefore not used in this study.

The choice of solvent also depends on what is planned with the extract. If the extract is to be used for antimicrobial screening, the effect of the solvent on subsequent bioassay is important. From previously published papers where authors screened plants for antimicrobial properties, it can be noted that the authors used a variety of extractants. These have varied from 80% ethanol [Vlietinck, 1995], methanol [Taylor, 1995], petroleum ether, chloroform, ethanol, methanol and water [Salie, 1996]. In previous studies it was found, however, that acetone extracted a greater number of inhibitors (14) than those extracted with methanol (9), methylene dichloride (11), ethanol (8) and water (1), and was therefore used in this study to extract the crude material [Eloff, 1998].

The leaves were carefully examined and old, insect-damaged; fungus-infected leaves, twigs and flowers were removed. Healthy leaves were spread out and dried in the laboratory at room temperature for 5-8 days or until they broke easily by hand. Once completely dry, leaf material was ground to a fine powder using a Jankel and Künkel Model A10 mill. Larger quantities were sent to Phytotron at the Department of Plant Production and Soil Science, where a Wiley mill was used to grind material to a fine powder of c. 1.0 mm diameter. Material was stored in a closed container at room temperature until required.

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Extraction was initially performed on a Labotec Model 20.2 shaking apparatus with a 10 ml: 1 g solvent to dry weight ratio. With larger quantities of material, a ratio of 5 ml: 1 g was used and shaken continuously for an hour each time. This procedure was repeated three times and all extracts were decanted and combined.

The extracts were filtered before drying using Whatman filter paper no. 2 on a Büchner funnel and the solvent removed by vacuum distillation in a Büchi rotary evaporator at 60ºC, care being taken to decrease the temperature to 40°C for the final drying. For quantitative determination, the extracts were placed in pre-weighed flasks before drying. Technical grade acetone [Merck] was used as solvent.

4.3 ANALYSIS BY TLC

Thin layer chromatography [usually 5 µl of a 100 mg extract/ml solution] was on Merck TLC F_{254} or Macherey-Nagel Alugram Sil G $_{254}$ or Polygram Sil G UV_{254} plates using chloroform:ethyl acetate:formic acid [5:4:1], acetone:methylene dichloride [2:3] and benzene:ethanol [9:1] as eluents. Streaking of polar components was minimised by the addition of 1% ammonium chloride to the benzene:ethanol solution. In some cases DC Alufolien RP18 F_{254} [Merck] plates were used, eluting with varying ratios of water: methanol. Samples were applied quickly and run without delay to minimise the possibility of oxidative or photo-oxidative change. Separated components were visualised under visible and ultraviolet light [254 and 360 nm, Camac Universal UV lamp TL-600]. Plates were also sprayed with 5% anisaldehyde in 5% sulphuric acid in ethanol and 0.36% vanillin in 3.6% sulphuric acid in methanol and heated for 2-5 minutes at 100°C to allow for development of colour changes [Carr and Rogers, 1986].

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4.4 BIOASSAY AND TEST ORGANISMS

The biological assays employed were chosen because of their simplicity, reproducibility, and sensitivity and relatively low cost while being rapid and simple at the same time.

4.4.1 Antibacterial activity

Minimum inhibitory concentration [MIC] was determined by serial dilution of extracts beyond the level where no inhibition of growth of test organisms was observed [Eloff, 1998]. This was performed in microplates by filling all wells, with 50 μl sterile Mueller Hilton broth. In row A, 50 μl [100%] of the extract was placed with a micropipette. From row A, 50 μl was transferred to row B after taking up and releasing three times to ensure adequate mixing. The process was repeated until all the rows were completed and the additional 50 μl from row H was discarded. Two wells were used as a sterility and growth control respectively with the sterility control containing only Oxoid® Mueller Hilton broth (MHB), whilst the growth control containing both MHB as well as test organism.

After adding 50 μl of the bacterial suspension to each row (except for the sterility control), the microplate was sealed and incubated at 37°C at 100% relative humidity overnight. The following morning 50 μl of a 0.2 mg/ml solution of INT (p-iodo-nitrotetrazolium violet) was added to each row and the plate was returned to the incubator for at least half an hour to ensure adequate colour development. p-INT is a dehydrogenase activity detecting reagent, which is converted into corresponding intensely coloured formazan by metabolically active micro-organisms [Navarro, 1998].

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Inhibition of growth was indicated by a clear solution or a definite decrease in colour reaction. This value was taken as the minimum inhibitory concentration [MIC] of the extract. Extracts used for MIC determination were either dissolved in acetone or solubilized in DMSO (100 to 200 µl/mg) and made up as a stock solution (200 µg/ml) with distilled water. Well A had typically a final concentration of 100 µg/ml. Positive controls for test organisms were usually made up to a concentration of 1000 µg/ml and are listed in section 4.4.3.

4.4.2 Bioautography

Chromatograms were dried overnight to remove solvents from the plate. In the case of TLC plates run in CEF, plates were dried for at least 40 hours to ensure adequate removal of formic acid. The plates were then sprayed with a suspension of actively growing *Staphylococcus aureus* bacterial cells and incubated overnight at 37°C in a chamber at 100% relative humidity. The following morning, the plates were sprayed with a 2 mg/ml solution of p-iodonitrotetrazolium violet [Sigma]. 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.

4.4.3 Test organisms used

The following test organisms were commonly used to test minimum inhibitory concentration of the extracts: *Staphylococcus aureus* (Gram-positive) [American Type Culture Collection number 29213] *Pseudomonas aeruginosa* (Gram-negative) [ATCC 27853], *Escherischia coli* (Gram-negative) [ATCC 25922] and *Enterococcus faecalis* (Gram-positive) [ATCC 21212]. *S. aureus* is considered to be one of the most important pathogenic bacteria and is used as primary bioassay organism for this reason.

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All these organisms are important nosocomial pathogens widely used in screening tests (Table 4.1) and are reference isolates recommended by the National Committee for Clinical Laboratory Standards, USA [NCCLS, 1990].

Table 4.1: Test organisms widely used in screening tests

Vlietinck [1995]	Fourie et al. [1992]	Wallhäuser [1966]
Microsporium canis		*Microsporium gypseum*
Trichophyton mentagrophytes		
Staphylococcus aureus	*Staphylococcus aureus*	*Staphylococcus aureus*
Pseudomonas aeruginosa	*Pseudomonas aeruginosa*	
Candida albicans	*Candida albicans*	*Candida albicans*
Escherischia coli	*Escherischia coli*	
	Streptococcus pyogenes	
	Aspergillus niger	
		Bacillus subtilis
		Micrococcus flavus
		Saccaromyces cerivisiae
		Sarcina lutea

Previous studies showed inhibition of these four test organisms to different degrees. The two Gram-positive organisms were more sensitive than the Gram-negative with *S. aureus* being the most sensitive (100%) followed by *E. faecalis* (36%), *E. coli* (11%) and *P. aeruginosa* (3%). It was therefore decided to include a larger spectrum of organisms (including a fungus) in order to obtain a wider knowledge of both the spectrum as well as the type of inhibition (bactericidal or bacteriostatic) that these compounds exhibit. For this reason *Klebsiella pneumoniae, Salmonella typhimurium, Streptococcus faecalis, Shigella sonei, Micrococcus luteus, Aspergillus niger* and *A.. fumigatus, Bacillus subtilis* and *Vibrio cholerae* were also included and were kindly supplied by Mrs. A. Lombard of the Department of Microbiology and Plant Pathology, University of Pretoria.

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Incubation times and positive controls used in MIC determinations for these organisms are listed below (Table 4.2).

The cultures were grown at 37ºC and regularly subcultured (1% inoculum) in Mueller Hilton broth. Every c. 3 months, new cultures of *S. aureus, P. aeruginosa, E. faecalis* and *E. coli* were obtained from Dr Flavia Huygens, Department Medical Microbiology, University of Pretoria.

Table 4.2: Incubation periods and positive controls for test organisms used in the bioassays

ORGANISM	POSITIVE CONTROL	INCUBATION PERIOD
Aspergillus	Amphotericin B	24 hours
Bacillus subtilis	Gentamicin	24 hours
Enterococcus faecalis	Gentamicin	4 hours
Escherischia coli	Gentamicin	4 hours
Klebsiella pneumoniae	Gentamicin	4-6 hours
Micrococcus luteus	Ampicillin	24 hours
Pseudomonas aeruginosa	Gentamicin	4 hours
Salmonella typhimurium	Ampicillin	4 hours
Shigella sonei	Ampicillin	4 hours
Staphylococcus aureus	Ampicillin	4 hours
Streptococcus faecalis	Ampicillin	4 hours
Vibrio cholerae	Chloramphenicol	12-24 hours

MHB is recommended by the NCCLS for broth dilution susceptibility testing and was therefore chosen for the bioassays.

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4.5 GROUP SEPARATION OF EXTRACTS

4.5.1 Solvent / solvent extraction

The purpose of this procedure is to simplify extracts by fractionating the chemical compounds into broad groups based on their solubilities. The solvent/solvent group separation procedure used by the USA National Cancer Institute as described by Suffness and Douros [1979] was applied with slight variation. After extracting the dry leaf material, the acetone extract was taken to dryness in a Büchi RE-120 rotary evaporator under reduced pressure, rotating at c. 100 rpm and the water bath temperatures initially at 60°C but not exceeding 40°C when the extract was nearly dry. This extract was dissolved in 1:1 mixture of chloroform and water and the two phases were separated in a separatory funnel. The water fraction was mixed with an equal volume of n-butanol in a separatory funnel to yield the water [W] and butanol [B] fractions. The chloroform fraction was taken to dryness in a rotary evaporator under reduced pressure as described above and extracted with an equal volume of hexane and 10% water/methanol mixture. This yielded the hexane [H] fraction and the 10% water/methanol mixture was diluted to 20% water/ methanol by the addition of water. This was then mixed with carbon tetrachloride in the separatory funnel to yield the carbon tetrachloride [CT] fraction. The 20% water/methanol was further diluted to yield a mixture of 35% water/ methanol and mixed with chloroform to yield the chloroform [CL] and 35% water in methanol [WM] fractions. In all cases, equal volumes of solvents were used and the process repeated until the extracting solution was light in colour. In some cases centrifugation had to be applied to separate the solvent phases.

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For quantitative determination, extractants were placed in pre-weighed flasks and the solvents removed under vacuum in the rotary evaporator. The extracts were later redissolved in acetone or acetone/water for further analysis. The process is schematically represented in Figure 4.1.

From previous studies with *S. aureus* as test organism, the chloroform soluble fraction contained by far the largest quantity of inhibiting components (100%), followed by the fractions soluble in water (23%), 35% methanol in water (18%), carbon tetrachloride (2%) and hexane (traces) [Martini, 1998]. Due to the relatively low complexity of the hexane fraction (one inhibitory compound) and the great diversity of inhibitory compounds present in the chloroform fraction, these two fractions were evaluated further. Bioassay-guided fractionation was used throughout the study.

4.5.2 Analysis and bioassay of fractions

The hexane, chloroform, carbon tetrachloride, butanol and 35% water/methanol fractions were dissolved in 100% acetone. The water fraction was dissolved in 30% acetone in water. Thin layer chromatography [5 µl of 100 mg/ml extract] was on Merck TLC F254 plates with acetone:methylene dichloride (2:3), chloroform:ethyl acetate:formic acid (5:4:1) and benzene:ethanol:ammonium chloride (9:1:1%). In some cases ethylacetate:methanol:water (40:5.4:4) and other variations thereof were used for comparison. Separated components were visualised under visible and ultraviolet light [254 and 360 nm, Camac Universal UV lamp TL-600]. Plates were then sprayed with 0.36% vanillin, 3.6% sulphuric acid in methanol and heated at 100°C for 2-5 minutes [Carr and Rogers, 1986]. Minimum inhibitory concentrations were determined by means of serial dilution as described in paragraph 4.4.1.

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SOLVENT/SOLVENT EXTRACTION

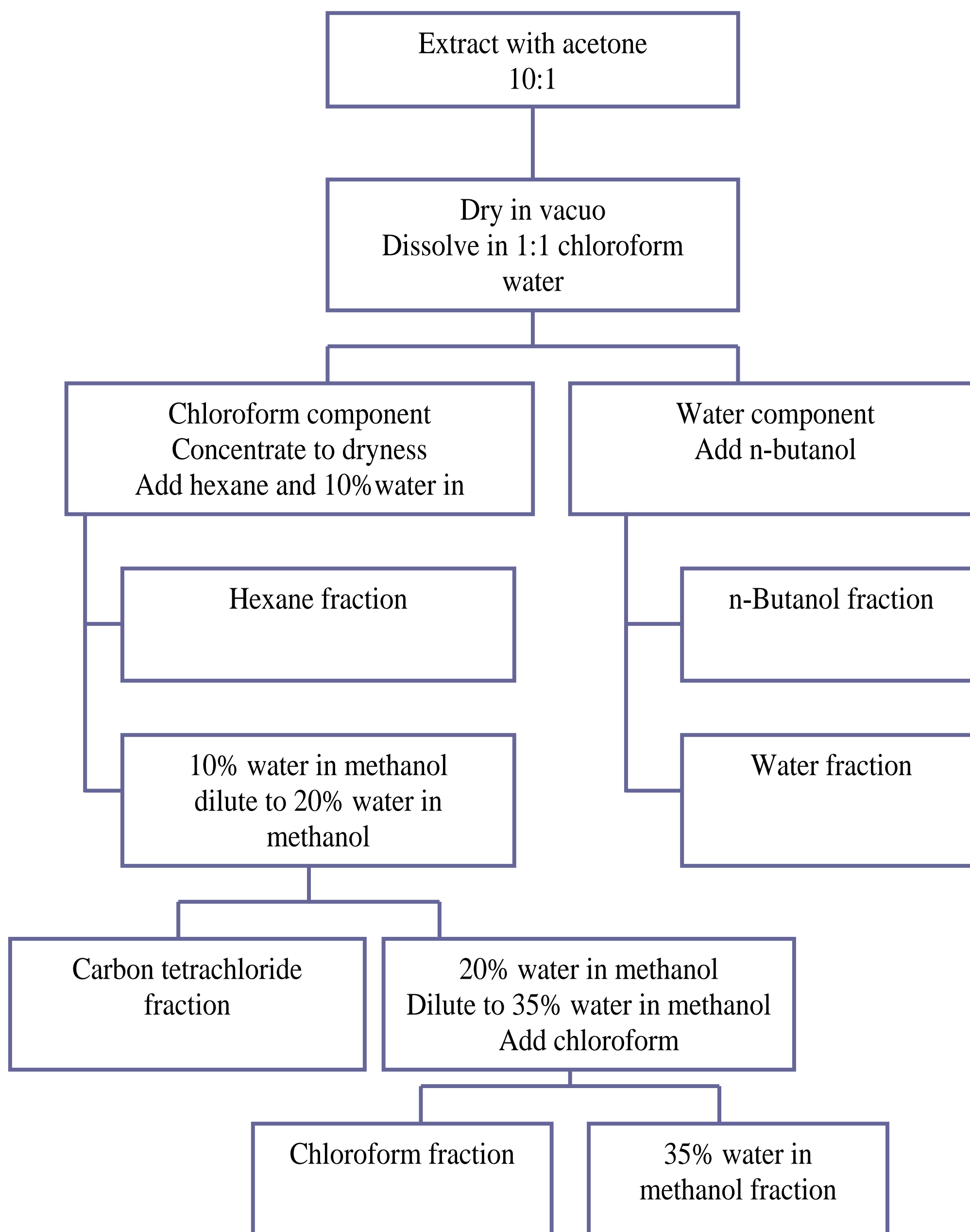

Figure 4.1: The procedure used for solvent/solvent fractionation

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4.6 ISOLATION OF BIOACTIVE COMPONENTS

4.6.1 Column chromatography

Column chromatography (CC) is the most widely used technique to isolate the components of complex mixtures (preparative chromatography). CC can also be used to determine the quantity of different compounds present in the mixture (analytical chromatography). Various stationary phases were used to separate compounds either according to polarity (normal and reverse phase silica gel, Sephadex) or size of compounds (Toyopearl - size-exclusion chromatography).

After solvent-solvent group fractionation (paragraph 4.5.1), the fractions were dried in the Büchi rotary evaporator to determine the mass extracted in each solvent. Initially the hexane fraction was chosen for column chromatography due to its relatively low complexity as seen with TLC and bioautography. Later the chloroform fraction was also employed in column chromatography.

In order to select the best mobile phase for eluting the hexane fraction, 5 μl of a 100 mg/ml solution was spotted on TLC and ran with combinations of solvents. In this way the solvent system that exhibited the most favourable separation of compounds was chosen.

In all cases a filter (Whatman filter no. 2) was cut to size and inserted at both the bottom and top of the stationary phase to prevent disruption during elution of the compounds.

Schematic presentations of the methods employed in separating and analysing the various fractions are depicted in Figures 4.2 and 4.3 at the end of the chapter.

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4.6.1.1 Silica gel

Silica gel (Kieselgel 60, 0.015-0.04 mm) was suspended in required solvent and left for c. 2 hours to swell after which it was poured into the column. The fraction obtained during solvent/solvent extraction was suspended in the minimum amount of particular solvent in which it would dissolve and filtered to remove impurities and any large particles which could cause diffusion problems whilst developing the column. This fraction was applied to the top of the column using a pipette with great care as not to disturb the top of the column. After application, the solvent flask was raised to facilitate solvent flow into the column and was run either using gravitational force or with pressure by connecting a Labotec pump to the column to facilitate elution. In the case where BEA was used as eluent, the column was developed in a fume hood to limit toxic benzene vapours in the atmosphere.

A concentration gradient of eluents was used. With the hexane fraction a 500 ml mixture of hexane:methylene dichloride (1:1) was used, gradually introducing a mixture of acetone:methylene dichloride (1:1). 100% acetone was introduced next and finally methanol was added to elute any components that could not be moved with the other solvents. The column was left to run overnight, connected to a fraction collector, at a flow rate of 0.6 ml/min.

The chloroform fraction was run initially with 40% hexane in dichloromethane (DCM), gradually increasing the DCM concentration to 100%. Methanol was introduced initially at 5% and increasing to a final concentration of 80% methanol in DCM.

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4.6.1.2 Sephadex

Sephadex LH-20 [Fluka®] was employed in separating the chloroform fraction. It was poured into the column in the dry state and the solvent added thereafter. A gradient was used starting with 100% hexane, 1:1 hexane to chloroform and then gradually increasing the chloroform to 100%. Methanol was added in small increments starting with 5% in chloroform and increasing to 100%.

4.6.1.3 Reverse phase

Bondesil C_{18} (40μm preparative grade) silica was used as reverse phase stationary phase. Reverse phase elutes the most polar compounds first and was thus employed in separating polar compounds not eluted with normal phase silica.

The stationary phase was mixed with 10% water in methanol and poured into a column. After a filter (Whatman filter paper no. 2) was cut to size and inserted at the top of the column, glass beads were thrown on top of the column to hold down the stationary phase. A small polypropylene pre-column was inserted on top of the main column containing the same stationary phase as that of the main column. The sample was applied to this pre-column and eluted with solvents to remove impurities and large particles thus preventing adsorption of compounds on the main column. Once fully eluted, the column was connected to the Labotec pump and solvent was pumped through to wash the column.

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4.6.1.4 Toyopearl®

Toyopearl HW-40F has a particle size of 45 µm and is supplied as a suspension in 20% ethanol. Toyopearl is a semirigid, macroporous, spherical resin synthesized from a hydrophilic vinyl copolymer exclusively composed of C, H and O atoms and is an excellent resin for the separation of biomolecules [Toyopearl information brochure].

A long burette was filled with Toyopearl and allowed to settle. Acetone (100%) was pushed through the burette using medium pressure (Labotec pump was connected to the burette) to replace the ethanol. The sample was dried using nitrogen gas to c. 0.5 ml and applied to the top of the column by means of a Pasteur pipette. The flow rate was adjusted to 100 drops/minute and samples were collected by means of fraction collector.

4.6.2 Analysis and concentration of fractions

As soon as column chromatography was completed, test-tubes were placed under a stream of air to facilitate concentration of the fractions for TLC analysis and bioassay. After c. 40% of the volume of the eluent had evaporated, every second fraction was analysed by TLC using c. 0.1% of each fraction. In some cases test-tubes were not pre-weighed and the fractions were not dried, therefore it was not possible to determine the exact concentration applied to the TLC plates. Fractions were analysed using BEA (9:1:1%), CEF (5:4:1) and 2A:3MDC as described in paragraph 4.3. EMW (45:5:4.4) was also attempted but with poor results. Separated components were sprayed with vanillin reagent.

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4.6.3 Combination of fractions

From TLC results, fractions were combined according to their separation profile. Combined fractions were placed under an air current to facilitate drying. Once dry, the components were weighed to calculate the total mass extracted, dissolved in the minimum amount of acetone possible and transferred to a glass vial with a screw top to prevent evaporation.

4.6.4 Preparative TLC

Fractions were dissolved in the smallest amount of solvent (usually acetone) in which it would dissolve (c. 1 ml) and applied in a band across the preparative TLC plate (Silica gel 60 F_{254}) starting and ending at least 1 cm from either side. The plate was developed repetitively (at least three times) in the mobile phase and the bands visualised under ultraviolet light (254 and 360 nm). A small section on the side of the plate was sprayed with vanillin and heated with a heating gun, whilst protecting the rest of the plate with foil. Components were easily visualised and marked using a soft 2B pencil. After spraying the plate with water to facilitate easier removal of components, the bands were scraped off the glass plate. The components were collected into separate beakers and crushed to a fine powder using a glass rod. The adsorbent powder was eluted with c. 5 ml acetone, depending on the quantity and pigment recovered by filtration using a sintered glass funnel. The process was repeated at least twice or until the powder regained its original white colour. This was followed by 1% acetic acid in methanol for a final rinse to remove any polar components not removed with acetone. Each component was collected into a separate vial and concentrated under a stream of cold air.

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4.7 GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

A splitless mode was chosen with helium as carrier gas. Initial temperature and pressure of the front inlet were 250 °C and 115.5 kPa respectively. Injection volume of the sample was 1.0 microliters and run period c. 48 minutes at a flow rate of 3.4 ml/min. No prior knowledge of compound structure was known and therefore a steroid method, recommended by Dr. T. Laurens, was chosen as it was thought to be the most effective in identifying plant-derived compounds. The column was a capillary column (Model no. J&W 122-1031) of 30.0 m in length, 250 µm diameter i.d. and 0.10 µm thickness.

Due to the minute quantities required for GC-MS analysis and the difficulty in weighing such small amounts, quantitative analysis was not possible. A small quantity of the fraction precipitate was taken, dissolved in the smallest quantity of chloroform into which would go, vortexed and injected onto the capillary column for detection. Later, 100µl of N-trimethylsilylimidazole (TMSI) was used to dissolve the dry precipitate. This was left overnight in an oven at 100°C and injected the following morning onto the GC-MS column. Polar compounds have a tendency to adsorb onto the support surface causing distorted peaks, which are broadened and often exhibit a tail. These support materials can be deactivated by silanization with TMSI, which has a strong affinity for polar organic molecules and tends to retain them by adsorption.
The supernatant, which was already dissolved in acetone, was also injected onto GC-MS to compare peaks found in the precipitate. The MS was programmed to incorporate the solvent after a delay of 3 minutes.

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4.8 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Analysis was performed on a Hewlett Packard 1050 HPLC with a Luna 5u C18 (250 mm x 4.60 mm i.d.) Phenomenex (U.K.) column. Fractions were diluted five to ten times before injection onto the column and eluted with 10% water: 90% methanol. Temperature was kept off during the run and average run time was 25 minutes. Parameters were set as follows: flow rate: between 1.0 and 2.0 ml/min with an average of 1.5 ml/min; minimum and maximum pressure limits at 20 and 400 bar respectively. The multiple wavelength detector (MWD) was set to measure signals at wavelengths of 204 nm and 254 nm. Fluorescence detection (FLD) was set at Em: 345 nm and Ex: 254 nm. Solvents used were Riedel-de-Häen HPLC grade [Merck].

4.9 NUCLEAR MAGNETIC RESONANCE (NMR)

After column chromatography, precipitation of some fractions began to take place. These precipitates were collected, cleaned using various solvent systems starting with non-polar solvents e.g. hexane and then introducing ethanol, methanol, ethyl acetate, chloroform and acetone. The samples were passed through a Pasteur pipette plugged with cotton wool to facilitate the removal of impurities. The clean samples were weighed and dissolved in maximum 2 ml deuterated solvents used for NMR [Merck]. In the studies, DMSO-d_6 was used as the solvent of choice, although other solvents were also attempted, because of its good ability to dissolve a wide range of compounds. The samples were then pipetted into NMR tubes [Wilmad, economy] with the aid of a Pasteur pipette and sent to Mr Eric Palmer of the Chemistry department, University of Pretoria. ^{1}H NMR was run at either 300 or 400 MHz and ^{13}C at 75 MHz using the solvent signal as reference. Structures were elucidated by Dr D. Katerere (Department of Pharmacology).

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4.10 MASS SPECTROMETRY (MS)

High Resolution Electron Impact Mass Spectroscopy (HREIMS) was performed on samples sent for analysis using a MASPEC II system [II32/A002]. Dr P. Boshoff from the Cape Technikon and Dr D. Katerere of the University of Pretoria, performed the analysis.

4.11 VARIATION IN BIOLOGICAL ACTIVITY

Previous studies compared the antibacterial activity of the leaves of seven different *C. erythrophyllum* trees situated in the University of Pretoria grounds. Results showed no difference in antibacterial activity but the TLC plates did show slight variations. Comparisons of trees growing in different areas of Pretoria as well as those present in other provinces were therefore carried out to determine whether other factors such as climactic and soil conditions would cause any differences in biological activity. Conditions of age and state of development among many other things can affect the bioactivity of plant material and the levels of secondary metabolites can differ with these parameters.

Plant material from each specimen was dried, ground to a fine powder (as described in section 4.1 and 4.2) and 500 mg was extracted with 5 ml acetone by shaking for 5 minutes on a shaking apparatus. The extracts were centrifuged for five minutes at 3500 rpm, the process repeated three times and the extracts decanted and combined. The extracts were transferred to pre-weighed glass vials and evaporated to dryness under a stream of air. Extracts were dissolved in acetone to produce a 100 mg extract/ml solution and 5 µl applied to Merck TLC F254 plates. These extracts were used in bioautography and MICs calculated according to the procedure described in sections 4.4.1 and 4.4.2.

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4.12 TOXICITY ASSAY

Toxicity of fractions was determined by the lactate dehydrogenase assay, which requires the use of human neutrophils at a concentration of 1 x 10^7 cells/ml. This assay was chosen for its simplicity and ease of determination as no specialised equipment is required. It is also a rather fast test, which allows for several determinations in a short span of time.

4.12.1 Cell separation

Heparinized blood was taken from healthy volunteers and diluted in a ratio of 1:2 with phosphate-buffered saline (PBS). The diluted blood was layered onto Ficoll (Histopaque 1077, Sigma Diagnostics) in a ratio of 2:1 and centrifuged for 30 minutes at 2100 rpm (1000xg). The serum was discarded up to 2 mm from the top layer and the mononuclear cells were aspirated and removed into a clean 50 ml tube. The suspension was centrifuged for 5 minutes at 1200 rpm (400xg) after adding 40 ml PBS and the resulting pellet (lymphocytes and monocytes) washed again with PBS.
Lysis buffer (0.83% (w/v) NH_4Cl) was added to the remaining cells to induce hypotonic lysis of the contaminating erythrocytes and suspended cells were incubated on ice for 5-6 minutes before centrifuging for 5 minutes at 1000 rpm. This procedure was repeated and the remaining pellet resuspended in 1-5 ml PBS after which cells were counted and adjusted to a concentration of 10^7/ml with PBS. All cells were kept in an ice bath prior to use.

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4.12.2 Lactate dehydrogenase assay (LDH)

Neutrophils (10^7 cells/ml) were incubated with Hanks' balanced salt solution (HBSS) for 5 minutes at 37°C in a ratio of 1:4 i.e. 20 µl cells + 80 µl HBSS. As a positive control, 20µl lysophosphatidylcholine (LPC) was added to one ependorff tube and the volume adjusted to 200µl with HBSS. Two other controls were selected, one with distilled water and the other with the same ratio of DMSO to water in which the compounds were brought to solution and 100 µl of each added to their respective tubes. In the other ependorffs 100 µl of the test compounds were added into their respective tubes to make up a final concentration of 100 µg/ml. The tubes were incubated for a further 10 minutes at 37°C after which they were centrifuged at 1000 rpm for 5 minutes. The supernatant (100 µl) of each was pipetted and transferred to a microplate into which 100 µl PBS was added. Into each well 20 µl of NADH (1.3 mg/ml) was added and just before reading 20 µl pyruvate (1.1 mg/ml) was added to initiate the reaction. The microplate was read at 340 nm at time 0, 1, 5 and 10 minutes and measurements were done in triplicate.

4.13 ANTIOXIDANT / ANTI-INFLAMMATORY ACTIVITY

4.13.1 Serum opsonization of Zymosan

Zymosan A (Sigma Chemical Co.) was mixed with distilled water (2.5 mg/ml), boiled for 5 minutes and centrifuged at 900 rpm for 5 minutes until sedimented. The zymosan was washed with 10 ml PBS and resuspended before spinning off again at 900 rpm for 5 minutes. Freshly pooled serum (10 ml) from a healthy volunteer was added to the zymosan and incubated for 60 minutes at 37°C.

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After centrifugation (900 rpm, 5 minutes), the supernatant was discarded and the pellet washed twice with PBS before finally resuspending in saline solution at a concentration of 2.5 mg/ml and used as the serum opsonized zymosan (SOZ).

4.13.2 Preparation of test compounds and chemiluminescence assay

Isolated test compounds were dissolved in DMSO and distilled water to yield a concentration of 200 µg/ml. The final concentration for each stimulant in solution with lymphocytes was: SOZ, 279 µg/ml and compounds, 50 µg/ml.

In each cuvette 100 µl of the cell suspension (2×10^6 cells/ml), was incubated with 100 µl luminol (5-amino-2,3-dihydro-1,4-phthlazindione; Sigma; final concentration 10^{-4} M) and the test compounds (100 µl) for 30 minutes at 37°C. The cuvettes were inserted in the chemiluminometer and the stimulant (SOZ) added just before recording. A sample that was not stimulated with SOZ but contained cells, luminol and RPMI to adjust the volume was included as the blank control. Two other controls, one containing RPMI instead of test compound and the other DMSO and water in the same concentration used to suspend compounds, were also included in the assay. Measurements were done in triplicate and each sample measured for 30 minutes using a luminescence assay. All buffers and reagents used from the same batch to limit variations in results. The peak response (peak CL) and the time for peak CL (peak time, minutes) were read from the recorder (BioORbit Oy, 1991).

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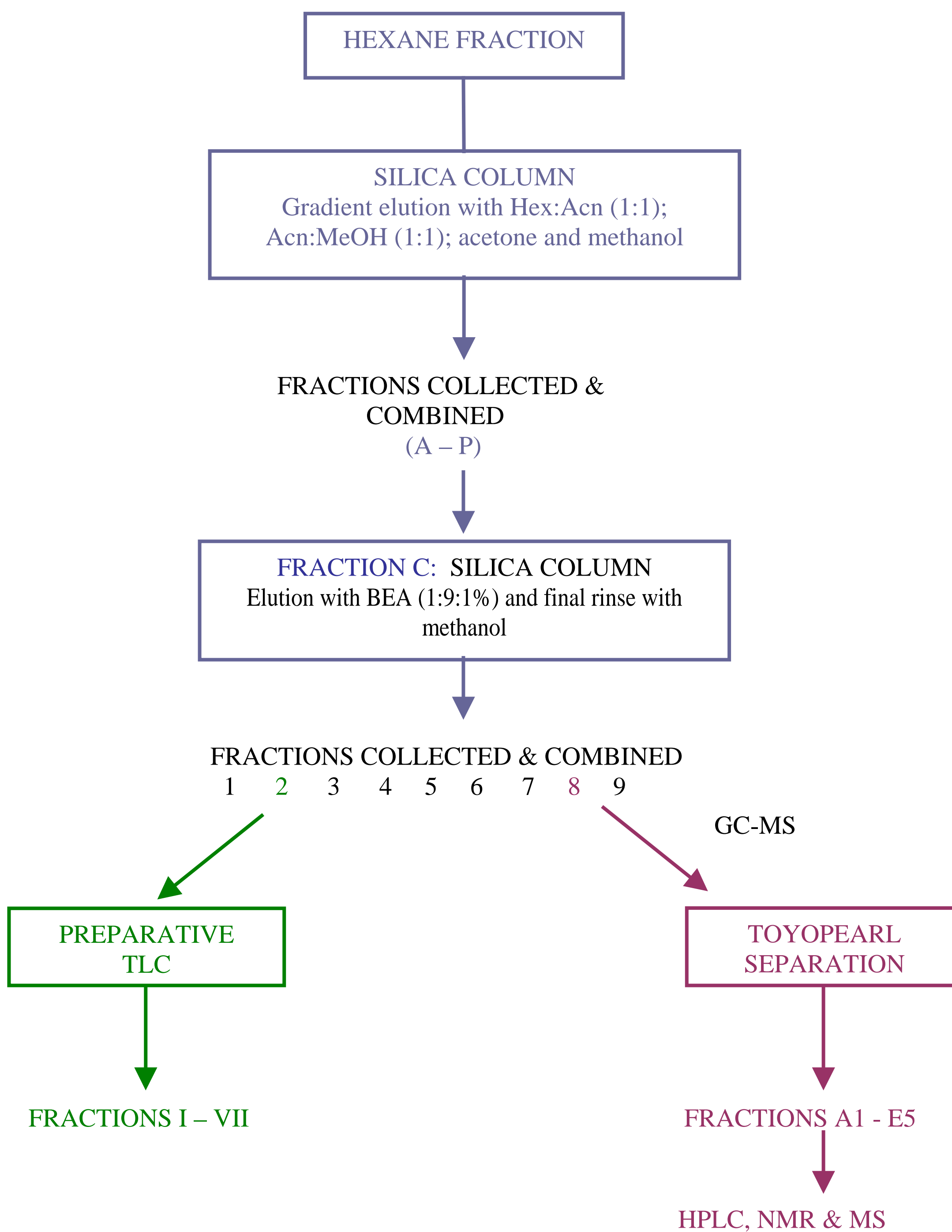

Figure 4.2: Schematic presentation of the compound isolation procedure followed with the hexane fraction of *C. erythrophyllum*, following solvent-solvent fractionation

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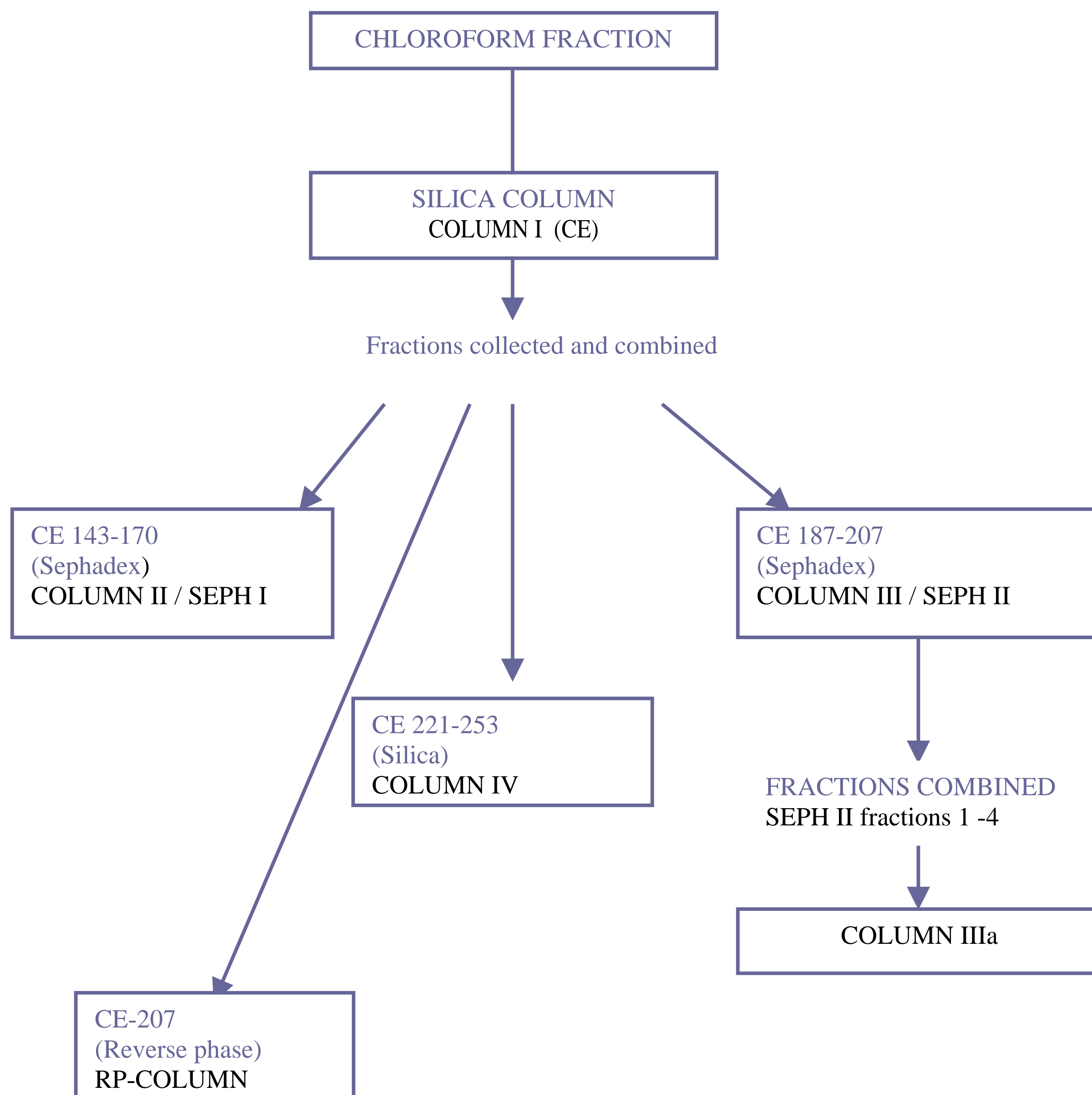

Figure 4.3: Schematic presentation of the compound isolation procedure followed using the chloroform fraction of *C. erythrophyllum*, following solvent-solvent fractionation