

Chapter 3

Materials and Methods

3.1. Plant collection

The leaves of selected plants were collected when they were still green from the Pretoria National Botanical Garden (Gauteng province) and the Lowveld National Botanical Garden in the Mpumalanga province of South Africa in the month of April 2007. Voucher specimens are deposited in the herbarium of the Department of Plant Sciences, University of Pretoria, South Africa.

3.2. Plant preparation and storage

Due to fewer complications when working with dried plant material (Eloff, 1998a), leaves of plant materials were carefully examined and dried leaves, or fungus-infected leaves and twigs removed. Leaves were then evenly spread out in a drying room at room temperature for about 15 days. When completely dried, the leaves were milled into fine powder in a Jankel and Kunkel mill (Model A10) and properly sealed airtight in glass jars and stored in the dark.

3.3. Extraction of plant material

Milled plant materials were individually extracted (1 g in 10 mL) using acetone, hexane, dichloromethane (DCM) and methanol (technical grade, Merck) in glass centrifuge tubes. Tubes were vigorously shaken for 30 min on a Labotec model 20.2 shaking machine at moderate speed. Thereafter, the tubes were centrifuged at 5000 rpm (Rotofix 32 A-Germany) for 5 minutes and filtered through Whatman No. 1 filter paper into pre-weighed labelled containers. The process was repeated three times on the marc to exhaustively extract the plant material and the extracts were combined. The extracts were dried under a stream of cold air in a fume cupboard at room temperature and the quantity extracted per solvent was measured.

3.4. Thin layer chromatography (TLC) analysis of crude extracts

Extracts were reconstituted in acetone (10 mg/mL) and 10 µL of the reconstituted extract was spotted onto aluminium-backed TLC plates (Merck, silica gel 60 F254). The plates were developed for separation of constituents under saturated conditions in eluent solvent systems of varying polarities developed in the Phytomedicine

Programme, namely: ethyl acetate/methanol/water [EMW] [40:5.4:5] (polar/neutral); chloroform/ethyl acetate/formic acid [CEF] [5:4:1] (intermediate polarity/acidic); benzene/ethanol/ammonium hydroxide [BEA] [90:10:1] (non-polar/basic) (Kotze and Eloff, 2002).

Separated constituents were visualised under UV light at wavelengths of 254 nm and 365 nm (Camac Universal UV lamp TL-600). Vanillin, 0.1 g, was dissolved in 28 mL methanol and 1 mL of sulphuric acid was carefully added. Plates were then sprayed with the vanillin sulphuric acid spray reagent and heated at 100°C for five minutes to allow for development of colour.

3.5. Determination of qualitative antioxidant activity of extracts

The qualitative antioxidant activity of selected plants was carried out using the method of Deby and Margotteaux (1970). Thin layer chromatographic plates were prepared as described (section 3.4) to separate active constituents in the different extracts. For the determination of antioxidant activity, the plates were sprayed with 0.2% 1,1-diphenyl-2-picryl-hydrazyl (DPPH) (Sigma®) in methanol as an indicator. A positive reaction is indicated by the appearance of a yellow spot against a purple background.

3.6. Solvent-solvent fractionation

The solvent-solvent group procedure employed by the United States National Cancer Institute as described by Suffness and Douros (1979) was used in this study. This procedure fractionates the components of an extract based on polarity and is represented in Fig 3.1.

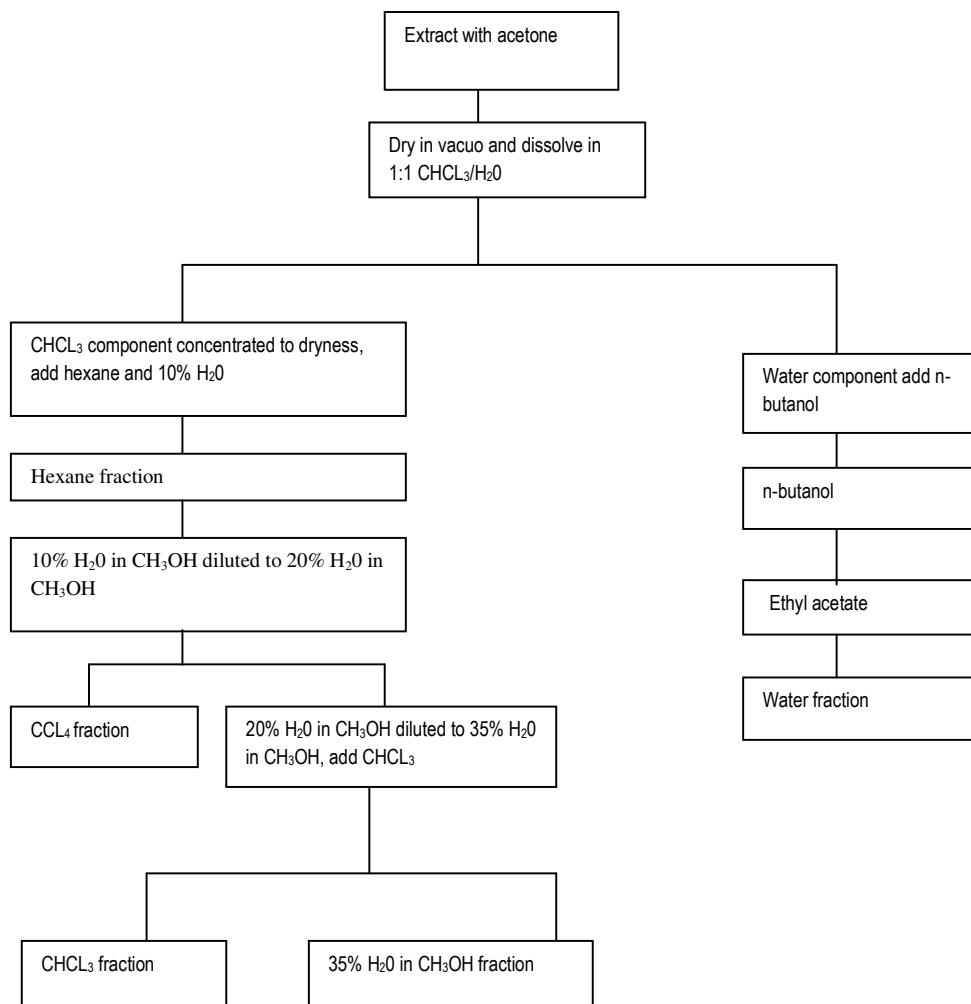


Figure 3.1: Flow chart of solvent-solvent fractionation of leaves

The grinded material (500 g) was extracted using acetone (1 g/10 ml) for 24 hours and dried in vacuo and dissolved in 1:1 chloroform/water. The water fraction was mixed with an equal volume of n-butanol in a separatory funnel to

yield the n-butanol fractions and water. Ethyl acetate was added to the water fraction to yield the ethyl acetate and water fractions. The chloroform fraction was dried in a vacuum rotary evaporator and extracted with an equal volume of hexane and 10% water: methanol mixture. This yielded the hexane fraction and water methanol mixture was diluted to 20% water: methanol by addition of water. This was then mixed with carbon tetrachloride in a separatory funnel giving the carbon tetrachloride fraction. The 20% water methanol fraction was further diluted to 35% water: methanol and mixed with chloroform to yield the chloroform and water: methanol fractions. In all cases, equal volume of solvents was used and separation repeated with small quantities of solvents to facilitate separation.

3.7. Antibacterial activity

3.7.1. Bioautography on TLC plates

Thin layer chromatography plates (10 x 10 cm) were spotted with 10 μl (10 mg/ml) of the extracts or fractions and eluted in the three different mobile eluting solvent systems of varying polarity: CEF, BEA and EMW (section 3.4). The chromatograms were dried for 5 days at room temperature under a stream of air to remove the eluent solvent system. Thereafter, the eluted TLC plates were sprayed with a concentrated suspension of the test organism prepared in Müller-Hinton (MH) broth in a Biosafety Class II cabinet (Labotec, SA). In this study, two Gram-positive bacteria *Enterococcus faecalis* (ATCC 29212) and *Staphylococcus aureus* (ATCC 29213), and two Gram-negative species, *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922) were used. The sprayed plates were placed in a humid chamber (100% relative humidity) and incubated overnight at 37°C. Plates were then sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (INT, Sigma) and further incubated at 37°C until a purple-red colour change was evident. Retardation factor (R_f) values of inhibitory zones, depicted as white areas (Begue and Kline, 1972) where reduction of INT to formazan did not occur, were recorded.

3.7.2. Microdilution assay for MIC determination

The serial microtitre dilution method described by Eloff (1998b) was used to screen the plant extracts for antibacterial activity. This method is used to determine the minimal inhibitory concentration (MIC) of plant extracts against bacteria by measuring reduction of INT to a red formazan in wells where biological activity against test organisms is evident. Growth inhibition is observed in wells where there is non-reduction of INT to a red colouration. The organisms (section 3.7.1) were used to test the MIC of the extracts, fractions or pure compounds. The bacterial cultures were incubated in Müller-Hinton (MH) broth overnight at 37°C. Overnight cultures were diluted 1:100 in MH and plated onto agar plates. The densities of the bacterial cultures before antimicrobial testing were approximately:

Enterococcus faecalis, 1.5×10^{10} cfu/ml; *Staphylococcus aureus*, 2.6×10^{12} cfu/ml, *Pseudomonas aeruginosa*, 5.2×10^{13} cfu/ml, and *Escherichia coli*, 3.0×10^{11} cfu/ml.

The assay was conducted in 96-well flat bottomed microtitre plates. Sterile water (100 µl) was added to all the wells of the microtitre plate. In row A, 100 µl of extract, fraction or pure compound was added using a micropipette. The contents of row A were thoroughly mixed using a micropipette and 100 µl transferred to row B. The process of dilution was continued until all the rows down the column were completed and 100 µl of the last dilution was discarded. After the serial dilution process, 100 µl of test organisms were added to all the wells except for the negative control wells and incubated at 37°C overnight. Thereafter, 40 µl of 0.2 mg/ml INT were added and reincubated to ensure adequate colour change. Two wells were used as sterile control containing only water. The growth control contained both water and test organism. Gentamicin was used as a positive control. MIC readings were recorded after 12 and 24 hours of incubation. Tests were carried out in triplicate and each experiment was repeated three times.

3.8. Antifungal assay

3.8.1. Bioautography on TLC plates

Thin layer chromatography plates (10 x 10 cm) were spotted with 10 µl (10 mg/ml) of the extracts or fraction and eluted in the three different mobile eluting solvent systems of varying polarity: CEF, BEA and EMW (section 3.4). The chromatograms were dried for 5 days at room temperature under a stream of air to remove the eluent solvent system. The fungal pathogens used in this study were *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. The pathogens were obtained from clinical cases of disease in animals and were kindly provided by Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. *C. albicans* was isolated from a Gouldian finch, *C. neoformans* from a cheetah, and *A. fumigatus* from a chicken. Eluted TLC plates were sprayed with a concentrated suspension of the actively growing organisms prepared in Sabouraud Dextrose (SD) broth in a Biosafety Class II cabinet (Labotec, SA). The sprayed plates were placed in a humid chamber (100% relative humidity) and incubated overnight at 30°C. Plates were then sprayed with a 2 mg/ml solution of INT and further incubated at 30°C until a purple-red colour change was evident. R_f values of inhibitory zones, depicted as white areas where reduction of INT to formazan did not occur, were recorded.

3.8.2. Microdilution assay for MIC determination

In the antifungal bioassay, the method described by Eloff (1998b) and modified by Masoko and Eloff (2005) using Sabouraud Dextrose (SD) broth as nutrient medium was used to test the activity of extracts, fractions or isolated compounds. Two-fold serial dilutions of test substances (10 mg/ml) dissolved in acetone were prepared in 96-well microtitre plates (section 3.9.1.1). Actively growing organisms (section 3.8.1) were transferred from SD agar plates using a sterile cotton swab into fresh SD broth. Densities of fungal cultures used in bioautography and for MIC determinations were as follows: *C. albicans*, $2.5 \times 10^6 \text{ cfu/ml}$; *C. neoformans*, $2.6 \times 10^6 \text{ cfu/ml}$; *A. fumigatus*, $8.1 \times 10^6 \text{ cfu/ml}$. *Candida albicans* was diluted to a density of about $2.5 \times 10^4 \text{ cfu/ml}$, *C. neoformans*, $2.6 \times 10^4 \text{ cfu/ml}$, and *A. fumigatus* $8.1 \times 10^4 \text{ cfu/ml}$. This suspension ($100 \mu\text{l}$) was added to each well. INT was then added and plates were incubated. Microtitre plates were incubated at 30°C for 24 to 48 hours. INT was used as an indicator of growth as previously described. Sterile controls were included as described (section 3.7.2). Amphotericin B (0.08 mg/ml), a standard antifungal agent, was included as a positive control. Each experiment was repeated three times.

3.8.3. Determination of total activity

The total activity of plant extracts and fractions was determined using the method of Eloff (2004). Total activities of an extract or fraction give an indication of the efficacy at which active constituents present in one gram can be diluted and still inhibit the growth of test organisms. This value is calculated in relation to the MIC value of the extract, fraction or compound and expressed mathematically as follows:

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

3.9. Determination of cytotoxicity of extracts, fractions and pure compounds (MTT)

The cytotoxic effect of extracts, fractions or pure compounds was tested against the Vero monkey kidney cell line, Crandell feline kidney cells (CRFK) and bovine dermis cells. The cells used in this study were kindly provided by the Department of Veterinary Tropical Diseases, University of Pretoria. Cells were maintained in minimal essential medium (MEM, Highveld Biological, South Africa) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Adcock-Ingram). Cultures for the assay were prepared from confluent monolayer cells, seeded at a density of 4.8×10^4 cells per well in 96 well microtitre plates and incubated overnight at 37°C in a 5% CO_2 atmosphere to allow attachment of the cells.

Dried crude plant extracts (100 mg) were reconstituted in 1ml DMSO and 10 fold serially diluted while pure compound (2 mg) was reconstituted in 0.1 ml of DMSO, and 2-fold serial dilutions of each test substance were prepared in growth medium. The growth medium on sub-confluent monolayer cells grown overnight in microtitre plates was removed and cells were exposed to 200 μl of the extracts, fractions or pure compound at different concentrations and incubated at 37°C in a 5% CO₂ atmosphere for 5 days. Thereafter, the extract-containing medium on the cells was replaced with fresh culture medium. Viability of cells was determined using the tetrazolium-based colorimetric assay (MTT assay) described by Mosmann (1983). The assay is based on mitochondrial dehydrogenase activity which is assessed by the reductive cleavage of the tetrazolium salt MTT (3- $\bar{Z}4$, 5-dimethyl thiazol-2-yl-2, 5-diphenyl tetrazolium bromide) by the succinic dehydrogenase present in living cells to yield a purple formazan dye. Cells were then exposed to 30 μl of 5 mg/ml MTT dissolved in phosphate buffered saline (PBS) and reincubated for four hours under the same conditions. The medium containing MTT was then removed and 50 μl DMSO was added to each well and the plates were gently rocked to dissolve the formazan crystals. The optical density (OD) was measured at a wavelength of 570 nm and a reference wavelength of 630 nm. The cytotoxicity was expressed as 50% cytotoxic concentration (CC₅₀) of substances to inhibit the growth of cells by 50%, when compared to untreated cells, calculated from the linear regression equation. Berberine chloride (Sigma) was used as a positive control; wells containing only cells without extract treatment were the negative control and a solvent control was also included.

3.10. Genotoxicity testing of isolated compounds

The isolated compounds were investigated for their potential mutagenic effect using the plate incorporation procedure described by Maron and Ames (1983). The assay was performed using *Salmonella typhimurium* strains TA98 and TA100. Aliquots of bacterial stock (100 μl) were incubated in 20 ml of Oxoid Nutrient broth for 16 h at 37°C on an orbital shaker. The overnight culture (0.1 ml) was added to 2 ml top agar (containing traces of biotin and histidine) together with 0.1 ml test solution (pure compounds, solvent control or positive control) and 0.5 ml phosphate buffer (for exposure without metabolic activation). The top agar mixture was poured over the surface of the agar plate and incubated for 48 h at 37°C. Following incubation, the number of revertant colonies (mutants) was counted. All cultures were prepared in triplicate (except the solvent control where five replicates were prepared) for each assay. The assays were repeated twice. The positive control used was 4-nitroquinoline-1-oxide (4-NQO) at a concentration of 2 $\mu\text{g}/\text{ml}$.

3.11. Antiviral assay

3.11.1. Cell cultures and viruses

Viruses and host cells were kindly provided by the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. Enveloped viruses, namely feline herpes virus-1 (FHV-1, dsDNA), canine distemper virus (CDV, ssRNA), canine parainfluenza virus-2 (CPIV-2, ssRNA) and lumpy skin disease virus strain V248/93 (LSDV, dsDNA) were used in the study. These groups of viruses were chosen for this study because entry of enveloped viruses into their host cells involves several successive steps, each one being open to therapeutic intervention. Inhibitors that prevent entry of the virus into host cells act by targeting viral and/or cellular components, through either the inhibition of protein-protein interactions within the viral envelope proteins or between viral proteins and host cell receptors, or through the inhibition of protein-lipid interactions. The susceptible cell types compatible for the growth of the viruses were Crandell feline kidney cells (CRFK), Vero cells and bovine dermis cells, respectively.

Cells were cultured in 75 cm³ culture flasks and maintained in minimum essential medium (MEM, Highveld Biological) containing 5% fetal calf serum (FCS, Highveld Biological) supplemented with 0.1% gentamicin (Virbac) and incubated at 37°C in an atmosphere of 5% CO₂. Compatible cells for the growth of each virus were inoculated using 0.5 mL of infective virus, reincubated and observed daily for evidence of cytopathic effect (CPE). Following the development of 90% CPE, infected culture flasks were frozen at -70°C for 20 min and thawed. The process was repeated three times to ensure adequate release of viruses from cultured cells into the growth medium. The contents of the flask containing cells, virus and medium were centrifuged at 800 rpm for 10 min. The supernatant containing the virus was collected in cryotubes and preserved in liquid nitrogen until use. The effective titre (TCID₅₀/mL) of each virus was determined using the method of Reed and Muench (1938) prior to each assay.

3.11.2. Virucidal assay

The virucidal activity of plant extracts was evaluated using the method described by Barnard *et al.* (1992) with slight modifications. Non-cytotoxic concentrations of extracts, solvent-solvent fractions or pure compound were serially diluted 10-fold in MEM containing 5% FCS in 2 mL Eppendorf tubes and an equal volume of virus (20 µL) at an infective titre of 10² TCID₅₀/mL was added to each concentration and incubated at 37°C for various time intervals ranging from 1 to 3 h. Growth medium on confluent cell monolayers grown in 96-well plates was removed and cells were exposed to 200 µL of the extract-virus mixture at each concentration in quadruplicate and incubated until CPE was observed, which was mostly between 1 and 5 days. The negative control comprised non-infected and untreated

cells while positive controls consisted of infected and untreated cells. The extent of cell damage was determined by the presence of CPE when compared to infected untreated and uninfected untreated controls by microscopic examination as well as the MTT colorimetric assay. The degree of cell destruction by microscopic examination was scored using reduction indexes described by Vanden Berghe *et al.*, (1993). Plant extracts exhibiting reduction of viral infectivity at concentrations of 10^3 and 10^4 dilutions indicate strong activity while those with 10^2 to 10 as moderate to weak activity respectively. For the MTT assay, antiviral activity was expressed as a selectivity index (SI), which is the value of cytotoxic concentration (CC_{50}) divided by effective concentration (EC_{50}). Selectivity index values of more than three indicate potential antiviral activity of the test extract (Chattopadhyay *et al.*, 2009). The EC_{50} was calculated from the regression equation as follows:

$$EC_{50} = [(ODt)v - (ODc)v] / [(ODc)mock - (ODc)v] * 100$$

where (ODt) v is the optical density of the cells treated with virus and substance, (ODc) v is the optical density of the cells treated with virus (virus control), and (ODc) mock is the optical density of the mock infected cells (cell control).

3.11.3. Attachment assay

The ability of the viruses to attach to the host cell was tested using the method of Barnard *et al.*, (1993) with slight modifications. Cells appropriate for the growth of each virus were seeded in 96-well flat-bottomed microtitre plates and incubated at 37°C in a 5% CO_2 incubator to attain an 80% confluent monolayer. Medium on the cells was removed and equal volumes of virus ($50 \mu\text{l}$) at an infective titre of $10^2 \text{ TCID}_{50}/\text{ml}$ was added to cells and incubated at different time intervals from 1-3 h. Thereafter, cells were washed with phosphate buffered saline (PBS) to remove the unattached virus. Ten-fold serially diluted extracts, solvent-solvent fractions or pure compound at non-cytotoxic concentrations were added to cells and incubated again at 37°C in an atmosphere of 5% CO_2 and observed daily for evidence of CPE. The ability of the extract to prevent subsequent replication of the virus in host cells was scored microscopically and by the MTT assay. Negative controls comprised non-infected and untreated cells while positive controls consisted of infected and untreated cells. Antiviral activity by CPE reduction and MTT assay was determined as previously described (section 3.11.2).