Chapter 2 – Materials and Methods

2.1 Plant material and isolation of essential oils

Mentha longifolia subsp. *polyadena* samples were collected in the 2004/2005 flowering season from eight different localities in the wild (Table 2.1) by obtaining distribution data from herbarium specimens housed at the South African National Biodiversity Institute (SANBI) and at the C.E. Moss Herbarium (Wits University).

Table 2.1 Localities from where plants were collected and corresponding voucher numbers.

Locality	Voucher number
Potchefstroom (North-West Province)	AV 1096
Lydenburg (Mpumulanga Province)	AV 1097
Dullstroom (Mpumalanga Province)	AV 1094
Komukwane (Botswana)	AV 1095
Prins Albert (Eastern Cape Province)	AV 1135
Wakkerstroom (Mpumulanga Province)	AV 1132
Clocolan (Free State Province)	AV 1133
Pretoria (Gauteng Province)	AV 1134

The fresh plant material was air-dried indoors at constant temperature and the essential oils obtained from the aerial parts by hydrodistillation in a modified Clevenger-type apparatus (Figure 2.1) for approximately three hours. The pure oils were collected in

amber vials to protect them from degradation by ultraviolet light. The tightly closed amber vials were stored at 4 °C to prevent the oils from possible decomposition.

Figure 2.1 Isolation of essential oil by hydrodistillation of chopped air-dried plant material.

2.2 Extraction of non-volatile compounds

The air-dried material was crushed and ground using a mortar and pestle into a fine powder, weighed and placed in a conical flask. A freshly prepared 1:1 mixture of methanol and chloroform (100 ml) was added to the crushed material. Extraction was done in a water-bath for about 3 hours at a constant temperature of 40°C. The extract was filtered using a glass funnel plugged with cotton wool. The filtered solution was collected in a pre-weighed petri-dish and placed in a fume-cupboard for one week until all the

solvent had evaporated. This process was repeated for all the samples. The weight of the resulting extract was recorded.

2.3 Gas chromatography coupled to mass spectroscopy (GC-MS)

The GC-MS unit consisted on a Perkin Elmer Autosystem XL gas chromatograph, equipped with DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific, Inc.), and interfaced with Perkin-Elmer Turbomass mass spectrometer (software version 4.1). Oven temperature was programmed 45° - 175°C, at 3°C / min, subsequently at 15°C / min up to 300°C, and then held isothermally for 10 min; injector and detector temperatures, 280°C and 290°C, respectively; transfer line temperature, 280°C; ion trap temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization energy, 70 eV; ionization current, 60 μA; scan range, 40-300 u; scan time, 1 s.

The identity of the components was assigned by comparison of their retention indices, relative to C_8-C_{17} *n*-alkanes, and GC-MS spectra with corresponding data of components of reference oils, laboratory-synthesized components and commercially available standards from a home-made library.

2.4 High performance liquid chromatography (HPLC)

The solvent extracts were analyzed by high performance liquid chromatography (HPLC) using a Waters Model 2690 HPLC system (Phenomenex Aqua C18 column, 250 mm x 2.1 mm at 40ºC) equipped with a Waters Model 996 photodiode array detector (PDA). Initially the mobile phase consisted of 10% acetonitrile, 90% water containing 10 mM formic acid. The solvent ratio was changed through a linear gradient to 90% acetonitrile, 10% water (with 10 mM formic acid) after 40 min. This ratio was maintained for 10 min

whereafter the solvent ratio was changed back to the initial starting conditions. The data was analysed using Empower® software.

2.5 Antimicrobial assays

Antimicrobial assays were performed on suitable dilutions of the essential oils and extracts at least in duplicate and where sufficient sample allowed in triplicate. Antimicrobial activity was conducted using selected yeasts, Gram-positive and Gram-negative bacteria based on the traditional uses of this plant. Traditional uses include the treatment of infected external wounds (Batten and Bokelmann, 1966), bronchial ailments (Watt and Breyer-Brandwijk, 1962), urinary tract infections and gastro-intestinal complaints (van Wyk *et al.*, 1997). The Gram- positive bacterial organisms were represented by *Staphylococcus aureus* (ATCC 12600), *Staphylococcus epidermidis* (ATCC 2223), *Enterococcus faecalis* (ATCC 29212) and *Bacillus cereus* (ATCC 11778), whereas the Gram-negative organisms were represented by *Escherichia coli* (ATCC 8739), *Klebsiella pneumoniae* (NCTC 9633), *Salmonella typhimurium* (ATCC 14028), *Moraxella catarrhalis* (clinical strain), and *Yersinia enterocolitica* (ATCC 23715). *Cryptococcus neoformans* (ATCC 90112) and *Candida albicans* (ATCC 10231) were selected to represent the yeasts. The microdilution assay was used to determine the minimum inhibitory concentration (MIC) of both the volatile and non-volatile samples (Eloff, 1998).

2.6 Minimum inhibitory concentration

The antimicrobial effectiveness of a chemical compound is often described in terms of its minimum inhibitory concentration, the lowest concentration of the compound capable of

inhibiting the growth of the challenging organism (Mann and Markham, 1998). Extracts and essential oil dilutions were prepared using acetone as the solvent to give starting concentrations of 64 mg/ml and 128 mg/ml respectively. The preparation of the micro-titre plates were done in a laminar flow unit. Each well was first filled with 100 μl of sterile water. In row A (Figure 2.2), 100 μl of oil / acetone extract was plated out for each sample to be analysed. Serial dilutions of the extract and oil samples were performed from wells A through to H. For example, 100 μl of contents from well A1 were withdrawn after the contents were thoroughly mixed and released through the micropipette, and transferred to well B1. This procedure was then repeated from well C1 to well H1, with the exception that the 100 μl withdrawn from well H1, was discarded. This dilution method (Eloff, 1998) was repeated in each column. The last two columns in Figure 2.2 represent the positive controls (Ciprofloxacin and Amphotericin B for the bacteria and yeasts respectively) and negative controls used for each pathogen tested. Each well was then filled with 100 μl of the culture medium containing the pathogen being studied at a concentration of approximately 1 x 10^6 colony forming units per millilitre, labelled and sealed. This step was performed out of the laminar flow unit in the laboratory. The micro-titre plate was incubated at 37°C for 24-48 hours, depending on the pathogen being studied. A 0.02 mg/ml ρ-iodonitrotetrazolium violet (INT) solution was prepared. Forty microlitres (40 μl) of this solution was added to each well and the micro-titre plate was allowed to stand for six hours in the case of the bacterial strains and 24 hours in the case of the yeast strains. Tetrazolium salts like INT, are used to indicate the biological activity because the colourless compound acts as an electron acceptor and is reduced to a coloured product by biologically active organisms (Eloff, 1998). The

contents of the well turned to red or purple if any microbial growth was present. This procedure was repeated at least twice on all samples.

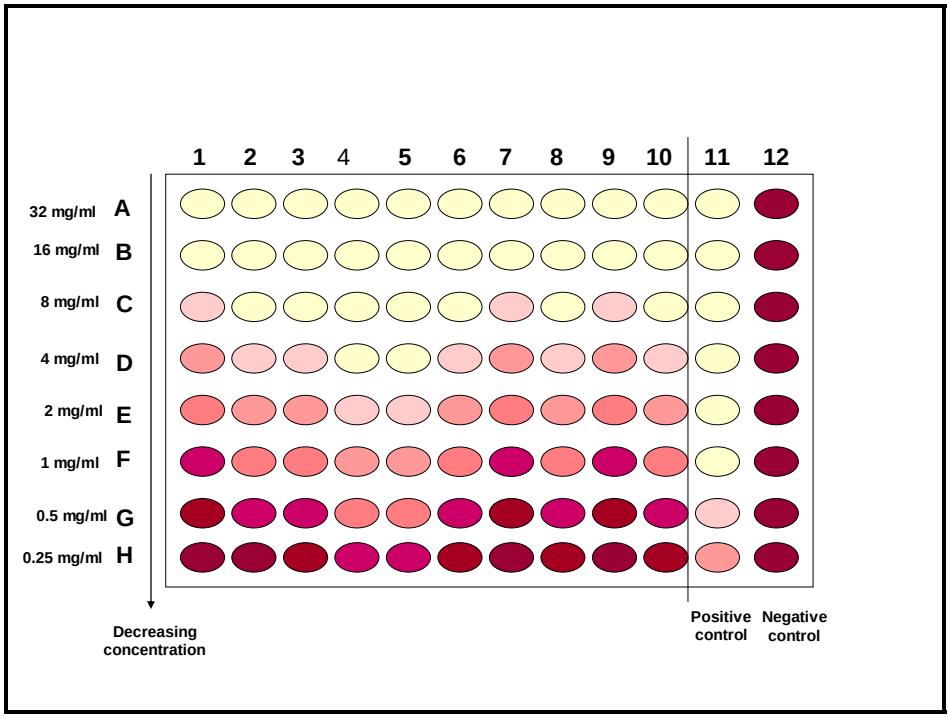

Figure 2.2 Diagram representing a 96 well micro-titre plate used to measure the minimum inhibitory concentration of the samples tested.

2.7 Statistical analysis

The percentage composition of the essential oil samples was used to determine the correlation between the different samples of *Mentha* species by cluster analysis using the NTSYS software. Correlation was selected as a measure of similarity, and the unweighted pair-group method with arithmetic average (UPGMA) was used for cluster definition (Rohlf, 1992).