The Antimicrobial Activity and Essential Oil Composition of Medicinal Aromatic Plants Used in African Traditional Healing

Sandy van Vuuren

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the Degree of Doctor of Philosophy

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Declaration

I, Sandy van Vuuren, declare that this thesis is my own work. It is being submitted for the Degree of Doctor of Philosophy, at the Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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Signature

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Date

Abstract

A study on the essential oil chemistry and microbiological activity of South African indigenous medicinal plants with the aim of establishing a scientific rationale for their anti-infective properties was undertaken. For the purpose of this study, nine medicinal aromatic plants were selected. The hydrodistilled essential oil was analyzed by gas chromatography combined with mass spectrometry (GC-MS) and various techniques were used to document the antimicrobial activity.

Disc diffusion studies on *Myrothamnus flabellifolius* indicated highest activities against the fungal test organisms *Cryptococcus neoformans* and *Aspergillus niger* with radial inhibition zones of 8 mm and 10 mm respectively. The highest antimicrobial activity noted in the minimum inhibitory concentration (MIC) assay was for *C. neoformans* (2 mg/mL). Time-kill studies demonstrated the death kinetic progression on *M. flabellifolius* essential oils where the killing rate was greatest for *Candida albicans*. *Osmitopsis asteriscoides*, a plant used traditionally for cuts and swellings showed highest disc diffusion antimicrobial efficacy against *Staphylococcus aureus* (3 mm) and *C. neoformans* (3 mm). The MIC study indicated highest susceptibilities (4 mg/mL) for *Escherichia coli* and *Staphylococcus epidermidis*. Death kinetics for the three organisms studied demonstrated that the killing rate was greatest for *C. albicans*. The role of the two major constituents (1,8-cineole and (-)-camphor) act synergistically to enhance antimicrobial activity. Disc diffusion assays undertaken on *Artemisia afra* showed highest activity against *Candida tropicalis* (5 mm). In the MIC assay the highest susceptibility was against *Serratia odorifera* (4 mg/mL). Time-kill assays on *Artemisia afra* showed a concentration dependent bactericidal activity, with evidence that the major constituents independently and in combination were not responsible for the overall activity of the plant. *Lippia javanica*, a plant used to treat coughs, colds and bronchitis, indicated highest susceptibility against the respiratory pathogen *Klebsiella pneumoniae* (5 mm) with the disc diffusion assay. The MIC assay indicated highest susceptibilities (4 mg/mL) against *C. neoformans* and *E. coli*. Death kinetic assays for three test organisms showed that the killing rate was the greatest for *K. pneumoniae*. The time-kill study for *L. javanica* in combination with *A. afra* demonstrated that the oils in combination act synergistically against *K. pneumoniae*. The antimicrobial activity of the essential oils and extracts were determined for *Helichrysum cymosum* subsp. *cymosum* where the extracts demonstrated at least a six times greater MIC efficacy than the essential oils. Using column

chromatography, the antimicrobially active compound was isolated from *H. cymosum* subsp. *cymosum* and identified as helihumulone. The traditional use of plants as a treatment for infectious diseases is not always restricted to a single part of the plant as was noted in the study on *Croton gratissimus* var. *subgratissimus*, where the leaf, bark and root extracts were investigated singularly and combined in various ratios to establish possible interaction. The MIC and fractional inhibitory concentration (FIC) results indicated variable efficacies for the plant combinations. The greatest synergistic profile was noted for *C. neoformans* in the leaf and root combination (MIC 0.4 mg/mL and FIC of 0.4). Further isobologram combination studies were thereafter conducted on varying ratios of leaf and root extracts, indicating greatest synergy for *Bacillus cereus, Enterococcus faecalis, C. albicans* and *C. neoformans*. While seasonal variation had very little impact on the MIC results obtained from *Heteropyxis natalensis*, the ratio of the two major compounds (1,8-cineol and limonene) fluctuated on a monthly basis. Moderate antimicrobial activity (3.0-16.0 mg/mL) was found for most pathogens with higher sensitivities for *C. neoformans*. The geographical variation of *H. natalensis* essential oil indicated similar profiles for Gauteng, Nelspruit and Waterberg samples. The Lagalametse sample, however, showed distinct variation both chemically and microbiologically where efficacy was higher than in all other samples. The impact of the enantiomeric configuration was investigated for limonene in combination with 1,8-cineole with (+/-)-limonene in combination with 1,8-cineole having the most significant synergistic ratios against *Pseudomonas aeruginosa.* The antimicrobial activities of the non-volatile and volatile fractions of *Tarchonanthus camphoratus* and *Plectranthus grandidentatus*, singularly and in combination demonstrated that the volatile constituents contribute to the total efficacy of the plant. Isobologram representation of the combination of various ratios of *T. camphoratus* and *P. grandidentatus* essential oil and non-volatile extracts devoid of essential oils present a predominant synergistic profile for all pathogens studied. A comparative study on five indigenous oils (*M. flabellifolius, O. asteriscoides, H. natalensis, A. afra* and *L. javanica*) was undertaken with five popular commercial oils (*Lavendula angustifolia, Thymus vulgaris, Melaleuca alternifolia*, *Mentha piperita* and *Rosmarinus officinalis*). The highest antimicrobial activity was noted for *Thymus vulgaris* in the MIC assay, followed by *M. flabellifolius, O. asteriscoides* and *M. alternifolia*. With the time-kill assay, *M. flabellifolius* showed the most rapid cidal effect against all three pathogens tested. The comparative evaluation of commercial essential oils with indigenous oils validated the use of South African aromatic plants for their anti-infective properties.

Publications arising from this study*

- AM Viljoen, MJ Klepser, E Ernst, D Keele, E Roling, **SF van Vuuren**, B Demirci, KHC Başer and B-E van Wyk. The composition and antimicrobial activity of the essential oil of the resurrection bush, *Myrothamnus flabellifolius*. South African Journal of Botany, 2002, 68, 100-105 **(abstract, pg 266)**.
- AM Viljoen, **SF van Vuuren,** B Demirci, KHC Başer and B-E van Wyk. *Osmitopsis asteriscoides* (Asteraceae) - The antimicrobial activity and composition of the essential oil of a Cape-Dutch remedy. Journal of Ethnopharmacology, 2003, 88, 137-143 **(abstract, pg 267)**.
- AM Viljoen, S Subramoney, **SF van Vuuren,** B-E van Wyk, KHC Başer and B Demirci. The composition, geographical variation and antimicrobial activity of *Lippia javanica* (Verbenaceae) leaf essential oils. Journal of Ethnopharmacology, 2005, 96, 271-277 **[recipient of best publication award, ICPPS conference, 2006]** **(abstract, pg 268)**.
- **SF van Vuuren**, AM Viljoen, RL van Zyl, FR van Heerden and KHC Başer. The antimicrobial, antimalarial and toxicity profiles of helihumulone, leaf essential oil and extracts of *Helichrysum cymosum* (L.) D. Don subsp. *cymosum*. South African Journal of Botany, 2006, 72, 287-290 **(abstract, pg 269)**.
- AM Viljoen, **SF van Vuuren**, T Gwebu, B Demirci and KHC Başer. The geographical variation and antimicrobial activity of African Wormwood (*Artemisia afra* Jacq.) essential oil. Journal of Essential Oil Research, Special Edition, 2006, 18, 19-25 **(abstract, pg 270)**.
- **SF van Vuuren** and AM Viljoen. A comparative investigation of the antimicrobial properties of indigenous South African aromatic plants with popular commercially available essential oils. Journal of Essential Oil Research, Special Edition, 2006, 18, 66-71 **(abstract, pg 271)**.
- **SF van Vuuren**, AM Viljoen, T Őzek, B Demirci and KHC Başer. Seasonal and geographical variation of *Heteropyxis natalensis* essential oil and the effect thereof on the antimicrobial activity. South African Journal of Botany, 2007, in press **(abstract, pg 272)**.

***Appendix B**

Conference proceedings relating to this thesis

- AM Viljoen, B-E van Wyk, KHC Başer, B Demirci and **SF van Vuuren**. The essential oil composition, medicinal uses and antimicrobial activity of the resurrection bush, *Myrothamnus flabellifolius* 31st International Symposium on Essential Oils, Hamburg, Germany, 2000. [poster]
- T Pelele, AM Viljoen, **SF van Vuuren,** B Demirci and KHC Başer. African wormwood – essential oil composition, geographical variation and antimicrobial properties of a coveted traditional herbal remedy. The 33rd International Symposium on Essential Oils, Lisbon, Portugal, 4-7 September, 2002. [poster]
- **SF van Vuuren**, AM Viljoen and ME Klepser. The antimicrobial activity of medicinal aromatic plants used in African traditional healing with special reference to method variability and essential oil composition. RAU Inter-University Post Graduate Symposium, 30 October, 2002. [**awarded a medal for best PhD podium presentation**]
- S Subramoney, **SF van Vuuren**, AM Viljoen, B Demirci and KHC Başer. Antimicrobial properties and geographical variation in essential oil composition of fever tea - *Lippia javanica* (Verbenceae). The 7th Conference of the International Society of Ethnopharmacology jointly with the South African Association of Botanists, University of Pretoria, 8-11 January, 2003.
- **SF van Vuuren,** AM Viljoen, E Ernst, B Demirci, T Özek and KHC Başer. The antimicrobial activity and death kinetics of the essential oil and chemical components of the South African endemic, *Osmitopsis asteriscoides*. The 7th Conference of the International Society of Ethnopharmacology jointly with the South African Association of Botanists, University of Pretoria, 8-11 January, 2003. **[awarded first prize for best ethnobotany poster]**
- LT Gwebu, **SF van Vuuren**, AM Viljoen, B Demirci and KHC Başer. The antimicrobial activity and essential oil chemistry of African wormwood (*Artemisia afra*). The South African Association of Botanists 30th Conference, Durban, 18-22 January, 2004. [podium presentation]
- **SF van Vuuren**, AM Viljoen, RL van Zyl and FR van Heerden. Biological activities of leaf essential oils and extracts of *Helichrysum cymosum* subsp. *cymosum*. The South African

Association of Botanists 30th Conference, Durban, 18-22 January, 2004. [**awarded first prize for best Ethnobotany poster**]

- **SF van Vuuren** and AM Viljoen. A comparative investigation of the antimicrobial properties of indigenous South African and commercially available essential oils. The 34th International Symposium on Essential Oils, Messina, Italy, 29 September – 2 October, 2004. [poster]
- **SF van Vuuren**, AM Viljoen, RL van Zyl, E Njenga and M Braithwaite. Some variables to be considered in pharmacognostic studies using South African medicinal plants as a demonstration model. Indigenous Plant Use Forum, Grahamstown, 27-30 June, 2005. [podium presentation]
- **SF van Vuuren** and AM Viljoen. Phyto-synergy: examples from indigenous aromatic medicinal plants used in antimicrobial therapy. University of Johannesburg Postgraduate Symposium, 2 November, 2005. [**awarded a medal for best PhD podium presentation**]
- **SF van Vuuren** and AM Viljoen. Exploring the Antimicrobial Activity of Indigenous Aromatic Plants. Agribusiness in Sustainable Natural African Plant Products. International Aromatic Plants and Essential Oil Mini-Symposium. Stellenbosch, 27 February - 1 March 2006. [podium presentation]
- **SF van Vuuren** and AM Viljoen. The biological activity of medicinal aromatic plants and their constituents. Indigenous Plant Use Forum, Botswana, 3-6 July, 2006. [podium presentation]
- **SF van Vuuren** and AM Viljoen. Aromatic plants and their constituents as a model to study phyto-synergy. The 37th International Symposium on Essential Oils, Grasse-Opio, France, 10-13 September, 2006. [podium presentation]

Publications submitted for review

- **SF van Vuuren** and AM Viljoen. *In vitro* evidence of phyto-synergy between two plants (*Lippia javanica* and *Artemisia afra*) and for plant part combinations (*Croton gratissimus* Burch. var. *subgratissimus*) in corroboration with their ethnobotanical use. Submitted to Journal of Ethnopharmacology.
- **SF van Vuuren** and AM Viljoen. The antimicrobial efficacy of limonene enantiomers and 1,8-cineole. Submitted to Flavour and Fragrance Journal.
- **SF van Vuuren,** AM Viljoen and KHC Başer *Tarchonanthus camphoratus and Plectranthus grandidentatus*; Antimicrobial activity and pharmacological interaction of the non-volatile and volatile fractions singularly and in combination. Submitted to South African Journal of Botany.

Dedication

To my husband Vernon for his continual support and encouragement,

and

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Abbreviations

AACHRD- African Advisory Committee for Health Research and Development

ADCAV- Plant collection by Antonio De Castro and Prof. Alvaro Viljoen

ATCC- American Type Culture Collection

CONC- Concentration

CFU- Colony forming units

DC- Combined volatile and non-volatile constituents that have been prepared from dried plant material

DD- Disc diffusion

DMSO- Dimethyl sulfoxide

EO- Essential oil

EMRSA- Epidemic methicillin resistant *Staphylococcus aureus*

ESCMID- European Society of Clinical Microbiology and Infectious Diseases

EX- Extract

FC- Combined volatile and non-volatile constituents that have been prepared with fresh plant material

FIC- Fractional inhibitory concentration

FID- Flame ionization detector

g- Gram

GC- Gas chromatography

GC-MS- Gas chromatography combined with mass spectrometry

HPLC- High performance liquid chromatography

hr- Hour

IC_{50}- The half maximum efficacy

ICU- Intensive care unit

INT- *p*-Iodonitrotetrazolium violet

IU- International units

JHB BG- Johannesburg Botanical Garden

JV- Plant collection by Jan Vlok

MIC- Minimum inhibitory concentration

min- Minutes

mg- Milligram

mL- Millilitre

mm- Millimetre

MRSA- Methicillin resistant *Staphylococcus aureus*

NCCLS- National Committee for Clinical Laboratory Standards

NaCl- Sodium chloride

NCTC- National Culture Type Collection

NHLS- National Health Laboratory Services

NMR- Nuclear magnetic resonance spectroscopy

NV- Non-volatile constituents

PB- Plant collection by Priscilla Burgoyne

PDA- Photodiode array detector

$\mathbf{R_f}$- Distance travelled by spot from base line with respect to solvent front.

RRI- Relative retention index

SABS- South African Bureau of Standards

SANBI- South African National Biodiversity Institute

Spp- Species

STI- Sexually transmitted infections

SVV- Plant collection by Sandy van Vuuren

TMD- Thermabeam mass selective detector

TIC- Total ion chromatogram

TLC- Thin layer chromatography

tr- Trace

µL- Microlitre

UV-Ultra violet

VRE- Vancomycin-resistant *Enterococcus faecalis*

WSBG- Walter Sisulu Botanical Garden

WHO- World Health Organization.