The antimicrobial interactions of *Agathosma crenulata, Dodonaea viscosa* and *Eucalyptus globulus* combination and their chemical profiling

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A thesis submitted in partial fulfilment of the requirements for the Degree of Master of Science (Botany) to the Faculty of Natural Sciences, Stellenbosch University.

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Declaration

I, Samkele Zonyane, declare that this thesis is my own work. It is being submitted in partial fulfilment for the degree of Master of Science at Stellenbosch University, Stellenbosch. It has not been submitted before for any degree or examination at this or any other University. Where the work of others has been consulted, it is duly acknowledged.

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Samkele Zonyane   Date

Declaration by Supervisors

We hereby declare that we acted as Supervisors for this Master of Science degree and regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the Faculty of Natural Sciences for examination by the University appointed examiners.

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Dr. N P Makunga   Date

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Abstract

In traditional medicine, there is a long-standing culture of combining herbal drugs to increase the therapeutic efficacy. The improved medical action is thought to be due to synergistic interactions between different plant bioactive components. The aim of this study was to test the pharmacological interactions in a medicinal plant combination which consisted of *Agathosma crenulata*, *Dodonaea viscosa* and *Eucalyptus globulus*. The rationale for the analysis of this particular mixture is that it had noteworthy antibacterial activity and exhibited the highest activity out of seven medicinal plant mixtures previously investigated. Using chromatographic analysis, the phytochemistry of the plants was also assessed.

The chloroform: methanol (1:1; v/v) extracts or hydo-distilled essential oils (*A. crenulata* and *E. globulus*) were screened individually and in combinations (double and triple plant combination) for activity against five respiratory pathogens using a microdilution assay. The antimicrobial interactions in combinations were assessed with the fractional inhibitory concentration (FIC) and the isobolograms. The organic extracts generally showed the highest antimicrobial activity with *E. globulus* having the highest activity with MIC values below 1 mg ml^{-1} representing noteworthy activity. The overall activity of the aqueous extracts was poor. The essential oil activity of *E. globulus* was mostly noteworthy (0.5 to 2 mg ml^{-1}) while *A. crenulata* essential oil displayed moderate activity (1 to 4 mg ml^{-1}).

The ΣFIC values for double combinations (1:1) of *A. crenulata* with *D. viscosa*, *A. crenulata* with *E. globulus* and *D. viscosa* with *E. globulus* were calculated from the minimum inhibitory concentration (MIC) data and the interactions were classified as synergistic, additive, indifferent and antagonistic. The highest synergistic interactions observed were for a 1:1 combination of *A. crenulata* with *E. globulus* against *K. pneumoniae*, *S. aureus* and *B. subtilis* with ΣFIC values of 0.07. There was only one incident of antagonism noted in the study for *D. viscosa* with *E. globulus* (1:1) against *C. neoformans* with ΣFIC value of 4.25. The double combinations against selective pathogens (*K. pneumoniae, S. aureus and E. coli*) were further analysed for interactions using isobolograms. Mostly, the antimicrobial interactions as presented by the isobolograms were congruent with FIC results which further validated the occurrence of relevant antimicrobial interactions in those

combinations. The ΣFIC values for triple combinations (1:1:1) revealed mostly synergistic interactions. When the triple combinations were analysed further against certain pathogens based on the predictions of the Design of Experiments software program (MODDE 9.1®), the MIC values remained the same despite the different combinations that were tested

Thin layer chromatography (TLC) was used for a quick chemical fingerprinting of the plant extracts. This was followed by a bio-autographic assay. The chemical profiles of the organic extracts and essential oils from two of the study aromatic plants (*A. crenulata* and *E. globulus*) were further analysed with liquid chromatography mass spectrometry (LC-MS) and gas chromatography mass spectrometry (GC-MS) respectively. For combined plant extracts, a multivariate data analysis using principal component analysis (PCA) and hierarchical clustering analysis (HCA) was used to determine the relationship of the chemical make-up of combinations with that of individual plant extracts. According to the TLC analysis, *E. globulus* extracts had more compounds than the other two plants in the study. For the bio-autographic assay, *E. globulus* and combinations that included this plant showed greater inhibition zones than *A. crenulata* and *D. viscosa*. For the LC-MS analysis, PCA and HCA showed a close relationship between *A. crenulata* with *D. viscosa*, *D. viscosa* with *E. globulus* and the triple combination. Twenty one components were identified in the essential oil of *A. crenulata* representing 88.83% of the total oil composition. The oil was dominated by oxygen-containing monoterpenes (46.25%). In the essential oil of *E. globulus*, twenty six compounds were identified making up to 95.62% of the oil composition. Oxygen-containing monoterpenes (32.98%) also dominated the *E. globulus* essential oil. There was no great variation in essential oil metabolites of the individual plants and their combination as shown by both PCA and HCA.

The enhanced *in vitro* antimicrobial activity and pharmacological interactions (synergy and additivity) in some of the combinations (double and triple) that were tested in this study adds scientific support to the use of medicinal plant combinations in Western Cape traditional medicine. The metabolic profiles of plants in combination might be unique due to interaction of the different plant bioactive molecules and thus result into defined antimicrobial activity.

Opsomming

In tradisionele geneeskunde is dit 'n lank bestaande kultuur om kruiemiddels te kombineer om die terapeutiese werking daarvan te verhoog. Dié verbeterde mediese werking word toegeskryf aan die oënskynlik sinergistiese interaksies tussen verskillende bioaktiewe plantkomponente. Die doel van hierdie studie was om die farmakologiese interaksies in medisinale plantkombinasies van *Agathosma crenulata*, *Dodonaea viscosa* en *Eucalyptus globulus* te bestudeer. Daar is op die ontleding van hierdie spesifieke mengsel besluit omdat dit oor beduidende antibakteriese waarde beskik en omdat dit uit sewe medisinale plantmengsels wat voorheen bestudeer is, as die doeltreffendste een aangewys is. Die fitochemie van die plante is ook met behulp van chromatografiese ontleding beoordeel.

Deur middel van 'n mikroverdunningstoets is die chloroform:metanol- (1:1; v/v-)ekstrakte of hidrogedistilleerde vlugtige olies (*A. crenulata* en *E. globulus*) individueel sowel as in kombinasie (dubbele en drievoudige plantkombinasies) nagegaan vir hul werking met betrekking tot vyf respiratoriese patogene. Die gekombineerde antimikrobiese interaksies is met behulp van fraksioneel stremmende konsentrasie (FIC) en isobologramme ondersoek. Die organiese ekstrakte het oor die algemeen die meeste antimikrobiese aktiwiteit by *E. globulus* getoon, met MIC-waardes onder 1 mg ml^{-1} wat as noemenswaardige aktiwiteit beskou is. Die algehele aktiwiteit van die waterekstrakte was swak. Die vlugtige-olieaktiwiteit van *E. globulus* was merendeels noemenswaardig (0,5 tot 2 mg ml^{-1}), terwyl die vlugtige olie van *A. crenulata* matige aktiwiteit getoon het (1 tot 4 mg ml^{-1}).

Die ΣFIC-waardes vir dubbelkombinasies (1:1) van *A. crenulata* en *D. viscosa*, *A. crenulata* en *E. globulus*, en *D. viscosa* en *E. globulus* is uit die minimum stremmende konsentrasie (MIC) bereken en die interaksies is as sinergisties, additief, neutraal en antagonisties geklassifiseer. Die sterkste sinergistiese interaksies is by 'n 1:1-kombinasie van *A. crenulata* en *E. globulus* met betrekking tot *K. pneumoniae*, *S. aureus* en *B. subtilis* opgemerk, met ΣFIC-waardes van 0,07. Die studie het slegs een geval van antagonisme opgelewer, naamlik by *D. viscosa* en *E. globulus* (1:1) met betrekking tot *C. neoformans*, wat 'n ΣFIC-waarde van 4,25 geregistreer het. Die werking van die dubbelkombinasies met betrekking tot gekose patogene (*K. pneumoniae*, *S. aureus* en *E. coli*) is voorts met behulp van isobologramme vir

interaksies nagegaan. Die antimikrobiese interaksies wat uit die isobologramme geblyk het, was meestal in pas met FIC-resultate, wat die bestaan van tersaaklike antimikrobiese interaksies in daardie kombinasies verder bevestig het. Die ΣFIC-waardes vir die drievoudige kombinasies (1:1:1) het meestal sinergistiese interaksies aan die lig gebring. Toe die drievoudige kombinasies verder op grond van die voorspellings van die sagteware Design of Experiments (MODDE 9.1®) met betrekking tot sekere patogene ontleed is, het die MIC-waardes onveranderd gebly, ondanks verskillende toetskombinasies.

Dunlaagchromatografie (TLC) is vir 'n vinnige chemiese ontleding van die plantekstrakte gebruik en is gevolg deur 'n bio-outografiese toets. Die chemiese profiele van die organiese ekstrakte en vlugtige olies van twee van die aromatiese plante in die studie (*A. crenulata* en *E. globulus*) is verder met vloeistofchromatografie-massaspektrometrie (LC-MS) en gaschromatografie-massaspektrometrie (GC-MS) onderskeidelik ontleed. Vir gekombineerde plantekstrakte is veelveranderlike-ontleding in die vorm van hoofkomponentontleding (PCA) en hiërargiese groepsontleding (HCA) gebruik om die verhouding van die chemiese samestelling van kombinasies in vergelyking met dié van individuele plantekstrakte te bepaal. Volgens die TLC-ontleding beskik *E. globulus*-ekstrakte oor meer verbindings as die ander twee plante in die studie. Vir die bio-outografiese toets het *E. globulus* en kombinasies daarmee groter stremmingsones as *A. crenulata* en *D. viscosa* getoon. In die LC-MS-ontleding het PCA en HCA op 'n hegte verhouding tussen *A. crenulata* en *D. viscosa*, *D. viscosa* en *E. globulus*, en die drievoudige kombinasie daarvan gedui. Een-en-twintig komponente is in die vlugtige olie van *A. crenulata* gevind, wat 88,83% van die algehele oliesamestelling uitmaak. Die olie is deur suurstofhoudende monoterpene (46,25%) oorheers. Die vlugtige olie van *E. globulus* het 26 verbindings opgelewer, wat 95,62% van die oliesamestelling uitmaak. Suurstofhoudende monoterpene (32,98%) het ook die vlugtige olie van *E. globulus* oorheers. Nóg PCA nóg HCA het op enige beduidende variasie in die metaboliete van die vlugtige olies van die individuele plante en hul kombinasies gedui.

Die verhoogde *in vitro*- antimikrobiese aktiwiteit en farmakologiese interaksies (sinergie en additiwiteit) in van die kombinasies (dubbel én drievoudig) wat in hierdie studie getoets is, bied wetenskaplike stawing vir die gebruik van medisinale plantkombinasies in Wes-Kaapse

tradisionele geneeskunde. Die metaboliese profiele van plantkombinasies kan verander weens die interaksie van die verskillende bioaktiewe plantmolekules, en kan baie bepaalde antimikrobiese aktiwiteit tot gevolg hê.

Scientific Outputs

Oral Presentations

Zonyane, S., Makunga, N.P. 2011. Antibacterial activity of some medicinal plant combinations used in the Greater Cape Town region. Oral presentation, 5th Annual Research Conference of Medical Research Council Early Career Scientist, 19-20 October 2011, Parow, Cape Town South Africa (Abstract in Appendix A1).

Zonyane, S., Van Vuuren, S.F., Makunga, N.P. 2012. Pharmacological and phytochemical analysis of a medicinal plant mixture that is used as traditional medicine in Western Cape. Oral presentation, 38th Annual Conference of the South African Association of Botanists, 15-18 January 2012, University of Pretoria, Pretoria South Africa (Abstract in Appendix A2).

Zonyane, S., Van Vuuren, S.F., Makunga, N.P. 2012. Unravelling antimicrobial interactions of a polyherbal combination used in traditional medicine. Oral presentation, 12th Annual Conference of the Indigenous Plant Use Forum, 02-05 July 2012, University of Venda, Thohoyandou South Africa (Abstract in Appendix A3).

Publication in peer-reviewed journal

Zonyane, S., Van Vuuren, S.F., Makunga, N.P. 2013. Antimicrobial interactions of Khoi-San poly-herbal remedies with emphasis on the combination; *Agathosma crenulata*, *Dodonaea viscosa* and *Eucalyptus globulus*. Journal of Ethnopharmacology 148, 144 - 151 (Abstract in Appendix A4).

This paper is a complilation of the results derived directly from the current MSc research project together with the results of the previous project (2010) which formed the basis of this MSc study.

Dedication

This thesis is dedicated to my adorable parents, Mr. and Mrs. Mluleki G. Zonyane.

Acknowledgements

- I wish to thank my lovely siblings for support; Sis Linda, Kosie and Hlumelo. I love you guys so much.

- Lastly, I thank my lovely parents, my mom and dad. Enkosi Xaba nawe mam'am. Your prayers, support, ambitious attitudes and hard work have really driven me to this point and beyond. Thank you so much for everything.

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Equation 3.1

$$FIC_{I} = \frac{MIC\,(A)\ in\ combination\ with\ (B)/2}{MIC\,(A)\ independently}$$

Equation 3.2

$$FIC_{II} = \frac{MIC\,(A)\ in\ combination\ with\ (B)/2}{MIC\,(B)\ independently}$$

Equation 3.3

$$\Sigma FIC = FIC_{I} + FIC_{II}$$

Equation 3.4

$$FIC_{I} = \frac{MIC\,(A)\ in\ combination\ with\ (B)\ and\ (C)/3}{MIC\,(A)\ independently}$$

Equation 3.5

$$FIC_{II} = \frac{MIC\,(A)\ in\ combination\ with\ (B)\ and\ (C)/3}{MIC\,(B)\ independently}$$

Equation 3.6

$$FIC_{III} = \frac{MIC\ (A)\ in\ combination\ with\ (B)\ and\ (C)/3}{MIC\ (C)\ independently}$$

Equation 3.7

$$\Sigma FIC = FIC_I + FIC_{II} + FIC_{III}$$

Equation 3.8

$$X = \frac{MIC\ of\ an\ extract/essential\ oil\ in\ combination}{MIC\ of\ an\ extract/essential\ oil\ independently}$$

Equation 3.9

$$Y = \frac{MIC\ of\ an\ extract/essential\ oil\ in\ combination}{MIC\ of\ an\ extract/essential\ oil\ independently}$$

List of Acronyms and Symbols

%	Percent
°C	Degree Celsius
µl	microlitre
µm	micrometre
ATCC	American type culture collection
CFR	Cape Floristic Region
CFU	Colony forming unit
eV	Electron volt
FIC	Fractional inhibitory concentration
g	Gram
GC-MS	Gas chromatography coupled to mass spectrometry
h	Hour
i.d	Inner diameter
INT	*p*-iodonitrotetrazolium salt
kV	Acceleration voltage
l	Litre
LC-MS	Liquid chromatography mass spectrometry
mg	Milligram
MH	Mueller Hinton
MIC	Minimum inhibitory concentration
min	Minute

ml Millilitre

m/z Mass to charge ratio

nm Nano-metres

NRF National Research Foundation

PCA Principal component analysis of variance

PDA Photodiode array

R$_f$ Retention factor

RI Retention index

rpm Revolution per minute

SANBI South African National Biodiversity Institution

TB Tuberculosis

TLC Thin layer chromatography

UV Ultra violet

V Volt

v/v Volume per volume

WHO World Health Organisation

Chapter 1

General Introduction

1.1 General introduction

Plants have been an important source of medicine since ancient times to the present day in nearly all cultures (Akerele *et al.*, 1991; Nostro *et al.*, 2000). Over centuries, experimentation by people living in intimate association with their environment has yielded knowledge of plant bioactivity (Sanz-Biset *et al.*, 2009). Products derived from plants have been used as a source of medication since the beginning of human civilization (Acharya and Rokaya, 2005). In South Africa about 27 million people consult with traditional herbal practitioners for physical and psychological health needs (Rabe and Van Staden, 1997; Light *et al.*, 2005, Street *et al.*, 2008). The use of traditional medicinal plants has persisted for ages even when the option of allopathic medicine is readily available (Kaido *et al.*, 1997). This is due to the fact that many people use traditional medicine in addition to the drugs prescribed by western medical practitioners (Thring and Weitz, 2006).

Recently, there has been intensive research on plants used as traditional medicine in terms of their antimicrobial activity and chemical constituents. This interest is based on the wide use of traditional medicines as an alternative form of healthcare and the potential they hold as sources of novel therapeutic agents (Nostro *et al.*, 2000; Samy and Ignacimuthu, 2000; Srinivasan *et al.*, 2001; Sanz-Biset *et al.*, 2009). Moreover, plant extracts are increasingly utilized in the pharmaceutical, cosmetic and food industries thus research on medicinal plants is essential in order to validate their immense value (Nostro *et al.*, 2000).

Furthermore, due to the fact that human pathogens are increasingly becoming more resistant to antibiotics and this problem has grown to very serious proportions (Kamatou *et al.*, 2006 and Gathirwa *et al.*, 2008), it is necessary that other efficient measures for therapeutic intervention are provided (Mitscher and Baker, 1998). It is important to establish scientific rationale of traditional medicines, as this lends impetus to the widespread use of these medicines in Africa. To have such information could lead to regulation and quality control of traditional medicine in South Africa which will scientifically validate and promote the use of medicinal plants which at this stage remains poorly legislated and controlled.

In most cases, the preparations of traditional medicine include combinations made from various plants (Van Vuuren and Viljoen, 2008) or even combinations of plants with animal parts (Louw *et al*., 2002). It is speculated that this method increases the therapeutic effect. However, this concept has not been scientifically investigated sufficiently. In South Africa, the number of studies focused on screening plant-based herbal mixtures is limited as many studies focus on validating the bioactivity of single plants utilised in traditional medicine.

Consequently, the focus and aim of this study was on antimicrobial interactions between different plants in a medicinal combination made up of *Agathosma crenulata* (L.) Pillans, *Dodonaea viscosa* Jacq. and *Eucalyptus globulus* Labill. These three plants are used in a combined form to treat respiratory ailments and they were administered by Mr. Paul Hattingh, a Rastafarian who practises Khoi-San traditional healing methods in the Western Cape. Traditionally, this combination is prepared by boiling the equal portions of the aerial parts of the three plants together in water, as recommended by the herbalist who administered them. Upon boiling the plants for few minutes, the medicinal solution is strained and can be drunk. The rationale behind the use of this particular combination in this study is that it displayed the best antibacterial activity out of six medicinal plant combinations tested in a previous study that was conducted in 2010 of which its results are included in Zonyane *et al*. (2013). As a result, there was motivation to further explore it in terms of its antimicrobial activity as well as its antimicrobial interactions.

Therefore the objectives of this study were:

- To test the plant extracts (*A. crenulata*, *D. viscosa* and *E. globulus*) and essential oils (*A. crenulata* and *E. globulus*) for antimicrobial activity individually and in various combinations using the minimum inhibitory concentration (MIC) technique.
- To test the possible pharmacological interactions in double (1:1) and in triple (1:1:1) plant combinations using the fractional inhibitory concentration index (FICI).
- To determine the interactive effects of selected combinations at various ratios and present this using isobolograms.

- To do a chemical fingerprinting of the study plants to get an understanding of the chemical modifications that take place when extracts or essential oils from different plants are combined:
  - ❖ Through conducting thin layer chromatography (TLC) on extracts and essential oils independently and in combinations.
  - ❖ Through carrying out gas chromatography coupled to mass spectrometry (GC-MS) on the essential oils.
  - ❖ The extracts were also subjected to liquid chromatography coupled to mass spectrometry (LC-MS) alone and in combinations.
- To examine the chemical fingerprinting of compounds demonstrating antimicrobial activity when the extracts are tested using bio-autographic analysis.

Chapter 2

Literature

2.1 Medicinal plants and bioactivity

Medicinal plants possess a wide range of bioactive molecules that make them therapeutic. These molecules have certain targets on which they act and help to maintain the normal human physiological state by interfering with infections, inflammation and organ malfunctions. Medicinal plant metabolites are in most cases multifunctional and because of this, plant extracts can affect more than one molecular target (Van Wyk *et al*., 2009).

South Africa has a rich floral diversity with nearly 30 000 plant species and of these about 3 000 (Light *et al*., 2005) to 4 000 (Mulholland, 2005) species are estimated as being important for benefiting health. There has been a longstanding culture of medicinal plant use to treat various diseases in this country (McGaw *et al*., 2008a). For example, the Western Cape Province is home to the Cape Floristic Region which alone has about 9000 plants. Some of these species such as *Agathosma betulina* (Berg.) Pillans, *A. crenulata*, *Dodonaea angustifolia* L.f., *Aloe ferox* Mill., *Artemisia afra* Jacq. ex Willd., *Elytropappus rhinocerotis* (L.f.) Less., *Helichrysum odoratissimum* Mill. and *Hoodia pilifera* (L.f.) are important as medicinal herbal products (Van Wyk, 2008a). In KwaZulu-Natal, approximately 1032 species from 147 families are administered by Zulu traditional healers (Mander, 1998). In the Eastern Cape, 166 plant species are gathered and used for medicinal, cultural and spiritual purposes (Dold and Cocks, 2002). South African medicinal plant species also bear economic value and some of these species are utilised in the formal phytotherapies sector. Several species such as *A. crenulata*, *A. betulina*, *Pelargonium reniforme* (Curt.), *A. ferox*, *H. odoratissimum*, and many more are readily available as commercial products and are exported to many countries (Dold and Cocks, 2002; Brendler and Van Wyk, 2008; Makunga *et al*., 2008, Van Wyk 2008b; Van Wyk, 2011).

2.2 Traditional medicine

An estimated 80% of the world's population depends on traditional medicine for healthcare needs (Acharya and Rokaya, 2005; Diederichs *et al*., 2006) and use of medicinal plants is more intense in developing countries. In Africa and Asia, traditional medicine forms an important part of primary healthcare and it is used intensively as these regions are faced

with challenges such as poorly developed medical facilities, shortages of economic resources and lack of access to western medicine. Therefore, people in such countries become more dependent on natural resources to support health requirements (Louw *et al.*, 2002). The health and economic benefits of traditional medicine, however, are acknowledged in both developing and developed countries, but formal recognition varies from region to region (Stafford *et al.*, 2005). The most popular forms of traditional medicine are remedies made up of crude plant material (Chhabra *et al.*, 1984).

On the African continent, the culture of traditional herbal medicine use for the prevention and treatment of diseases spans over several centuries. The ancient African healers employed a detailed *materia medica* (mixtures of various herbs, animal parts, minerals and clays) in their healing practices. The reality of African medicine is not a matter of a medicine man dispensing medicine to a patient but it is a complex relationship between man and plants and the cosmic balance of natural forces as tools in healing (Iwu, 1993). Apart from treating the disease which is the main method employed in Western medicine, in some cases, additional contribution from spiritual guidance is often an important element in the healing process. This gives more meaning to healing within the context of African culture. The pharmacopoeia of African traditional medicine includes cures for a variety of diseases such as cough, snakebites, diabetes, infectious diseases and many more illnesses. Claims of these African herbs curing more complex diseases such as cancer (Iwu, 1993) are reported.

South Africa has an estimated number of 200 000 indigenous traditional healers who employ medicinal plants remedies for healing (Diederichs *et al.*, 2006). In addition to the high diversity of medicinal plants, the country is also endowed with a rich cultural diversity and traditional healing therefore, is often specifically associated with each ethnic group (Van Vuuren, 2008). The different types of traditional healers in the country include; "isangoma" for the Zulu culture, "ixhwele/igqirha" for the Xhosa culture, "inqaka" for the Sotho culture and "bossiedokter" for the Khoi-San culture (Van Wyk *et al.*, 2009). Traditional herbal medicines form an important part of healthcare in South Africa and it is often preferred because of its cultural importance (Light *et al.,* 2005; Diederichs *et al.*, 2006). Most of this traditional medicine is derived from plant species indigenous to the region. These may be obtained on prescription from a traditional healer, purchased from herb

sellers or gathered in the wild for self-medication. Trade of herbal remedies contributes also to the livelihood of many, including those that harvest plant material from the wild and those that then sell these wild-crafted materials in informal herbal markets (Mander *et al*., 2006).

2.2.1 Traditional medicine in Western Cape and Khoi-San traditional healing system

Due to a vast diversity of medicinal plants and many cultural groups there is a history of diverse medicinal plant use in this province. There are two main groups of traditional healers found in the Western Cape; Xhosas and 'bossies-dokters' (bush-doctors). These groups have different ideologies and knowledge of plants used in the province (Makunga *et al*., 2008). The Xhosa traditional healers who originate from Eastern Cape believe that they are called by their ancestors to practise traditional medicine and therefore they claim to be spiritually empowered, while the latter group practise the Khoi-San traditional healing system.

Historically, the Khoikhoi herders and San hunter-gatherers have been the inhabitants of the Cape region of South Africa for centuries. These two cultural groups are usually collectively referred to as Khoi-San people (Van Wyk, 2008a). In a review by Van Wyk (2008b), 170 medicinal plants were reportedly used by this group prior to 1932 while Donaldson and Scott (1994) revealed the ethnobotanical use of 533 plants by Khoi-San people. A large fraction of "bossiedokters" practice Rastafarianism and they also practise a cross-cultural method of healing. Aston-Philander (2011) published an ethnobotanical survey on the use of plants by these "Rastafarian bossie-dokters" and emphasised that these "Rasta" herbalists gained their knowledge of medicinal plant use from elderly people of the previous generations in the late 1970s. Rastafarians are known to be committed to using materials in their natural or organic state in herbal healing. This group of people use various herbs as to promote well-being (Aston-Philander, 2011). Similarly to other herbalists, they will often combine different herbs and plant parts and sell these to their clients.

2.2.2 Preparation and dosage of traditional medicinal remedies

Traditional medicine is prepared and consumed in various ways that include teas, extracts, mixtures, tinctures, macerations, snuffs, medicinal oils, mixtures; and many other forms. Plant parts used as a medicine vary from one plant to another. Some plants are used as a whole while others, only a specific part is active. Infusions including teas, extracts and mixtures are prepared by soaking the plant material in water for few minutes and the solution is strained thereafter. Traditional herbal medicine can also be prepared with alcohol, known as a tincture. Extractions prepared with organics such as brandy are thought to be of a more superior preparation compared to other means of preparation. Alcohols easily extract alkaloids from the plant material and can also serve as a storage medium. As a common practice, the plant material is usually soaked in hot or cold water and administered orally (Van Wyk *et al*., 2009).

Proper preparation of herbal drugs, dosage and the form of administration are important factors to be considered to achieve the optimal effect in medicinal preparations. The amount administered may vary in the range of 5- 50 g of dry plant material per litre of water. Higher doses of plant material are seldom used or recommended. Dose variation has greater effects on children due to their smaller size. The concentration of active compounds and other plant metabolites in harvested herbal plants also determine the efficacy. This is affected by several factors which include variation in the plant part used, maturity and the period of harvesting the plant, topographical conditions such as soil acidity, water condition and contaminants in the soil, weather conditions and other growth factors (Roulet *et al*., 1988).

2.3 Combination therapy in traditional medicine and phyto-synergism

In traditional medicine, different parts of the plant or either different plant species which are not related in any way are combined with the intention of increasing efficacy of the medicinal preparation (Van Vuuren and Viljoen, 2008). This method of combining different herbal extracts to treat diseases has been practised for decades in various traditional medicine groups and has been effective in treating diseases. Hence, there is an increased interest to find a scientific rationale for the therapeutic superiority in multi-herb extracts.

Clinical studies have been conducted to examine the action of the plant combinations (Wagner and Ulrich-Merzenich, 2009). It is believed that the enhanced efficacy in combined extracts is attributable to synergistic interactions between different plant bioactive constituents and their by-products (Van Vuuren and Viljoen, 2008; Wagner and Ulrich-Merzenich, 2009).

Synergy is defined as the mechanism where the overall cumulative effect of a combination is greater than that expected from the sum of their individual effects (Williamson, 2001). The increase in potency may be explained by the mechanism whereby various plant bioactive constituents affect several target sites and work co-operatively in a synergistic manner (Al-Bayati, 2008). The polyvalence of plant extract constituents is due to their abilities to bind to various molecular structures such as proteins or glycoproteins and such constituents possess great affinities for cell membranes that ultimately cause the plant bioactives to pass through cell walls with great ease. Consequently, the overall efficacy is enhanced (Wagner and Ulrich-Merzenich, 2009).

Natural drug combinations are speculated to suppress bacterial resistance which usually develops when single drugs are used (Al-Bayati, 2008). The underlying mechanism may include intervention at the active site of the bacteria such as the penicillin-binding proteins by directly or indirectly attacking the peptidoglycan part of the bacterial cell wall. Other agents act by inhibiting topoisomerase IV or RNA synthesis. Natural products can also inhibit lactam or ester-cleaving enzymes that are produced by a bacterium to deactivate antibiotics. Another mechanism is attributed to combined agents blocking the pumping system that is developed by many bacteria allowing better penetration of therapeutic agents into bacterial cells. This pumping system also eliminates the antibiotics that have already passed through the cell wall (Wagner and Ulrich-Merzenich, 2009).

In phyto-therapy, certain compounds in an extract, such as polyphenols or saponins often do not possess specific pharmacological effects themselves. However, these may increase the solubility and the reabsorption rate of key components in the extract and thereby enhancing the extract's bioavailability. This results in a higher effectiveness of the extract than an isolated constituent of the extract itself (Wagner and Ulrich-Merzenich, 2009).

Combination therapy is not only confined to traditional medicine but is also employed in modern medicine for cancer chemotherapy, HIV treatment and other diseases (Williamson, 2001). As part of allopathic medical practices, drug combination is also known to reduce side effects (Briskin, 2000). For instance, treatment of tuberculosis involves multiple drugs. Since many antimicrobial agents produce excess toxicity when taken in high doses, it is advantageous to use multiple drugs in low doses so as to reduce adverse effects that might be caused by high dose of single antimicrobial agents (Eliopoulos and Moellering, 1996).

Some studies have investigated the efficacy of medicinal plant combinations as well as their antimicrobial interactions, however, there is still a vast gap in this research area. South African studies have mostly focused on studying the pharmacological effects of individual plants. Therefore further scientific evidence needs to be provided to confirm this concept (Al-Bayati, 2008; Van Vuuren and Viljoen, 2008).

Van Vuuren and Viljoen (2008) examined the combination of different plant parts of *Croton gratissimus* and these were investigated for antimicrobial interactions *in vitro*. Various plant part combinations showed different degrees of activity with a combination of leaf and root (1:1) exhibiting synergism against *Cryptococcus neoformans*. In another study, the essential oil combinations of *Thymus vulgaris* L. and *Pimpinella anisum* L. as well as methanol extracts combinations were tested for activity against nine bacteria. The combinations demonstrated higher antibacterial activity compared to individual plants against most bacteria (Al-Bayati 2008). The medicinal plant combination of *Salvia chamelaeagnea* (K. Bergius) and *Leonotis leonurus* (L.) R. Br. at various ratios displayed some synergistic interactions particularly on Gram-positive bacteria when it was tested against Gram-positive and Gram-negative bacteria (Kamatou *et al.*, 2006). *A. afra* which is widely used in medicinal plant combinations with *A. betulina*, *E. globulus* and *Osmitopsis asteriscoides* Berg. to treat respiratory ailments was studied for against respiratory pathogens. A combination of *A. afra* with *O. asteriscoides* at the ratio of 8:2 resulted in synergism (Suliman *et al.*, 2010). Ncube *et al.* (2012) tested the leaves and bulbs of *Tulbaghia violacea* Harv., *Hypoxis hemerocallidea* Fisch.Mey. & Avé-Lall. and *Merwilla plumbea* (Lindl.) Speta and various organic solvents combinations of different plant parts were compared. Many synergistic interactions were noted for various extract combinations.

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These studies present *in vitro* findings that scientifically validate the use of medicinal plant combinations for increased therapeutic activity. Understanding of pharmacological interactions is important as a platform to develop novel phyto-medicines that are effective.

2.4 Progress and importance of ethnopharmacology research globally and in Africa

Although traditional medicine is gaining popularity and is widely accepted, it is still faced with certain challenges such as lack of policy, safety and unsustainable use (World Health Organization (WHO), 2002). The situation is even critical in the African continent as most of African traditional medicine have very little recorded documentation. Worldwide research in the ethnopharmacological field has been on the increase over the past years. Even so, most of African indigenous knowledge of traditional medicines has not been studied sufficiently compared to other traditional medicinal systems such as India Ayurvedic and Chinese traditional medicines (Light *et al.*, 2005). Africa is lagging behind in terms of ethnopharmacological research output.

There are only a few examples of African medicinal plants that are recognized in modern pharmacopoeias, such as *Catharanthus roseus* (L.) G. Don., *Salix* L. species, and *Harpagophytum procumbens* (Burch.) DC. ex Meisn. *Salix capensis* L. which has been serving as a pain killer and an antipyretic agent is one of the few medicinal plant species from which a universal analgesic, aspirin, has been developed. Recording this valuable indigenous knowledge before it is lost without scientific validation needs to be prioritized (Iwu, 1993).

Documentation of medicinal uses of African plants is further necessary as there is a high rate of natural habitat loss and degradation of some species before their therapeutic properties are evaluated. It is not only the loss of plants threatened by habitat degradation but traditional community life and culture are also affected.

However, organizations such as WHO are influential in research of traditional medicine. The WHO promotes use of evidence in ensuring safety, efficacy and product quality of

traditional medicine. This organization is also working towards ensuring the safety and quality of traditional medicine through regulation of products, practices and providers and thereby integrating traditional medicine into national health systems. Lastly, the organization is instrumental in preserving indigenous knowledge and it acknowledges traditional medicine as part of primary health care and promotes increased access to medicinal care (WHO, 2002).

In South Africa, antimicrobial plant research was very poorly studied. It is only in the last decade that an effort has been made to document the anti-infective properties of African medicinal plants. The South African government and the National Research Foundation (NRF) made efforts to encourage research in the area of pharmacological analysis (Light *et al*., 2005). The South African NRF considers studying of indigenous knowledge a high priority (Mulholland, 2005) and therefore a steady rise in ethnopharmacology research in South Africa over the past 14 years is evident in the literature.

2.5 Medicinal plants for the treatment of respiratory diseases

Respiratory ailments are a major health problem that is encountered worldwide, particularly in low income countries. They result in high mortality rates and may further result in morbidity through acute respiratory infections. Co-occurence with human immunodeficiency worsens the situation with pneumonia being the major cause of high mortality rates in adults (Feikin *et al*., 2004). In developing countries four million children die annually from respiratory infections (Shann *et al*., 1999). Among respiratory diseases, tuberculosis (TB) and influenza have caused many deaths in South Africa (York *et al*., 2011) with TB ranked fourth on the list of highest TB countries in the world (USAID, 2008). The emergence of multidrug-resistant (MDR) TB is further complicating the extent of the TB epidemic (USAID, 2008). Globally medicinal plants are traditionally used in the treatment of respiratory diseases (Caceres *et al*., 1991; Lall and Meyer, 1999; Njoroge and Bussmann, 2006; Ballabh and Chaurasia, 2007; Eldeen and Van Staden, 2007; Gautam *et al*.,2007; Savithramma *et al*., 2007; Green *et al*., 2010; Mohamad *et al*., 2011). It is thus important to screen these plants as this may lead to the discovery of novel therapeutic agents.

Worldwide research on medicinal plants used to treat respiratory infections is evident. In Guatemala, 68 plants were screened for inhibition activity against three respiratory bacteria; *S. aureus*, *Streptococcus pneumoniae* and *S. pyogenes*. Almost half of the plants screened showed antibacterial activity inhibiting one or more of the test bacteria (Caceres *et al.*, 1991). Certain plants (*Gnaphalium oxyphyllum*, *Gnaphalium americanum*, *Cunila lythrifolia* Benth., *Gossypium hirsutum* L., *Bougainvillea glabra* Choisy and *Crescentia alata* Kunth) used in Mexican traditional medicine to treat respiratory ailments were evaluated for antibacterial activity against a number of bacteria responsible for respiratory infections. The extracts prepared from these plants showed some antimicrobial activity (Rojas *et al.*, 2001). Disengomoka *et al.* (1983) conducted an ethnobotanical survey of the traditional herbs that cure children's respiratory infections. The survey gives details of the use of each plant, plant part used and method of administration. In China, a traditional combination remedy called, TanReQing which is made from a mixture of natural products derived from five Chinese traditional medicines (*Scutellaria baicalensis* Georgi (radix), Bear Gall powder, Goral horn, *Lonicera japonica* Thunb (flos) and *Forsythia suspensa* (Thunb.) Vahl. (fructus) was found to have anti-biofilm activity as demonstrated by its ability to prevent the formation of *S. aureus* biofilm (Wang *et al.*, 2011).

Many of South African medicinal plant species have also been screened for respiratory conditions such as antimycobacterial activity. Green *et al.* (2010), screened plants used in Venda in treating TB related symptoms for activity against H_{37}Ra *Mycobacterium tuberculosis* strain which is susceptible to all first line drugs. In a study by Bamuamba *et al.* (2008), five of the medicinal plants used in Western Cape for treatment of TB; *Olea capensis* L., *Tulbaghia alliacea* L.f., *Dittrichia graveolens* L., *Leysera gnaphaloides* and *Buddleja saligna* Willd. were screened for anti-TB activity. Extracts of *B. saligna* and *L. gnaphaloides* exhibited significant antimycobacterium activity (Bamuamba *et al.*, 2008). Detailed information about South African plants that possess anti-TB properties is also steadily documented, McGaw *et al.* (2008a) have collated all plants used for treating TB in South Africa and almost 180 plant species have been documented in their review. This review reports that it is only about 30% of these plants that have been scientifically validated for their efficacy. To mention a few, plants species such as *Euclea natalensis* A.DC., *Ekebergia capensis* Sparrm., *Helichrysum melanacme* DC., *Polygala myrtifolia* L., *Helichrysum*

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caespititium DC., *Pelargonium sidoides* DC., *P. reniforme*, *Arctotis auriculata* Jacq., *Eriocephalus africanus* L., *Dodonaea viscosa* demonstrated some inhibitory activity against certain *Mycobacterium* species using different *in vitro* bio-assays. Some of these tested species are reported to have exhibited a remarkable antimycobacterial activity. *E. natalensis*, *E. capensis*, *H. melanacme*, *P. myrtifolia* and *H. caespititium* were active against certain *M. tuberculosis* strains at the MIC values as low as 0.1 mg ml^{-1}, using a radiometric method by Lall and Meyer (1999) and Meyer *et al*. (2002). *E. natalensis* also demonstrated high activity when it was screened for inhibitory effects against *Mycobacterium bovis* at an MIC of 0.026 mg ml^{-1} (McGaw *et al*., 2008b)*.* The extracts of the aerial parts of *Salvia radula* (Benth.), *Salvia verbenaca* L. and *Salvia dolomitica* Codd displayed the MIC of 0.1 mg ml^{-1} against *M. tuberculosis* H37Ra using the BACTEC method (Kamatou *et al*., 2007). *Coleonema album* (Thunb.) Bartl. & J.C. Wendl. showed inhibitory effect of more than 99% on a population (MIC$_{99}$) of drug-sensitive strain of *M. tuberculosis* (Esterhuizen *et al*., 2006). Plants used for the treatment of respiratory diseases and growing in Maputaland, a rural region in KwaZulu Natal, were also recorded in terms of method of preparation, dosage, plant part used and vernacular names. However, there are few plant combinations that have been tested for TB, it is mostly single plant extracts investigated for activity (York *et al*., 2012; Zonyane *et al*., 2013).

Many South African plants species which are used for the treatment of respiratory infections can also be administered as combined remedies. Table 2.1 summarizes the uses of some of the medicinal plant combinations used to treat respiratory infections.

Table 2.1 Medicinal plant mixtures used for the treatment of respiratory infections.

Plant mixture	Ethnobotanical use	Method of administration	References
Agathosma betulina and *Artemisia afra*	Respiratory ailments	Vinegar infusion	Watt and Breyer-Brandwijk, (1962).
Eucalyptus globulus and *Artemisia afra*	Respiratory ailments	Steam inhaled or decoctions	Watt and Breyer-Brandwijk, (1962); Hutchings *et al*. (1996).

Plant mixture	Ethnobotanical use	Method of administration	References
Eucalyptus globulus, Artemisia afra, and *Leonotis microphylla*	Respiratory ailments	Infusion , tincture	Watt and Breyer-Brandwijk, (1962); Van Wyk *et al*. (2009).
Eucalyptus globulus, Artemisia afra and *Osmitopsis asteriscoides*	Respiratory ailments	Tincture	Watt and Breyer-Brandwijk, (1962); Suliman *et al*. (2010).
Cheilanthes hirta and *Mohria cafforum*	Cold and sore throat in children	Burned and inhaled	Hutchings *et al*. (1996).
Cannabis sativa and *Warburgia salutaris*	Dry cough, asthma	Snuff	Hutchings *et al*. (1996); Bryant, (1970).
Nymphaea nouchali and *Tulbaghia species*	Cough and cold	Decoctions	Hutchings *et al*. (1996).
Ranunculus multifidus and *Helichrysum nudifolium*	Severe cough and sore throat	Milk infusions of roots	Hutchings *et al*. (1996).
Ozoroa paniculosa and *Berchemia zeyheri*	Acute inflammatory conditions of the chest	Taken orally	Hutchings *et al*. (1996).
Pterocelastrus echinatus and *Alepidea amatymbica*	Respiratory ailments	Decoction	Hutchings *et al*. (1996); Pujol, (1990).
Cucumis hirsutus and *Aster bakeranus*	Chronic cough	Decoctions	Hutchings *et al*. (1996); Bryant, (1970).
Warburgia salutaris and Acorus calamus	Pneumonia	Infusion	Felhaber, (1997).
Lippia javanica and Aloysia triphylla	Cold, fever, asthma	Tea	Van Wyk and Wink, (2004).

2.6 Medicinal plants metabolites

Medicinal plants are known to be a rich source of various beneficial compounds including the flavonoids, terpenoids, phenolics, and many more (Douglas and Soejarto, 2002). It is these compounds that are responsible for the biological activities noted. Plant secondary compounds, unlike primary compounds, are not involved in the primary metabolism which is necessary for the immediate survival of a plant, however, they play various ecological roles such as pollination, defence against herbivores and pathogens, protection from abiotic stresses and interactions with competitors and mutualists (Kennedy and Wightman, 2011). They are differentially distributed among taxonomic groups in the plant kingdom (Buchanan *et al*, 2000).

Phytochemistry is one of the most important focus areas in medicinal plant research (Briskin, 2000). There is a need to screen the herbal plants for the composition and abundance of secondary compounds as this determines their pharmaceutical and economic value (Shyur and Yang, 2008; Wishart, 2008). For example, some modern drugs have their origins from plants, where plants have been used either directly to provide pure compounds for the synthesis of drugs or have been used indirectly as models for new drug targets (Gautam *et al*., 2007). A few examples of popular drugs derived solely from plant resources include vinblastine and paclitaxel (taxol) which are anticancer drugs. Vinblastine, a drug used for treatment of Hodgkin's disease; lung cancer and lymphoma, is derived from *C. roseus* (Tripathi, 2003). Paclitaxel, derived from *Taxus brevifolia* Nutt. is used to treat ovarian and breast cancer. The other popular drug of plant origin is aspirin and its sources are *Salix* species (Tripathi, 2003).

There are various chromatographic methods used to study the chemical profile of medicinal plants due to diverse nature of plant secondary compounds. The most commonly used techniques include gas chromatography mass spectroscopy (GC-MS), liquid chromatography mass spectroscopy (LC-MS) and thin layer chromatography (TLC). For GC-MS, the separation of analytes is carried through a silica column using helium or nitrogen as a carrier gas and chemicals migrate at a rate proportional to their masses. Gas chromatography mass spectroscopy (GC-MS) is mostly used for analysis of volatile compounds. Non-volatile

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compounds are better analysed with LC-MS technique. The LC-MS technique is more suitable for separation of polar compounds such as phenols, alkaloids, flavonoids and caffeic acids (Allwood *et al*., 2011). The thin layer chromatography (TLC) assay is a quick and simple method of separating bioactive compounds in a plant extract and other chemical mixtures. It localizes active compounds very accurately. Although the TLC method is simple and quick, its resolution is lower than other methods used in chemistry to identify chemical compounds (Diederichs, 2006).

2.7 Study plants

2.7.1 *Agathosma crenulata*

2.7.1.1 Botanical description and geographical distribution

Agathosma crenulata (L.) Pillans belongs to the Family Rutaceae and is commonly known as 'Buchu' in English and Khoi; 'Boegoe' in Afrikaans; and 'Ibhuchu' in Xhosa (Van Wyk and Wink, 2004; Moolla, 2006; Van Wyk *et al*., 2009). 'Buchu' species, especially *A. crenulata* and *A. betulina* are some of the most popular herbs used in South Africa, particularly around the Western Cape and internationally (Lis-Balchin *et al*., 2001). Buchu is a perennial aromatic shrub with delicate stems, it can grow up to 2.5 m and it is characterised by dark green glossy, oval leaves that bear oil glands (Figure 2.1).

Figure 2.1 Leaves and flowers of *Agathosma crenulata* (www.plantzafrica.com).

Its flowering season is between June and November and it produces one to three white or mauve flowers per stem in the leaf axils (Van Rooyen and Steyn, 1999). *A. crenulata* with about 150 other *Agathosma* species occur and are restricted in the mountainous areas of the Cape region of South Africa (Van Wyk and Gericke, 2000) (Figure 2.2).
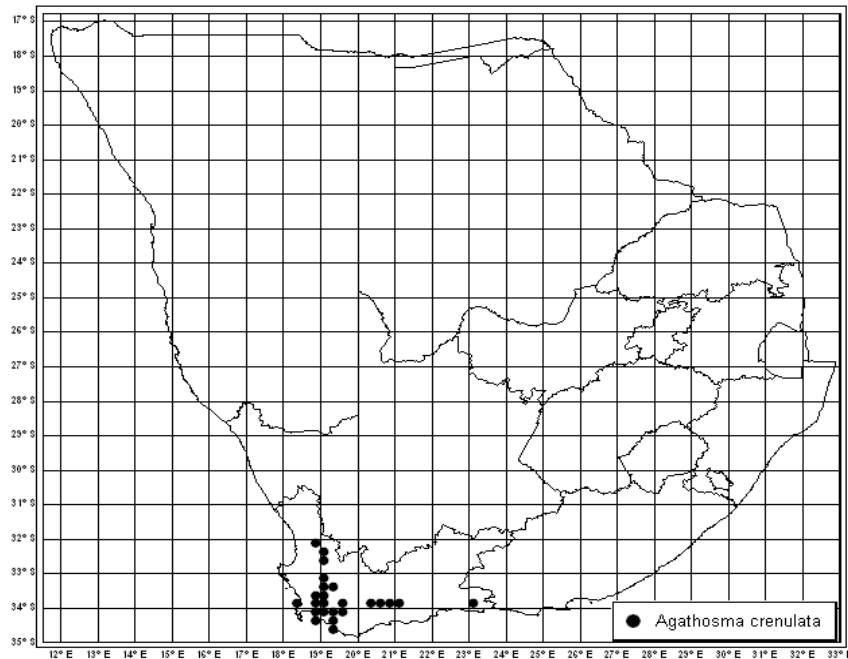
Figure 2.2 Geographical distribution of *Agathosma crenulata* in South Africa (SANBI).

2.7.1.2 Medicinal uses

'Buchu' was first introduced by indigenous people of the Cape to European colonists as a medicinal plant. It was then widely used, mostly as diuretic and antiseptic in Europe and America (Lis-Balchin *et al.*, 2001) to treat variable diseases including kidney stones, inflammation, stomach complaints and catarrh of the bladder (Grieve, 1937; Watt and Breyer-Brandwijk 1962; Simpson, 1998). This herb has been an important part of the Khoi-San culture whereby the aromatic plants were mixed with fat and the preparation was applied to the skin to keep it soft, or used as an antibacterial and antifungal agent, insect repellent and deodorant. Other methods of medicinal preparation that were used include tinctures whereby leaves were mixed with brandy and alternatively, the leaves would be chewed (Moolla *et al.*, 2007).

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It is still used today as a general tonic and medicine throughout South Africa, treating urinary tract infections, kidney disease, rheumatism and gout. The leaves can also be soaked in vinegar to clean wounds. It has become increasingly important due to an essential oil preparation which is of commercial significance. The 'Buchu' essential oil which is characterised by strong pulegone scent and pale colour (Van Rooyen and Steyn, 1999) is known to have a variety of therapeutic properties, treating fever, coughs, colds and flu. It is also used in the flavour and fragrance industry to enhance fruit flavours (Moolla and Viljoen, 2008).

2.7.2 *Dodonaea viscosa*

2.7.2.1 Botanical description and geographical distribution

Dodonaea viscosa Jacq., is an evergreen shrub that belongs to the family Sapindaceae (Getie *et al*., 2003). The common name is 'sand olive' in English and 'ysterhouttoppe' in Afrikaans (Van Wyk *et al*., 2009). This species can reach 8 m in height and the stem has a diameter of 20 cm with light grey and finely fissured bark (Liu and Noshiro, 2002). It is characterised by shiny pale green long and narrow leaves and produces small yellowish-green flowers (Figure 2.3). The flowering season is between April and August (Van Wyk *et al*., 2009).

Figure 2.3 Leaves of *Dodonaea viscosa* (S. Zonyane).

D. viscosa has a cosmopolitan distribution in tropical, subtropical and warm temperate regions of Africa, North and South America, southern Asia and Australia (Wagner *et al.*, 1987; Shanmugavasan and Ramachandran, 2011; Wabo *et al.*, 2012). In South Africa, it is distributed throughout the country except the central part (Figure 2.4).
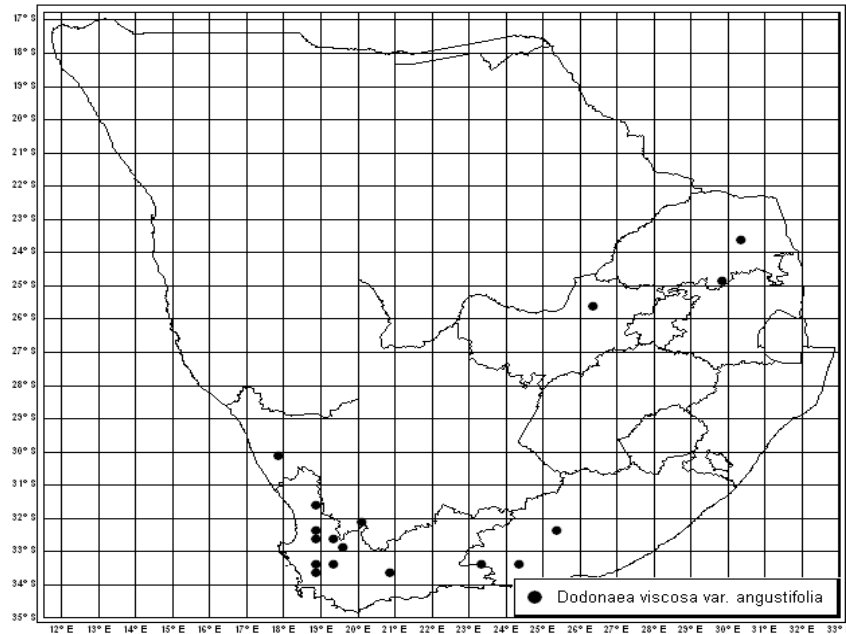
Figure 2.4 Geographical distribution of *Dodonaea viscosa* in South Africa (SANBI).

2.7.2.2 Medicinal uses

In traditional medicine, it is used to treat skin infections, pain and swelling that is caused by rheumatism, gout, waist pain, stomach-ache, colds, influenza, fever, inflammation, measles, and chest complaints (McGaw *et al.*, 2008a; Teffo *et al.*, 2010; Shanmugavasan and Ramachandran, 2011). The plant is also used as a gargle for throat infections and oral thrush. It has been reported to have different pharmacological activities including anti-inflammatory, gastro-protective, antipyretic, analgesic, anti-HIV and other antimicrobial properties (De Oliveira, 2012; Wabo *et al.*, 2012). Historically, it was used by Khoi-Khoi as a treatment for colds and influenza and by the early Cape settlers for the treatment of fever. In Namaqualand, leaves are soaked and boiled in water before the solution is strained and the extract is used to treat colds and influenza. It is also known to induce sweating. The leaves are applied externally to relieve skin rashes. Other historical uses of *D. viscosa*

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include being used as a cure for pneumonia and tuberculosis. In southern Africa, it is considered one of the most important traditional medicines and it can be used in combination with other medicinal plants (Harris, 2012).

2.7.3 *Eucalyptus globulus*

2.7.3.1 Botanical description and geographical distribution

Eucalyptus globulus Labill. is a tall and evergreen tree from the family Myrtaceae (Tyagi and Malik, 2011) which consists of 140 genera and approximately 3800 species (Bachir and Benali, 2012). Eucalyptus trees have a smooth ash-grey bark and these plants can grow up to 30 m high. They have bluish-white cordate-ovate leaves that grow on juvenile shoots and adult leaves which are alternate and lanceolate (Figure 2.5).

Figure 2.5 Leaves of immature *Eucalyptus globulus* tree (S. Zonyane).

E. *globulus* is native to Australia, however it is now naturalized in many other countries (Figure 2.6) and it is one of the most important and commonly cultivated genera in the world (Mulyaningsih *et al.*, 2010).
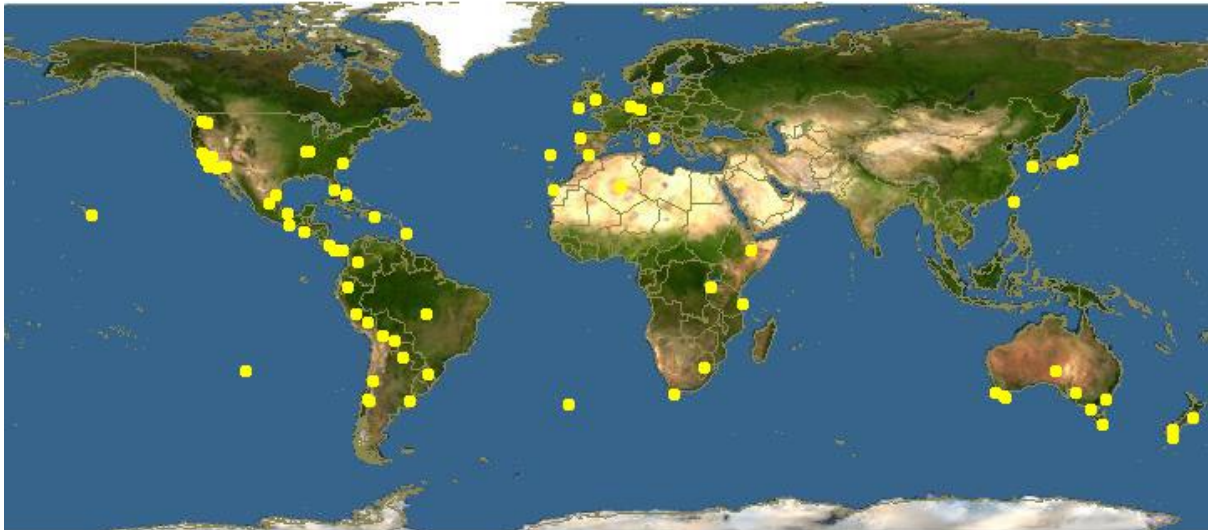
Figure 2.6 Geographicaldistributionof *Eucalyptus globulus* in the world (www.discoverlife.org).

2.7.3.2 Medicinal uses

Many *Eucalyptus* species possess biological and pharmacological properties and are thus used in folk-medicine throughout the world to treat a wide range of diseases including flu, fever, cold, bronchial infections, influenza, pulmonary tuberculosis, fungal infections and diabetes (Silva *et al.*, 2003; Boulekbache-Makhlouf *et al.*, 2012). In China, for example, leaves are dried and extracted with boiled water and the preparation is used as an anti-inflammatory, analgesic and antipyretic remedy to relieve symptoms related to respiratory infections (Tyagi and Malik, 2011). These species have a wide range of biological activities including antibacterial, antifungal, analgesic and anti-inflammatory properties (Navarro *et al.*, 1996; Srinivisan *et al.*, 2001; Cimanga *et al.*, 2002; Silva *et al.*, 2003). *E. globulus* is the most popular species of its genera and its essential oils are widely used both by local communities and industries for medicinal purposes. These essential oils are in high demand in the market because of their wide range of application that includes use as; "anaesthetic, anodyne, antiseptic, astringent, deodorant, diaphoretic, disinfectant, expectorant, febrifuge, fumigant, hemostat, inhalant, insect repellent, preventative, rubefacient, sedative yet stimulant, vermifuge, for a folk remedy for abscess, arthritis, asthma, boils, bronchitis, burns, cancer, diabetes, diarrhoea, diphtheria, dysentery, encephalitis, enteritis, erysipelas,

fever, flu, inflammation, laryngalgia, laryngitis, leprosy, malaria, mastitis, miasma, pharyngitis, phthisis, rhinitis, sores, sore throat, spasms, trachalgia, worms, and wounds" (Bachir and Benali, 2012). The essential oils are also used in modern food and cosmetic industries (Silva *et al*., 2003).

This study aimed to better define the synergistic actions of these three study plants when combined into an herbal remedy.

Chapter 3

Materials and methods

3.1 Collection of plant material

A. crenulata, D. viscosa and *E. globulus* were collected from the wild in the Stellenbosch mountainous regions (33°93'614"S 18°86'019"E), between May and August (2011) with the assistance of Mr Paul Hatting (the traditional healer from whom the mixture of these plants was obtained in the preliminary study (Zonyane *et al.*, 2013). Botanical identification was conducted at the Department of Botany and Zoology, Stellenbosch University. Voucher specimens (*Agathosma crenulata* - M7PN 42, *Dodonaea viscosa* - M7PN 44, *Eucalyptus globulus* - M7PN 40) are housed at the Stellenbosch University herbarium.

3.2 Preparation of non-volatile and volatile extracts

3.2.1 Organic and aqueous extracts

The aerial parts of each plant were oven-dried at 50 °C for 12 h and pulverized into a fine powder using a pestle and mortar. Twenty grams of each pulverized plant was extracted with chloroform: methanol (1:1; v/v) (Merck) and sonicated for 40 min (Rabe and Van Staden, 1997; Ncube *et al.*, 2012). The extraction process was repeated twice and the extracts were filtered through Whatman® No. 1 filter paper (pore size: 20- 25 µm). Each filtrate was concentrated to dryness by rotary evaporation (Buchi 41) at 40 °C. Extracts were stored in the dark at 4 °C while not in use. The combination of chloroform: methanol was used to extract compounds over a range of polarities.

The aqueous extracts were prepared by boiling 20 g of each powdered plant in distilled water and this was left to soak overnight. The extracts were then filtered using Whatman® No. 1 filter paper, placed in a Hirsch funnel for vacuum filtration and frozen at -80 °C. Thereafter, these were lyophilised using a CHRIST® LOC- 1M freeze-drier.

3.2.2 Hydro-distillation for essential oils

The essential oils from two of the aromatic plant species (*A. crenulata* and *E. globulus*) in the study were hydro-distilled using a Clevenger-type apparatus (Figure 3.1) for 6 h at 70 °C. For every 1 kg of fresh leaf material used, 1.5 l distilled water was added in a round bottomed

flask. After 6 h, the essential oils were collected in vials and the yield was recorded. They were kept at 4 °C in the dark until further analysis.
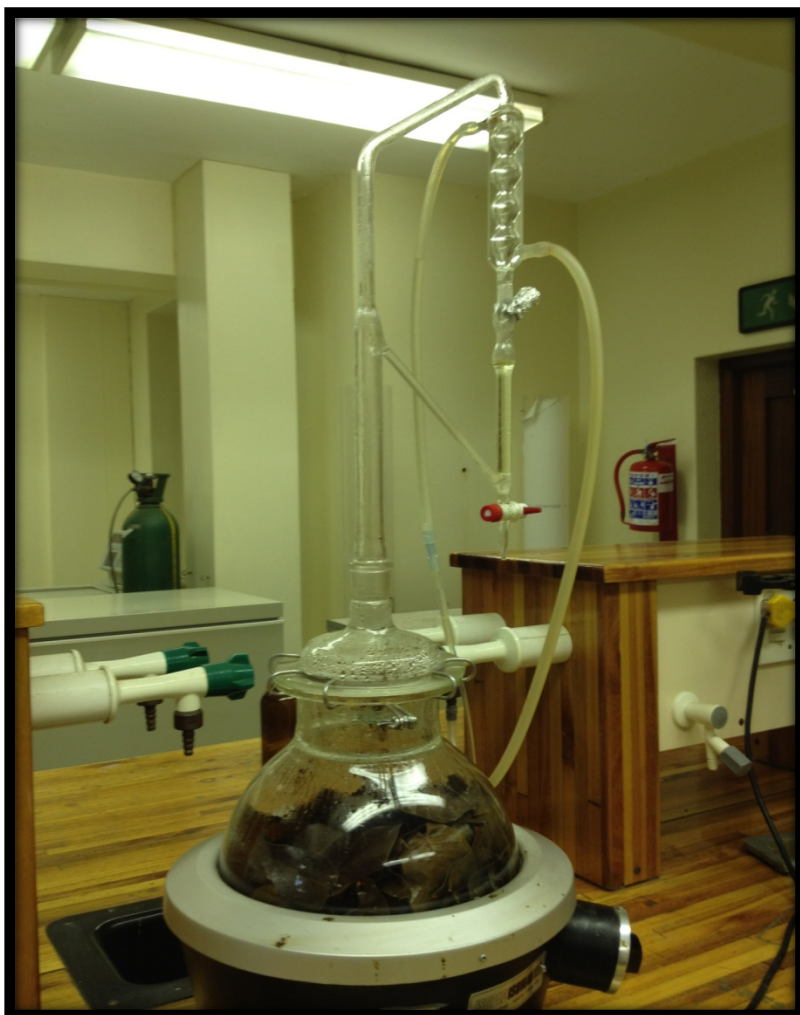
Figure 3.1 Clevenger-type apparatus extracting essential oil from *E. globulus* leaves (S. Zonyane).

3.2.3 Resuspension of extracts, essential oils and controls for bio-assay analysis

The dried plant extracts and concentrated essential oils were re-dissolved to a final concentration of 64 mg ml^{-1} prior to microdilution analysis using methanol for organic extracts and distilled water for aqueous extracts.

The positive controls used, ciprofloxacin (Sigma-Aldrich) and amphotericin B (Sigma-Aldrich), were dissolved to make stock solutions of 0.1 mg ml^{-1} and 1.0 mg ml^{-1} respectively,

using sterile distilled water for ciprofloxacin and dimethyl sulphoxide for amphotericin B. The antibiotics were further diluted with distilled water to final concentrations of 0.01 mg ml^{-1} and 0.1 mg ml^{-1} respectively.

3.3 Antimicrobial activity

3.3.1 Test micro-organisms and growth conditions

For the antimicrobial analysis, attention was not only given to pathogens associated with respiratory infections, but also given to a general screening, as the study is composed of plants which are reportedly used to treat other microbial non-respiratory diseases. Pathogens used were the Gram-negative bacterial strains; *Escherichia coli* ATCC 11775, *Klebsiella pneumoniae* ATCC 13883 (respiratory pathogen), *Moraxella catarrhalis* ATCC 23246 (respiratory pathogen). Gram-positive strains included; *Bacillus subtilis* ATCC 6051, *Mycobacterium smegmatis* ATCC 14468 (non-pathogenic strain used to represent other pathogenic species of the *Mycobacterium* genus), *Staphylococcus aureus* ATCC 12600 (respiratory pathogen) and a yeast, *Cryptococcus neoformans* ATCC 90112 (respiratory pathogen).

The bacterial cultures, *B. subtilis* ATCC 6051, *E. coli* ATCC 11775, *K. pneumoniae* ATCC 13883, *M. catarrhalis* ATCC 23246 and *S. aureus* ATCC 12600 were grown in sterile Mueller Hinton (MH). *M. smegmatis* was grown in Middlebrook 7H9 broth (Difco) with Middlebrook OADC growth supplement (Fluka analytical; Germany) and *C. neoformans* was grown in tryptone soya broth (Oxoid). These microbial suspensions were grown overnight at 37 °C on an orbital shaker at 200 rpm with the incubation of *C. neoformans* extending to 48 h. The bacterial and fungal suspensions were adjusted to 1×10^{6} colony forming units (CFU)/ml using the respective medium depending on the type of pathogen. Optimal incubation conditions were followed for each pathogen.

3.3.2 Microdilution assay

The *in vitro* antimicrobial activity of the extracts and essential oils were evaluated by the microdilution technique as described by Eloff (1998). Under aseptic conditions, 100 µl of sterile distilled water was added into all the wells of a 96-well, flat bottomed, microtitre plate. One hundred microlitres of the test agent (extract or essential oil) were then added in wells A and two-fold serially diluted in vertical orientation until the last wells. From the last wells, 100 µl was discarded from each well. The concentration range from well A to well H was from 16 to 0.125 mg ml^{-1}. Further serial dilutions were made when the MIC of the concerned extract was not found within the concentration range of 16 to 0.125 mg ml^{-1} and in such cases the concentration ranged from 0.063 to 0.0005 mg ml^{-1}. Positive, negative and culture controls were included. The antibiotics, ciprofloxacin at 0.01 mg ml^{-1} and amphotericin B at 0.10 mg ml^{-1} were utilized as positive control for the bacteria and fungi, respectively. The negative control, methanol was used to test if as a solvent it does not contribute to the antimicrobial activity itself. Bacteria-free or fungi-free microbial growth media, specific to the type of micro-organism tested, acted as a culture control to test if the microbial cultures were able to grow in the media. One hundred microlitres of the microbial suspension, with the inoculum size of about 1×10^{6} CFU/ml, was then added into all the wells of the plate. The plates were covered with sterile adhesive sealing films and incubated at 37 °C for 18 h and 48 h for bacteria and yeast respectively. The purity of the cultures used in the assay was confirmed by incubating the bacterial suspensions streaked onto agar plates. The growth of the microbes was detected by the addition of 50 µl of *p*-iodonitrotetrazolium chloride (INT) (Sigma-Aldrich; Germany) at 0.2 mg ml^{-1}, placed into all wells after incubation. This is used to visualize the microbial growth as this reagent acts as an electron acceptor and is reduced into a pink colour by biologically active micro-organisms. The results were recorded in terms of the minimum inhibitory concentration (MIC), which is regarded as the lowest concentration of the sample which does not show bacterial growth. All the assays were done in triplicate.

All the MIC values were interpreted according to Aligiannis *et al*. (2001) and Van Vuuren (2008) where plant extracts exhibiting activity lower than 1 mg ml^{-1} are considered as having

noteworthy activity. The antimicrobial activity of essential oils that is equal to or below 2 mg ml^{-1} is considered as noteworthy (Van Vuuren, 2008).

3.4 Combination study

The fractional inhibitory concentration (FIC) and the isobolograms were employed to establish the type of pharmacological interactions that occur in various combinations and ratios tested. The Design of Experiments (MODDE 9.1®) software predictions method was used for further analysis of triple combinations where necessary.

3.4.1 Fractional inhibitory concentration (FIC) determination

The fractional inhibitory concentration (FIC) is widely used in assessing antimicrobial interactions of combinations of antimicrobial agents (Kamatou *et al*., 2006; Sibandze *et al*., 2010; Suliman *et al*., 2010; Ncube *et al*., 2012). It is the interaction of two agents where the concentration of each agent in a mixture is expressed as a fraction of the concentration that would produce the same effect when used independently (Van Vuuren and Viljoen, 2008). The correlation between the two plant extracts or essential oils is classified as either synergistic, additive, indifferent or antagonistic based on the calculations obtained from comparing the MIC values of the plant extract or essential oil alone and in combination.

Once the independent MIC values were determined for each of the extracts or essential oils of the study plant, a two-plant combination study and a three-plant combination study were designed. For the two-plant combination study, the plants were combined in three possible ways: *A*. *crenulata* with *D*. *viscosa* (1:1), *A*. *crenulata* with *E*. *globulus* (1:1), *D*. *viscosa* with *E*. *globulus* (1:1). The antimicrobial activity of each two-plant combination was tested and the sum of the fractional inhibitory concentration (ΣFIC) was calculated for each of these double combinations using Equations 3.1 to 3.3.

$$FIC_I = \frac{MIC \, (A) \text{ in combination with } (B)/2}{MIC \, (A) \text{ independently}}$$

Equation 3.1

$$FIC_{II} = \frac{MIC \text{ (A) in combination with (B)/2}}{MIC \text{ (B) independently}}$$

Equation 3.2

$$\Sigma FIC = FIC_{I} + FIC_{II}$$

Equation 3.3

For the three-plant combination study, the equal portions (33.3 µl) of each plant: *A. crenulata*, *D. viscosa* and *E. globulus* (1:1:1) were combined and the antimicrobial activity thereof was determined. For triple combinations, the FIC was calculated as follows:

$$FIC_{I} = \frac{MIC \text{ (A) in combination with (B) and (C)/3}}{MIC \text{ (A) independently}}$$

Equation 3.4

$$FIC_{II} = \frac{MIC \text{ (A) in combination with (B) and (C)/3}}{MIC \text{ (B) independently}}$$

Equation 3.5

$$FIC_{III} = \frac{MIC \text{ (A) in combination with (B) and (C)/3}}{MIC \text{ (C) independently}}$$

Equation 3.6

$$\Sigma FIC = FIC_{I} + FIC_{II} + FIC_{III}$$

Equation 3.7

The letters A represents *A. crenulata*, B represents *D. viscosa* and C represents *E. globulus*. The summation of FIC values were interpreted as follows: a sum of ≤0.5 is a synergistic

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interaction, >0.5- 1 is an additive interaction, >1- ≤4 is an indifferent interaction, and >4 is an antagonistic interaction (Van Vuuren and Viljoen, 2011).

3.4.2 Isobologram construction

The isobologram provides a visual assessment of the pharmacological interactions in combination where two plants are in various ratios (Gennings *et al.*, 1990). It is a more insightful approach in determining correlation between different agents in a mixture as it graphically displays interactions (Tallarida, 2006). The two plants in various ratios are combined and the FIC of each ratio is plotted in a graph.

Table 3.1 Concentration of each of the two plants combined in ratios from 9:1 to 1:9.

Ratios		Concentrations (mg ml^{-1})	
Plant A	**Plant B**	**Plant A**	**Plant B**
1.0	0.0	32.0	0.0
0.9	0.1	28.8	3.2
0.8	0.2	25.6	6.4
0.7	0.3	22.4	9.6
0.6	0.4	19.2	12.8
0.5	0.5	16.0	16.0
0.4	0.6	12.2	19.2
0.3	0.7	9.6	22.4
0.2	0.8	6.4	25.6
0.1	0.9	3.2	28.8
0.0	1.0	0.0	32.0

The two-plant combinations in Section 3.4.1 were combined in nine ratios: 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 (v/v) that have varying concentrations of each plant (Table 3.1). These ratios and individual plants were screened for their antimicrobial activity against the pathogens *K. pneumoniae* ATCC 13883, *S. aureus* ATCC 12600 and *E. coli* ATCC 11775, using the microdilution assay as described in Section 3.3.2. The ratio values, which are the MIC of

the combined test substances in relation to the MIC of the test substance alone, were calculated using Equations 3.8 and 3.9.

$$X = \frac{\textit{MIC of an extract/essential oil in combination}}{\textit{MIC of an extract/essential oil independently}}$$

Equation 3.8

$$Y = \frac{\textit{MIC of an extract/essential oil in combination}}{\textit{MIC of an extract/essential oil independently}}$$

Equation 3.9

Then, the ratio values were plotted on an isobologram (Figure 3.2) to provide a graphical representation of interactions taking place between various two-plant extract ratios. The isobolograms were constructed with GraphPad Prism® version 5 software. The data points for each ratio were interpreted as determined in Figure 3.2.
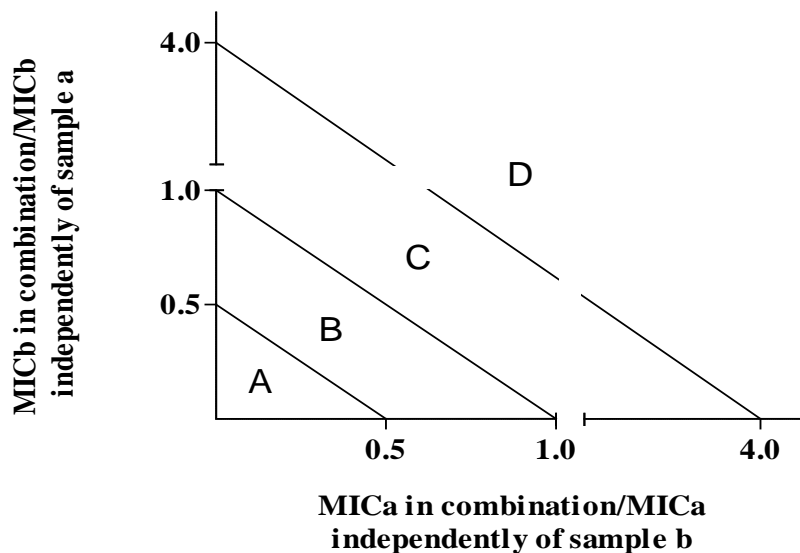
Figure 3.2 An illustration of an isobologram where ratio points falling in area A represent synergy, in B, C and D they represent additive, indifferent and antagonistic interactions respectively (Van Vuuren and Viljoen, 2011).

3.4.3 The Design of experiments (MODDE 9.1®) predictions

For triple combinations, further analysis against selected micro-organisms was conducted using the Design of Experiments software program (MODDE 9.1®). The selected micro-organisms were the micro-organisms against which the extracts were not highly active (MIC > 0.5 mg ml^{-1}) except the triple combination. When the MIC data was obtained for micro-organisms concerned, a worksheet (Table 3.2) was created using MODDE 9.1® software.

Table 3.2 Worksheet generated by MODDE 9.1® for the analysis of triple combinations against selected micro-organisms.

Run	Items of combination (µl)			MIC (mg ml^{-1})
	Extract A	Extract B	Extract C	
1	100	0	0	
2	0	100	0	
3	50	50	0	
4	0	0	100	
5	50	0	50	
6	0	50	50	
7	33	33	33	
8	33	33	33	
9	33	33	33	
10	33	33	33	
11	100	0	0	
12	0	100	0	
13	50	50	0	
14	0	0	100	
15	50	0	50	
16	0	50	50	
17	33	33	33	
18	33	33	33	
19	33	33	33	
20	33	33	33	

MICs were inserted after microdilution assays

This software program is a novel approach system that determines the best ratio at which the three plants should be combined that will give the lowest MIC value. This is done by

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comparing the MIC data of double combinations and triple combination against the MIC of each individual plant. In addition to the ratio predicted by the software, some extra ratios were designed for comparative purposes whereby the volumes of each plant in combination were altered slightly in relation to the original volume combinations that were predicted by the software.

3.5 Chemical analysis of plants used in the triple combination

The organic extracts and essential oils were investigated for their chemical profiles individually and in various combinations to test for possible chemical modifications that might occur when samples from different plant species are combined.

3.5.1 Thin layer chromatography and bio-autographic assay

A chemical finger-printing of the organic extracts was carried out individually and in combination using a thin layer chromatography (TLC) technique. TLC analysis was performed only for the organic extracts as they had demonstrated mostly noteworthy antimicrobial activity compared to aqueous extracts and essential oils. Twenty microlitres of plant extracts and extract combinations were applied as thin bands at the bottom of an aluminium Merck TLC plate coated with silica gel (F_{254}). The TLC plate was then developed in an eluent that is made up of toluene: ethyl acetate (93:7 v/v). At the end of each run, the chromatograms were visualized with UV light at wavelengths of 254 nm and 366 nm. *p*-Anisaldehyde (0.5% v/v) (Wagner and Bladt, 1996) was used to further visualise the bands whereby the plates were developed at 100 °C for 5 min after application of the staining agent.

Bio-autographic assays were conducted according to the method by Reid *et al*. (2005) and Stafford *et al*. (2005). *S. aureus* ATCC 12600 was used as the test organism. An extract loaded plate was developed in toluene: ethyl acetate (93:7 v/v) eluent. The TLC plate was then sterilized by placing it under the UV light (24 nm) for 1 h before spraying it with *S. aureus* bacterial suspension. The plates were then incubated at 37 °C for 18 h in a sterilized

moisturized container. After incubation, INT at 2 mg ml^{-1} was sprayed evenly on the plates to expose bacterial growth. Areas of bacterial growth are purple in colour as the actively growing bacteria are able to metabolise the tetrazolium salt, whereas those that are white are indicating bacterial growth inhibition (Begue and Kline, 1972).

3.5.2 Phytochemical analysis of extracts by liquid chromatography mass spectrometry

Liquid chromatography mass spectrometry (LC-MS) was used to analyse the metabolite profiles of organic extracts. Chromatographic separations and determination were performed on a Waters Synapt G2 quadrupole time-of-flight mass spectrometry (Milford, MA, USA) equipped with a Waters Acquity ultra-perfomance liquid chromatography (UPLC LG 50 nm) with an Acquity photo diode array (PDA) detector and an autosampler. Two solvents; 0.1% formic acid (A) and acetonitrile (B) were used for a mobile phase according to the gradient demonstrated in Table 3.3 at a flow rate of 350 µl min^{-1} and each run took 17 min. For mass separation of analytes, positive and negative modes were utilized at a cone voltage of 15 V and a capillary voltage of 2.5 kV. The data was acquired with MassLynx 4.1 software. LC-MS analysis was conducted in three replicates whereby the leaves obtained from various regions of the plant were mixed randomly and divided into three parts which were extracted separately following the same method.

Table 3.3 A gradient chart of the mobile phase that is made up of 0.1% formic acid (A) and acetonitrile (B) for LC run.

Time (min)	% A	% B
0.00	90	10
0.20	90	10
7.00	83	17
15.00	20	80
15.10	90	10
17.00	90	10

3.5.3 Phytochemical analysis of the essential oils by gas chromatography mass spectrometry

The chemical analysis of the essential oils was carried on an Agilent Technologies 6890N gas chromatography (GC) instrument coupled directly to the Agilent Technologies 5975B mass spectrometry (MS) (Agilent Technologies Inc., Palo Alto, CA). The separation of the volatile components was performed in a capillary column (60.0 m x 250.0 μm i.d, film thickness 0.50 μm, J&W 122-3263 FFAP model). The column was maintained at 70 °C after injection for 1 min. It was then ramped up to 142 °C at a rate of 3 °C/min. The temperature finally rose up to 225 °C at a rate of 5 °C/min and it was then maintained for 3 min. Helium was used as a carrier gas at a flow of 1.9 ml/min. The mass spectrometer was operated under the electron impact mode at ionization energy of 70 eV, scanning from 25 to 650 m/z. The total run time was 44.60 min. The MSD Chemstation software was used to acquire the identity of the essential oil components. The data resulting from the MSD Chemstation was converted and the peaks were identified and quantified with MassLynx 4.1 by matching their mass spectra recorded to the NIST05® chemical library and their GC retention indices. The assessment of essential oil composition from the two aromatic plants (*A. crenulata* and *E. globulus*) was done in triplicate.

3.5.4 Multivariate data analysis

To assess the inter-relationship between the chemical make-up of combined solvent extracts and essential oils, and, that of single plants extracts and essential oils, multivariate data analysis, using principal component analysis and hierarchical clustering analysis, was employed. Retention indices and abundance percentage of all the components were noted and the data was analysed with MassLyx 4.1 software program and Statistica 11.0 for the construction of PCA and HCA plots, respectively.

Chapter 4

Results and discussion

4.1 Antimicrobial activity

The chloroform: methanol extracts of the individual plants showed varying degrees of activity with *E. globulus* having the highest potency. The MIC range was between 0.001 mg ml^{-1} (against *M. catarrhalis*) to 0.500 mg ml^{-1} (*M. smegmatis* and *C. neoformans*) (Table 4.1). *A. crenulata* was the least active of the three plants (MIC values of 2 to 4 mg ml^{-1}) as no noteworthy activity (<1 mg ml^{-1}) was noted against any of the test micro-organisms. *D. viscosa* was active against *E. coli*, *S. aureus* and *B*. *subtilis* (MIC of 0.500, 0.500 and 0.250 mg ml^{-1} respectively).

The aqueous plant extracts studied independently generally showed non-significant to poor activity. However, *D. viscosa* aqueous extracts demonstrated better antimicrobial activity than *A. crenulata* and *E. globulus*, having the MIC values ranging from 1 to 2 mg ml^{-1}. *B. subtilis* showed the highest sensitivity (MIC was recorded at 1 to 2 mg ml^{-1} for the aqueous extracts) (Table 4.1).

Essential oils from the aromatic plants exhibited moderate activity against most pathogens tested. Noteworthy antimicrobial activity, however, was observed against *C. neoformans* (1 and 0.500 mg ml^{-1} respectively). *M. catarrhalis* was less susceptible with the MIC values of 4 mg ml^{-1} for both essential oils (Table 4.1). Out of the aromatic oils tested, the *E. globulus* oil had better antimicrobial activity than the *A. crenulata* essential oil.

Overall the organic extracts exhibited better antimicrobial activity (0.001 to 4 mg ml^{-1}) followed by the essential oils (MIC values 0.500 to 4 mg ml^{-1}) and lastly the aqueous extracts with the MIC range of 1 to 16 mg ml^{-1}. Many studies have established that aqueous extracts exhibit lower activity (Karaman *et al*., 2003; Natarajan *et al*., 2005; Er *et al*., 2007). Even so, the water extracts were tested because anthropological botanical remedies are generally prepared with water. The better antimicrobial activity displayed by the organic extracts in this study suggests that tinctures are more suitable for preparation of traditional medicinal plants in order to achieve optimal efficacy.

Table 4.1 MIC (mg ml^{-1}) values of chloroform: methanol extracts, aqueous extracts and essential oils of *A. crenulata*, *D. viscosa* and *E. globulus*.

Plant species	*K. pneumoniae* (ATCC 13883)	*M. catarrhalis* (ATCC 23246)	*E. coli* ATCC 11775	*S. aureus* (ATCC 12600)	*M. smegmatis* (ATCC 14468)	*B. subtilis* (ATCC 6051)	*C. neoformans* (ATCC 90112)
A. crenulata							
Chloroform :methanol extract	2.000	4.000	1.000	2.000	2.000	2.000	2.000
Aqueous extract	8.000	8.000	16.000	4.000	8.000	2.000	8.000
Essential oil	4.000	4.000	4.000	4.000	**2.000**	**2.000**	**1.000**
D. viscosa							
Chloroform :methanol extract	1.000	2.000	**0.500**	**0.500**	1.000	**0.250**	1.000
Aqueous extract	2.000	2.000	1.000	1.000	2.000	1.000	1.000
E. globulus							
Chloroform :methanol extract	**0.060**	**0.001**	**0.008**	**0.060**	**0.500**	**0.060**	**0.500**
Aqueous extract	8.000	8.000	4.000	2.000	8.000	1.000	16.000
Essential oil	**1.000**	4.000	**1.000**	**1.000**	**2.000**	**1.000**	**0.500**
Controls							
Ciprofloxacin/Amphotericin B	1.56×10^{-4}	6.25×10^{-4}	2.5×10^{-3}	2.5×10^{-3}	1.25×10^{-3}	6.25×10^{-4}	1.25×10^{-2}
Methanol	8.000	8.000	16.000	16.000	8.000	8.000	8.000

Values in boldtype face indicate noteworthy antimicrobial activity

All the three study plants, which are traditionally used in a combined form, are well established as botanical medicines. For instance, *A. crenulata* (buchu) is one of South Africa's most popular plants which are traded commercially for its essential oil. This plant remains important as a Khoi-San medicinal plant due to its proven antiseptic action wherein it has a broad ethnobotanical use, such as acting as an antispasmodic, antipyretic agent, cough remedy, a diuretic and for curing fever amongst other ailments (Moolla and Viljoen, 2008). The findings of this study thus support some of the ethnobotanical uses for *A. crenulata* as it inhibited the growth of pathogens responsible for respiratory ailments. Some previous studies have also demonstrated the antimicrobial activity of *A. crenulata*. In a study by Moolla *et al*. (2007) the methanol: dichloromethane extracts of *A. crenulata* were screened for activity against *B. cereus*, *S. aureus*, *K. pneumoniae* and *C. albicans*, using a microdilution method. The extracts showed the highest activity against *B. cereus*, *S. aureus* and *C. albicans* with MIC of 2 mg ml^{-1}. The *A. crenulata* organic extract has also shown the MIC of 2 mg ml^{-1} for *S. aureus* in this study. The other study where *A. crenulata* oil was screened for activity against *E. coli*, *Enterococcus hirae*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *S. aureus* was undertaken by Lis-Balchin *et al*. (2001) using the agar diffusion method. The oil showed some activity only against *E. coli*, *S. cerevisiae*, *S. aureus* with 5.1, 6.1 and 5.4 mm zones of inhibition respectively. Due to the problems associated with volatile oils and diffusion studies, a more recent quantitative method (Viljoen *et al*., 2006) may be a more accurate representation of activity. The essential oil of *A. crenulata* was found to be active on all pathogens tested with the highest activity against *B. cereus* at 3 mg ml^{-1}.

D. viscosa displayed good antibacterial activity in this study, confirming results from previous studies on its antibacterial properties (Rojas *et al*., 1992; Thring *et al*., 2007; Getie *et al*., 2003; Mothana *et al*., 2008; Khurram *et al*., 2009; Teffo *et al*., 2010). *D. viscosa* is also known to have a wide variety of pharmacological activities such as anti-inflammatory, antifungal and antiviral activity (Getie *et al*., 2003; Patel and Coogan, 2008). Studies that have investigated the antimicrobial activity of *D. viscosa* include one by Mothana *et al*. (2008) whereby the methanol and aqueous extracts of *D. viscosa* were tested for activity against *S. aureus*, *B. subtilis*, *Micrococcus flavus*, *E. coli*, *P. aeruginosa*, *Candida maltosa*,

Staphylococcus epidermidis and *Staphylococcus haemolyticus*. The highest activity was against *M. flavus* with 21 mm of inhibition zone. In this study, *D. viscosa* organic and water extracts generally showed higher inhibitory activity on Gram-positive bacteria than on Gram-negative bacteria. Thring *et al*. (2007) tested the antimicrobial activity of aqueous, methanol, ethanol and ethyl-acetate leaf extracts of *D. viscosa* against *S. aureus*, *P. aeruginosa*, *C. albicans* and *M. smegmatis* using the disc-diffusion assay and microdilution method. According to the disc diffusion assay, the extracts were only active against *S. aureus*. For microdilution assay, antimicrobial activity was improved and observed against *S. aureus*, *C. albicans* and *M. smegmatis*. Rojas *et al*. (1992) also conducted screening of methanol extracts against selected Gram-positive, Gram-negative bacteria and *C. albicans*. The extracts showed antimicrobial activity with 6, 8, 5, 5 and 5 mm against *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa* and *C. albicans* respectively. The inhibitory effects of *D. viscosa* methanol extracts have also been noted on *S. aureus*, *S*. *pyogenes* and *Corynebacterium diphtheriae* (Getie *et al*., 2003). Khurram *et al*. (2009) noted high antimicrobial activity of ethanol extracts of *D. viscosa* against *S. aureus*, *Micrococcus luteus*, *B. subtilis*, *B. cereus*, *E, coli*, *P. aeruginosa* and *Salmonella typhi*. The highest activity was against *B. subtilis* with the inhibition zone of 13.3 mm.

Eucalyptus species are a rich source of essential oils with antibacterial, antifungal, analgesic and anti-inflammatory medicinal value (Ramezani *et al*. 2002; Silva *et al*. 2003; Sartorelli *et al*. 2007). Microbial inhibition is thought to be facilitated by compounds such as monoterpenes in several *Eucalyptus* species including *E. globulus* (Hasegawa *et al*., 2008). The antibacterial activity of *E. globulus* is documented in many other studies (Navarro *et al*., 1996; Srinivasan *et al*., 2001; Cimanga *et al*., 2002; Silva *et al*., 2003; Bussmann *et al*., 2010). The methanolic organic extracts were evaluated for inhibitory activity against *S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans* where some antimicrobial activity (MIC value of 5 mg ml^{-1} for *S. aureus* and 10 mg ml^{-1} for *E. coli*) was observed (Navarro *et al*., 1996). *E. globulus* ethanol and aqueous extracts were also studied by Bussmann *et al*. 2010 for activity using microdilution method against *E. coli* and *S. aureus*. Only ethanol extracts exhibited antimicrobial activity for *S. aureus* and no activity was observed for water extracts for any of the pathogens. The *E. globulus* aqueous extracts were also investigated (Srinivasan *et al*., 2001), for inhibitory activity against a wide range of micro-organisms that included four of

the current study pathogens (*K. pneumoniae, E. coli, S. aureus and B. subtilis*). The water extracts showed good activity on both Gram-positive bacteria (*S. aureus and B. subtilis*) and Gram-negative bacteria (*K. pneumoniae, E. coli*) with the highest activity on *B. subtilis* at 31 mm of inhibition zone. The *E. globulus* essential oils have also been screened for activity against many micro-organisms. Recently, Bachir and Benali (2012) screened *E. globulus* essential oil for activity against *E. coli* and *S. aureus* using dilution broth method. The essential oil was active against both pathogens depending on its concentration and the size of the inocula. The *E. globulus* essential oil inhibitory activity have also been studied by Tohidpour *et al*. (2010) against *K. pneumoniae*, *S. aureus*, *E. coli* and *B. cereus* through agar dilution method. The inhibitory action of the oil was good showing the highest activity against *S. aureus* with the MIC value of 51.36 µg ml^{-1}. This activity is higher than the one recorded in this study and differences in activity might be influenced by components of essential oils obtained from plants growing in different types of soils.

In this study, it has been frequently observed that Gram-positive bacteria are more susceptible to the test samples compared to Gram-negative bacteria. This is a common trend, which has been noted in other studies which have been discussed above. Gram-negative bacteria have an outer membrane whose major components are lipopolysaccharides with proteins and phospholipids and these prevent the passage of some bioactive components of plant extracts, (Kumar et al., 2006).

4.2 Two-plant combination study

4.2.1 *A. crenulata* with *D. viscosa* in combination

4.2.1.1 MIC and FIC determination

For a combination of *A. crenulata* and *D. viscosa* organic extracts, a noteworthy antimicrobial activity was noted against *K. pneumoniae*, *E. coli*, *S. aureus* and *B. subtilis* (0.500 mg ml^{-1}) (Table 4.2). The antimicrobial interactions were predominantly additive with one synergistic interaction recorded for *K. pneumoniae* (ΣFIC of 0.380).

Table 4.2 MIC (mg ml^{-1}) values of double combinations (1:1) of *A. crenulata* with *D. viscosa* and sum FIC values thereof.

Activity and interaction	*K. pneumoniae* ATCC 13883	*M.catarrhalis* ATCC 23246	*E. coli* ATCC 11775	*S. aureus* ATCC 12600	*M. smegmatis* ATCC 14468	*B. subtilis* ATCC 6051	*C. neoformans* ATCC 90112
MIC							
Chloroform: methanol	**0.500**	2.000	**0.500**	**0.500**	2.000	**0.500**	1.000
Aqueous	2.000	4.000	1.000	2.000	2.000	**0.500**	4.000
Essential oil	**0.500**	4.000	**2.000**	**1.000**	**2.000**	0.500	**0.500**
ΣFIC							
Chloroform: methanol	**0.380**	0.750	0.750	0.630	1.500	1.130	0.750
Aqueous	0.630	1.250	0.530	1.250	0.630	**0.380**	2.250
Essential oil	**0.310**	1.500	2.250	1.130	1.500	1.130	**0.500**
Antimicrobial interaction							
Chloroform: methanol	**Synergistic**	Additive	Additive	Additive	Indifferent	Indifferent	Additive
Aqueous	Additive	Indifferent	Additive	Indifferent	Additive	**Synergistic**	Indifferent
Essential oil	**Synergistic**	Indifferent	Indifferent	Indifferent	Indifferent	Indifferent	**Synergistic**

Values in boldtype face indicate noteworthy antimicrobial activity and significant interactions

The combination of the aqueous extracts of *A. crenulata* and *D. viscosa* showed moderate activity with *B. subtilis* demonstrating the highest sensitivity towards the combination with the MIC value of 0.500 mg ml^{-1} (Table 4.2). Synergism was noted against *B. subtilis* (ΣFIC of 0.380).

The combination of *A. crenulata* essential oil and *D. viscosa* organic extracts showed moderate antimicrobial activity and was mostly active with the MIC of 0.500 mg ml^{-1} against *K. pneumoniae*, *B. subtilis* and *C. neoformans*. This combination exhibited synergistic interactions when investigated against *K. pneumoniae* (ΣFIC of 0.310) and *C. neoformans* (ΣFIC of 0.500) (Table 4.2).

Very importantly, no antagonism was observed when *A. crenulata* and *D. viscosa* were examined in 1:1 combinations.

4.2.1.2 Ratio combination analysis

To determine the interactive effects of double combinations at various ratios, two respiratory pathogens (*K. pneumoniae* and *S. aureus*) and one non-respiratory pathogen (*E. coli*) were selected and the interactions were presented on an isobolograms. Various ratios of a combination of *A. crenulata* with *D. viscosa* displayed synergistic interactions against *K. pneumoniae* with the exception of one ratio which was additive (Figure 4.1 A). The most synergistic ratios were 8:2, 7:3 and 6:4; *A. crenulata*: *D. viscosa*. The general trend is that, more of *A. crenulata* volume in a ratio facilitates synergism for this combination as it was observed that as the volume of *A. crenulata* decreases in ratio, the synergy becomes less stronger.

The ratio combinations of *A. crenulata* with *D. viscosa* showed synergistic and additive interactions against *S. aureus* (Figure 4.1 B). Even though the 1:1 combination interaction of *A. crenulata* with *D. viscosa* did not result into synergism (Table 4.2), combining these plants into nine ratios revealed that the optimum antimicrobial activity is achieved when *A. crenulata* is in majority and there is less of *Dodonaea viscosa*.

Figure 4.1 Isobolograms of the nine ratios of *A. crenulata* (AC) with *D. viscosa* (DV) chloroform: methanol extracts in combination against *K. pneumoniae* (A), *S. aureus* (B) and *E. coli* (C).

When *A. crenulata* was combined with *D. viscosa* and tested against *E. coli*, various interactions were noted ranging from synergistic (three ratios where *A. crenulata* is in majority) to additive (five ratios where *D. viscosa* is in majority) interactions, and even one non-interaction was noted (Figure 4.1 C).

4.2.2 *A. crenulata* with *E. globulus* in combination

4.2.2.1 MIC and FIC determination

The combination of *A. crenulata* with *E. globulus* organic (solvent extracts) was highly active

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Table 4.3 MIC (mg ml^{-1}) values of double combinations (1:1) of *A. crenulata* with *E. globulus* and sum FIC values thereof.

Activity and interaction	*K. pneumoniae* ATCC 13883	*M.catarrhalis* ATCC 23246	*E. coli* ATCC 11775	*S. aureus* ATCC 12600	*M. smegmatis* ATCC 14468	*B. subtilis* ATCC 6051	*C. neoformans* ATCC 90112
MIC							
Chloroform: methanol	**0.008**	**0.002**	**0.008**	**0.008**	1.000	**0.080**	1.000
Aqueous	8.000	8.000	4.000	2.000	2.000	1.000	16.000
Essential oil	**2.000**	4.000	**2.000**	**1.000**	**2.000**	1.000	1.000
ΣFIC							
Chloroform: methanol	**0.070**	1.000	**0.500**	**0.070**	1.250	**0.070**	1.250
Aqueous	1.000	1.000	0.630	0.750	0.630	0.750	1.500
Essential oil	1.250	1.000	1.250	0.630	1.000	0.750	1.500
Antimicrobial interaction							
Chloroform: methanol	**Synergistic**	Additive	**Synergistic**	**Synergistic**	Indifferent	**Synergistic**	Indifferent
Aqueous	Additive	Additive	Additive	Additive	Additive	Indifferent	Indifferent
Essential oil	Indifferent	Additive	Indifferent	Additive	Additive	Indifferent	Indifferent

Values in boldtype face indicate noteworthy antimicrobial activity and significant interactions

against all micro-organisms (0.002 to 0.008 mg ml^{-1}) with the exception of *M. smegmatis* and *C. neofo*rmans against which it showed moderate activity (MIC of 1 mg ml^{-1}) (Table 4.3). The lowest MIC observed was against *M. catarrhalis* (0.002 mg ml^{-1}). This combination also yielded the strongest synergistic interactions (ΣFIC values of 0.070 against *K. pneumoniae*, *S. aureus* and *B. subtilis*) and ΣFIC 0.500 against *E. coli* and was additive when tested against *M. catarrhalis* (ΣFIC of 1) Indifferent interactions were recorded against *M. smegmatis* and *C. neoformans* with ΣFIC values of 1.250.

Overall the antimicrobial activity of the aqueous extracts of, *A. crenulata* and *E. globulus* in combination, was moderate to poor. All pharmacological interactions were additive when subjected to the test micro-organisms with the exception of studies on *C. neoformans* which demonstrated an indifferent interaction.

Essential oils of *A. crenulata* and *E. globulus* in combination were active against all micro-organisms (1 to 2 mg ml^{-1}) with the exception of *M. catarrhalis* against which the essential oil combination was moderate (4 mg ml^{-1}) (Table 4.3). The antimicrobial interactions were either additive or indifferent with no antagonism encountered.

4.2.2.2 Ratio combination analysis

The nine ratios of a combination of *A. crenulata* with *E. globulus* organic extracts displayed highly synergistic interactions for *K. pneumoniae* (Figure 4.2 A). The interactions reflected by the isobologram were congruent with the FIC value obtained for this pathogen as the ΣFIC was 0.070 demonstrating high synergy.

When various mixture variations of *A. crenulata* with *E. globulus* were investigated against *S. aureus,* all interactions were synergistic irrespective of the ratio in which they were combined except one ratio which was additive (8:2; *A. crenulata*: *E. globulus*). The ratio points were closely clustered together (Figure 4.2 B), and from this it can be concluded that the ratios had a similar pattern of activity and pharmacological interaction irrespective of the various volumes of each plant extract in each ratio.
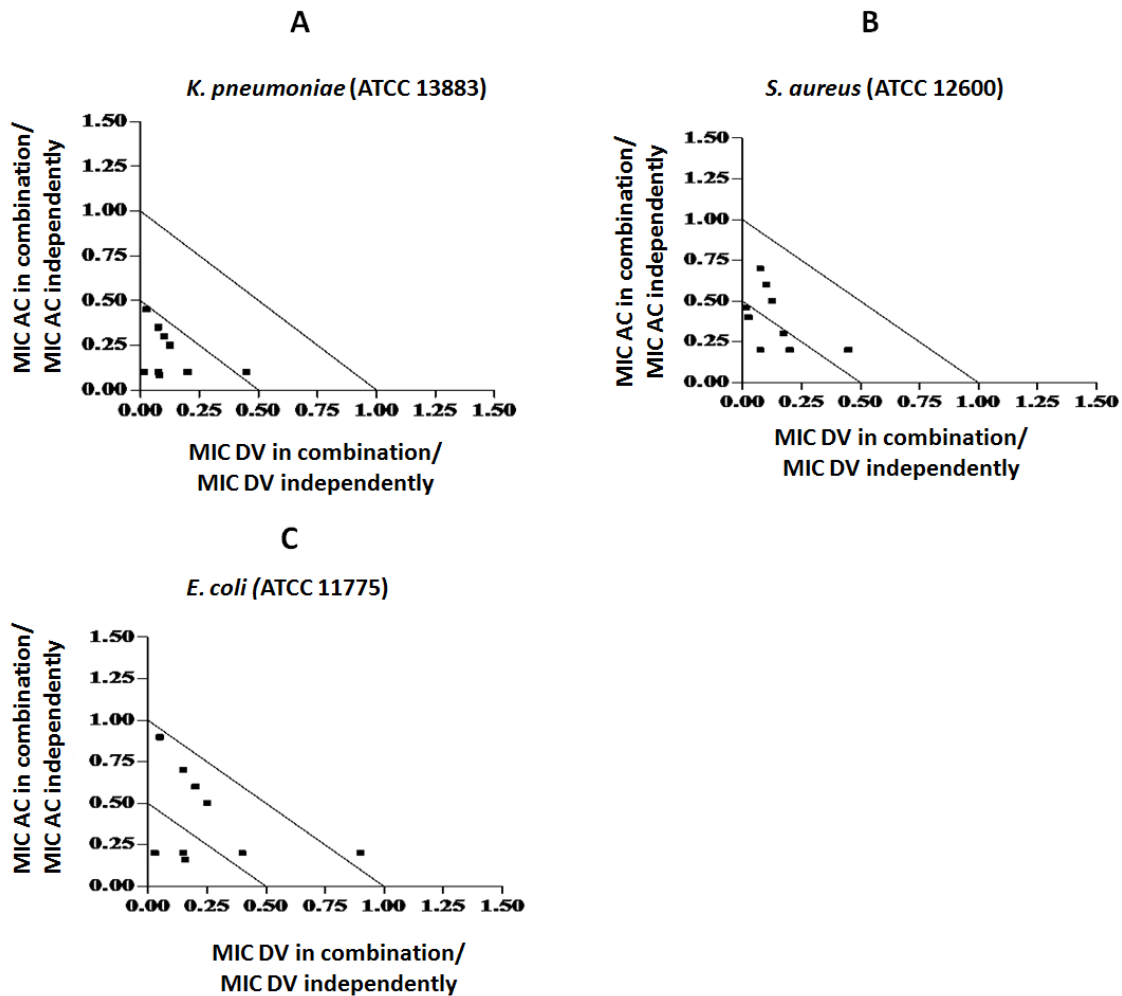
Figure 4.2 Isobolograms of the nine ratios of *A. crenulata* (AC) with *E. globulus* (EG) chloroform: methanol extracts in combination against *K. pneumoniae* (A), *S. aureus* (B) and *E. coli* (C).

All the combinations where *A. crenulata* was combined with *E. globulus* against *E. coli* displayed additive interactions except the ratio, where *A. crenulata* and *E. globulus* were combined in equal quantities, which was synergistic at FIC 0.500 (Figure 4.2 C).

4.2.3 *D. viscosa* with *E. globulus* in combination

4.2.3.1 MIC and FIC determination

The chloroform: methanol extracts of *D. viscosa* and *E. globulus* in combination exhibited very high antimicrobial activity (0.002 to 0.250 mg ml^{-1}) (Table 4.4) against all pathogens,

Table 4.4 MIC (mg ml^{-1}) values of double combinations (1:1) of *D. viscosa* with *E. globulus* and sum FIC values thereof.

Activity and interaction	*K. pneumoniae* ATCC 13883	*M.catarrhalis* ATCC 23246	*E. coli* ATCC 11775	*S. aureus* ATCC 12600	*M. smegmatis* ATCC 14468	*B. subtilis* ATCC 6051	*C. neoformans* ATCC 90112
MIC							
Chloroform: methanol	**0.063**	**0.002**	**0.008**	**0.015**	1.000	**0.008**	**0.250**
Aqueous	2.000	8.000	8.000	1.000	2.000	**0.500**	8.000
Essential oil	**1.000**	**2.000**	**1.000**	**1.000**	**2.000**	0.500	1.000
ΣFIC							
Chloroform: methanol	0.530	1.000	0.510	**0.130**	1.500	**0.080**	**0.370**
Aqueous	0.630	1.000	2.500	0.750	0.630	**0.500**	4.250
Essential oil	1.000	0.750	1.500	1.500	1.500	1.250	1.500
Antimicrobial interaction							
Chloroform: methanol	Additive	Additive	Additive	**Synergistic**	Indifferent	**Synergistic**	**Synergistic**
Aqueous	Additive	Additive	Indifferent	Additive	Additive	**Synergistic**	Antagonistic
Essential oil	Additive	Additive	Indifferent	Indifferent	Indifferent	Indifferent	Indifferent

Values in boldtype face indicate noteworthy antimicrobial activity and significant interactions

except the moderate activity noted against *M. smegmatis* (1 mg ml^{-1}). With the exception of only one indifferent reaction for *M. smegmatis*, antimicrobial interactions were equally additive and synergistic.

The antimicrobial activity of the aqueous extracts of *D. viscosa* and *E. globulus* was generally moderate. The lowest MIC (0.500 mg ml^{-1}) was recorded for *B. subtilis* (Table 4.4). Additive or synergistic pharmacological interactions occurred for *K. pneumoniae* (ΣFIC of 0.630), *S. aureus* (ΣFIC of 0.750), *B. subtilis* (ΣFIC of 0.500) and *M. smegmatis* (ΣFIC of 0.630). Only one antagonistic interaction was observed in *D. viscosa* with *E. globulus* combination against the pathogen *C. neoformans* (Table 4.4).

The combination of *E. globulus* essential oil with the organic extract of *D. viscosa* demonstrated interactions that were either additive or indifferent.

4.2.3.2 Ratio combination analysis

Against *K. pneumoniae*, the ratio combinations of *D. viscosa* with *E. globulus* showed highly synergistic actions with one additive ratio (5:5; *D. viscosa*: *E. globulus*) (Figure 4.3 A). The overall interaction was more enhanced compared to interaction presented by ΣFIC where the two plants were combined in equal proportions (Table 4.4).

A combination of *D. viscosa* with *E. globulus* organic extracts displayed synergistic interactions for all nine ratios against *S. aureus* (Figure 4.3 B) with ratio 9:1; *D. viscosa* with *E. globulus* having the highest synergy.

On the other hand, the graphical interpretation of the interactive effects of *D. viscosa* with *E. globulus* against *E. coli* revealed mild synergism (Figure 4.3 C). This was not very different from the ΣFIC recorded for this micro-organism (Table 4.4).

In most cases, the representation of the antimicrobial interactions were congruent between two techniques (FIC and isobologram) that were used for analysis of interactions.

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A

K. pneumoniae (ATCC 13883)

B

S. aureus (ATCC 12600)

C
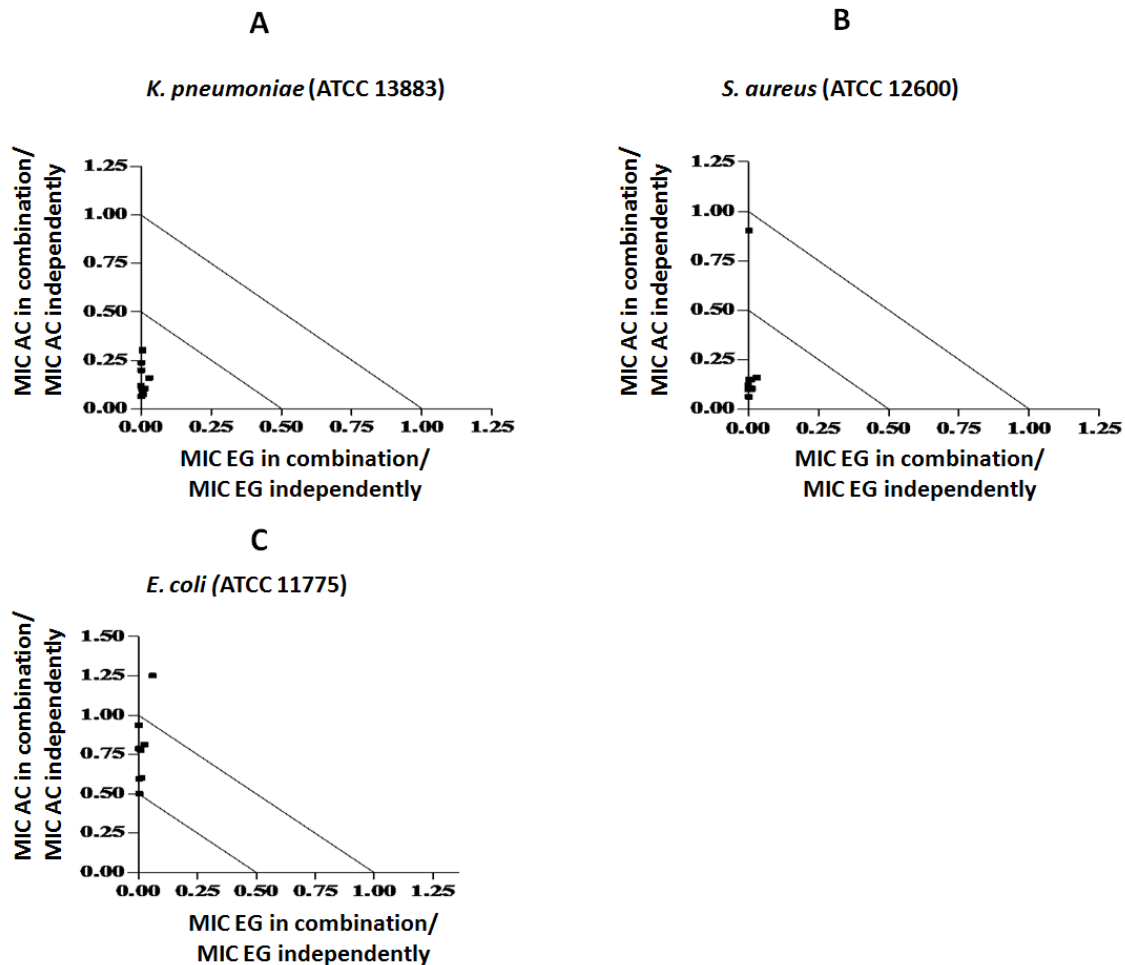
E. coli (ATCC 11775)
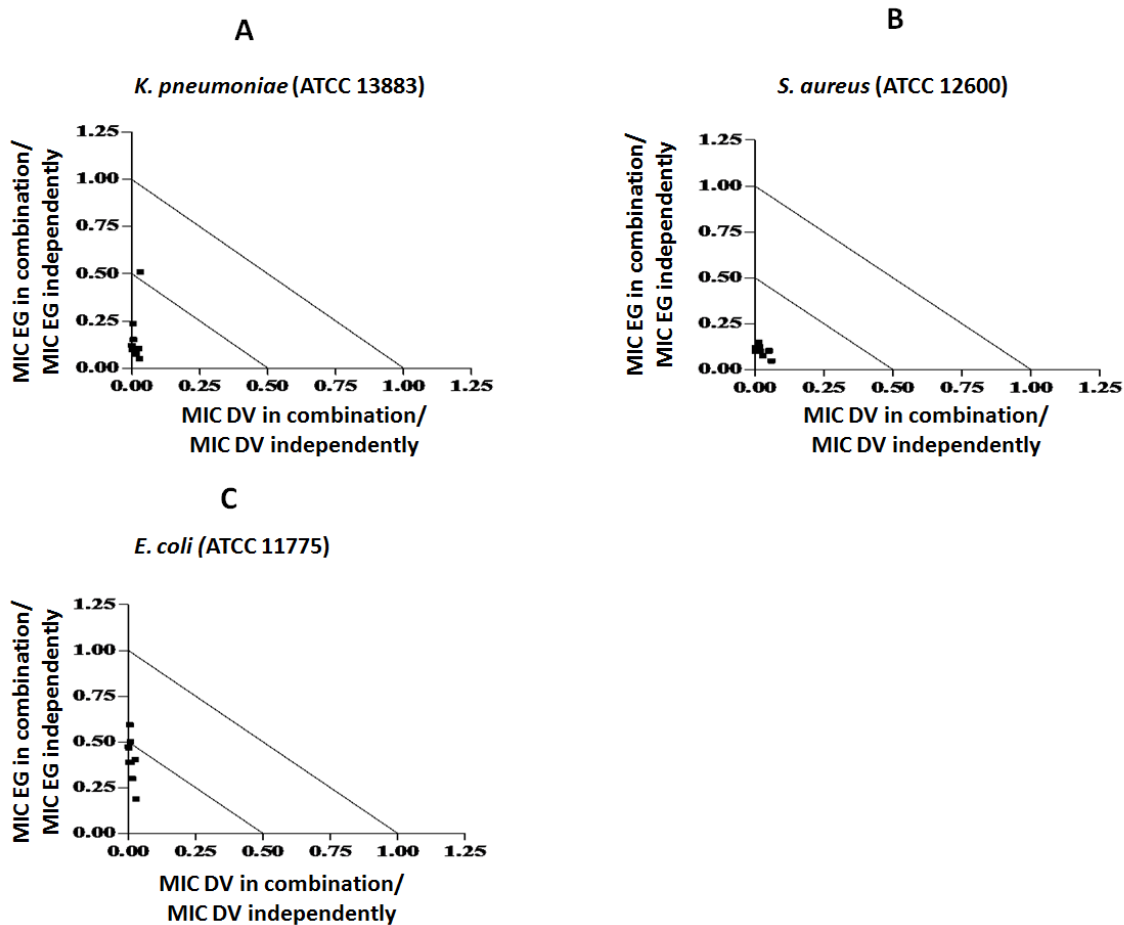
Figure 4.3 Isobolograms of the nine ratios of *D. viscosa* (DV) with *E. globulus* (EG) chloroform: methanol extracts in combination against *K. pneumoniae* (A), *S. aureus* (B) and *E. coli* (C).

This further validated the occurrence of a certain observed pharmacological interaction of a given combination. Isobolograms were even more effective in identifying the best fractions of combinations that may give optimum antimicrobial efficacy.

4.3 Three-plant combination study

4.3.1 MIC and FIC determination

The chloroform: methanol extracts of a triple combination had a noteworthy activity against all micro-organisms with the lowest MIC for *M. catarrhalis* and *B. subtilis* (0.004 mg ml^{-1}) (Table 4.5).

Table 4.5 MIC (mg ml^{-1}) values of triple combinations (1:1:1) of *A. crenulata, D. viscosa* with *E. globulus* and sum FIC values thereof.

Activity and interaction	*K. pneumoniae* ATCC 13883	*M.catarrhalis* ATCC 23246	*E. coli* ATCC 11775	*S. aureus* ATCC 12600	*M. smegmatis* ATCC 14468	*B. subtilis* ATCC 6051	*C. neoformans* ATCC 90112
MIC							
Chloroform: methanol	**0.015**	**0.004**	**0.008**	**0.008**	**0.500**	**0.004**	**0.500**
Aqueous	2.000	4.000	8.000	1.000	2.000	**0.500**	**8.000**
Essential oil	**0.500**	**2.000**	**1.000**	**1.000**	**0.500**	**0.500**	**0.500**
ΣFIC							
Chloroform: methanol	**0.090**	1.330	0.340	**0.050**	0.580	**0.030**	0.580
Aqueous	**0.500**	1.250	2.000	1.130	1.500	**0.420**	3.170
Essential oil	**0.380**	0.700	1.080	1.080	**0.330**	0.920	0.670
Antimicrobial interaction							
Chloroform: methanol	**Synergistic**	Indifferent	**Synergistic**	**Synergistic**	Additive	**Synergistic**	Additive
Aqueous	**Synergistic**	Indifferent	Indifferent	Indifferent	Indifferent	**Synergistic**	Indifferent
Essential oil	**Synergistic**	Additive	Indifferent	Indifferent	**Synergistic**	Additive	Additive

Values in boldtype face indicate noteworthy antimicrobial activity and significant interactions

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The highest MIC recorded, which still demonstrated noteworthy activity was for *M. smegmatis* and *C. neoformans* (0.500 mg ml^{-1}) (Table 4.5). The antimicrobial activity of the triple combination was enhanced for *M. smegmatis* and *B. subtilis* (0.500 and 0.004 mg ml^{-1} respectively) compared to double combinations. The pharmacological interactions were mostly synergistic (*K. pneumoniae*, *E. coli*, *S. aureus* and *B. subtilis*). Additive interactions were noted for *M. smegmatis* and *C. neoformans* and only one indifference was recorded (*M. catarrhalis*) (Table 4.5).

The enhanced antimicrobial activity when the plants were combined may be explained by the mechanism whereby various plant bioactive constituents affect several target sites and work cooperatively in a synergistic manner (Al-Bayati, 2008). Natural drug combinations to treat complex diseases (Gathirwa *et al*., 2008) are well accepted even by traditional healers as this approach is speculated to suppress bacterial resistance which usually develops when single drugs are used (Al-Bayati, 2008).

Some Southern African studies on traditional medicine have highlighted the possibility of synergistic interactions that occur in plant combinations. For example, Sibandze *et al*. (2010) proved the synergistic action of *Breonadia salicina* (Vahl) Hepper & J.R.I.Wood, *Syzygium cordatum* Hochst ex Sond and *Ozoroa sphaerocarpa* R.Fern. & A.Fern. as a multiple decoction that is beneficial for treating diarrhoea. Even different parts of the same plant enhance antimicrobial activity when used in combination as indicated by Van Vuuren and Viljoen (2008) for leaves, roots and bark of *Croton gratissimus.*

4.3.2 Antimicrobial interactions interpretation by MODDE 9.1® predictions

Further analysis of the triple combination for the organic extracts was conducted against *C. neoformans* and *M. smegmatis* using the Design of Experiments (MODDE 9.1® software program). *C. neoformans* and *M. smegmatis* were selected as they were some of the micro-organisms against which most extracts were not highly active (MIC > 0.500 mg ml^{-1}) but only the triple combination (1:1:1); *A. crenulata*, *D. viscosa* and *E. globulus* (Table 4.1). Thus, this

experiment was done to explore the best possible antimicrobial activity of various ratio combinations of the study triple mixture.

4.3.2.1 *Cryptococcus neoformans*

The program predicted that the combination that will yield the best MIC for activity against *C. neoformans* will be *A. crenulata*, *D. viscosa* and *E. globulus* at the volumes of 8.5:43.4:48.1 µl respectively (Table 4.6). The predicted MIC value was 0.287 mg ml^{-1}. Other combinations, that closely resemble the one from the program, were also designed for comparative purposes. These included *A. crenulata*, *D. viscosa* and *E. globulus* (8.5:45.4:46.1), (6.5:45.4:48.1), (6.5:43.4:50.1), (9.5:35.4:55.1), (5:35:60), (10:30:60), (10:40:50), (15:40:45) and (10:20:70) µl (Table 4.6). All these combinations were then screened for activity against *C. neoformans* using microdilution assay.

The MIC values were recorded and surprisingly, irrespective of the fact that combinations were made up of different volumes of each plant, all the combinations had the same MIC value of 0.500 mg ml^{-1} except a combination of (5:35:60) µl which had the MIC of 0.250 mg ml^{-1} (Table 4.6) that closely resembled the predicted MIC of 0.287 mg ml^{-1}. This MIC of 0.500 mg ml^{-1} was similar to the triple combination which had equal portions (1:1:1) of each plant. Even though, the predicted MIC (0.287 mg ml^{-1}) for the ratio that was designed by the software did not match the MIC (0.500 mg ml^{-1}) that was obtained in the experiment, their activities were relatively similar considering non-significant difference of one dilution factor.

Table 4.6 MIC value(s) (mg ml^{-1}) of a combination predicted by MODDE 9.1® program and combinations prepared in relation to the program-predicted combination against *C. neoformans*.

Combinations ratios (µl)	MIC
A. crenulata + D. viscosa + E. globulus (8.5:43.4:48.1) (original from MODDE software)	0.500
A. crenulata + D. viscosa + E. globulus (8.5:45.4:46.1)	0.500
A. crenulata + D. viscosa + E. globulus (6.5:45.4:48.1)	0.500

Combinations ratios (µl)	MIC
A. crenulata + *D. viscosa* + *E. globulus* (9.5:35.4:55.1)	0.500
A. crenulata + *D. viscosa* + *E. globulus* (5:35:60)	0.250

The combination in which the MIC was 0.250 mg ml^{-1} had the lowest volume of *A. crenulata* at 5 µl (Table 4.6). In addition to that, in all cases (predictions from MODDE 9.1®) *A. crenulata* was always at the lowest volume and this is not surprising considering that *A. crenulata* is the least active of all three plants.

4.3.2.2 *Mycobacterium smegmatis*

The best combination predicted by MODDE software was the combination of volumes *A. crenulata*, *D. viscosa and E. globulus* at 5.7:29:65.3 µl respectively (Table 4.7) with the MIC of 0.541 mg ml^{-1}. The combinations that were closely related to this, were designed for comparative purposes (3.7:31:65.3), (4.7:27:68.3), (8.7:27:64.3) and (4.7:29:66.3) µl (Table 4.7). All these combinations were then screened for activity using microdilution assay. The MIC was the same, 0.5 mg ml^{-1}, for all the designed combinations and program predicted combination (Table 4.7).

Table 4.7 MIC value(s) (mg ml^{-1}) of a combination predicted by MODDE 9.1® program and combinations prepared in relation to the program-predicted combination against *M. smegmatis*.

Combinations	MIC
A. crenulata + *D. viscosa* + *E. globulus* (5.7:29:65.3) (original from MODDE software)	0.5
A. crenulata + *D. viscosa* + *E. globulus* (3.7:31:65.3)	0.5
A. crenulata + *D. viscosa* + *E. globulus* (4.7:27:68.3)	0.5
A. crenulata + *D. viscosa* + *E. globulus* (8.7:27:64.3)	0.5
A. crenulata + *D. viscosa* + *E. globulus* (4.7:29:66.3)	0.5

This was also similar to the MIC of triple combination organic extracts with equal quantities (1:1:1) (Table 4.5). Furthermore, the MIC recorded for the predicted combination together with other combinations was very close to the program-predicted MIC of 0.541 mg ml^{-1} (Table 4.7).

In general, the trend for *M. smegmatis* was the same as in *C. neoformans*. The fact that the MIC remained the same despite changes in volumes may validate the traditional way of combining plants, as in traditional medicine the portions of the plants in combinations are not in the exact same quantities. For example, in a survey conducted by York *et al*. (2011) in rural Maputaland of KwaZulu-Natal, the amount of medicinal plant material to be used in a mixture was often referred to as "a handful" and that indicates some inaccuracy in measurements of the dosage. However, based on the results obtained from this study, the expected outcome by traditional medicine users might not vary significantly. Thus this study is able to show that pharmacological action remains constant irrespective of the informal way of mixing plants, that is, a handful of each, where quantities are not measured to the exact gram.

4.4 Chromatographic analysis

4.4.1 Thin layer chromatography and bio-autography

A quick chemical finger-printing, a thin layer chromatography (TLC) of the solvent extracts, was investigated. The locality of the various compounds in TLC plates was determined using retention factor (R_f) values. Under normal light (Figure 4.4 iii), relatively few compounds were visible, however, assessment of the chromatograms under the UV light at 254 nm showed good separation of the plants constituents where all the three individual plants A, B and C (*A. crenulata*, *D. viscosa* and *E. globulus* respectively) had a characteristic pink band which occurred at the R_f value of 0.4 in lanes A, B and C respectively (Figure 4.4 i). *D. viscosa* (lane B) was characterised by a thick dark blue band at R_f value of 0.13 while *E. globulus* (lane C) had a white band at the top of the plate (R_f value 0.81) (Figure 4.4 i). *E. globulus* (lane C) had the highest number of compounds separated among the three plants. In the chromatograms of the combined extracts (lanes A + B, A + C, B + C and A + B + C), all the bands of each plant that is in a combination were visible signifying the complexity of chemistry in combined extracts (Figure 4.4 i).
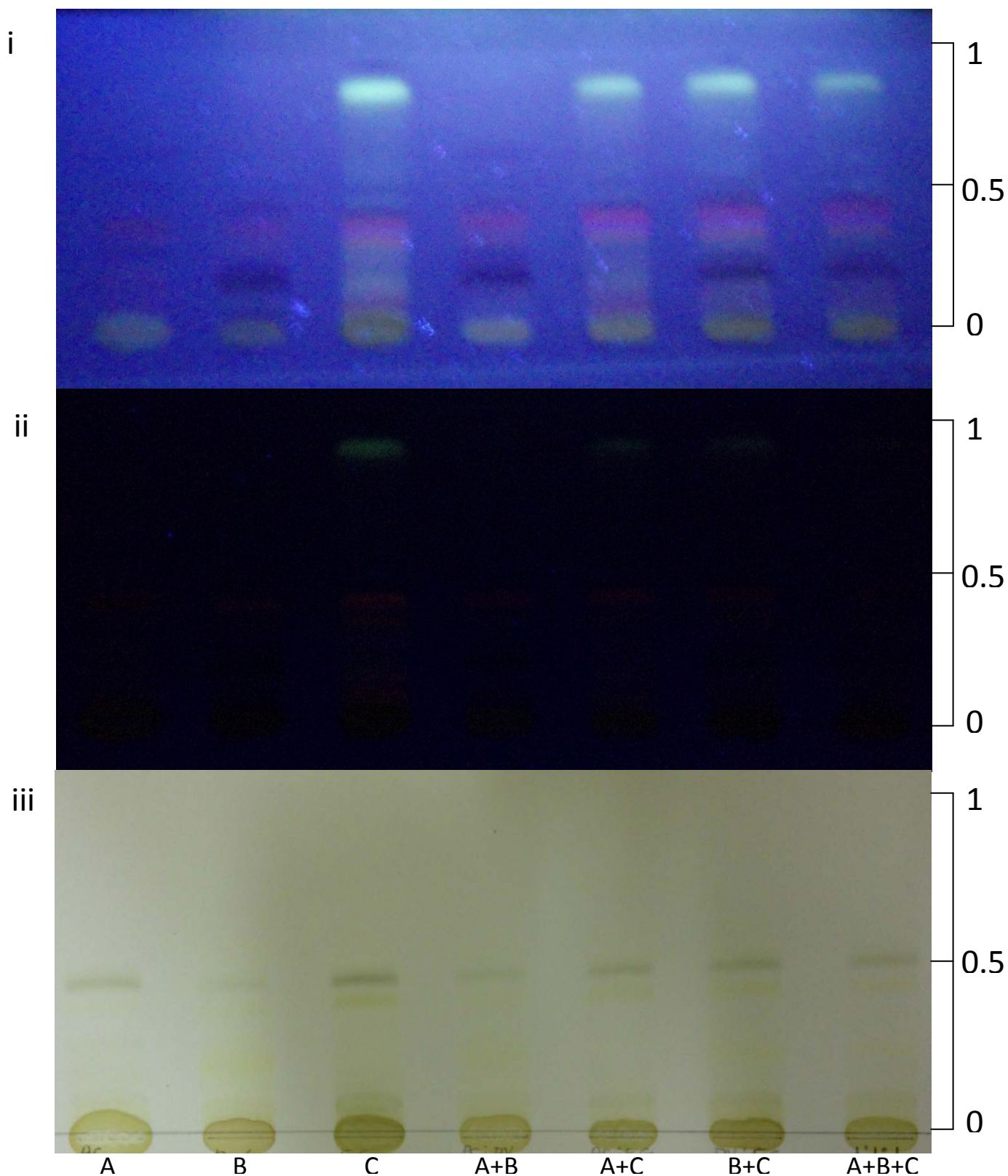
Figure 4.4 TLC of the organic extracts of *A. crenulata*, *D. viscosa* and *E. globulus* individually and in combinations visualized under the UV lights of 254 nm (i), 365 nm (ii), normal light (iii).
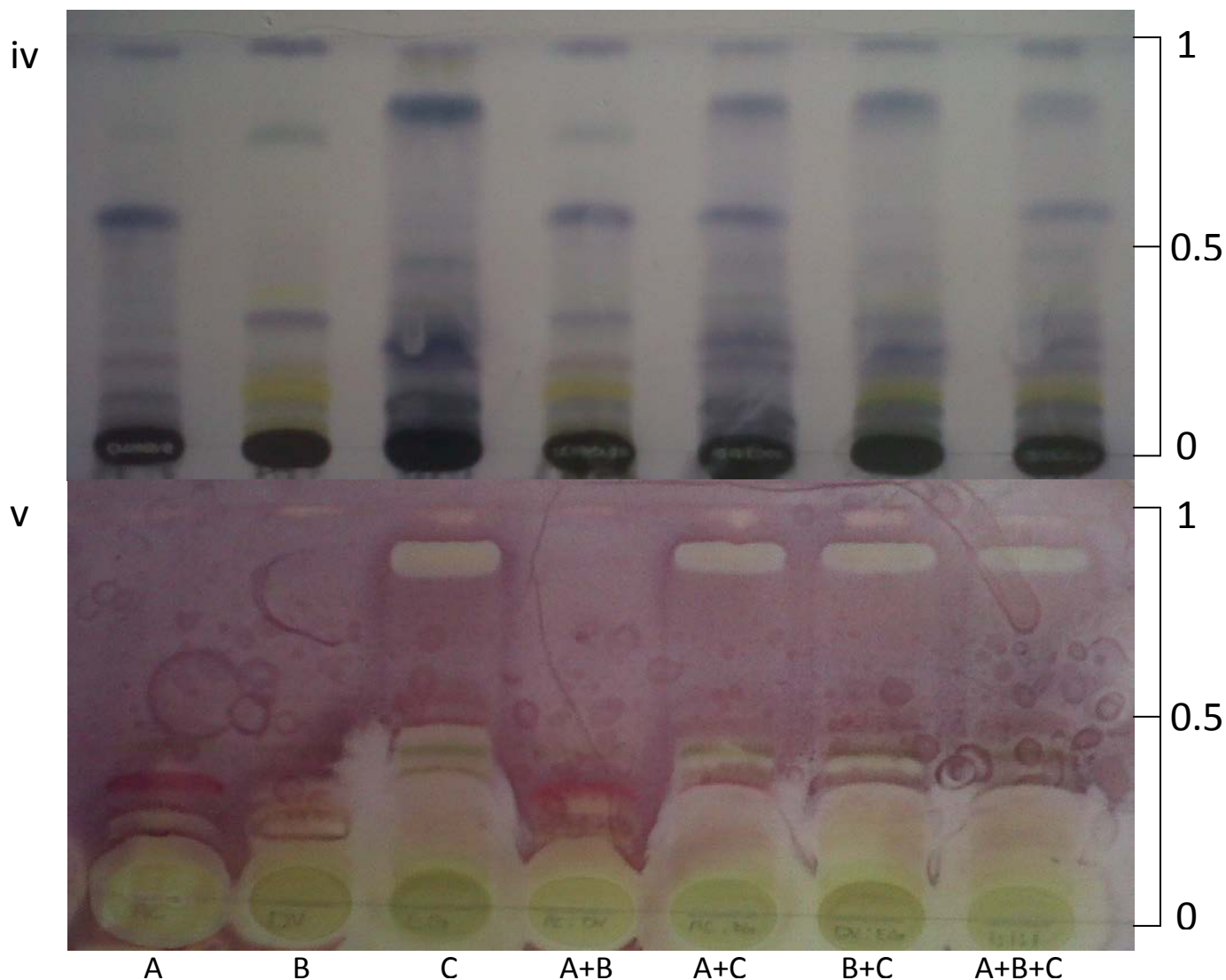
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Figure 4.4 TLC of the organic extracts of *A. crenulata*, *D. viscosa* and *E. globulus* individually and in combinations visualized after derivatization (iv) and bio-autographic analysis (v). A- *A. crenulata*; B- *D. viscosa*; C- *E. globulus*; A + B- *A. crenulata* with *D. viscosa*; A + C- *A. crenulata* with *E. globulus*; B + C- *D.viscosa* with *E. globulus*; A + B + C- *A. crenulata*, *D.viscosa* and *E. globulus*.

At the UV light of 365 nm, not many compounds could be detected clearly (Figure 4.4 ii). After viewing with UV light, the TLC plates were derivatized with *p*-anisaldehyde and derivatization revealed more compounds that were not visible in normal light and under UV light at both wavelengths. The dark blue band that was characteristic of *D. viscosa* was changed into a yellow colour (Figure 4.4 iv; lane B) after staining. This compound which is

characterised by a yellow band might possibly be a flavonoid glycoside and the two grey zones in *D. viscosa* (Figure 4.4 iv; lane B) might be representative of the saponin components (Wagner and Bladt, 1996).

The literature supports this as *D. viscosa* is known to be rich in flavonoids and saponins (Sachdev and Kulshreshtha, 1983; Wagner *et al*., 1987; Getie *et al*., 2003; Patel and Coogan, 2008).

Staining also revealed a common minor band at the solvent front which occurred in all the three plant extracts and their various combinations at R_f value of 0.96 (Figure 4.4 iv). These are non-polar compounds since they migrated to the top of the TLC plate which is typical of terpene alcohols (Wagner and Bladt, 1996).

A bio-autographic assay was conducted to determine which particular bands, among the separated compounds have antimicrobial activity. Zones of bacterial inhibition are represented by white spots against a pink background (Figure 4.4 v). The strongest inhibition zones were noted for *E. globulus* (lane C) mostly at the bottom and in the white band (when viewed at 254 nm at R_f values of 0.81 (Figure 4.4 i). This inhibitory trend was noted in all the combinations that constituted *E. globulus* (lanes A + C, B + C and A + B + C). Zones of inhibition were also spotted in the other two plants (lanes A and B) by bands at the lower part of the plate and slightly by the top band that was common to all extracts ran. Antibacterial activity in *D. viscosa* was exhibited by the compound (R_f value of 0.13) speculated to be a flavonoid glycoside.

The presence of a greater number of bands in the *E. globulus* extract and all combinations that included it in the TLC plate shows a complex phytochemical pool which may possibly lead to its significant antimicrobial activity that has been noted in this study. For the other two plants, *A. crenulata* and *D. viscosa* and their combination (A, B, and A + B respectively), the antimicrobial activity was exhibited predominantly by the compounds found at the lower part of the plate and some minor activity from compounds at R_f value of 0.96 at the top of the plate was noted. The compounds at the bottom of the TLC plate which exhibited

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antimicrobial activity are possibly polar compounds since they did not run to the top parts of the plate.

4.4.2 The metabolite profiles of extracts individually and in combination by liquid chromatography mass spectrometry

The metabolite profiles of single and various extract combinations were acquired using LC-MS whereby the chemical make-up of the combinations were compared with that of individual plants. Unlike GC-MS, LC-MS does not have a comprehensive library, however, the main purpose for using this metabolomic analytical tool was to compare the metabolite profiles of extract combinations to individual plants extracts. A total dataset of 1113 was used to generate a PCA score plot. Variance of the principal components was noted at 42.63% for PC 1 and for PC 2 at 36.03% (Figure 4.5). The LC-MS analysis was run in triplicate but for the purpose of clarity of points, only one point is shown. The plot showed separation of individual plants and various combinations into two clusters with *A. crenulata* (A), *E. globulus* (C), the combination thereof (A + C) in Cluster I and *D. viscosa* (B), *A. crenulata* with *D. viscosa* (A + C), *D. viscosa* with *E. globulus* (B + C), triple combination (A + B + C) grouped in Cluster II (Figure 4.5). Both plants (*A. crenulata* and *E. globulus*) in Cluster I are aromatic, therefore, the similarity in their metabolites might be due to the presence of highly non-polar compounds. A double combination of these plants (A + C) showed equal distance from each individual plant (A) and (C), possibly showing equal portions of the chemical makeup of each plant. The chemistry of this double combination (A + C) was very distinct from the other two-plant combinations (A + B and B + C) and this could explain its best antimicrobial activity and enhanced pharmacological interaction compared to the other double plant combinations (Section 4.2; Tables 4.2 to 4.4). The loading plot indicated that the cluster of *A. crenulata*, *E. globulus* and their combinations could be differentiated from the other cluster based on the presence of the unidentified compound which eluted at the retention time of 3.43 min and with a mass of 431.1992 (circled in green) (Figure 4.6). The compound circled in red might be responsible for separation of the second cluster (Figure 4.6). All the other extract combinations clustered very close to *D. viscosa* (B) (Figure 4.5). No distinct trend could be correlated to the antimicrobial activity of the extracts in Cluster II.

Figure 4.5 Score plot of principal component analysis showing the inter-relationships between the various combinations of three study plants (*A. crenulata* (A), *D. viscosa* (B) and *E. globulus* (C)) and the individual plants comprising the combinations based on LC-MS profiles.
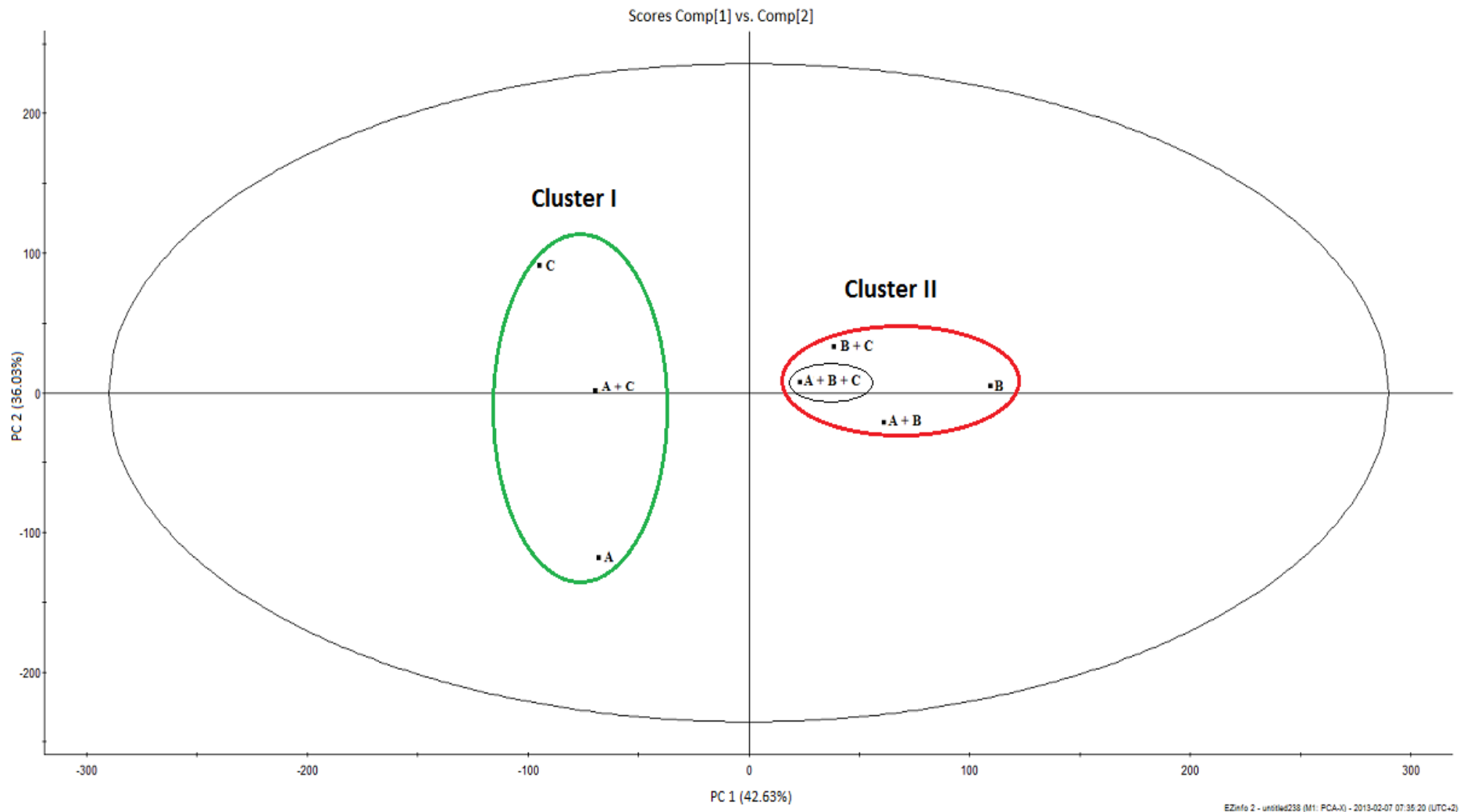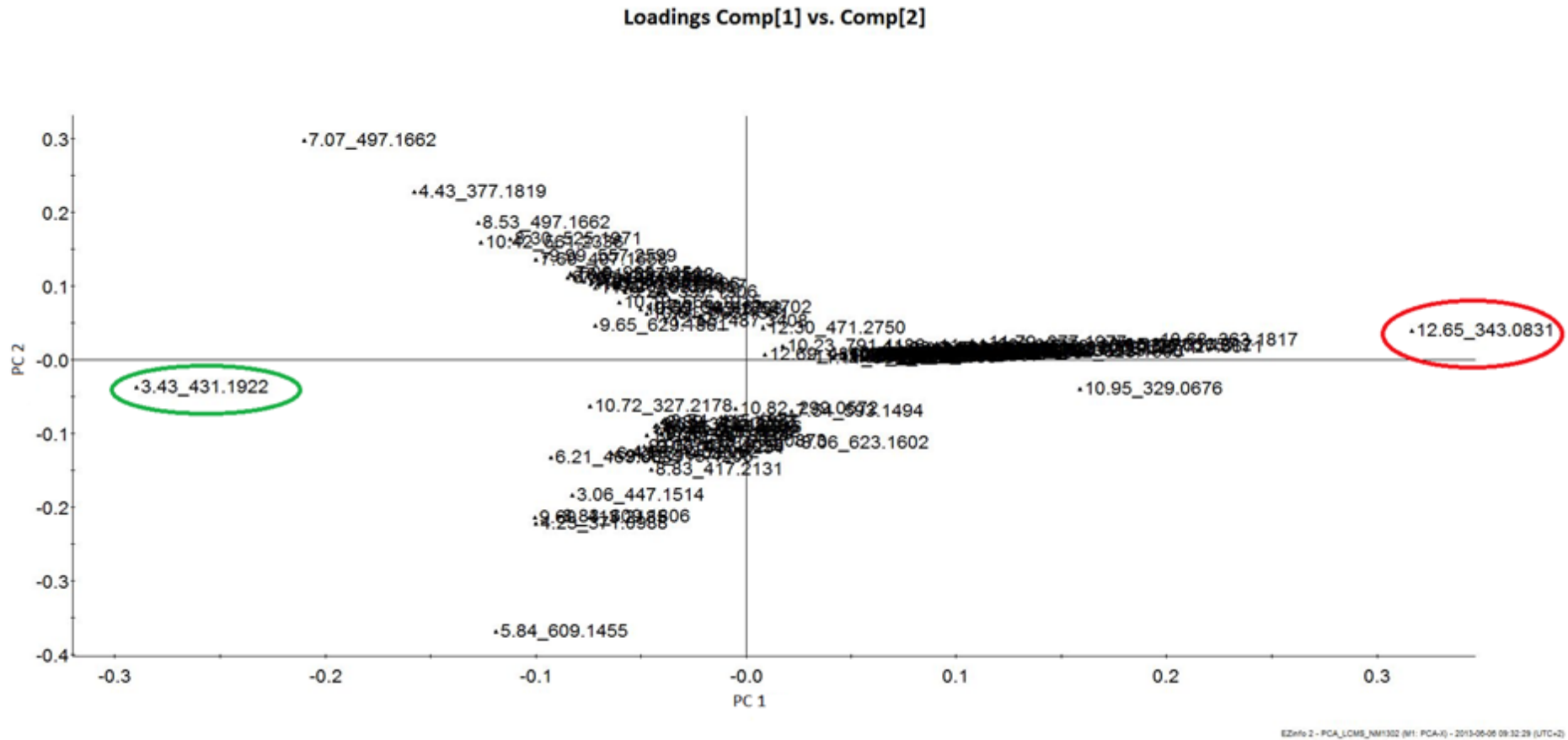
Figure 4.6 Loading of principal component analysis showing the inter-relationships between the various combinations of three study plants (*A. crenulata*, *D. viscosa* and *E. globulus*) and the individual plants comprising the combinations based on LC-MS profiles.

The PCA plot contained relatively highly non-polar compounds (represented by aromatic *A. crenulata* and *E. globulus*) on the negative score of PC 1 and more polar compounds which are speculated to be represented by *D. viscosa* and all its combinations on the positive score of PC 1 (Figure 4.5). It can be suggested that the negative score plot of PC 2 (Figure 4.5) contained metabolites which did not exhibit best antimicrobial activity in this study as it was noted for *A. crenulata* (A; the least active among the individual plants) and for the combination of *A. crenulata* with *D. viscosa* (A + B; the least active among extract combinations) (Tables 4.1 and 4.2).

On the other hand the metabolites of plant extracts which showed relatively good activity appeared on the positive score plot of PC 2 (Figure 4.5). It is clear from the PCA plot that, although the metabolite profiles of the combinations and individual plants grouped together, they were certainly unique from each other. This shows that combining different plants extracts creates a unique chemistry which determines the extent of pharmacological activity.

The HCA dendrogram plot demonstrated that the highly related chemical profiles were between the combinations; *D. viscosa* with *E. globulus* (B + C) and a triple combination (A + B + C) with a linkage distance value of approximately 277 (Figure 4.7). This is congruent with the PCA results (Figure 4.5). *A. crenulata* (A) formed a highly distinct cluster and had the furthest relationship from the various extract combinations compared to the other two individual plants. Its distinct chemistry from other extracts might explain why this extract has lower activity compared to the others. Another considerable similarity was observed between *E. globulus* (C) and *A. crenulata* with *E. globulus* (A + C) with a linkage distance of approximately 791. This further validates high interaction of *A. crenulata* (A) with *E. globulus* (C) (both aromatic plants) when combined which is speculated to have caused high antimicrobial activity that was observed in this double combination.

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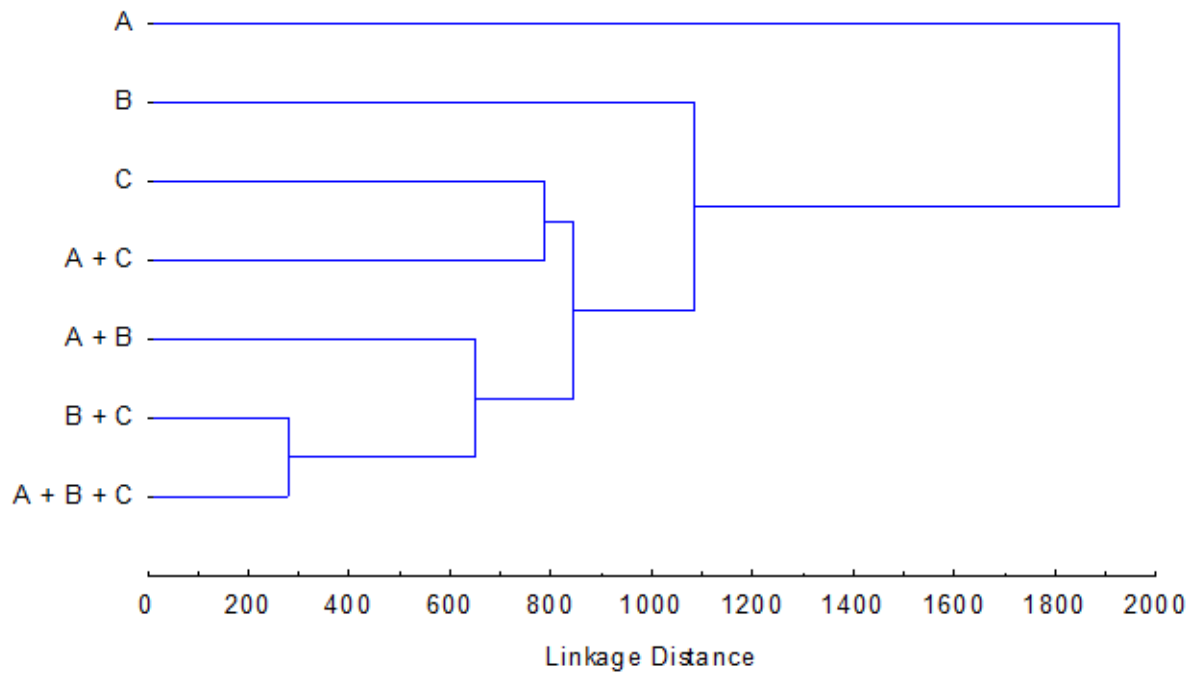
Figure 4.7 Hierarchical cluster analysis (HCA) dendrogram of the organic extracts metabolites of *A. crenulata*, *D. viscosa* and *E. globulus* in various combinations.

4.4.3 Essential oils composition individually and in combination by gas chromatography mass spectrometry

The *A. crenulata* and *E. globulus* essential oils constituted a complex mixture of monoterpene hydrocarbons, oxygen-containing monoterpenes, sesquiterpene hydrocarbons and oxygen-containing sesquiterpenes. *A. crenulata* oils abundantly contained monoterpene hydrocarbons and oxygen-containing monoterpenes while *E. globulus* oils had monoterpene hydrocarbons, oxygen-containing monoterpenes and oxygen-containing sesquiterpenes in high abundance (Table 4.8).

Twenty one compounds were identified in the essential oil of *A. crenulata* making up 88.83% of the total composition of the oil (Table 4.8). Major compounds present were: D-limonene (5.90 ± 0.07%), menthone (19.61 ± 0.26%), pulegone (44.11 ± 1.01%) and p-menthon-8-thiol (11.19 ± 0.46%) (Table 4.8). The results of this study are generally consistent with previous reports (Fluck *et al*. 1961; Kaiser *et al*. 1975; Blommaert and Bartel, 1976; Collins and Graven, 1996; Posthumus and Van Beek, 1996; Sandasi *et al.*, 2010) on the chemical profiles of *A. crenulata* essential oils. In a recent study, Sandasi *et al*. (2010)

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recorded high content of pulegone at 58.5% in the essential oil of *A. crenulata* using vibrational spectrometry confirming previous data by Posthumus and Van Beek (1996), who recorded this volatile compound at a high abundance of 54%.

Other reports also confirmed the occurrence of high amounts of pulegone as a marker compound for identifying *A. crenulata* oil (Fluck *et al*. 1961; Blommaert and Bartel, 1976; Collins and Graven, 1996). Kaiser *et al*. (1975) observed that the sharper, minty note of *A. crenulata* is caused by very high amounts of pulegone and isopulegone isomers which characterise the oil. Other chemical compounds such as isomenthone, limonene, menthone and isomenthone were identified in the commercial essential oil of this species by Viljoen *et al*. (2006) with the use of GC and GC-MS. Two 8-acetylthio-*p*-menthan-3-one isomers were also identified. The abundance of major compounds recorded in the present study varied from the abundance of the same chemical constituents in the literature. This was seen with the essential oil of *A. crenulata* pulegone content which varied by more than 9% from essential oils studied by Posthumus and Van Beek (1996) and Sandasi *et al*. (2010).

E. globulus essential oil showed the presence of 26 components, which formed 95.62% of the total oil composition with α-Pinene (6.31 ± 0.46%), D-limonene (4.57 ± 0.12%), eucalyptol (70.68 ± 0.81%), p-menth-1-en-8-ol (6.65 ± 0.22%) as major compounds (Table 4.8). The oils extracted from the *Eucalyptus* species are among the world's top traded oils as they are relatively superior in quality and find application in perfumery, pharmaceutical and food industries. The major volatile constituents found in the *E. globulus* essential oils are known to possess toxicity against a wide range of bacteria and fungi (Batish *et al*., 2008). Many of the compounds identified in the essential oil of *E. globulus* in this study have been reported in the literature. For example, Kumar *et al*. (2012) identified 31 volatile compounds in the essential oil of *E. globulus* of which, 11 were also identified in this study. Eucalyptol had the highest abundance (33.62%) of all the compounds even though the abundance is not the same as the area of eucalyptol in this study. In another study by Tyagi and Malik (2011) 20 compounds were identified and 10 of those also occurred in the essential oil investigated in this study. The abundance of the eucalyptol in *E. globulus* essential oil investigated in this study also varied considerably from the abundance recorded in other

studies (Batish *et al.*, 2008; Tyagi and Malik, 2011; Kumar *et al.*, 2012). The variation in chemical compositions can be due to differences in environmental factors, soil composition, part and age of the plant as well as method of extraction (Maciel *et al.*, 2010).

Table 4.8 Phytochemical composition of the essential oils of *A. crenulata*, *E*. *globulus* and the combination (1:1) thereof.

Compound	RI[a]	Relative abundance (%)[b]		
		A. crenulata	*E. globulus*	*A. crenulata* with *E. globulus* (1:1)
α-Pinene	**948**	0.35 ± 0.01	**6.31 ± 0.46**	**2.75 ± 0.04**
β-Pinene	943	-	0.37 ± 0.02	0.60 ± 0.05
α-Thujone	1062	-	0.13 ± 0.05	-
Myrcene	958	0.67 ± 0.02	-	-
D-limonene	**1018**	**5.90 ± 0.07**	**4.57 ± 0.12**	**5.23 ± 0.02**
Eucalyptol	**1059**	0.56 ± 0.06	**70.68 ± 0.81**	**33.92 ± 0.64**
β-trans-ocimene	976	0.52 ± 0.04	0.25 ± 0.01	0.11 ± 0.01
3-carene	948	0.52 ± 0.03	-	-
γ-Terpinene	998	-	0.23 ± 0.01	-
o-Cymene	1042	0.10 ± 0.01	0.49 ± 0.02	0.25 ± 0.01
Menthone	**1148**	**19.61 ± 0.26**	-	**10.90 ± 0.26**
Linalool	1082	0.57 ± 0.01	-	0.34 ± 0.02
Isopulegol	1196	0.18 ± 0.01	-	0.1 ± 0.01
Isopulegone	1179	2.95 ± 0.03	-	-
(-)-4-Terpineol	1137	0.11 ± 0.00	0.64 ± 0.23	-
Dihydrocarvone	1179	0.16 ± 0.00	-	0.11 ± 0.01

Compound	RI[a]	Relative abundance (%)[b]		
		A. crenulata	*E. globulus*	*A. crenulata* with *E. globulus* (1:1)
Pinocarvone	1114	-	0.12 ± 0.01	-
Neoisocarvomenthol	1164	0.27 ± 0.02	-	-
Aromadendrene, (+)-	1386	-	0.83 ± 0.02	-
Alloaromadendrene	1386	-	0.21 ± 0.01	0.28 ± 0.01
Pulegone	**1212**	**44.11 ± 1.01**	-	**25.16 ± 0.76**
L-Pinocarveol	1131	-	0.55 ± 0.00	-
2H-Inden-2-one, 1,4,5,6,7,7a-hexahydro-7a-methyl-, (S)-	1237	0.13 ± 0.02	-	-
(-)-Myrtenyl acetate	1314	0.14 ± 0.01	-	-
10-Pinalol	1126	0.32 ± 0.03	-	-
p-Menthon-8-thiol	**1431**	**11.19 ± 0.46**		**5.78 ± 0.38**
p-menth-1-en-8-ol	**1143**	-	**6.65 ± 0.22**	3.17 ± 0.09
Borneol	1138	-	0.13 ± 0.01	-
1,5,7-Octatrien-3-ol, 2,6-dimethyl-	1120	-	0.21 ± 0.01	-
Cyclohexanol, 2-methylene-5-(1-methylethenyl)-	1201	-	0.31 ± 0.01	-
2,6-Octadien-1-ol, 3,7-dimethyl-	1228	-	0.24 ± 0.01	-
2-Hexadecanol	1774	0.22 ± 0.02	-	-
Coumarin	1374	-	0.26 ± 0.01	-

Compound	RI[a]	Relative abundance (%)[b]		
		A. crenulata	*E. globulus*	*A. crenulata* with *E. globulus* (1:1)
Epiglobulol	1530	-	0.13 ± 0.01	0.12 ± 0.01
Globulol	1530	-	0.42 ± 0.27	0.37 ± 0.06
Cubenol	1580	-	-	0.12 ± 0.01
Geranyl isovalerate	1586	-	0.24 ± 0.04	-
Butyl stearate	2375	0.25 ± 0.19	-	0.16 ± 0.04
β-Maaliene	1432	-	0.24 ± 0.02	-
Hinesol	1598	-	0.14 ± 0.05	-
α-Eudesmol	1598	-	0.44 ± 0.02	0.26 ± 0.02
β-Eudesmol	1593	-	0.83 ± 0.01	0.41 ± 0.29
Total		**88.83**	**95.62**	**90.14**
Monoterpene hydrocarbons		25.44	25.82	21.10
Oxygen-containing monoterpenes		46.25	32.98	42.21
Sesquiterpenes hydrocarbons		-	11.04	2.72
Oxygen-containing sesquiterpenes		17.14	25.78	24.11

[a] Retention index
[b] Values represent mean ± SD (n=3)
[c] Compounds highlighted in bold typeface are major constituents in the essential oil

These compounds have a wide application in the pharmaceutical, cosmoceutical, food industry and they are of great commercial value (Laude *et al.*, 1994; Whysner and Williams, 1996; Allahverdiev *et al.*, 1999; Zhao *et al.*, 2001; Pillai and Panchagnula, 2003).

When the essential oils of *A. crenulata* and *E. globulus* were combined (1:1), GC-MS revealed a total of 20 volatile compounds (Table 4.8). The identified compounds made up to 90.14% of the oil composition. The compounds identified in a mixture were the same as the compounds found in either *A. crenulata* or *E. globulus*. However, the combination of the two oils created a unique chemistry as the abundances of the constituents in the combination varied from those of individual plants essential oils. Intergration between compounds from both species may impact pharmacological activity of the essential oil combination.

Some studies that have investigated the chemical profiles of multi-herb extracts mainly aimed at quality assessment for standardization purposes. They therefore used a target approach to quantify the chemical groups that are known to occur in the plants involved in a herbal combination without an attempt to study the overall chemistry profile in those combinations (Lin *et al*., 2006; Xiaohui *et al*., 2006; Chen *et al*., 2009).

To demonstrate the relationship of the metabolite profile of the essential oil mixture to the individual plants essential oils, PCA and HCA were also used for visual representation of this relationship. The pattern represented by the PCA plot (Figure 4.8) was relatively consistent with the results shown in Table 4.8 as the point for the mixture (A + B) was in the middle of the two points for the individual plants essential oils. There was no great variation in essential oils metabolites of the individual plants and their combination as they clustered together (Cluster I). This was further confirmed by an even distribution of analytes which does not show any particular pattern (Figure 4.9).

Similarly, the antimicrobial efficacies of the combination of these two essential oils did not differ from the individual plant essential oils and no synergistic combination was recorded for the essential oil combination on any pathogen (Tables 4.1 and 4.3). Furthermore, the sample variance for the two principal components was 100% (PC 1, 99.81% and PC 2, 0.19%) (Figure 4.8). Even though the point for the mixture was in the middle, it was slightly above the two points representing the individual plants.
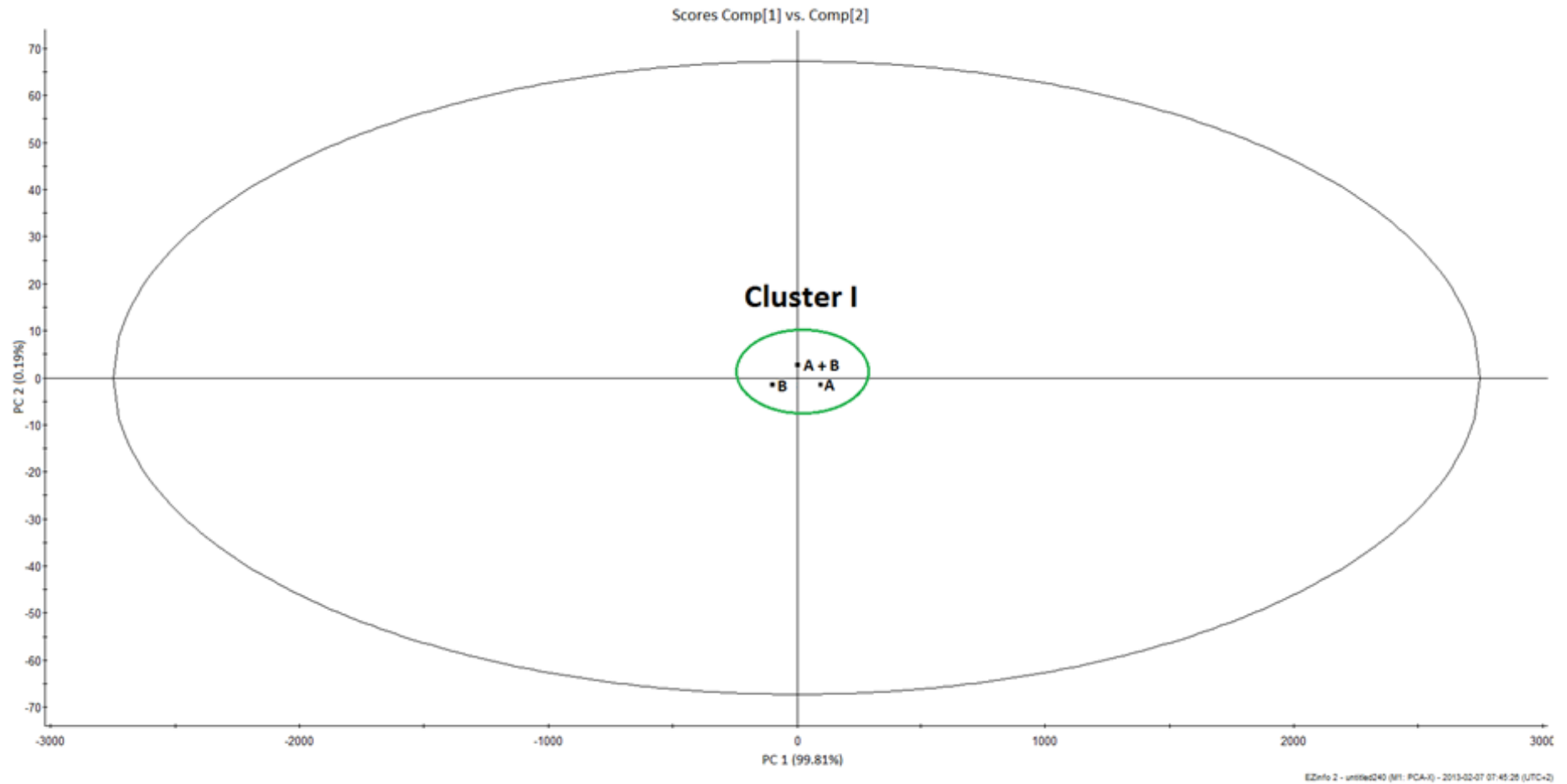
Figure 4.8 Score plot of principal component analysis showing the inter-relationships between the essential oils mixture (*A. crenulata* essential oil with *E. globulus* (A + B)) and the individual plants comprising the mixture based on GC-MS profiles.
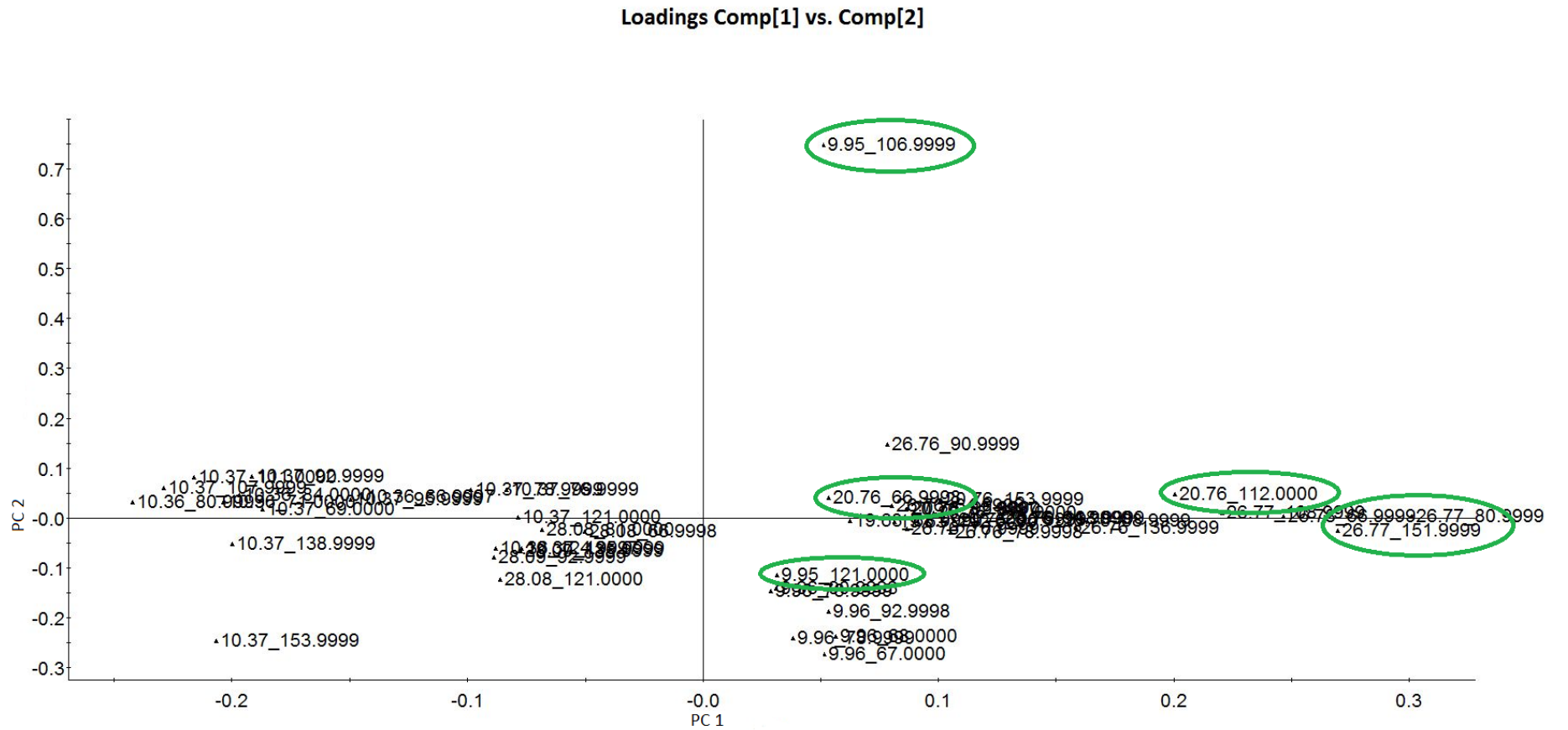
Figure 4.9 Loading of principal component analysis showing the inter-relationships between the essential oils mixture (*A. crenulata* essential oil with *E. globulus* (A + B)) and the individual plants comprising the mixture based on GC-MS profiles.

A hierarchical cluster analysis (HCA) was conducted using retention times and abundance of essential oils components identified in the study plants and their combinations. Contrary to the extracts, HCA revealed less interaction of the essential oil components with the linkage distance that only ranged from 80 to 100 (Figure 4.10). One major cluster was identified, wherein *E. globulus* formed the first lineage and the second lineage sub-divided into *A. crenulata* and a double-plant combination (*A. crenulata* and *E. globulus*) (Figure 4.10). The strong correlation (linkage distance value of approximately 82) demonstrated by the HCA for *A. crenulata* and the mixture was based on the abundance of D-limonene, menthone and pulegone (Table 4.8). These compounds (circled in green) occurred on the positive score of PC 1, positive and negative scores of PC 2 of the loading plot (Figure 4.9) distinguishing the *A. crenulata* oil and the essential oil combination from *E. globulus*. D-limonene, menthone and pulegone were represented as compounds with retention times of 9.95, 20.76 and 26.77 min respectively and their masses differed (Figure 4.9).

In general, the correlation of the essential oils and their combination demonstrated by the HCA was relatively similar to the trend demonstrated by the PCA, as in both methods only one major cluster could be identified.
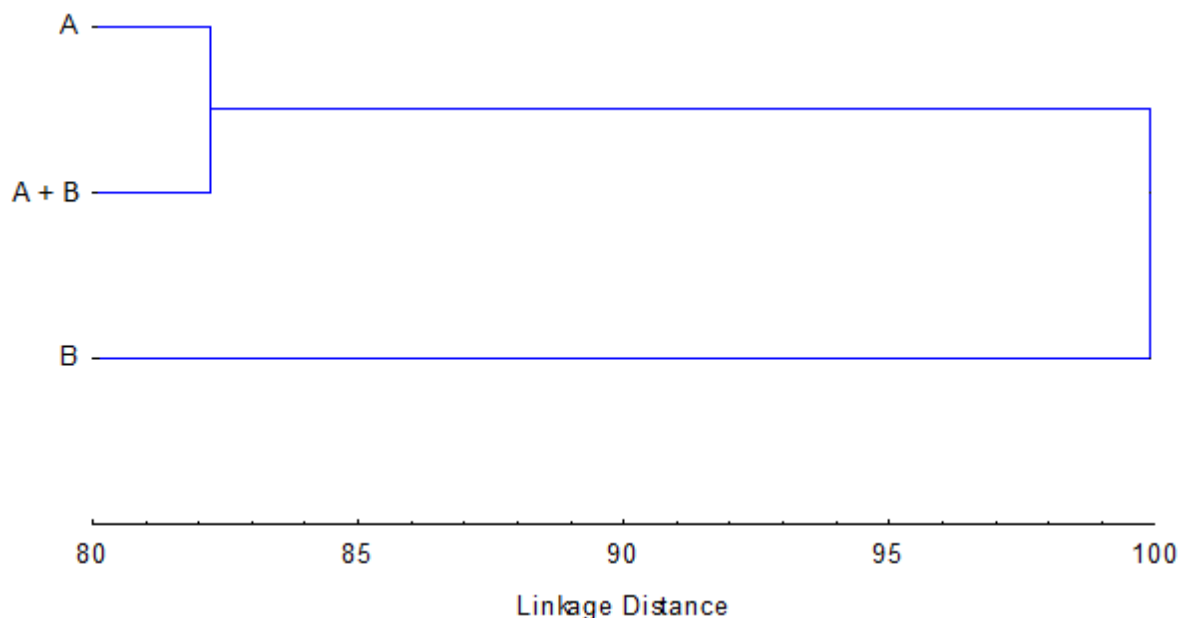
Figure 4.10 Hierarchical cluster analysis (HCA) dendrogram of the essential oils components of *A. crenulata* (A), *E. globulus* (B) and the combination thereof (*A. crenulata*: *E. globulus,* A + B).

It can be concluded that the interaction of the different plant bioactive components in plant combinations may alter the metabolic profiles, promoting the abundance of chemicals, as demonstrated in LC-MS analysis of organic extracts (Figure 4.5), which in turn produces defined antimicrobial properties, described in Sections 4.2 and 4.3.

Chapter 5

Conclusions and Recommendations

5.1 Conclusions

Research on medicinal plant combinations is beneficial to improve our understanding of pharmacological interactions taking place in herbal mixtures. This study was conducted with the aim of finding the scientific rationale for the use of *A. crenulata*, *D. viscosa* and *E. globulus* in combination for the treatment of respiratory infections. This was done by evaluating the antimicrobial efficacy of these three plants in combination and testing for the presence of synergistic interactions and other pharmacological interactions (additivity, indifference and antagonism). In addition to that, the phytochemical profiles of the study plants were investigated. The main objectives of the study were achieved and the major findings are listed hereafter.

Objective 1: To test the plant extracts and essential oils for antimicrobial activity individually and in various combinations using the minimum inhibitory concentration (MIC) technique.

The three study medicinal plants, *A. crenulata*, *D. viscosa* and *E. globulus* exhibited distinct antimicrobial activities with the highest activity recorded for *E. globulus* followed by *D. viscosa* and lastly *A. crenulata*. The organic solvent extracts, aqueous extracts and essential oils had remarkable differences with regards to their efficacy against micro-organisms. This highlights the importance of the method of preparation of medicinal plants in determining efficacy. From these findings tinctures are suggested to be the best way of medicinal plant administration as organic solvent extracts showed improved antimicrobial activity compared to aqueous extracts and essential oils. Furthermore, based on the performance of essential oils observed in this study, the inhalation method of medicinal plant is also justifiable as an effective means of administration. Overall, the three study plants demonstrated some antimicrobial activity and the activities were variable dependent on pathogen tested. However, their activities when studied individually were not as high as when they are combined which suggests that, to achieve optimum effect these plants have to be combined.

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Objective 2: To test the possible pharmacological interactions in double (1:1) and in triple (1:1:1) plant combinations using the the sum fractional inhibitory concentration (ΣFIC).

Overall, triple combinations had more synergism than any of the double combinations with ΣFIC values as low as 0.03 and 0.05 for *B. subtilis* and *S. aureus*, respectively. *E. globulus* had a contributory effect in enhancing the potency of organic extracts combinations. Synergistic interactions were mostly displayed for *K. pneumoniae* and *B. subtilis*. Among the double (1:1) combinations, the organic extracts of *A. crenulata* with *E. globulus* (1:1) combination displayed relatively enhanced antimicrobial activity and stronger pharmacological interactions. Both *A. crenulata* and *E. globulus* are aromatic plants, therefore the enhanced activity observed in the combination of these particular plants might also be caused by the interaction of volatile compounds contained within them. Although aqueous extracts did not show significant activity, most of their combinations displayed additive interactions and two synergistic interactions were also noted in the double combinations of *A. crenulata* with *D. viscosa* (1:1) and *D. viscosa* with *E. globulus* (1:1) against *B. subtilis*. Only one antagonistic interaction was recorded (aqueous extracts of *D. viscosa* with *E. globulus* (1:1) against *C. neoformans*) out of several combinations investigated. The lack of antagonism observed in the study of the medicinal plant mixture possibly reflects that the combination of these plants is effective and might not cause adverse effects.

Objective 3: To determine the interactive effects of selected combinations at various ratios and present this using isobolograms.

The pattern of antimicrobial interactions displayed by isobolograms was mainly congruent with the interactions when tested in 1:1 combinations and expressed as ΣFIC, further validating synergism of the combinations concerned. Isobolograms displayed pharmacological interactions, more effectively as they were able to show strength of interactions of each ratio in a combination, for example to show that other ratios are synergistic while some are additive.

Objectives 4 and 5: To do a chemical fingerprinting of the study plants to get an understanding of the chemical modifications that take place when extracts or essential oils from different plants are combined and to conduct bio-autographic analysis.

Different chromatographic techniques; LC-MS, GC-MS and TLC revealed a complex phytochemical profile in each plant indicating the presence of many compounds which might be responsible for their pharmacological activities. The chemical fingerprint of each plant's polar fraction (organic extracts) was unique as reflected by LC-MS chromatograms. All the double and triple combinations clustered next to *D. viscosa* with the exception of a double combination of *A. crenulata* with *E. globulus* as expected. The essential oils of *A. crenulata* and *E. globulus* had the highest contents of volatile compounds such as pulegone (44.1%) and eucalyptol (70.7%) respectively, acting as marker compounds which are routinely used for the identification of these oils. Monoterpenes, particularly the oxygen-containing monoterpenes constituted the greatest part of the essential oils studied here. This group encompasses many of the volatile compounds that are known to have antimicrobial activity against Gram-positive and Gram-negative bacteria. The TLC assays showed good separation of the plants organic extracts compounds and active compounds responsible for antibacterial activity were shown through bio-autographic assay with *E. globulus* bands exhibiting more activity. *E. globulus* generally had a high number of compounds for all chromatographic analyses conducted. This might explain its significant antimicrobial activity.

Main conclusion

Even though *A. crenulata*, *D. viscosa* and *E. globulus* are traditionally used as a triple combination, it was interesting to analyse their antimicrobial efficacy when they are mixed in various double combinations to discover the best possible combination that will give the best antimicrobial activity. Nevertheless, the triple combination generally displayed higher antimicrobial activity and promising antimicrobial interactions. Overall, the *in vitro* antimicrobial activity and pharmacological interactions (synergism and additivity) were

enhanced in various combinations of the study plants and that scientifically validates the use of the study medicinal plants combination for the treatment of respiratory infections.

5.2 Recommendations

Further research should consider *in vivo* investigation of the combinations that demonstrated synergistic effects. It is often difficult to predict interactions of herbal extracts in humans on the basis of *in vitro* results (Gathirwa *et al*., 2008). However, the current study using *in vitro* approach has shed some light on which combinations might increase therapeutic efficacy when used for the treatment of respiratory infections in the human body. These should be the mixtures which may be further examined for efficacy in an *in vivo* model.

Based on the observations of the survey that was conducted in 2010 as a preliminary study, the traditional way of preparing the medicinal plant mixtures involves combining the plants and extract them together rather than extracting the plants separately and combining them thereafter. It is the latter method that was used in this study in order to measure the extraction yield of each plant, however future investigations must consider conducting bio-assays on the samples prepared in the exact same way as in traditional medicine. For both organic solvent and aqueous extraction that were employed in the study, the extracts were filtered through Whatman® No. 1 filter paper. While in traditional medicine, upon boiling the plant material the solution is strained which might not exclude some larger molecules that cannot go through a filter paper. The impact of such material needs to be investigated. Future studies also need to analyse the chemical profiles of the samples prepared in a traditional way.

Moreover, medicinal plants are assumed to be safe as they are considered natural and pure. However, plants produce secondary metabolites as part of their defence against predators. Some medicinal plants used as traditional medicines may damage genetic material in cells and have long-term genetic effects which induce mutations, and increasing the incidence of cancers (Fennell *et al*., 2004; Street *et al*, 2008). An increase in the number of scientific studies investigating toxicity is thus urgently needed.

The implications of the combination on toxicity also needs to be explored. Sometimes the combination of two drugs may have a synergistic effect for the treatment of a microbial infection, but prove to be toxic to the host's cells even though the individual doses may not amount to such toxicity (Horn, 2007). This is not limited only to clinical drugs but herbal extracts can also interact and ultimately result in adverse effects (Naidoo *et al.*, 2013). Therefore, the potential toxic effects that may result when combining plants in the treatment of disease needs to be investigated.

The investigation of chemical modifications, that take place as a result of interaction between bioactive molecules of different plants in combination, is recommended. This can be done by subjecting a triple plant combination extract to bio-autographic-guided fractionation wherein the fractions will be studied to see if any known marker compounds of the individual plants are still in their original chemical form if they still occur at all.

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Appendix A: Abstracts of Oral Presentations and Publication in peer-reviewed journal

Appendix A1: (Oral presentation: Medical Research Council Early Career Scientist, Cape Town 2011)

ANTIBACTERIAL ACTIVITY OF SOME MEDICINAL PLANT COMBINATIONS USED IN THE GREATER CAPE TOWN REGION

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Human pathogens are increasingly becoming more resistant to antibiotics, raising a need to search for novel therapeutic compounds from other sources. In traditional medicine, different plant species which are often not related are combined as to enhance the efficacy of a medicinal preparation and this has been practiced for decades. As part of modern medicine, combination therapy regime is known to prevent antibiotic resistance by pathogens, a problem which is becoming more pressing with the higher incidence of infectious diseases. As part of indigenous herbal knowledge, it is believed that better therapeutic effects are derived from synergistic interactions between different plant constituents. However, there is inadequate scientific evidence to confirm this practice as many ethnopharmacological studies focus on validating efficacy of single plant extracts in South Africa. We thus screened plant mixtures used as traditional medicine in peri-urban and urban centres around Cape Town for antibacterial activity, individually and as combinations. Methanol and aqueous extracts of eight plant mixtures were tested for activity against two Gram-negative bacteria; *Escherichia coli* and *Klebsiella pneumoniae* and two Gram-positive bacteria; *Staphylococcus aureus* and *Bacillus subtilis* using the microdilution method. Those plant mixtures with the most potent bacterial inhibition became the targets for the isolation and putatively identification of active chemical ingredients. Variation in bacterial inhibition was evident with minimum inhibitory concentration (MIC) values ranging from 0.049 to 12.5 mg ml^{-1}. Combining plants into a mixed herbal preparation was beneficial for improving the action of plant mixtures as individual plants were less active on their own. This is one of few studies focussing on the pharmacological effects of ethnoherbal combinations prescribed by traditional herbal practitioners in South Africa. An in-depth investigation of phyto-synergism in a three-plant medicinal mixture (the most potent of the eight tested) is now in progress.

Appendix A2: (Oral presentation: South African Association of Botanists, Pretoria 2012)

Oral

Theme: Phytochemistry and biological activity

SAAB student award: Young botanist, MSc student

Pharmacological and phytochemical analysis of a medicinal plant mixture that is used as traditional medicine in Western Cape

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In traditional medicine, different plant species which are often not related are combined to enhance the efficacy of a medicinal preparation. As part of indigenous herbal knowledge, it is believed that better therapeutic effects are derived from synergistic interactions between different plant constituents. However, there is inadequate scientific evidence to confirm this practice as many ethnopharmacological studies focus on validating efficacy of single plant extracts in South Africa. We thus investigated a plant mixture which consist of three plant species; *Agathosma crenulata*, *Dodonaea viscosa* and *Eucalyptus globulas* for synergistic interactions. The individual plant extracts and their different combinations and ratios were screened for antibacterial activity against bacterial pathogens using the microdilution method to determine the type of chemical interaction between these plants. The fractional inhibitory concentrations (FIC) were recorded for all two-plant combinations. It was evident from the FIC values that the overall pharmacological activity is synergistic (FIC values ranged from 0.079 to 0.750). Additive and few antagonistic interactions were also observed. The combination of *D. viscosa* and *E. globulas* exhibited the strongest synergistic interaction with FIC values as low as 0.079 while the combination of *D. viscosa* and *A. crenulata* was mildly synergistic. Combining plants into a mixed herbal preparation was beneficial for improving the action of plant mixtures as individual plants were less active on their own. This finding scientifically validates the use of plant combinations in traditional medicine for improved efficacy.

Appendix A3: (Oral presentation: Indigenous Plant Use Forum, Thohoyandou 2012)

Unravelling antimicrobial interactions of a polyherbal combination used in traditional medicine

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The use of natural drug combinations to treat diseases is a well accepted concept, even in traditional medicine. This approach has been speculated to suppress bacterial resistance which usually develops when single drugs are used. Out of seven plant mixtures tested, the combination of *Agathosma crenulata, Dodonaea viscosa* and *Eucalyptus globulus* was the most potent against all test bacteria. This mixture is used as a single remedy to treat respiratory ailments and was formulated by a herbalist that professes to practice the Khoi-San routed herbalism. To test this remedy for antimicrobial interactions, chloroform: methanol (1:1;v/v) extracts and essential oils (aromatic plants only) were prepared. These were assayed for activity against a number of respiratory pathogens, independently and in various combinations. The fractional inhibitory concentration indices were determined for double (1:1; v/v) and triple (1:1:1; v/v) plant combinations to establish the nature of antimicrobial interactions. The efficacy of plants was enhanced when combined (lowest MIC value obtained: 0.002 mg/ml). This may be explained by the mechanism whereby various plant bioactive constituents affect several target sites and work cooperatively in a synergistic manner. Overall, the interactions against different pathogens demonstrated additive (FIC range: 0.58 to 1.00) with some synergistic interactions (FIC range: 0.05 to 0.38) and a few indifferent reactions (FIC range: 1.00 to 1.50). Interestingly, no antagonistic reactions were evident for any of the ratios. The positive microbial inhibition recorded explains why traditional healers sometimes administer multiple decoctions to their patients, especially for treating complex diseases. Moreover, research on medicinal plant combinations is beneficial as a platform for developing plant-based therapies where drug resistance is becoming a problem.

Appendix A4: (Publication: Journal of Ethnopharmacology 148, 144 – 151: 2013)

Antimicrobial interactions of Khoi-San poly-herbal remedies with emphasis on the combination; *Agathosma crenulata*, *Dodonaea viscosa* and *Eucalyptus globulus*

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ARTICLE INFO

ABSTRACT

Ethnopharmacological relevance: Plants are often combined in traditional herbal remedies to increase medicinal efficacy, thus this investigation provides some insight into the antimicrobial efficacies of selected combinations.

Aims of the study: The first aim was to scientifically validate antibacterial efficacy of plant mixtures that are traded within peri-urban centres of Cape Town (Western Cape, South Africa). This was followed by an in-depth evaluation of the most antimicrobially active mixture; *Agathosma crenulata*, *Dodonaea viscosa* with *Eucalyptus globulus*.

Materials and methods: Methanol and aqueous extracts of six plant mixtures were screened for antibacterial properties against two Gram-negative and two Gram-positive bacteria using the minimum inhibitory microdilution method. Thereafter, chloroform: methanol (1:1; v/v) extracts, essential oils and aqueous extracts of *Agathosma crenulata*, *Dodonaea viscosa* and *Eucalyptus globulus* were assayed for antimicrobial activity independently and in various combinations. The fractional inhibitory concentration indices (ΣFIC) were determined for double and triple plant combinations to establish antimicrobial interactions.

Results: From the six plant mixtures prepared by herbalists, a methanol extract derived from combining *Agathosma crenulata*, *Dodonaea viscosa* and *Eucalyptus globulus* showed the best antibacterial activity. The MIC values of 49 µg/ml for *Staphylococcus aureus* and *Bacillus subtilis*, and 98 µg/ml for *Klebsiella pneumoniae* and *Escherichia coli* were recorded. When *Agathosma crenulata*, *Dodonaea viscosa* and *Eucalyptus globulus* were mixed in various 1:1 combinations, mostly additive and synergistic interactions were noted. The most noteworthy synergistic (ΣFIC value 0.07) 1:1 combinations were observed for the chloroform: methanol extracts of *Agathosma crenulata* mixed with *Eucalyptus globulus* against *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Bacillus subtilis*. When combined in a mixture of three plants (1:1:1), enhanced efficacy was evident against most of the pathogens, for both organic and aqueous extracts. The triple combination against *Bacillus subtilis* demonstrated the greatest synergy (ΣFIC values of 0.03).

Conclusions: The enhanced antimicrobial efficacy and synergistic interactions noted for some of the mixtures, particularly the combination of *Agathosma crenulata*, *Dodonaea viscosa* and *Eucalyptus globulus* support the Western Cape Khoi-San traditional medicinal practices of combining plants for enhanced efficacy.

The paper can be accessed by following this link:

http://www.sciencedirect.com/science?_ob=ArticleListURL&_method=list&_ArticleListID=-359759315&_sort=r&_st=13&view=c&_acct=C000032099&_version=1&_urlVersion=0&_userid=613892&md5=9983c68e79f57d89f7eda56ef4935b08&searchtype=a

Appendix B: Chromatograms of the organic solvent extracts and essential oils metabolite profiles
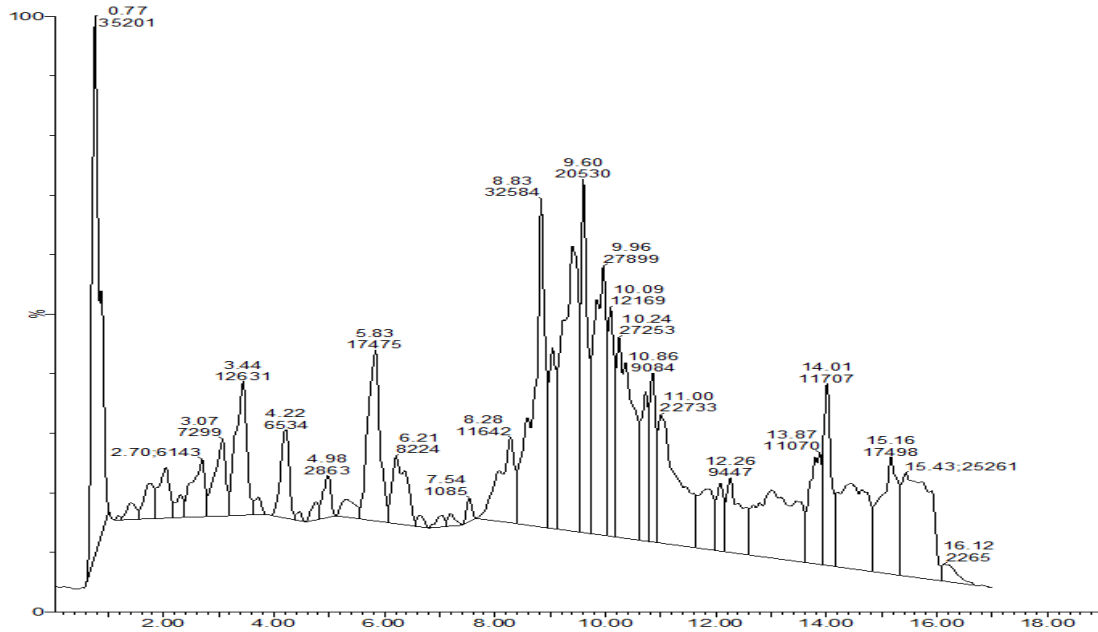

Figure B1 LC-MS chromatogram of the solvent extract of *A. crenulata*.
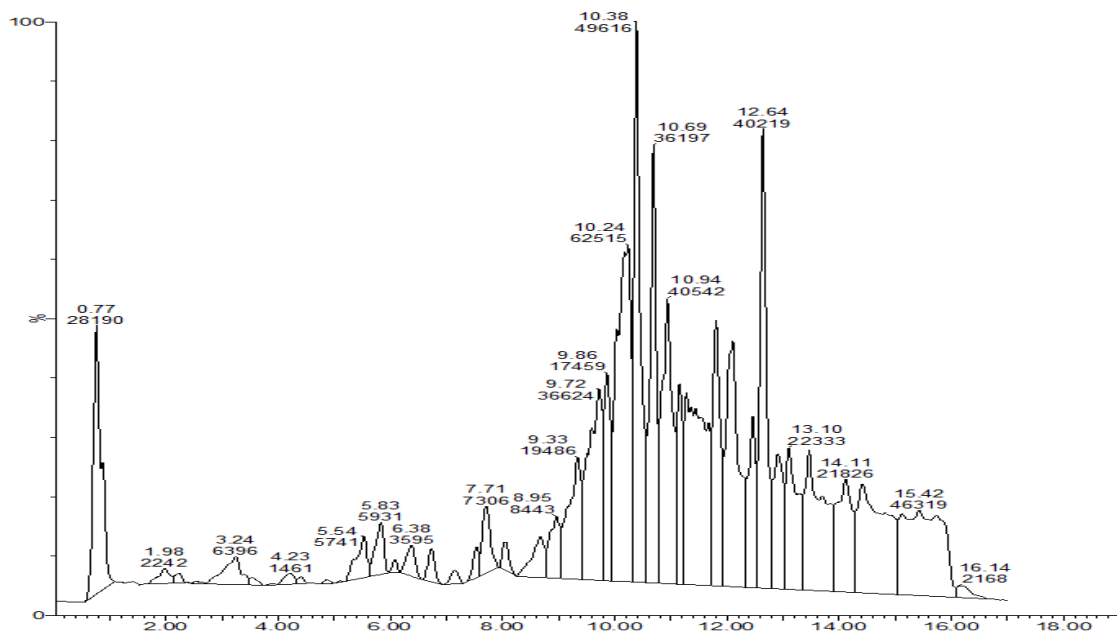

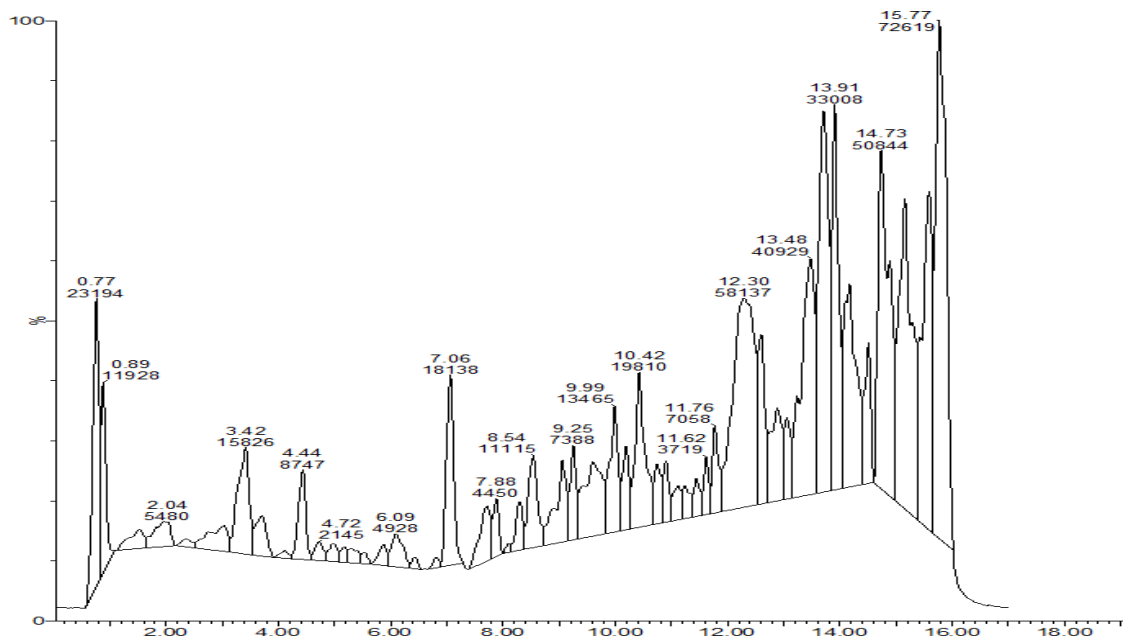**Figure B2** LC-MS chromatogram of the solvent extract of *D. viscosa*.

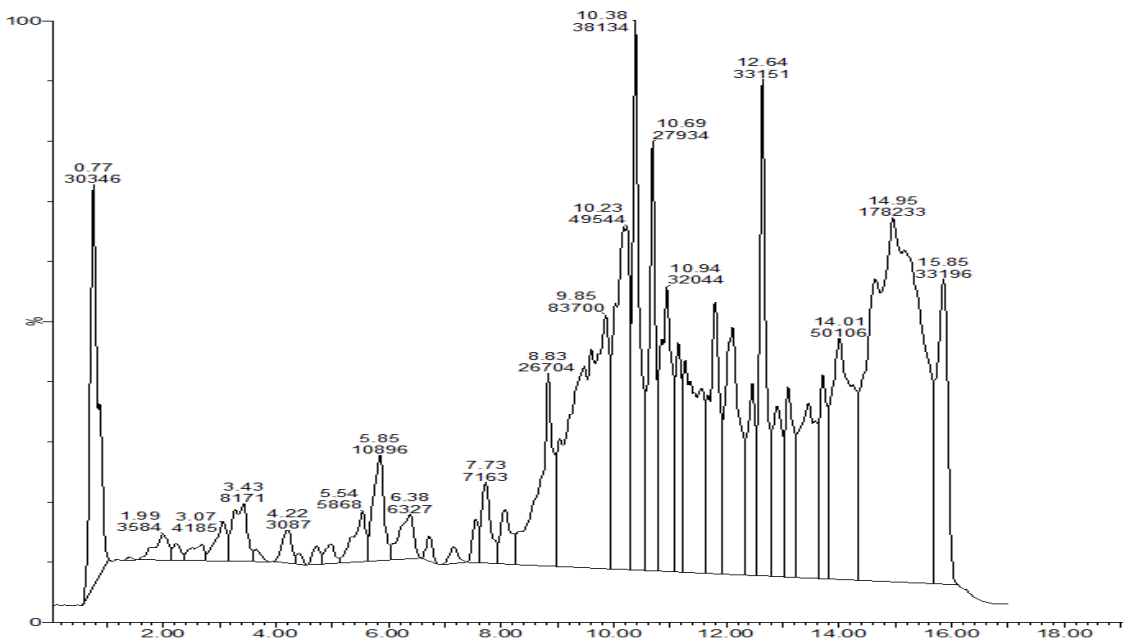**Figure B3** LC-MS chromatogram of the solvent extract of *E. globulus*.

Figure B4 LC-MS chromatogram of the solvent extract of *A. crenulata* with *D. viscosa*.
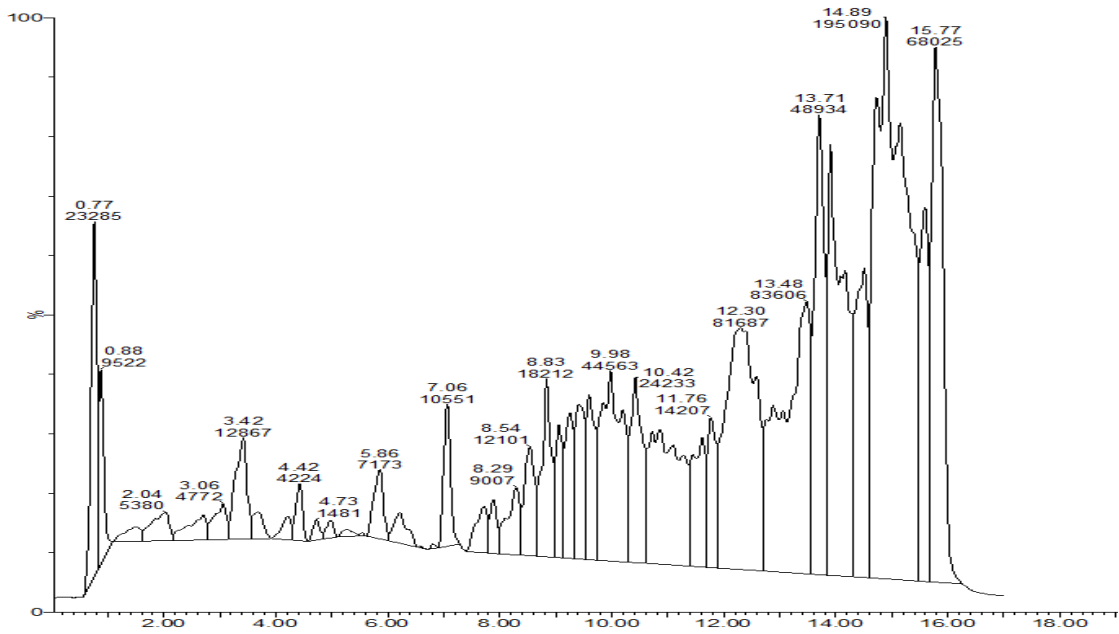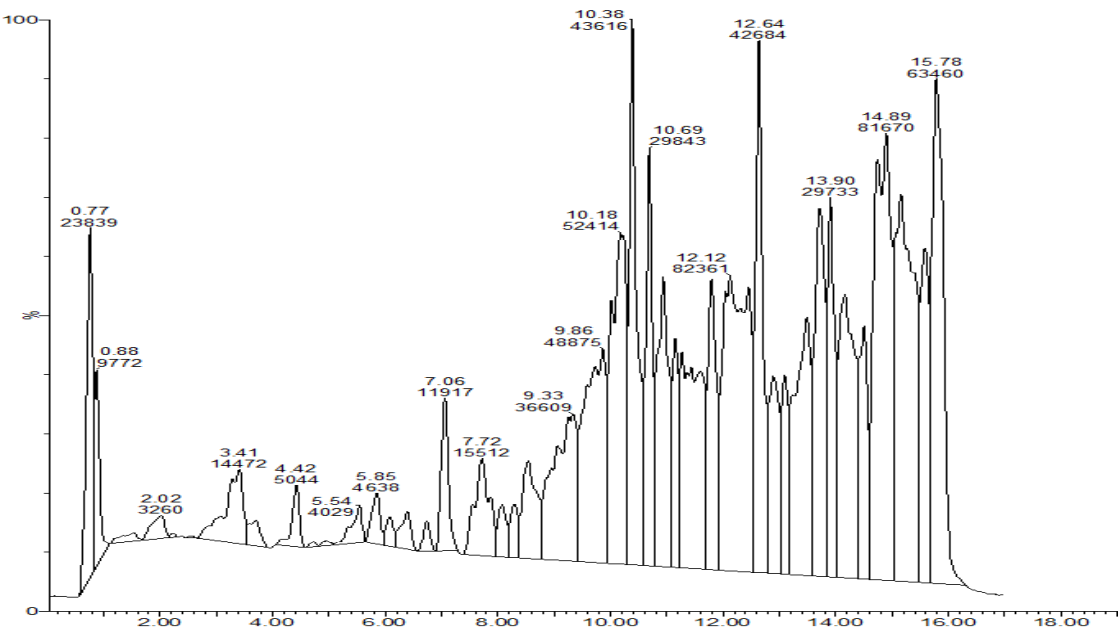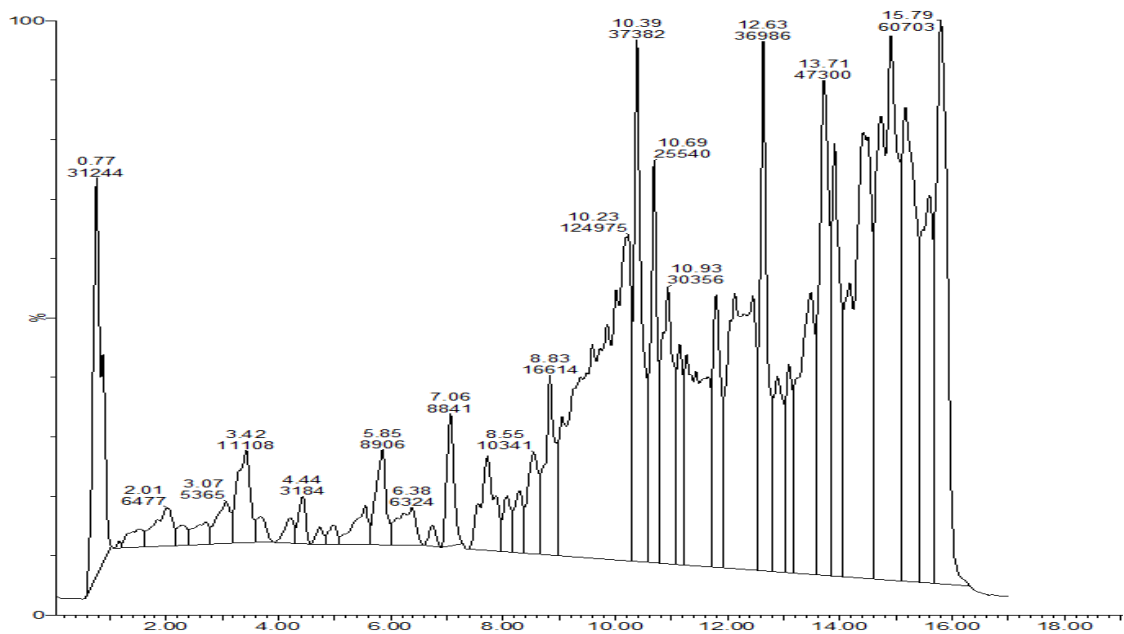
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Figure B5 LC-MS chromatogram of the solvent extract of *A. crenulata* with *E. globulus*.

Figure B6 LC-MS chromatogram of the solvent extract of *D. viscosa* with *E. globulus*.

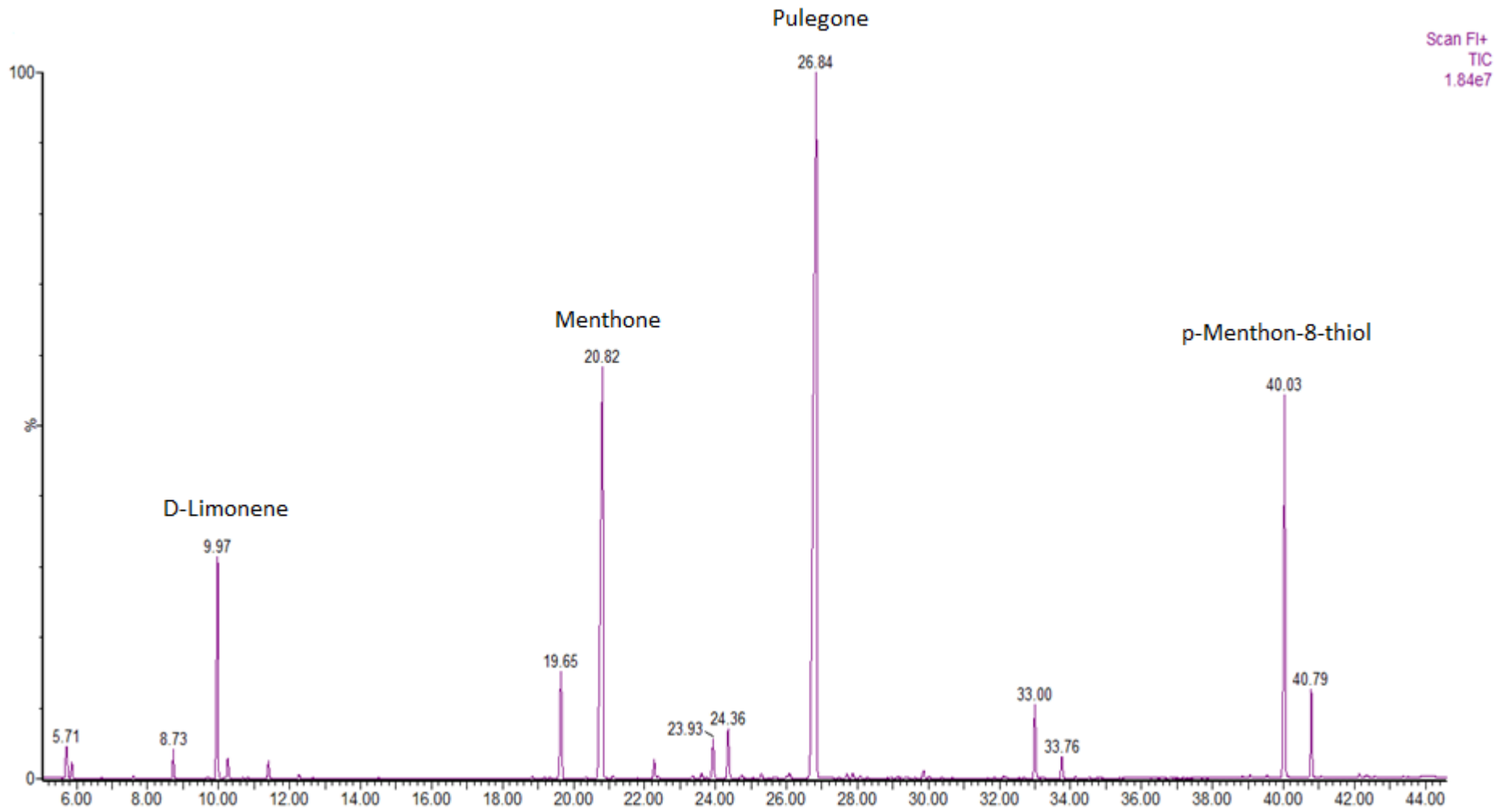**Figure B7** LC-MS chromatogram of the solvent extract of *A. crenulata*, *D. viscosa* and *E. globulus*.

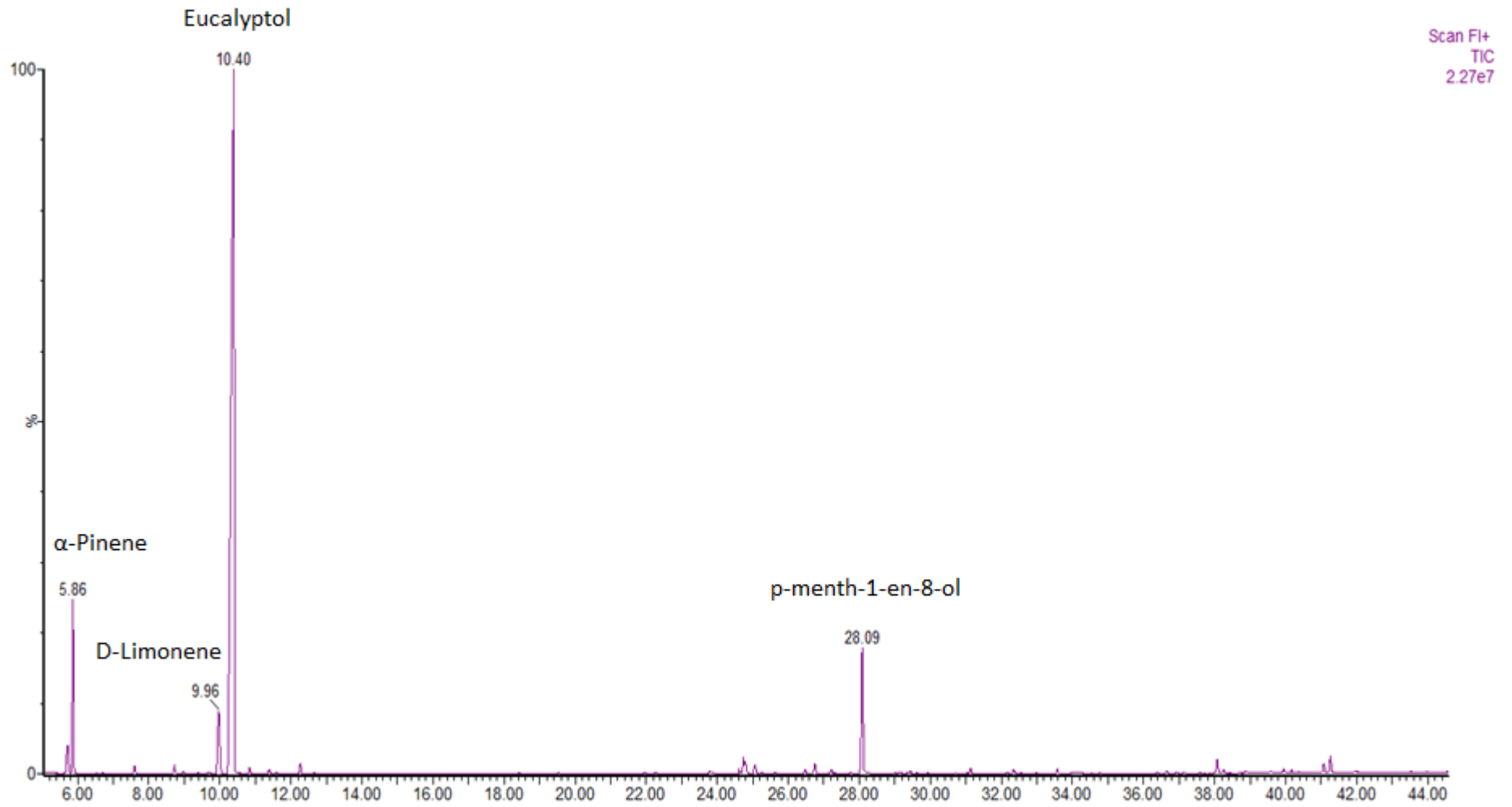**Figure B8** GC-MS chromatogram of the essential oil of *A. crenulata*

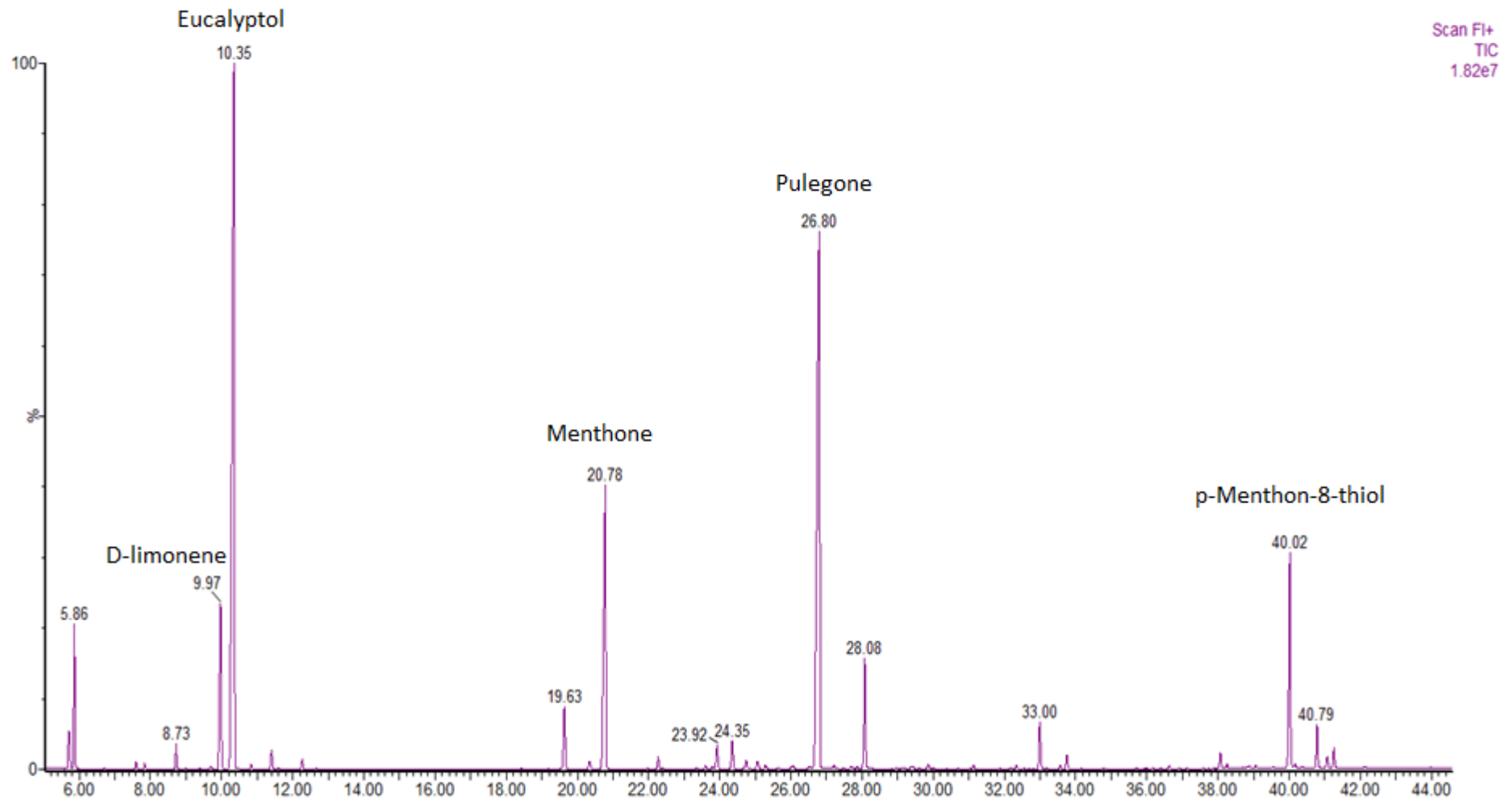**Figure B9** GC-MS chromatogram of the essential oil of *E. globulus*.

Figure B10 GC-MS chromatogram of the essential oil of the double combination of *A. crenulata* with *E. globulus*.