

Metabolomic profiling and micropropagation of *Salvia africana-lutea* L. potent against *Fusarium verticillioides*

by **Mpumelelo Nkomo**

*Dissertation presented for the degree of Doctor of Philosophy in the
Faculty of Science, Department of Botany and Zoology at Stellenbosch
University*



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F gego dgt'2014

Declaration by student

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Declaration by promoters

We hereby declare that we acted as promoters for this PhD 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 Science for examination by the University appointed examiners.

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Dedication

This publication is a dedication to Mr Ndabezinhle Nkomo and Mrs Getrude Nkomo; you believed in me and have pushed me to achieve more than I could ever have dreamt.

Ngiyabonga bazal' bam', ngiyimi lamuhla ngenxa yani. Unkulunkulu alibusise.

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List of outputs from the study

Publications

1. **Nkomo M**, Katerere D R, Vismer H K, Cruz T, Balayssac S, Malet-Martino M, Makunga N P. (Submitted) *Fusarium* inhibition by wild populations of the medicinal plant *Salvia africana-lutea* L. linked to metabolomic profiling. BMC Complementary and Alternative Medicine. MS ID: 1201356122924702

Provisional patent application

Anti-*Fusarium* bioactives identified in *Salvia africana-lutea*

An application has been put through, to protect some of the findings from the study. As this study reports on the first antifungal activity of compounds isolated from *S africana-lutea* extracts.

Conferences

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3. **Nkomo M**, Makunga N, Katerere D.R, Vismer H.F, Malet-Martino M. Antifungal activity of Western Cape *Salvia africana-lutea* populations against *Fusarium* species. Medical Research Council (MRC) Research Day Bellville 24 – 25 October 2011.

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List of Acronyms

%	Percent
>	Greater than
©	Copyright
®	Registered trademark
$\mu\text{g mL}^{-1}$	Micrograms per millilitre
μL	Microliters
BA	Benzyl adenine
CAM	Complementary and Alternative medicine
CD	Circular dichroism
CLA	Carnation leaf agar
DCC	Droplet counter current
DMSO	Dimethyl Sulfoxide
ELEM	Equine leukoencephalomalacia
ESI	Electron spray ionization
FIC	Fractional inhibitory concentration
FID	Free induction decay
g Kg^{-1}	Grams per kilogram
g L^{-1}	Grams per liter
g mL^{-1}	Grams per milliliter
g	Gram
GC-MS	Gas chromatography mass spectrometry
HPLC	High performance liquid chromatography
HSCC	High-speed countercurrent
IR	Infra-red
LC-MS	Liquid chromatography
MHz	Megahertz
MIC	Minimum inhibitory concentration
min	Minutes
mm	Millimeters
MPLC	Medium pressure liquid chromatography
MS	Mass spectrometry

MS media	Murashige and Skoog media
NAA	Napthalene acetic acid
NIST	National institute of standards and technology
NMR	Nuclear magnetic resonance
°C	Degree Celsius
PCA	Principal component analysis
PGR	Plant growth regulators
PLS-DA	Partial least squares discriminant analysis
ppm	Parts per million
rpm	Revolutions per minute
SFE	Supercritical fluid extraction
TM	Traditional medicine
UHPLC-MS	Ultra-high performance liquid chromatography
UPLC-MS	Ultra-performance liquid chromatography
UV	Ultra violet
v/v	Volume to volume
VIP	Variable importance in the projection
w/v	Weight to volume
WHO	World Health Organization
RPMI	Roswell Park Memorial Institute

Abstract

Salvia africana lutea is one of 26 Sage species indigenous to Southern Africa from a total of 900 worldwide. The genus *Salvia* belongs to the Lamiaceae family. Labeled a ‘broad spectrum remedy’ *S. africana lutea* amongst other sage species is medicinally important. Reports are many highlighting its benefits, which include from alleviating coughs and colds to gynaecological complaints. Studies have revealed *in vitro* antimicrobial, anti-cancer and antioxidant activity.

Plant secondary metabolites fundamentally have a strong bearing on the phytochemical activities a plant may possess. Consequently the environment indirectly affects the phytochemical properties as it influences the variation in the plant metabolome via plant-environment interactions. Five *S. africana lutea* plant populations, within the Western Cape province of South Africa were sampled and chemotypes and bioactivity tested. Four populations were wild growing in protected areas namely; Brackenfell, Koeberg, Silwerstroomstrand and Yzerfontein, while the fifth was a garden growing population from Stellenbosch.

Using gas chromatography hyphenated with mass spectrometry (GC-MS), compounds such as monosaccharides, carboxylic acids and fatty acids were detected. Variation of compounds identified with 80% certainty was compared across all populations. Stellenbosch population showed some compounds that were not present in the other four sites. These compounds were namely; propanoic acid, rythronic acid, 2-keto-1-gluconic acid and 1,3-dibromobicyclon, while this population also did not have xylitol that was detected in all the other four populations.

To consolidate the GC-MS findings, analysis on the metabolite profiles (utilizing liquid chromatography linked with mass spectrometry (LC-MS) and nuclear magnetic resonance (^1H NMR)) was done. Principal component analysis (PCA) was applied to the NMR data. The partial least squares-discriminant analysis (PLS-DA) was used to integrate LC-MS and NMR data sets. All statistics were performed with the SIMCA-P+ 12.0 software. By integrating LC-MS and ^1H NMR analyses, large chemotype differences leading to samples grouping by site, suggested strong plant-environment interactions as factors influencing metabolite composition. Signals distinguishing the Stellenbosch profile were in the aromatic part of the ^1H NMR spectra.

Antimicrobial activity was tested against two *Fusarium* species. *Fusarium* is a plant pathogenic species that causes large agricultural losses particularly in the maize crop, one of the staple foods in the African continent. Some species also produce mycotoxins in infected crop and lead to a significant increase in the risk factor of cancers when contaminated foods are consumed. Four high-mycotoxin producing strains from two species *F. verticillioides* (MRC 826 and MRC 8267); *F. proliferatum* (MRC 7140 and MRC 6809) were utilized in all *in vitro* antifungal assays in this study. A preliminary assay using dichloromethane: methanol (1:1 v/v) crude plant extracts of the five populations; Stellenbosch, Brackenfell, Koeberg, Silwerstroomstrand and Yzerfontein, from 2009 and 2011. All test samples exhibited good activity as the minimum inhibitory concentrations (MIC) values ranged from 0.031 mg ml⁻¹ to 0.5 mg ml⁻¹, values below the latter are regarded as strong inhibitors. The Stellenbosch extracts were the most active for both 2009 and 2011 collections, with the best activity against *F. verticillioides* MRC 8267 and MRC 826 at 0.031 mg ml⁻¹. While the least activity, albeit still a strong inhibitor, was observed from the Yzerfontein extracts with an MIC value of 0.5 mg ml⁻¹. Generally comparison between the two years revealed that samples collected in 2011 were more potent than those in 2009, possibly due to prolonged storage that may have resulted in chemical decomposition.

As the Stellenbosch population had shown the best activity as well as a relatively different chemical profile, leaves from these plants were then introduced into tissue culture conditions. Leaf explants were placed on solid plant growth regulator (PGR)-free Murashige and Skoog media and that supplemented with hormones in various combinations. (two concentrations of benzyl adenine (BA) utilized individually and in combination with naphthalene acetic acid (NAA) 4.4 and 8.8, while for NAA 0.27, 2.7 and 5.4.) Namely: 4.4 BA, 8.8 BA, 0.27 NAA: 4.4 BA, 2.7 NAA: 4.4 BA, 5.4 NAA: 4.4 BA, 2.7 NAA: 8.8 BA and 5.4 NAA: 8.8 BA. The PGR combinations did not induce shooting nor rooting, only callus on PGR-free MS media. Antifungal activity of the callus extract was in the same range as the whole plant extracts from which the leaf explants were harvested from, showing no 'loss' of activity after introduction to tissue culture conditions. Metabolite profiles using LC-MS, however, did reveal qualitative and quantitative differences, though they appear to not have any bearing on the activity.

A bioassay-guided fractionation was then conducted on samples collected from Stellenbosch. This led to the identification of carnosol and carnosic acid being involved in the anti-*Fusarium* activity of *S. africana-lutea*. A combinational study revealed no synergistic activity of the two compounds against four *Fusarium* test strains, with fractional inhibitory concentration (FIC) values of 1.5 and 3.0.

Antifungal activity of carnosol and carnosic acid was observed to be in the same range (strong inhibitor) as was the callus and whole plant extracts.

The study showed variation in population chemotypes and identified two compounds that are involved in *S. africana-lutea* activity against *Fusarium* species. It also provided a tissue culture system onto which mass production of the two bioactives may be achieved from, in the development of new fungicides.

Abstraksie (Samevatting of Opsomming)

Salvia Africana lutea is een van die 26 Salie spesies wat inheems aan Suid-Afrika is uit 'n totaal van 900 spesies wêreldwyd. Die genus *Salvia* hoort tot die Lamiaceae familie. *S. africana lutea* word geklassifiseer as 'n "breë spektrum geneesmiddel", en medisinaal as belangrik geag tussen die ander salie spesies, want volgens verslag word dit gebruik vir die verligting van hoes en verkoues tot selfs ginekologiese ongesteldhede. Definitiewe biologiese aktiwiteit eksperimente het anti-mikrobiese, anti-kanker en anti-oksidadant aktiwiteite aan die lig gebring.

Plant sekondêre metaboliete het fundamenteel 'n baie sterk verband met die fitochemiese aktiwiteite van 'n plant. Gevolglik affekteer die omgewing indirek die fitochemiese eienskappe, want dit beïnvloed die variasie in die plant metaboolom deur die interaksies van die plant met die omgewing. In vyf streke binne die Wes-Kaap van Suid-Afrika waar *S. africana lutea* bevolkings voorkom, is steekproewe gedoen en chemotipes en bioaktiwiteit getoets. Vier bevolkings was wild-groeiende bevolkings in beskermdede areas, naamlik; Brackenfell, Koeberg, Silwerstroomstrand en Yzerfontein, terwyl die vyfde uit 'n tuingroeiende bevolking in Stellenbosch geneem is.

Deur gas chromatografie gekoppel met massa spektrometrie te gebruik, is primêre samestellings soos monosakkariede, karboksielsure en vetsure gevind. Variasies van samestellings wat met 80% sekerheid geïdentifiseer is, is oorkruis met al die bevolkings vergelyk. Die Stellenbosch bevolking het 'n paar samestellings geopenbaar wat nie aanwesig was in die ander vier terreine nie. Hierdie samestellings was: propanoësuur, erythroniese suur, 2-keto-1-glukoniese suur en 1,3-dibromobicyclon. Verder het hierdie bevolking geen xylitol gehad nie en dit is in al vier die ander bevolkings gevind.

Verdere studies was gedoen met die gebruik van vloeibare chromatografie gekoppel met massa spektrometrie (LC-MS) sowel as kern magnetiese resonansie (^1H NMR). Chemiese profiele het hoë variasies getoon, en dus deur te fokus op die aromatiese samestelling streke, het die Stellenbosch terrein duidelik merkbare verskille en punte op die PLS-DA aangetoon. Met die koppeling van NMR data met LC-MS data, is daar gevind dat onderskeidende punte van die NMR PLS-DA wat gegropeer is met retensie tye die skeiding van die Stellenbosch terrein van ander terreine gedryf het. Dit het onweerlegbaar bewys dat daar variasie binne die vyf bevolkings voorkom en dat Stellenbosch die mees noemenswaardige chemotipe variasie het.

Dit blyk uit die anti-mikrobiëse eksperimente dat aktiwiteit teen *Fusarium* heel nuwe belangstelling wek. *Fusarium* is 'n plant-patogeniese spesie wat groot landbou verliese veroorsaak veral in die mielie gewasse, een van die stapelvoedsels van die Afrika kontinent. Dit produseer ook mikotoksiene in aangetaste gewasse en hierdie kan lei tot die ontstaan van kankers wanneer besmette voedsel op groot skaal verbruik word. Vier hoë-mikotoksien produserende swamlyne van twee spesies, naamlik *F. verticillioides* (MRC 826 en MRC 8267) en *F. proliferatum* (MRC 7140 en MRC 6809) is gebruik in alle *in vitro* anti-swam ondersoeke in hierdie studie. Die eerste analise het dichloromethan: methanol (1:1 v/v) ongesuiwerde plant ekstrakte bevat van die vyf bevolkings: Stellenbosch, Brackenfell, Koeberg, Silwerstroomstrand en Yzerfontein, geneem gedurende 2009 en 2011. Al hierdie toets monsters het goeie aktiwiteit getoon waar die minimum beperkende konsentrasie (MIC) waardes van 0.031 mg ml^{-1} tot 0.5 mg ml^{-1} gevarieer het. Waardes laer as laasgenoemde word beskou as sterk inhibeerders. Die Stellenbosch ekstrakte was die mees aktief vir albei jare hierbo genoem, met die beste aktiwiteit teen *F. verticillioides* MRC 8267 en MCR 826 by 0.031 mg ml^{-1} . Die minste aktiwiteit (hoewel nog 'n sterk inhibeerder) waargeneem was van die Yzerfontein ekstrakte, met 'n MIC waarde van 0.5 mg ml^{-1} . Oor die algemeen het 'n vergelyking tussen die twee jare aangetoon dat die monsters wat in 2011 versamel is veel sterker was dan dié van 2009, moontlik te wyte aan 'n verlengde bewaringstyd wat moontlik 'n chemiese dekomposisie ten gevolge gehad het.

Omdat die Stellenbosch bevolking die beste aktiwiteit getoon het sowel as 'n relatief afwykende chemiese profiel, is blare van hierdie plante toe bekendgestel aan weefselkultuur kondisies. Blaar eksplante is op soliede hormoonvrye Murashige en Skoog media geplaas en dit is aangevul met sintetiese auksien Naftaleen asynsuur (NAA) en sitokien Bensiel adenien (BA) individueel en in verskillende kombinasies. Geen wortels of uitloopsels is waargeneem in al die hormoon kombinasies nie maar in die hormoonvrye media het daar egter Kallus in twintig persent van die eksplante voorgekom. Kallus is toe as subkultuur van hormoonvrye MS media gekweek en saamgevoeg en dichloromethan: methanol (1:1v/v) ekstrakte is getoets teen die volgende *Fusarium* swamlyne MRC 826; MRC 8267; MRC 7140 en MRC 6809. MIC waardes het sterk inhiberende eienskappe getoon met die laagste waarde as 0.025 mg ml^{-1} teen drie swamlyne: MRC 1740, MRC 8267 en MRC 826, en die hoogste was 0.25 mg ml^{-1} na 48 uur. Die minimum inhiberende konsentrasie waardes het gestyg na 0.5 mg ml^{-1} na 60 uur, wat 'n fungistatiese aksie getoon het. Maar van 60 tot 92 uu het waardes egter 'n swamdodende aksie aangetoon met geen verandering van 0.5 mg ml^{-1} nie.

In die identifisering van die bioaktiewe komponente, is die ekstraksie van Stellenbosch se bevolking in dichloromethan: methanol (1:1 v/v) uitgevoer, en met gebruik van vyftig gram van die ekstrak is bioanalise-geleide fraksionering gedoen deur gebruik van 'n VersaFlash[®]. Die mees aktiewe fraksie is verder gefraksioneer deur die gebruik van 'n konvensionele silikajel kolom. Aktiewe fraksies is getoets deur LC-MS te gebruik, en twee verbindings, carnosol en carnosic suur, is geïdentifiseer. Voorbereidende TLC is gebruik om identiteit te bevestig, want fraksies was naas die kommersiele standaard van die twee verbindings getoets. Sinergistiese aktiwiteit van die twee samestellings is ondersoek deur 'n antiswam ontleding teen die vier swamlyne uit te voer.

Hierdie studie het dus die veronderstelde bestaan van verskillende chemotipes tussen die bevolkings waarvan voorbeelde geneem is, bekend gestel. Veral die Stellenbosch se bevolking het die meeste verskil, heel moontlik omrede die verlengde en hoër versteurings deur die nabyheid van mense. Plant-omgewing interaksies speel 'n belangrike rol in die metaboolom van plante, wat dan indirek hul eienskappe verander, en in hierdie geval die antiswam aktiwiteit. Die tuingroeiende bevolking was die mees aktief, heel moontlik omrede hierdie aspek. Nietemin was geen bioaktiwiteit verloor waar die mees kragtige bevolking met weefsel kultuur kondisies in aanraking gebring is nie. Dus is dit ideaal vir kommersialisering. Een nuwe belangrike bevinding was die carnosol en carnosic suur wat twee welbekende samestellings is wat meesal geassosieer is met *Rosmarinus officinalis* en gedokumenteer is vir antioksidant aktiwiteit. Hier dui laasgenoemde samestellings 'n antiswam aktiwiteit aan teen die getoetste *Fusarium* swamlyne. Met 'n gevestigde weefsel kultuur sisteem alreeds in plek, voorsien dit 'n beginpunt vir die bestudering van hoe hierdie bioaktiewe komponente in massa geproduseer kan word in die ontwikkeling van nuwe swamdodende produkte.

Chapter One

1.1 General introduction

1.2 Traditional, complementary and alternative medicine

The term traditional medicine (TM) in industrialized countries was at some point known as complementary and alternative medicine (CAM) (Hasan et al., 2009). Though more recently CAM has been defined as an umbrella term referring to a broad group of healthcare systems, therapeutic practices and products (including acupuncture, chiropractic, naturopathy, herbal medicine and yoga) that are not traditionally associated with the conventional medical profession (Adams et al., 2011). Some researchers refer to TM as being part of CAM while others use the term traditional, complementary and alternative medicine (TCAM). Traditional, complementary and alternative medicine (TCAM) is then defined as broadly comprising herbal remedies, spiritual practices and prayer, traditional Chinese medicines, acupuncture, acupressure, chiropractic care, massage therapy, meditation, visualization, therapeutic touch and micronutrients (vitamins, minerals, and multivitamins) (Peltzer et al., 2010). The use of TCAM is widespread world over (Shelley et al., 2009) and steadily increasing especially in the developed or high-income countries (Hasan et al., 2009; Quartey et al., 2012).

Developing countries in Africa, Asia and Latin America use TM. It is estimated that over 80% of the populations of these low-income countries are using herbal medicine as part of their basic healthcare (Quartey et al., 2012). In response to the increasing demand for herbal remedies, medicinal plant research has increased (Nkomo and Kambizi 2009). The drive to understand the chemistry behind the herbal remedies has taken precedence in the late 1990s.

1.3 Phytochemistry and herbal medicine

Data from the World Health Organization (WHO) show that 25% of modern medicines are made from plants that were first used traditionally, the most famous being morphine, taking into account the rate of technological advancement in the 21st century there are endless opportunities of discovering novel bioactive compounds (Liu, 2011). Chemical studies of herbal medicines thus

provide fundamental substances for further studies of biological and pharmacological activity (Liu, 2011). This may be achieved through extraction, isolation and structure identification of bioactive compounds. Since the early 1950s, chromatography, including medium pressure liquid chromatography (MPLC) and high performance liquid chromatography (HPLC), and other methods in particular supercritical fluid extraction (SFE), droplet countercurrent (DCC), and high-speed countercurrent (HSCC) have been applied for isolation of natural products. While spectral equipment such as infrared (IR), ultraviolet (UV), nuclear magnetic resonance (NMR), circular dichroism (CD), and mass spectrometer (MS), as well as MS coupled with gas chromatography (GC), have been commonly utilized for structure elucidation. More recently, LC-MS and LC-NMR have become available and gradually more popular as well as other hyphenated forms such as LC-UV/MS (Liu, 2011). With all these advancements, has come with the 'omics' era of importance in this regard is metabolomics.

Metabolomics aims at quantitative analysis and/or qualitative profiling of large numbers of cellular metabolites. It compliments other 'omics' disciplines, like proteomics and transcriptomics, to obtain a holistic view of living systems (Roessner et al., 2001; Bajad and Shulaev 2011). There are essentially two approaches: targeted and untargeted methods. The targeted approach focuses on identifying and quantifying selected metabolites (or metabolite classes), such as substrates of an enzyme, direct products of a protein, a particular class of compound or members of a particular pathway. While the targeted approach is usually hypothesis-driven, untargeted analysis can generate a new hypothesis for further tests by measuring (ideally) all the metabolites of a biological system (Lommen et al., 2007).

Untargeted metabolomics studies typically apply mass spectrometry coupled to a range of diverse chromatographic platforms, including GC and comprehensive GC x GC (Huege et al., 2011), LC-MS, and related advanced hardware including ultra-performance liquid chromatography (UPLC-MS), also referred to as ultra-high performance liquid chromatography (UHPLC-MS) (Spagou et al., 2010). In the use of LC-MS in non-targeted profiling, identification and quantification of all peaks in the chromatogram is attempted even if the chemical structures of many compounds remain unknown. While the use of GC-MS in non-targeted profiling is minor as it is predominately dependent on the mass spectrum reference libraries such as the National Institute of Standards and Technology (NIST) for metabolite identification and tracking (Zhou et al., 2012). However, it provides an important tool when used in the multivariate data analysis to identify compounds detected using other methods such as LC-MS and NMR.

The major advantage of NMR-based metabolomics studies is the general ease and simplicity of the methodology (Powers, 2009). In general biofluids are added to a deuterated aqueous buffer to maintain pH and lock signal, then transferred to an NMR tube and analyzed. The spectra obtained can thus be subjected to multivariate analysis and presented in statistical techniques such as principle component analysis (PCA) thus enabling cross referencing to biological activity and other methods such as LC-MS. This link then provides a starting point in the isolation and identification of bioactive compounds discovered from the metabolite profiling.

Modern extraction, isolation and identification of compounds from herbal remedies are often guided by bioassays to identify bioactives more accurately. This effectively provides the direct link in the chemistry of the herbal remedies to their applications in alleviating ailments. However, it is important to note that while isolating the compounds there is a risk of losing activity. A study by Zonyane and others (2013) showed a cocktail or suite of compounds maybe responsible for perceived activity of a herbal remedy. Contrary to what mainstream drugs usually have a single purified compound being the active ingredient, traditional medicines are usually used as mixtures, thus a suite of compounds is more than likely to be responsible for the activity. However, it essentially becomes of paramount importance in any given study to incorporate bioassay-guided isolation prior to identification of the active compound(s). In the event that activity is due to a suite of compounds their synergistic activity can thus also be investigated.

1.4 Chapter synopsis

The thesis comprises of six chapters, chapters one and two being none experimental chapters and three to five involving the experimental work. Experimental chapters three, four and five are written and presented as articles targeted for journal publication. The contents of the chapters are given below:

Chapter one: General introduction

Here a brief introduction on traditional, complementary and alternative medicine has been given as well as the link between phytochemistry and the herbal medicine. Lastly, there is the thesis structure, and breakdown, dictating what to expect in each of the subsequent chapters.

Chapter two: Literature review

Starting on the medicinal plant use in South Africa to the main plant species studied *Salvia africana-lutea* this chapter paints a picture of the uses and distribution of this plant species as well as the effect of *Fusarium* species. Last but not least, how this may be achieved simultaneously without endangering the wild population by way of tissue culture and the aims and objectives of the project are then listed.

Chapter three: *Fusarium* inhibition by wild populations of the medicinal plant *Salvia africana-lutea* L. linked to metabolomic profiling

This is the first experimental chapter and involves the metabolite profiling of five *S.africana-lutea* populations utilizing GC-MS, LC-MS and NMR, examining the differences in the chemical profiles and later linking this with their bioactivity. Antifungal assays against two *Fusarium* species *F. verticillioides* and *F. proliferatum* are reported in the minimum inhibitory concentrations (MIC).

Chapter four: Identification of bioactives in *Salvia africana-lutea* L. inhibiting *Fusarium* growth.

Upon identifying the most bioactive population from the five sites, deemed the “elite chemotype” isolation of compounds via a bioassay-guided fractionation are reported and discussed in this chapter. Tentative identification using LC-MS after isolation using a VersaFlash® system followed by column fractionation. There after the identified compounds are tested against the same *Fusarium* strains to compare activity with fractions and different combinations evaluated for synergistic properties.

Chapter five: Callus induction from wild *Salvia africana-lutea* L. leaf explants linked to inhibitory activity against two *Fusarium* species

This chapter details the initiation of a tissue culture system of the most bioactive population from the five study sites. The tissue culture protocol adopted one that has been developed for *S. africana-lutea* however, as earlier work was on plant derived from seeds purchased; instead in this chapter explants were obtained from plants from the wild. Products from the tissue culture system namely the callus are tested for bioactivity and compared with results from chapters three and four.

Chapter six: General discussion and conclusion

This chapter sums up all the findings and seeks to tie up the results obtained and match with the objectives and identifying the key findings and future work that may be done.

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Chapter Two

2.1. Literature review

2.2. Medicinal plants in South Africa

South African inhabitants have a long history of exploiting plant diversity, with herbal medicines since time immemorial (Rybicki et al., 2012). This may be supported by current speculation as to the origins of modern humans (Henn et al., 2011), this further re-enforces the use of plants dating long back. The country is well endowed with about 30 000 species of higher plants comprising about 10% of the world total. Around 80% of these are endemic species (Rybicki et al., 2012). From the gross total of higher plant species 10% are used as herbal medicines for a steadily increasing huge market of 27 million people (Light et al., 2005), raising concern on conserving the flora. Plant species that have since become world famous from South Africa, albeit, some controversy with bio-prospecting laws and patent infringements, include; *Pelargonium sidoides*, *Combretum caffrum*, *Sutherlandia frutescens*, and *Hoodia gordonii* to mention a few (Moyo et al., 2013; Drewes and Drewes 2012; Knight et al., 2012). It is no surprise that there is continuously an upsurge of vigorous research in exploiting the rich phytochemical treasure trove in South Africa.

2.3 *Salvia africana-lutea* use and distribution

Salvia species belong to the Lamiaceae family (formerly Labiatae) (Kamatou et al., 2008), encompassing 900 species worldwide of which about 26 are endemic and medicinally important to the Southern African region (Jäger and Van Staden, 2000; Kamatou et al., 2012). The genus name *Salvia* L. is derived from the Latin *salvare* which means ‘to heal or to be safe and unharmed’ referring to the medicinal properties of some of the species (Kamatou et al., 2008). This name was corrupted to *sauge* in French and *sawge* in Old English then eventually to sage, as we commonly know it today (Blumenthal et al., 1998). Common names include; ‘geelblom-salie’ in Afrikaans and beach, dune or golden sage in English (Codd, 1985). Growing two metres tall, this aromatic woody shrub is highly branched with grey-green aromatic leaves that are compacted on hairy or velvety glandular stems. The plant displays a long floral period (June to December) in which inflorescences undergo colorimetric changes from bright yellow to a rusty-orange or mustard colour prior to wilting (Makunga and van Staden, 2008) (**Figure 2.1**).



Figure 2.1 An example of a flowering *Salvia africana-lutea* part[†]

Distribution of *Salvia* species worldwide is sub-cosmopolitan, however, these plants are largely absent in the North and most of the low-lying tropical areas of such as the Amazon basin and central and West Africa (Paton, 1991). Mexico having the highest number of species (approximately 250), the center of origin of the genus is speculated to be Afghanistan and Soviet Central Asia where a larger range of primitive morphological types occur (Paton, 1991; Kamatou et al., 2008). In South Africa, the geographical distribution of *Salvia* species is limited to the southwestern coastal area, extending from Namaqualand to the Cape peninsula and eastwards to the Eastern Cape Province in Port Alfred (Makunga and van Staden, 2008) (**Figure 2.2**).

[†] Picture taken by Mpumelelo Nkomo 2011

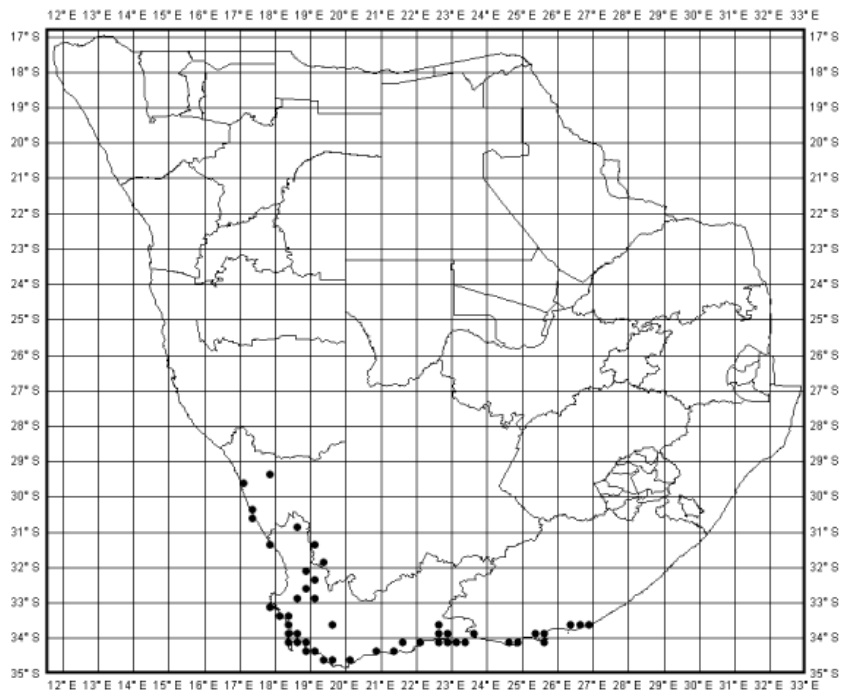


Figure 2.2. Distribution of *Salvia africana-lutea* populations along the coastal regions of South Africa[‡]

Documented traditional uses include use for coughs and colds, sexual debility, mental and nervous conditions, throat inflammation, bronchitis and gynaecological complaints (Watt and Breyer Brandwijk, 1962). These plants are also used as flavoring agents in culinary dishes, aromatherapy, perfumery and cosmetics, as well as insecticides and disinfectants. Studies have shown antimicrobial, anti-cancer and antioxidant activity (Kamatou et al., 2008), to name a few.

2.4 Effects of *Fusarium* species in South Africa

Fusarium species are a widely studied genus due to their ability to infect and cause severe destruction on some small grain crops and maize (*Zea mays* L.). They are ubiquitous hylohyhomycete fungi widespread in a myriad of environments, as source species capable of producing mycotoxins on crops in both the field and in storage grains (Dambolena et al., 2012). These mycotoxins mainly fumonisins and zearalenone are considered to be the main contributors to maize production losses (Queiroz et al., 2012). Fumonisins are produced by a variety of *Fusarium* species with *Fusarium verticillioides* and *F. proliferatum* (Voss, Smith & Haschek 2007) being

[‡] PRECIS Map obtained from the South African Biodiversity Institute (SANBI) 2011

prolific producers of these food-borne carcinogenic mycotoxins. These have been found in products from maize and maize in the field as well as in stored maize products. Maize serves as a major staple food in South Africa. Thus it is a dormant source of mycotoxin exposure, to both humans and animals (Chilaka et al., 2012).

The effects of mycotoxins on animals and humans is collectively termed mycotoxicoses that include carcinogenesis, teratogenicity, immune suppression and growth retardation (Chilaka et al., 2012). The severity of mycotoxin effect is dependent upon the type of mycotoxin and exposure amounts as well as the duration of exposure (Chilaka et al., 2012). These mycotoxins are known to cause several diseases in animals such as equine leukoencephalomalacia (ELEM) and porcine pulmonary oedema (Queiroz et al., 2012). They have also been reported to induce liver and kidney tumours in rodents, and fumonisin B1 is classified as Group 2B “possibly carcinogenic to humans” (International Agency for Research on Cancer, 2002).

Thus the effects of *Fusarium* mycotoxins in both animals and humans, has prompted many countries to implement legislations to regulate acceptable levels of mycotoxins in small grain cereals and maize (Verstraete, 2006; Van Egmond et al., 2007). In South Africa, however, this is not the case as there is no legislation or enforcement to regulate the acceptable levels as with other countries (Fandohan et al., 2003; Shephard 2008); Wagacha and Muthomi, 2008). However, a more recent survey has shown some shifts on *F. verticillioides* being the predominant species requiring attention (Boutigny et al., 2012). The survey further reveals that the shifts may be due to the extensive use of antifungal agents coupled with fluctuations in the climatic conditions being unfavourable for species such as *F. verticillioides*.

2.5 Possible interventions to curb *Fusarium* problem using phytochemicals

Fungicides are biocidal chemical compounds or biological organisms used to kill or inhibit fungi or fungal spores (Haverkate et al., 1969). Currently synthetic fungicides are mostly used. In South Africa *Fusarium* is mostly controlled in maize farming. The most important method of protecting maize against fungal attack is the use of synthetic fungicides, but residues of chemical fungicides in maize and its processed products may cause damage to the health of animals and humans and also affect export potential of these commodities (Deng et al., 2011).

Furthermore, development of resistance of *F. verticillioides* towards synthetic fungicides is of great concern (Mdee et al., 2009). In an attempt to reduce the use of synthetic fungicides, extensive investigations towards the possible exploitation of plant extracts which are safe for human and the environment as alternative to synthetic chemical, have been undertaken over the past two decades worldwide (Quiroga et al., 2009; Weerakkody et al., 2010).

Plants produce biochemicals pivotal in healthcare, food, flavor and cosmetics industries. A wide range of pharmaceuticals, are derived from secondary metabolites of plants. Examples include digitalis, L-DOPA, morphine, codeine, reserpine and the anticancer drugs vincristine, vinblastine and taxol (Onxal[™]) used in the treatment of ovarian and breast cancer (DiCosmo and Misawa, 1995). Thus there seems to be an exponential increase in metabolite research for bioactives to offset the ever-increasing demand for new pharmaceutical drugs. One such group of compounds are the phenolic compounds, they have been studied mostly for their antioxidative properties (Rice-Evans et al., 1997). However, more recent work has shown that phenolic compounds have many other properties, such as anti free radical and antioxidant activity (Baydar and Baydar 2013) and antifungal activity (Dambolena et al., 2012).

Several publications have reported the antifungal activity of some phenolic components of essential oils such as thymol, carvacrol and eugenol on *Fusarium verticillioides* (Menniti et al., 2010; Dambolena et al., 2012). However, little is known about the molecular properties related to the antifungal activity. *Salvia* species have been used in the treatment of a myriad of diseases and ailments. This has been attributed to phenolic compounds in abundance along other secondary metabolites from reviews done by Kamatou and others in 2008. Sage medicinal properties as well as the known phenolic compounds they possess thus provide an unlimited source of novel antifungal bioactives for development into drugs. Thus investigating the metabolome of *S. africana-lutea* is of paramount importance in order to further understand and identify the bioactives pivotal in the growth inhibition (if any) of *Fusarium* species. Upon identification it is then prudent to utilize both sustainable and commercially viable means to mass-produce the bioactives without depleting the wild populations. This may be achieved via means of micro propagation such as tissue culture.

2.6 Possible contribution of tissue culture

Plant tissue culture is fundamentally the aseptic cultivation of any plant section (explants) such as single cell, a tissue or an organ in an enclosed vessel under controlled conditions (Hartmann et al.,

2002). The utilization of such applications has many advantages as reliable and stable high quality bio-actives are produced under controlled conditions without any climatic and geographical limitations (Collin, 2001). Moreover, the yield of the bioactives can be manipulated through genetic alterations (Nigro et al., 2004). These attributes fundamentally lay a good foundation for commercializing the production of *S. africana-lutea* antifungal bioactives industrially.

A micropropagation protocol for *S. africana-lutea* (Makunga and van Staden, 2008) has already been developed, facilitating a starting point. Follow-up work by (Kamatou et al., 2008) showed that induction of hairy root cultures from *S. africana-lutea* by A4T (an *Agrobacterium rhizogenes* strain) resulted in hairy root lines with variable *Fusarium* growth inhibition activity, a phenomenon that the author attributed to differential transgenics, differences in genetic makeup that resulted from insertion of the of bacterial T-DNA on different loci on the plant's genome. However, comparison of these minimum inhibitory concentrations (MIC) with *ex situ* samples from different locations translating to different chemotypes to identify the elite chemotypes is essential. Identification of elite chemotypes to be targeted for micropropagation would provide a wider platform with vast possibilities from more bioactives.

Industrial production to meet commercial demands in such micropropagation systems may be up-scaled by using bioreactor systems. This allows for easily mechanized plant growth and metabolite extraction, ensuring uniformity of final product. The second advantage of rapid large-scale production system of *S. africana-lutea* would be conserving the wild populations.

2.7 Aim and objectives

The aim of the project is to evaluate the chemotypes that may be present in different plant populations and identify the set of metabolites responsible for perceived antifungal activity and to establish a tissue culture system of the most active population.

Objectives:

- Conduct phytochemical profiling using LC-MS, GCMS and NMR techniques on five target populations;
- Conduct *in vitro* antifungal assays on the different populations and identify the most potent;
- Initiate bioassay guided isolation of bioactive compounds from the elite chemotype population; and,
- Establishment and maintenance of a prolific tissue culture system of non-transgenic plants from chemotypes most biologically active.

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Chapter Three

Fusarium inhibition by wild populations of the medicinal plant *Salvia africana-lutea* L. linked to metabolomic profiling

Submitted in this format to BMC Complementary and Alternative medicine Journal and currently undergoing the 2nd revision prior to publication.

Abstract

Background

Salvia africana-lutea L., an important medicinal sage used in the Western Cape (South Africa), can be termed a 'broad-spectrum remedy' suggesting a multiplicity of bioactive metabolites. This study aimed at assessing wild *S. africana-lutea* populations for chemotypic variation and anti-*Fusarium* properties.

Methods

Samples were collected from four wild growing population sites (Yzerfontein, Silwerstroomstrand, Koeberg and Brackenfell) and one garden growing location in Stellenbosch. Their antifungal activities against *Fusarium verticillioides* (strains: MRC 826 and MRC 8267) and *F. proliferatum* (strains: MRC 6908 and MRC 7140) that are aggressive mycotoxigenic phytopathogens were compared using an *in vitro* microdilution assay. To correlate antifungal activity to chemical profiles, three techniques *viz.* Gas chromatography-mass spectrometry (GC-MS); Liquid chromatography-mass spectrometry (LC-MS) and ¹H Nuclear Magnetic Resonance (NMR) were employed. Principal Component Analysis (PCA) was applied to the NMR data. The partial least squares-discriminant analysis (PLS-DA) was used to integrate LC-MS and NMR data sets. All statistics were performed with the SIMCA-P+ 12.0 software.

Results

The dichloromethane:methanol (1:1; v/v) extracts of the plant species collected from Stellenbosch demonstrated the strongest inhibition of *F. verticillioides* and *F. proliferatum* with minimum inhibitory concentration (MIC) values of 0.031 mg ml⁻¹ and 0.063 mg ml⁻¹ respectively. GC-MS

showed four compounds which were unique to the Stellenbosch extracts. By integrating LC-MS and ^1H NMR analyses, large chemotype differences leading to samples grouping by site when a multivariate analysis was performed, suggested strong plant-environment interactions as factors influencing metabolite composition. Signals distinguishing the Stellenbosch profile were in the aromatic part of the ^1H NMR spectra.

Conclusions

This study shows the potential of chemotypes of *Salvia africana-lutea* in controlling fungal growth and consequently mycotoxin production. Products for use in the agricultural sector may be developed from such chemotypes.

Keywords

Salvia africana-lutea, chemotypes, *Fusarium* species, Gas Chromatography-Mass spectrometry (GC-MS), Liquid Chromatography-Mass spectrometry (LC-MS), ^1H Nuclear Magnetic Resonance (NMR)

3.1 Background

Products of secondary metabolism are influenced by different environmental regions and plant-environment interactions [1,2]. Coupled with other factors such as genetic hybridization, studying wild populations for novel bioactives is of paramount importance. Studies of this nature may provide information that can be used in the improvement of the compositional quality of crops and medicinal plants that are utilized as phytomedicines.

Since the 1990s, metabolomics has been employed to hasten discovery of industrially useful chemicals [3] and also as a tool to provide deeper understanding of plant metabolism using systems biology approaches [4]. Since individual plant species have been known to manufacture over 100 000 secondary metabolites [5], robust techniques that are able to analyze these large metabolite numbers in the shortest time possible [3] are useful. The vast numbers also pose a problem in the identification of known and novel compounds. Nuclear magnetic resonance (NMR) is a convenient method for confirming the presence of known biomolecules and for assigning the chemical structure of novel ones. It can measure compounds in crude extracts or *in vivo* in a non-destructive nature [6]. There have been several NMR-based geographical characterizations of plant species, e.g. studies on *Coffea arabica* [7] and on *Withania somnifera* [8]. The advantage of using NMR is that no prior knowledge of the identity or quantity of the individual chemicals within the metabolomic pool is necessary. Therefore, the comparison of NMR profiles to differentiate groups is fast, convenient and a reproducible tool especially since the development of databases and software packages that can handle large datasets [9]. To consolidate NMR metabolite profiles, separation techniques such as gas chromatography and liquid chromatography hyphenated to mass spectrometry (GC-MS and LC-MS) can be utilized. Data generated from these techniques may yield additional information, providing a deeper view of a particular plant metabolome.

In this study, we focused on *Salvia africana-lutea* L. (Lamiaceae; commonly known as the beach or dune sage) which is one of 27 South African sages and 3 naturalized species [10]. In South Africa, the geographical distribution of this species is mainly limited to the south-western coastal area extending from the south-western part of the country through to the Cape Peninsula, and also, eastwards to the Eastern Cape Province in Port Alfred [11,12]. This grey-green aromatic woody shrub, growing to about 2 m, has leaves that store the essential oil in glandular trichomes and it has flowers that are easily recognizable in its reproductive phase (June to December). This plant displays mustard-yellow flowers that progressively become a burnt-orange colour as they senescence [12,13]. Studies on the phytochemical constituents of *Salvia* species originating from

Africa has largely focused on profiling the compounds in the volatile essential oil fraction [14] but few studies have closely examined the non-volatile secondary metabolites.

This particular species is important to the ethnobotanical pharmacopoeia of the Western Cape because it is utilized for a myriad of disease symptoms such as coughs, sexual debility, mental and nervous conditions, throat inflammation, chronic bronchitis, tuberculosis, influenza, stomach ache, diarrhea, and urticaria, amongst others [14,15]. Biological properties reported mainly from *in vitro* experiments include: antibacterial, anti-inflammatory, anticancer and antioxidant activities [16, 17,18,19,20,21,22]. Due to its phytomedicinal properties, commercialization and domestication has been proposed.

Ramogola [23] reported that extracts of *Salvia africana-lutea* inhibited *Fusarium* species. *Fusarium* infections may result in large agricultural production losses and potential contamination with mycotoxins, particularly in maize crops. This paper is a lead up from the study by Ramogola [23] and a more intensive metabolomic investigation of the species. We thus analyzed several different populations of *S. africana-lutea* to determine the extent of chemical variation. We also examined their efficacy against four strains of two *Fusarium* species. Biological activity is an expression of genotypic and phenotypic plasticity that leads to a changed secondary metabolite composition, often influenced by environmental perturbations [24]. This study aimed to assess the different metabolite profiles from the five populations and identify the most biologically active population, to link the activity with the elite chemotype.

3.4 Methods

3.2.1 Plant material

Salvia africana-lutea samples were collected from five different sites: Stellenbosch (S 33° 55.120' E 18° 51.360'), Brackenfell Nature Reserve (S 33° 52.845' E 18° 42.784'), Koeberg Nature Reserve (S 33° 40.128' E 18° 26.524'), Silwerstroomstrand (S 33o 34.632' E 18o 22.349') and Yzerfontein (S 33° 22.309' E 18° 10.871'). All locations are situated in the Western Cape Province of South Africa (Additional file 3.1). In total, 25 samples were collected from the different sites. Samples were identified by Dr Petra Wester and voucher specimens were deposited at the Stellenbosch University Herbarium. The arboreal plant parts were collected at two different times: April 2009 and June 2011. The plant material was oven-dried in closed brown bags at 50 °C then ground to a

fine powder using a mortar and pestle with liquid nitrogen prior to storage in the dark at room temperature.

3.2.2 Extraction of plant material

For each site, extraction was carried out on the dried powdered aerial parts (5 g) with 20 ml of a 1:1 (v/v) methanol:dichloromethane mixture in a 60 ml glass test tube. These were then sonicated for 35 min (Bransonic 220, USA) before filtering with Whatman filter paper number 1. The extraction was repeated twice and pooled extracts were dried using a rotary evaporator (Buchi, Germany) at 55 °C. Extracts were then stored in a desiccator prior to use. Micro-extraction was done on 0.5 g of dried powder using 10 ml of solvent mixture. These were then vortexed for 1 min and sonicated for 30 min. This step was repeated twice prior to centrifuging for 2 min at 4750 revolutions per minute (rpm). All extracts were filtered using cotton wool in a Pasteur pipette and collected in a 10 ml tube. Thereafter, they were evaporated to dryness *in vacuo*. Five extractions were performed for each site at one particular time. The experiments were repeated at least twice; unless otherwise stated.

3.2.3 Fungal isolates and microtitre assay

Isolates of two fungal species *Fusarium verticillioides* (MRC 826 and 8267) and *F. proliferatum* (MRC 7140 and 6908) kept at the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) Unit Culture Collection of the South African Medical Research Council (MRC) were used. The *Fusarium* isolates utilized are classified as high fumonisin B₁ producers. Fungal isolates were grown on Carnation Leaf Agar (CLA) slants for 21 days at 25 °C to induce spore production and stored in a cold room at 4 °C prior to use. Fungal suspensions were prepared by dislodging the conidia in a 20 ml sterile 0.85% (w/v) saline solution. Conidia suspensions were standardized to a 0.5 McFarland concentration. The reference method for broth dilution antifungal susceptibility testing of filamentous fungi as described by the M38-A2 guide of the Clinical and Laboratory Standards Institute [25] was used to determine the minimum inhibitory concentration (MIC) for plant extracts. Each plant extract was resuspended in dimethyl sulfoxide (DMSO) to obtain stock solutions at a concentration of 50 mg ml⁻¹. These were further diluted in the Roswell Park Memorial Institute RPMI-1640 medium at a 1:50 (v/v) ratio to obtain final concentrations of 1.0, 0.5, 0.25, 0.125, 0.063, 0.031, 0.016, 0.008, 0.004 and 0.002 mg ml⁻¹ in the 10 wells. Voriconazole (Vfend[®], Pfizer) was used as a positive control. A row of DMSO and medium was used as a solvent control, while the last negative control had the medium only (growth control).

3.2.4 LC-MS analysis

Five extracts from the different study sites were resuspended in 1 ml of a 50% (v/v) mixture of acetonitrile and H₂O containing 0.1% (v/v) formic acid. The suspensions were vortexed for 1 min then sonicated for 5 min, vortexed again for 1 min prior to spinning at 10 000 rpm for 10 min. The supernatant (3 μ l) was injected into the LC-MS instrument. Metabolites were separated using a gradient of H₂O with 0.1% formic acid (solvent A) and acetonitrile (solvent B), using a Waters UPLC at a flow rate of 0.4 ml min⁻¹ on a Waters BEH C18, 2.1x50 mm column. Mass spectrometry was obtained on a Waters SYNAPT™ G2 MS (Manchester, England) using electron spray ionization (ESI) running in positive mode with a cone voltage of 15 V. The injections were repeated once to ensure repeatability.

3.2.5 NMR analysis

Twenty one dried micro-extracts from five different locations (four from Stellenbosch, four from Yzerfontein, three from Silwerstroomstrand, five from Koeberg and five from Brackenfell) were vortexed for 15 s after the addition of 2.5 ml of DMSO-d₆ (Eurisotop, France). The mixture was filtered and 550 μ l of the filtrate were analyzed. A 10 mM solution of sodium 2,2,3,3-tetradeutero-3-trimethylsilylpropionate (TSP) (Sigma-Aldrich, St. Louis MO, USA) (10 μ l) was added as an internal chemical shift reference before the NMR analysis. One dimensional (1D) ¹H NMR spectra were recorded at 298 K on a Bruker Avance 500 NMR instrument operating at 500.13 MHz, equipped with a 5-mm TCI cryoprobe. The ¹H NMR experiments were acquired using a relaxation delay-pulse-acquisition sequence. Acquisition parameters were as follows: pulse width of 2.8 μ s (flip angle \approx 30°), relaxation delay of 4 s, 64 K data points, spectral width of 9500 Hz (19 ppm) and 128 scans. All FIDs were processed using the Bruker TopSpin 2.1 software with one level of zero-filling and a line broadening of 0.7 Hz. Baseline correction was performed on each spectrum and spectra were referenced to the signal of TSP at δ 0.00 ppm.

3.2.6 Chemometric analysis of the data

The 1D ¹H NMR spectra were transferred to the KnowItAll® software (Bio-Rad, USA). The bin area method was used to segment the spectra between 0 and 13.1 ppm with the variable size intelligent bucketing tool included in the KnowItAll® package. Bucket sizes ranged from 0.01 to 0.30 ppm. The spectral regions containing the NMR signals of DMSO (δ 2.47-2.57 ppm) and its ¹³C satellites (δ 2.36-2.40 ppm, and 2.63-2.67 ppm), methanol (δ 3.17-3.20 ppm), H₂O (δ 3.33-3.45 ppm) and dichloromethane (δ 5.75-5.80 ppm) were removed. A manual filtering procedure was

applied to the whole spectrum to exclude buckets that contained only noise. A total of 119 variables were considered for subsequent statistical analyses. For spectrum normalization, integrated regions were divided by the total area of the spectrum and multiplied by the mean value of the corresponding family previously calculated. Data were preprocessed by mean-centering. The unit variance (UV) scaling method was applied prior to analysis.

Both principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were performed with the SIMCA-P+ 12.0 software (Umetrics, Umeå, Sweden) and for the t-tests, the R software (R Development Core Team, 2012) was used. The predictive ability of the PLS-DA models was assessed from the values of Q^2_{cum} (> 0.5), R^2Y_{cum} (> 0.7) and R^2X_{cum} parameters. The statistical significance of R^2Y and Q^2 parameters was also estimated through the response permutation test where the Y matrix was 999 times randomly permuted when the X matrix was fixed [26]. For determining the discriminating variables between classes, loading plots, coefficient plots, variable importance in the projection (VIP) from PLS-DA models, and p-values ($< 10^{-3}$) of the t-tests on the variables arising from the coefficient plot and VIP were considered. Several PLS-DA models were built: (i) from variables of the whole 1H NMR spectrum (119); (ii) from variables of the 13-5 ppm region of the 1H NMR spectrum (69); and, (iii) from a combination of the 69 variables of the 13-5 ppm region of the 1H NMR spectrum with the 39 variables corresponding to the main peaks of the LC-MS chromatograms.

3.2.7 GC-MS analysis

For each sample 100 mg of ground plant material was utilized. A similar protocol to that used by Glassop et al. [27] was employed with only a minor change for the derivatization of solvent extracts. Myo-inositol (2 mg ml^{-1}) was added to the ground plant material then dissolved in 350 μl of methanol:chloroform (1:1, v/v) in a 2 ml microcentrifuge tube. All sample tubes were placed in a sonicator (Bransonic 220, USA) at room temperature for 45 min. Samples were then centrifuged at 1 200 rpm for 10 min at room temperature in a centrifuge (Biofuge pico, Germany). Ribitol (Sigma-Aldrich; Germany) was included as an internal standard after derivatization. One μl of the samples was injected for a splitless run with an initial temperature of $70 \text{ }^\circ\text{C}$ (5 min) and a maximum oven temperature of $330 \text{ }^\circ\text{C}$ (equilibration time of 0.25 min) was used. Analysis was performed using a network GC system (6890N) coupled to inert XL EI/CI Mass Selective Detector (MSD) 5975B (Agilent Technologies Inc., Palo Alto, CA) equipped with a CTC Analytics PAL autosampler had. Separation was achieved with a capillary column (Restek RTX200; trifluoropropylmethyl (30 m in length; 250 μm diameter; 1 μm in thickness)).

The temperature was increased from 76 °C (1 ramp min⁻¹) to 320 °C (4 ramp min⁻¹) The run time was 72 min and helium gas was used as a carrier at a flow rate of 53.7 ml min⁻¹. The instrument was set to the following conditions: pressure of 62.6 kPa, purge flow of 50 ml min⁻¹ for 2 min, flow rate of 1 ml min⁻¹ (37 cm sec⁻¹) and a data rate of 20 Hz.

The mass spectrometer was operated in electron ionization (EI) mode at ionization energy of 70 eV, scanning from 35 to 600 m/z in positive mode. Caffeic acid, rosmarinic acid, myo-inositol, glucose, galactose and mannose were used as standards to aid with identification of constituents in the extracts. All standards were purchased from Sigma-Aldrich (Germany) except for the mannose which was provided by Merck (Germany). Data were analyzed using the MSD Chemstation software which was linked to the National Institute of Standards and Technology (NIST) mass spectral search program library ver. 2.0 d (2005; standard reference data program of the National Institute of Standards and Technology, USA) for peak identification of metabolites. A library match of 80% and above for metabolites from the NIST library were regarded as likely hits. The relative abundance of metabolites was recorded using the total ion chromatogram peak integration (Additional file 2).

3.3 Results and Discussion

All test plant samples showed good *in vitro* antifungal activity against the test strains of *Fusarium*, with MIC values between 0.031 mg ml⁻¹ and 0.5 mg ml⁻¹ (**Table 3.1**). Although there is no congruency in terms of the classifications used for antifungal *in vitro* assays, several authors [28,29] have suggested that MIC values below 0.5 mg ml⁻¹ should be regarded as representing phytochemical extracts with strong inhibition and above 1.6 mg ml⁻¹ are regarded as weak inhibitors. The Stellenbosch site extract was the most active against the tested fungal strains for the two years (2009 and 2011). It showed the best activity against *F. verticillioides* MRC 8267 and MRC 826 at 0.031 mg ml⁻¹ (**Table 3.1**). This activity compared favourably with the positive control, voriconazole (MIC - 0.0156 mg ml⁻¹) (**Table 3.1**). Our data was similar to that of Ramogola [23] where the strongest inhibition against *F. verticillioides* MRC 8267 (MIC - 0.02 mg ml⁻¹) was reported for extracts produced from Stellenbosch plants. In this study, the least activity against *F. proliferatum* (MRC 6908) at 0.5 mg ml⁻¹ was exhibited by the extract from the Yzerfontein population but this still falls into the “strong inhibitor” category according to the recommendations by Souza et al. [29]. Differences in the MIC values were also associated with years of harvesting. Thus plant material harvested in 2011 was generally more potent than that collected in 2009 (**Table 3.1**). This is likely to be as a result of chemical decomposition due to prolonged storage [30]. This result is not surprising as Kamatou et al. [20] demonstrated the

correlation of seasonal variation on the essential oil composition and biological activity of *S. africana-lutea*.

Table 3.1 Minimum inhibitory concentrations (MIC) values observed after 48 h from crude plant extracts obtained from the five study sites.

Extract/Isolate	Collection year	<i>F. proliferatum</i>		<i>F. verticillioides</i>	
		MRC 6908	MRC 7140	MRC 8267	MRC 826
Stellenbosch	2009	0.125	0.063	0.125	0.125
	2011	0.125	0.125	0.031	0.031
Brackenfell	2009	0.250	0.125	0.125	0.250
	2011	0.250	0.250	0.125	0.250
Koeberg	2009	0.250	0.250	0.125	0.250
	2011	0.250	0.250	0.250	0.250
Silwerstroomstrand	2009	0.250	0.125	0.125	0.250
	2011	0.250	0.250	0.063	0.125
Yzerfontein	2009	0.125	0.250	0.125	0.250
	2011	0.500	0.250	0.063	0.125
Voriconazole		0.0156	0.0156	0.0156	0.0156

MIC results observed after 48 h are regarded as: strong inhibitors ($\text{MIC} < 0.5 \text{ mg ml}^{-1}$); moderate inhibitors ($0.6 \leq \text{MIC} \leq 1.5 \text{ mg ml}^{-1}$) and weak inhibitors ($\text{MIC} > 1.6 \text{ mg ml}^{-1}$) [29].

Seasonal-climatic influences may thus cause great changes in the phytochemical profiles of these plants. In the present study the impact of temporal variation was compared to assess differences between the 2 years in terms of anti-fungal actions as well as chemical constituents bioactivity.

In assessing the chemotype variations, we first analyzed the extracts using GC-MS. Compounds detected were mostly primary metabolites such as monosaccharides, organic acids and fatty acids. This was expected as the derivatization protocol favoured these. **(Fig. 3.1)** displays the distribution of some of the compounds in the sampled sites using an 80% identification limit, while **(Table 3.2)** shows the retention times and Kovats indices. Interestingly, those samples extracted from Stellenbosch exhibited several remarkable differences which discriminated them from the other locations. They contained propanoic acid, rythronic acid, 2-keto-1-gluconic acid and 1,3-dibromobicyclon not detected in the other locations. In contrast, some compounds that were common to all the other samples, such as xylitol, were not observed in the Stellenbosch samples. These compounds may not be directly linked to the difference in antifungal activity from the other sites, but serve to highlight metabolic signatures that distinguish the Stellenbosch samples from the others.

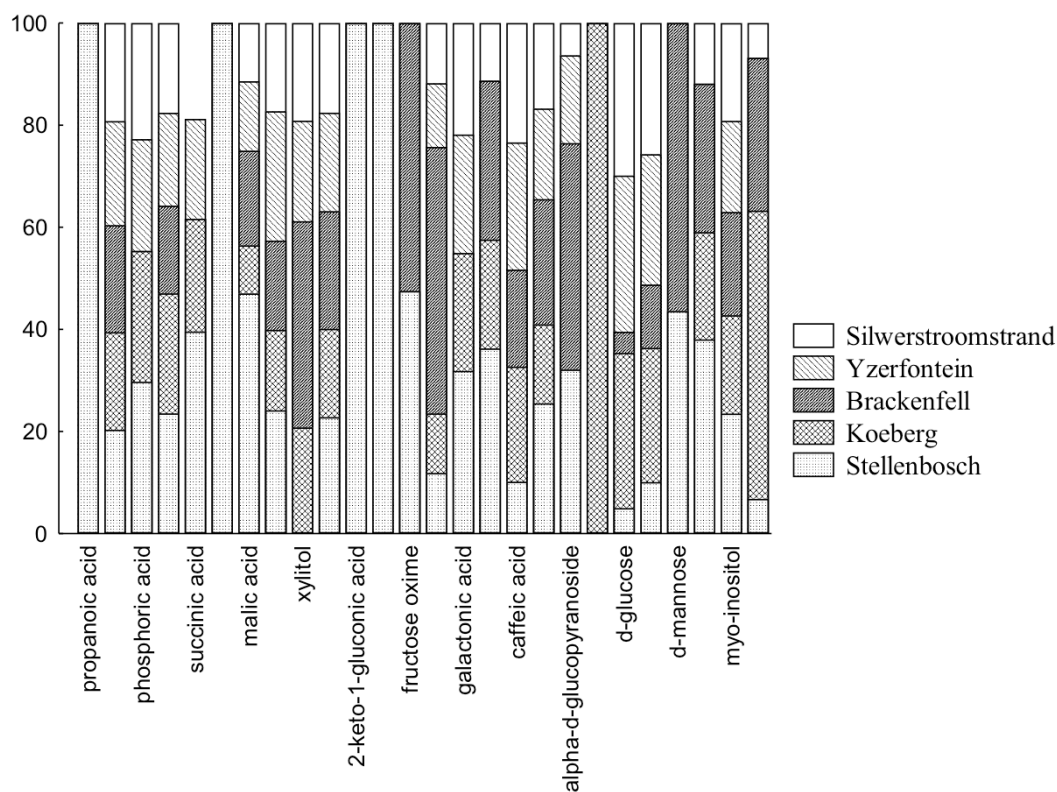


Figure 3.1 Distribution of compounds detected using GC-MS across all sites

Table 3.2 Kovats Indices of compounds identified using GC-MS

Compound	Retention time (min)	Kovats index
Propanoic acid	12.847	1057
Dodecane	19.403	1214
Phosphoric acid	22.188	894
Glycerol	22.386	940
Succinic acid	23.836	1132
Malic acid	29.751	1294
Pentadecane	30.149	1500
Rythronic acid	31.958	1518
Xylitol	36.487	1491
Ribitol	36.641	1491
2-keto-1-gluconic acid	37.616	1748
1,3-dibromobicyclon	38.996	1674
D-fructose	40.452	1726
Fructose oxime	40.709	2323
D-glucose	40.888	1698
D-mannose	41.074	1698
D-galactose	41.408	1698
Sedoheptulose, o-methyloxime	41.812	2533
Galactonic acid	43.403	1991
Hexadecanoic acid	44.737	2047
Myo-inositol	45.706	2152
Mannitol	46.559	1752
Caffeic acid	46.681	1985
Octadecanoic acid	49.138	2238
Alpha-D-glucoopyranoside	56.798	3552
Octacosane	59.922	2800

Chemicals were identified using the NIST library and ribitol was used as the internal standard

For in-depth metabolite profiling especially on secondary metabolites, ^1H NMR and LC-MS were performed (Fig. 3.2 and 3.3).

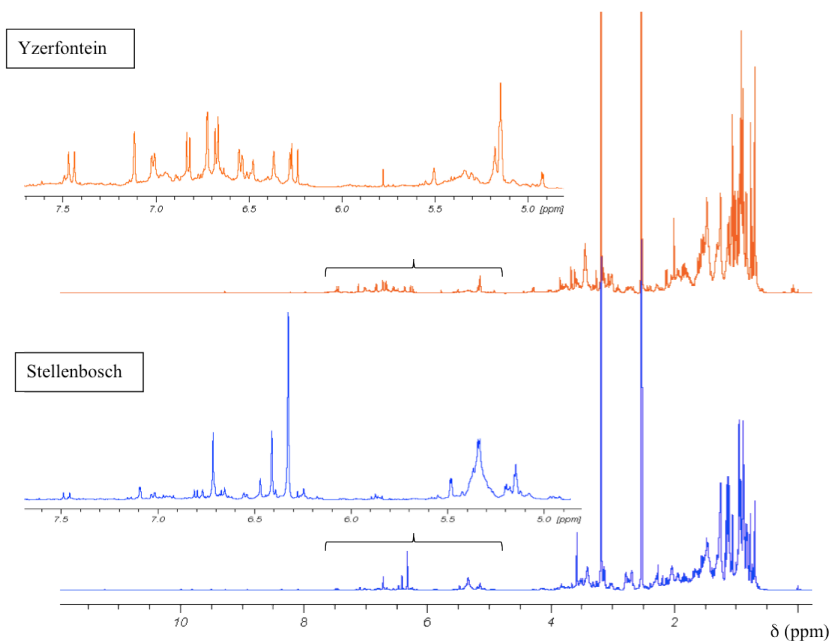


Figure 3.2 Nuclear magnetic resonance spectra of the two most different sample sites

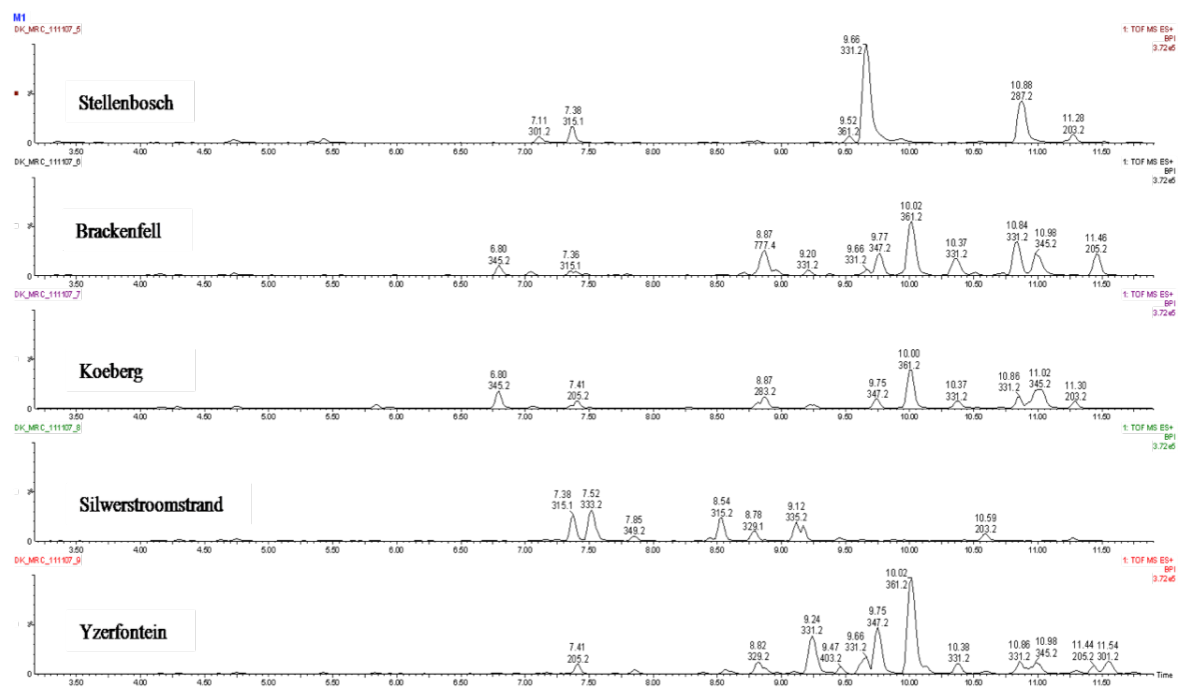


Figure 3.3 LC-MS spectra of all the different sites

Chemical profiles were highly complex especially with NMR and all populations showed differences with both techniques presented for instance on the score plots of the PCA and PLS-DA of ^1H NMR data where 4 and 5 clusters could be observed respectively (Fig. 3.4).

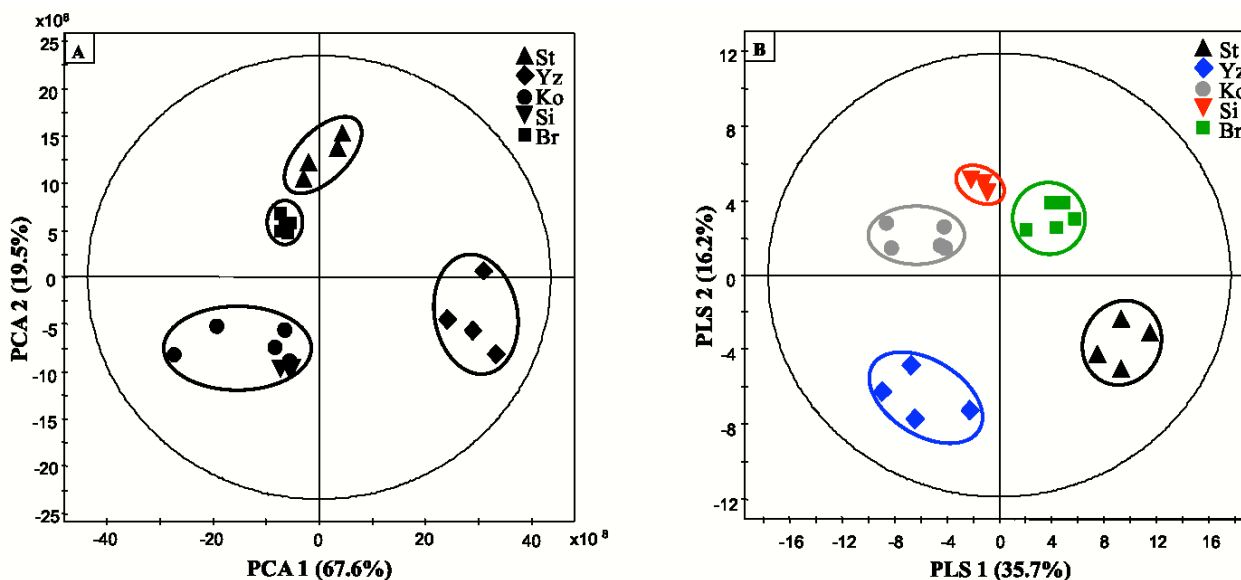


Figure 3.4 Score plots of the PCA (A) (6 axes, R^2X 0.995) and PLS-DA (B) (6 axes, Q^2_{cum} 0.89, R^2X_{cum} 0.88, R^2Y_{cum} 0.97) on ^1H NMR data (entire spectrum) of samples from the 5 sites.

Plants from Stellenbosch are subjected to constant perturbations as they are in close proximity to human dwellings while the other locations are in more protected areas with less interaction. It has been reported that accumulation of aromatic compounds, mainly phenylpropanoids, flavonoids and other such metabolites, which have high antimicrobial action, becomes favoured when plants are under stress [1]. We thus performed a PLS-DA on the 69 variables of the 13-5 ppm region of the ^1H NMR spectra of Stellenbosch samples versus all other samples. The main signals distinguishing the Stellenbosch profile from the others were located at 8.36, 8.28, 7.79, 6.32 and 5.48 ppm (Fig. 3.5). On combining ^1H NMR (69) and LC-MS (39) variables, the separation was driven mainly by the same NMR signals along with five LC peaks with retention times of 7.11, 9.66, 9.93, 10.88 and 12.13 min. We therefore can tentatively state that the Stellenbosch site is markedly different from other sites both in chemical composition and biological (antifungal) activity. The challenge thus far is in linking the discriminating peaks to bioactivity. Work is in progress to determine the structure of these compounds, which might be at least for some of them, flavonoids, and to further confirm the antifungal activity of these pure compounds.

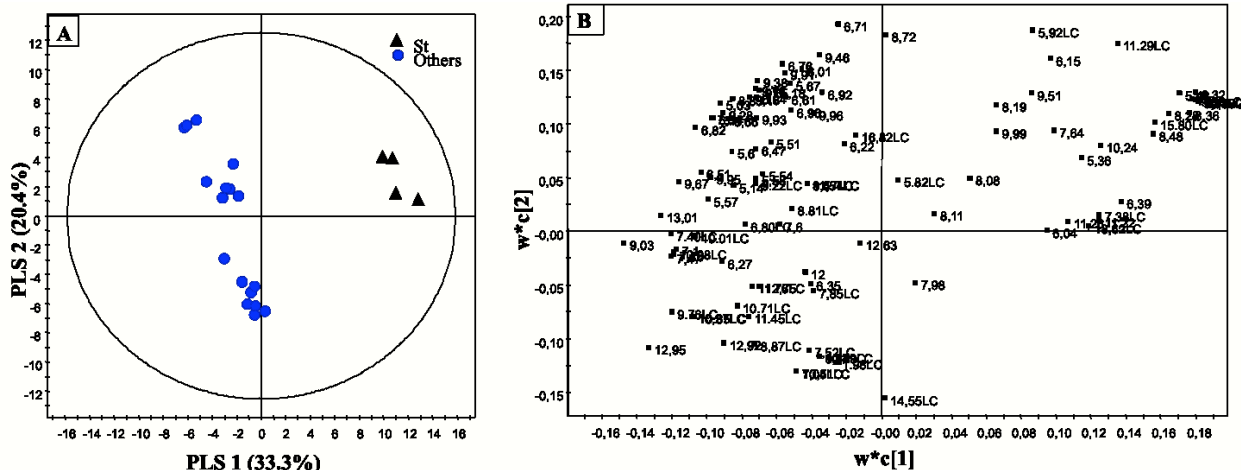


Figure.3.5 Score plot (A) and loading plot (B) of the PLS-DA on ^1H NMR (13-5 ppm region) and LC-MS data of samples from Stellenbosch (denoted as St) versus other sites (validation parameters of the model: 3 axes, Q^2_{cum} 0.96, R^2X_{cum} 0.60, R^2Y_{cum} 0.9

3.4 Conclusion

Salvia africana-lutea extracts have strong anti-*Fusarium* properties and this activity holds potential for product development. This may be of particular interest to manufacturers of agrochemicals. It becomes imperative to follow this study with one that will rigorously correlate the chemical differences to bioactivity in an effort to identify the metabolites responsible for it. Apart from this, a deeper understanding of the chemical constituents which compose the Stellenbosch extracts will be beneficial as part of a commercial and domestication platform for *S. africana-lutea*. Indeed, plant extracts that possess such antimicrobial qualities show great potential for development into chemotherapeutic or preventive drugs and might ultimately replace the current choices at our disposal especially because many of the antifungal agents available in the market may become redundant as microorganisms develop drug tolerance.

3.5 List of Abbreviations

CLA	Carnation leaf agar
DMSO	Dimethyl sulfoxide
ESI	Electron Spray Ionization
MIC	Minimum Inhibitory Concentration
MRC	Medical Research Council
NIST	National Institute of Standards and Technology
PROMEC	Programme on Mycotoxins and Experimental Carcinogenesis
rpm	Revolutions per minute
TSP	Sodium 2,2,3,3-tetradeutero-3-trimethylsilylpropionate
UV	Unit variance
VIP	Variable importance in the projection

3.6 Competing interest

The authors declare that they have no competing interests.

3.7 Acknowledgements

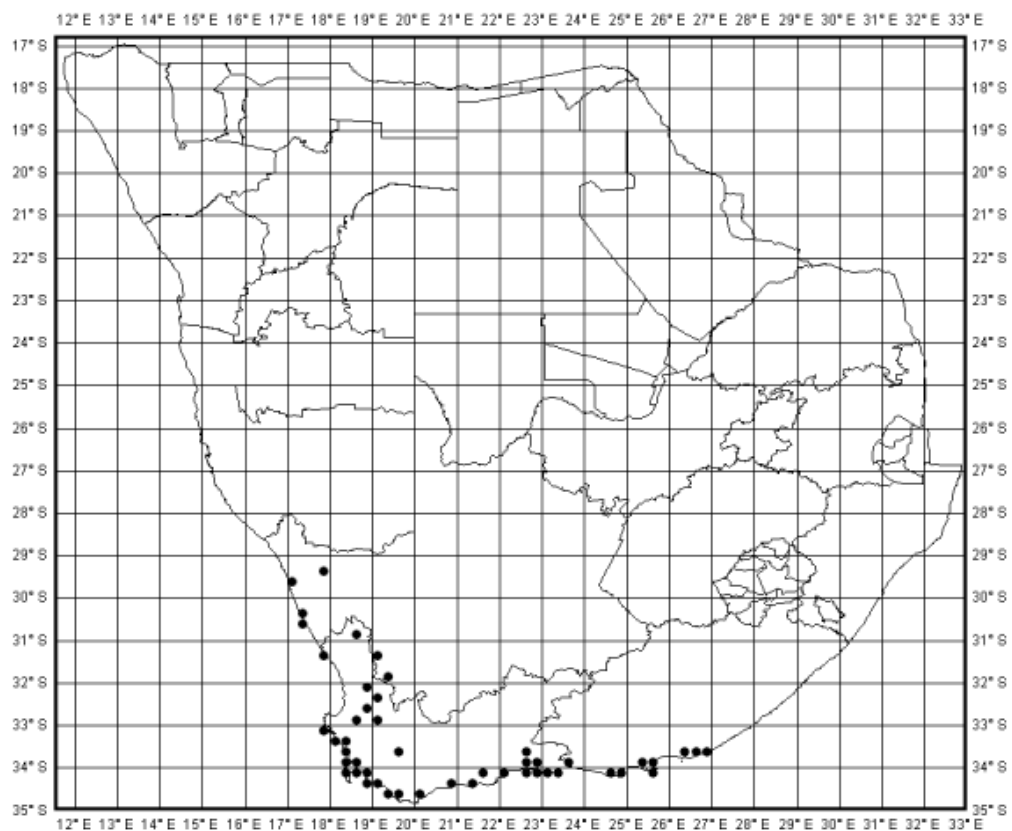
Financial support was obtained from the National Research Foundation (Pretoria; South Africa) and the Division of Research Development (Stellenbosch University; SU). The NRF travel mobility grant was used to visit the NMR Biomedical Laboratory at Toulouse III University. Mr M. Nkomo was a recipient of a postgraduate Department of Botany and Zoology bursary. Cape Nature is thanked for the plant collection permit. Dr Petra Wester, Mr Lucky Mokoena and Mr Fletcher Hiten of the central analytical facility (SU) are thanked for their assistance. Professor Véronique Gilard (Toulouse III) assisted with analyzing the LC-MS data. This work was partly conducted at the Medical Research Council (Tygerberg, Cape Town) and the PROMEC unit is thanked for use of their facilities. We are grateful to Ms Lorraine Moses and Ms Gail Imrie for assistance with technical aspects related to antifungal assays.

3.8 References

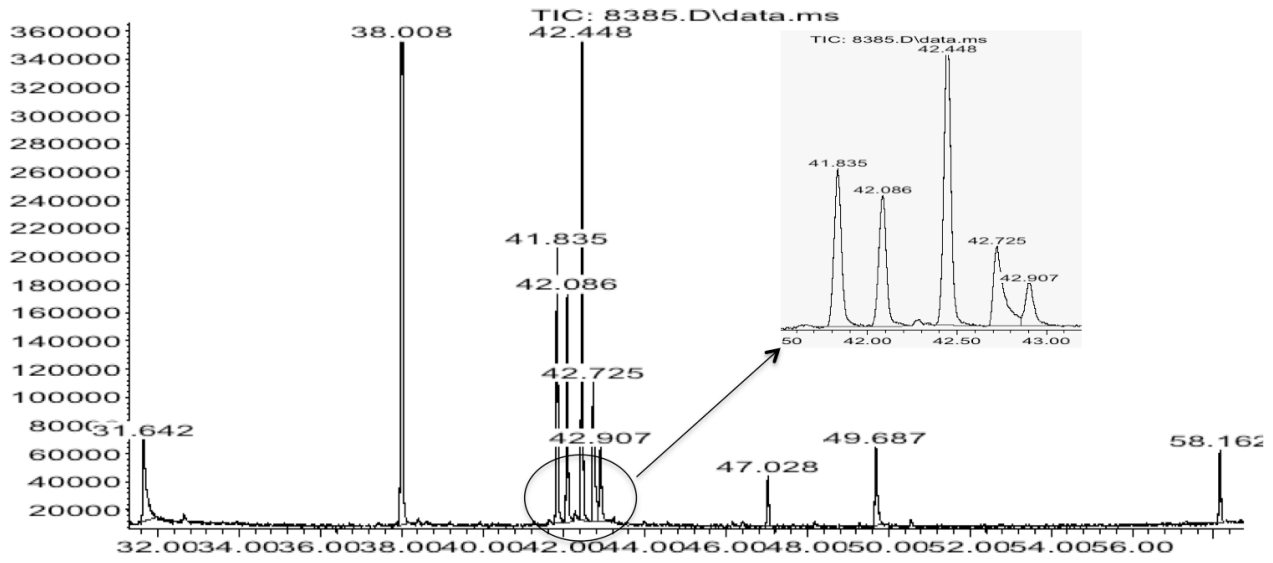
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Additional file 3.1 Distribution of *Salvia africana-lutea* populations along the coastal regions of South Africa



Additional file 3.2 Total ion chromatogram peak integration of Gas chromatography-mass spectrometry

Chapter Four

Anti-fungal activity of *Salvia africana-lutea* L. extracts inhibiting *Fusarium* growth.

Abstract

Salvia africana-lutea L., a medicinal dune sage used in the Western Cape (South Africa), known to possess a multiplicity of bioactive metabolites, was studied with the aim of identifying bioactives against *Fusarium verticillioides* (strains: MRC 826 and MRC 8267) and *F. proliferatum* (strains: MRC 6908 and MRC 7140) through a bioassay guided fractionation. The chosen strains are aggressive mycotoxigenic phytopathogens and there were compared using an *in vitro* micro-dilution assay. Activity ranged between 0.031 mg ml⁻¹ minimum inhibitory concentration (MIC) to 0.500 mg ml⁻¹, was followed in the resulting fractions and two compounds were ultimately identified; carnosol and carnosic acid. After, a combinational test was done, and the fractional inhibitory concentrations (FIC) of 1.5 to 3.0 rendering the two 'indifferent' or not having any synergism. The study did reveal the bioactives responsible for the fungistatic activity, and the potential for fungicide production.

Keywords

Salvia africana-lutea, chemotypes, *Fusarium* species, carnosol, carnosic acid, Liquid Chromatography-Mass spectrometry (LC-MS), Fractional inhibitory concentrations (FIC), synergism

4.1 Introduction

The plant kingdom represents an extraordinary reservoir of novel molecules (Hostettmann and Wolfender, 1997). One characteristic of complex natural products derived from the plant kingdom is their diversity of secondary metabolites (Dobson, 2004; Koch et al., 2005), which co-exist with the complex primary metabolome of the living world (Inui et al., 2012).

The role of plants as a drug discovery source has been increasingly acknowledged in terms of bioactive (ethno) botanicals and complementary medicines (Balubas and Kinghorn, 2005; Tyler, 1999), previously discussed in Section 1.2. In identifying bioactives, isolation and characterization of individual active compounds is key in the evaluation of these biologically active mixtures. The most widely accepted method is bioassay-guided fractionation (BGF) (Inui et al., 2012).

Bioassay-guided fractionation involves the use of biological activity to direct prioritization of fractionation prior to compound identification. In this study fractionation was achieved using a VersaFlash® system to fractionate the extracts prior to a silica column. While in the identification of the major bioactive principles thin layer chromatography (TLC), preparative TLC, liquid chromatography coupled to mass spectroscopy (LC-MS) and nuclear magnetic resonance (NMR) were utilized. Upon separation, identification of compounds becomes an achievable feat. Thus one part of this chapter aimed at identifying the bioactive compounds responsible for the antifungal activity of the Stellenbosch extracts.

In this study, the bioactivity of interest is against *Fusarium* species. As has been mentioned earlier *Fusarium* effects on both humans and animals after ingestion of contaminated food(s). The most common way of intervention is the use of fungicides and/fertilizers. However, this has been shown in some cases that if not applied properly may actually worsen the situation as mycotoxin production is triggered when fungi are under stress (Edward, 2004). The other problem that arises with use of such synthetic chemicals (fungicides) is that they are expensive and usually not biodegradable raising environmental threats and are prone to increasing resistance by the fungi (Wilson et al., 1997).

Plants have been used in combination in traditional and alternative medicine with great success (Gathirwa et al., 2008). Drugs are also used in combinations called cocktails to treat disease, particularly cancer and viral infections. Most antimicrobial combinations are known to prevent antibiotic resistance (Wagner and Ulrich-Merzenich, 2009). Any two or more drugs, or for that matter, two or more bioactive plant compounds, will either interact in some way or fail to interact (Boik et al., 2009). It is postulated that the myriad of bioactives in the multi-herb extracts target or interact with different parts of collective system. Some bioactives may for example disrupt the cell membrane, while another interferes with the biosynthesis of vital compounds.

If an interaction produces an effect greater than that expected for each individual drug, the interaction is termed synergistic. “Synergy” is also generally defined as the mechanism where the overall cumulative effect of a combination is greater than expected from the sum of their individual effects (Williamson, 2001). If the effect is less than expected, it is termed antagonistic. If the effect is equal to the expected effect (i.e., there is no interaction), the interaction is termed additive (Cseke et al., 2006).

Owing to this, interest in seeking a scientific rationale for superior therapeutic multi-herb extracts has risen (Zonyane et al., 2013). Thus the last part of this chapter, examines the antifungal activity of carnosol and carnosic acid, identified from fractions obtained via a bioassay-guided fractionation of a methanol: dichloromethane 1:1 (v/v) *Salvia africana-lutea* extract. Lastly, comparing their individual activities and combinations to assess whether there is any synergistic, additive or antagonistic relationship.

4.2 Materials and methods

4.2.1 Plant material

This section is similar to 3.2.1 however, only *S. africana-lutea* samples from Stellenbosch (S 33° 55.120' E 18° 51.360'), Western Cape Province of South Africa in June 2011 were used. Plant material was oven-dried in closed paper bags at 50 °C, ground to a fine powder using a mortar and pestle with liquid nitrogen prior to storage in the dark at room temperature.

4.2.2 Extract preparation

Ground plant powder weighing 400 g was dissolved in 2000 mL of a 1:1 (v:v) solvent solution of dichloromethane (DCM): methanol and placed on an orbital shaker for 48 h, followed by filtration using a Buchner funnel and Whatman filter paper No 1. These two steps were repeated three times. Pooled filtrates were concentrated to dryness under reduced pressure at a maximum of 55 °C using a rotating evaporator (Buchi, Germany).

4.2.3 Fractionation

Fifty grams of plant extract was resuspended in 150 mL of a 1:1 (v/v) solvent solution of acetone: methanol and sonicated for 30 min (Branson 220, USA). Diaion (Sigma-Aldrich, St. Louis MO, USA) was added to bind with the non-polar compounds. This was placed in a rotary evaporator (Buchi, Germany) to form a paste. These steps were repeated twice. The paste was then filtered in 6 different solutions consecutively in the following order: Solution A [2 000 mL (10% acetone in methanol)], B [1 000 mL (25% acetone in methanol)], C [1 000 mL (50% acetone in methanol)], D [1 000 mL (50% acetone in dichloromethane)], E [1 000 mL of dichloromethane] and F [1 000 mL of hexane]. Filtrates were concentrated to dryness under reduced pressure at 55 °C using a rotor vapour (Buchi, Germany). Thin layer chromatography (TLC) profiles of the 6 different filtrate profiles were ran in 2 different separation systems: CEF [chloroform (5): ethyl acetate (4): formic acid (1)] and EMW [ethyl acetate (4): methanol (5.4): water (4)]. The dried filtrate A product was ran in a Versa Flash (Sigma-Aldrich, Supelco, USA), with 20 g loaded onto a silica filled cartridge. Fraction A was chosen as it had the largest quantity in comparison to the rest although they all had similar activity Eight solvents were ran as follows: A1 [hexane: DCM (80:20)], A2 [hexane: DCM (50:50)], A3 [hexane: DCM (20:80)], A4 [DCM: methanol (90:10)], A5 [DCM: methanol (80:20)] and A6 [DCM: methanol (50:50)]. Filtrates were collected in numbered 50 mL test tubes that were left at room temperature to dry in a fume hood. Changing between solvents was done only when the filtrate TLC profile remained unchanged after 10 test tubes and appeared as the solvent was.

The product of A4 [DCM: methanol (90:10)] collected in test tubes 15 to 28 was run in a small silica column. With 0.7 g mixed with to methanol to dissolve then in chloroform to form a slurry was poured onto the column and ran with four different eluents: A5.1 chloroform (1): ethyl acetate (3)], A5.2 [chloroform (1): ethyl acetate (9)], A5.3 ethyl acetate and A5.4 [ethyl acetate (19): methanol (1)].

Filtrates collected in numbered 25 mL test tubes, which were left at room temperature in a fume hood to dry.

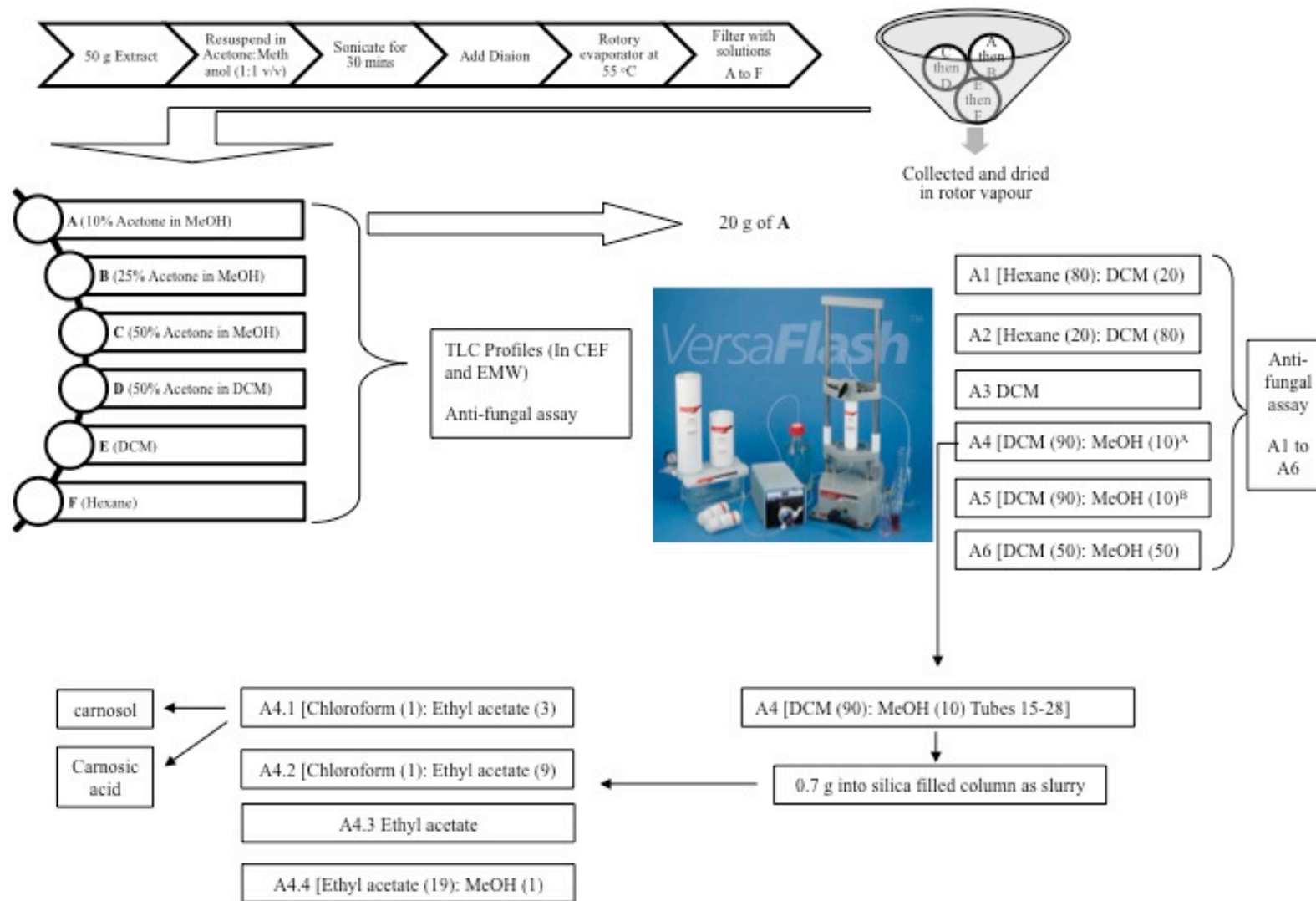


Figure 4.1 Fractionation schematic flow chart

4.2.4 Fungal isolates and antifungal assay on fractions

The method used is as described in section 3.2.3. Isolates of two fungal species *Fusarium verticillioides* (MRC 826 and 8267) and *F. proliferatum* (MRC 7140 and 6908) kept at the Program on Mycotoxins and Experimental Carcinogenesis (PROMEC) Unit Culture Collection, of the African Medical Research Council (MRC) were used. The *Fusarium* isolates utilized are classified as high fumonisin B1 producers. Fungal isolates were grown on Carnation Leaf Agar (CLA) slants for 7 days at 25 °C to induce spore production and stored in a cold room at 4 °C prior to use. Conidia suspensions were prepared by dislodging the conidia in a 20 ml sterile 0.85% (w/v) saline solution. Conidia suspensions were standardized to a 0.5 McFarland concentration.

The reference method for broth dilution antifungal susceptibility testing of filamentous fungi is described by the M38-A2 guide of the Clinical and Laboratory Standards Institute (2008) was used to determine the minimum inhibitory concentration (MIC) for fraction extracts. Extract fractions obtained with solvents A to F all were tested while, only six of the eight obtained from Fraction A were tested (excluding the two 50:50 ratio combinations and the 35:65). Each extract was resuspended in dimethyl sulfoxide (DMSO) to obtain stock solutions of 50 mg ml⁻¹. These were further diluted in the RPMI-1640 medium at a 1:50 (v/v) ratio to obtain final concentrations of 0.5, 0.25, 0.125, 0.063, 0.031, 0.016, 0.008, 0.004 and 0.002 in the 10 wells. Voriconazole (Vfend, Pfizer) was used as a positive control. A row of DMSO and medium was used as a solvent control while the last negative control had the medium only. With 50 µl of 0.2 mg ml⁻¹ *p*-iodonitrotetraazol violet being added after 48 h as an indicator.

4.2.5.1 Antifungal assay of pure compounds

The method is similar to that described in section 3.2.3 with slight alterations. Three isolates of fungal species, two from *Fusarium verticillioides* (MRC 826 and 8267) and one strain *F. proliferatum* (MRC 7140) were used. The reference strain used was *Fusarium verticillioides* (Saccardo) Nirenberg (ATCC[®] MYA-3629[™]) obtained from the American Type Culture Collection in Manassas, Virginia. Fungal suspensions were prepared by dislodging the conidia in a 10 ml sterile 0.85% (w/v) saline solution. Conidia suspensions were standardized to a 0.5 McFarland concentration.

The reference method described by the M38-A2 guide of the Clinical and Laboratory Standards Institute (2008) was used to determine the minimum inhibitory concentration (MIC), with alterations on to the dilution series. Carnosol and carnosic acid obtained from Sigma-Aldrich ®. were resuspended in dimethyl sulfoxide (DMSO) to obtain a 1000 $\mu\text{g ml}^{-1}$ stock solution. This was further diluted in the Roswell Park Memorial Institute RPMI-1640 medium at a 1:2 (v/v) ratio to obtain final concentrations of 500, 250, 125, 62.5, 31.25, 15.625, 7.8125, 3.9063, 1.9531 and 0.9766 $\mu\text{g ml}^{-1}$. These concentrations were further diluted with the inoculum consisting of the fungal spores in RPMI-1640 media, to final concentrations in the wells: 250, 125, 62.5, 31.25, 15.625, 7.8125, 3.9063, 1.9531, 0.9766 and 0.4883 $\mu\text{g ml}^{-1}$. Voriconazole (Vfend®, Pfizer) was used as a positive control. The stock solution was also made by dissolving in DMSO to form a 32 $\mu\text{g ml}^{-1}$ solution. Serially diluted in 10 test tubes to a concentration of 0.0625 $\mu\text{g ml}^{-1}$. This was further diluted with the inoculum to achieve the following concentrations in the 10 wells: 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.0313 $\mu\text{g ml}^{-1}$. Row 11 comprised, inoculum: RPMI (1:50) and DMSO: RPMI (1: 50), while row 12 had inoculum: RPMI (1: 50) and RPMI media. While the same indicator volume and concentration was used after 48 h.

4.2.5.2 Compound combination study

Pure compounds were obtained from Sigma-Aldrich ®. Stock solutions were made to a concentration of 50 mg ml^{-1} and further diluted in RPMI-1640 media at a 1:50 (v/v) ration, were mixed as follows; (1:1; v/v), (2:1; v/v), (1:2; v/v) and tested individually. The MIC values were recorded for each of the combinations whereby the pharmacological interaction was calculated using the following equations;

Equation 4.1 Fractional inhibitory concentration of carnosic acid

$$FIC_I = \frac{\text{MIC (Carnosol) in combination with (Carnosic acid)}}{\text{MIC Carnosol independently}}$$

Equation 4.2 Fractional inhibitory concentration of carnosol

$$FIC_{II} = \frac{\text{MIC (Carnosic acid) in combination with (Carnosol)}}{\text{MIC Carnosic acid independently}}$$

The sum of the FIC (Σ FIC) was calculated as:

Equation 4.3 Sum of the Fractional inhibitory concentration (Σ FIC)

$$\Sigma\text{FIC} = \text{FIC}_I + \text{FIC}_{II}$$

The summation of the FIC values, were interpreted as synergistic ($\Sigma\text{FIC} < 0.5$), additive ($0.5 > \Sigma\text{FIC} > 1$), indifferent ($1 > \Sigma\text{FIC} > 4$), or ($\Sigma\text{FIC} > 4$) as antagonistic interactions (Van Vuuren and Viljoen, 2011).

4.2.6 LC-MS analysis

This method is as described above in section 3.2.4. Extracts and pure compounds were re-suspended in 50% (v/v) mixture of acetonitrile and H₂O containing 0.1% (v/v) formic acid, and then further diluted in 50% (v/v) mixture of acetonitrile (H₂O). Metabolites were separated using a gradient of H₂O with 0.1 % formic acid (solvent A) and acetonitrile (solvent B), using a Waters UPLC at a flow rate of 0.4 ml min⁻¹ on a Waters BEH C18, 2.1x50 mm column. Mass spectrometry was obtained on a Waters SYNAPT™ G2 MS (Manchester, England) using electron spray ionization (ESI) running in a negative mode with a UPLC (Waters).

4.2.7 Preparative Thin Layer Chromatography

To confirm the identity of carnosol and carnosic acid results obtained from LC-MS, two dried extract fractions were re-suspended separately in methanol, along with carnosol and carnosic acid (Merck, Germany). Twenty microliters of the suspensions were spotted on PLC Silica gel 60 F254 plates, (Merck, Germany) and placed in a tank containing a solution of hexane and ethyl acetate in the ration

of 7:3. Bands that eluted with an R_f value of 0.40 were scraped off and dissolved in 400 µL of methanol. The solutions were then filtered with 0.20 micron non-pyrogenic 25 mm Corning syringe filters (Sigma, Germany.) The remaining residue was diluted once more in 400 µL of methanol and filtered again. Pooled filtrates were placed into LC-MS vials.

4.3 Results and discussion

The identification and characterization of desirable bioactives from natural products continues to receive significant attention. In this study, a bioassay-guided fractionation was used to achieve this.

Fraction A, extracted using 10% (v/v) acetone in methanol, produced more than 20 grams from the initial whole 50 grams starting extract weight. Moreover, as being the most polar it also exhibited strong antifungal properties, and there were more bands on a TLC plate system for this fraction (**Figure 4.2**). Minimum inhibitory concentrations (MIC) values of the six fractions ranged from the 0.063 mg ml⁻¹ to 0.25 mg ml⁻¹ (Table 1.) In comparison with the whole extract all fractions showed activity very close within the same antifungal strength ranges. Secondly all extracts exhibited fungistatic activity, as there was a steady rise in the MIC values with time, showing extracts being less effective as time progressed. Thus fraction A, having favourable activity and a higher yield, was then further run through the Versa Flash system for further separation.

Table 4.1 Minimum inhibitory concentrations (MIC; mg ml⁻¹) of fractions A to F after 48, 60 and 96 h

Fractions/ Strain	MIC mg ml ⁻¹											
	48 h				60 h				96 h			
	<i>F. proliferatum</i>		<i>F. veriticilliodes</i>		<i>F. proliferatum</i>		<i>F. veriticilliodes</i>		<i>F. proliferatum</i>		<i>F. veriticilliodes</i>	
A	7140	6908	826	8267	7140	6908	826	8267	7140	6908	826	8267
B	0.031	0.125	0.063	0.125	0.250	0.250	0.125	0.250	0.250	0.250	0.250	0.250
C	0.031	0.125	0.063	0.125	0.250	0.250	0.250	0.250	0.250	0.250	0.250	0.250
D	0.031	0.125	0.063	0.125	0.250	0.250	0.125	0.250	0.250	0.250	0.250	0.250
E	0.031	0.125	0.063	0.063	0.125	0.125	0.250	0.250	0.250	0.250	0.250	0.250
F	0.031	0.125	0.063	0.063	0.125	0.125	0.250	0.250	0.250	0.250	0.250	0.250
Whole extract	0.031	0.125	0.063	0.063	0.125	0.125	0.250	0.250	0.250	0.250	0.250	0.250
Voriconazole	0.063	0.125	0.125	0.125	0.250	0.250	0.250	0.250	0.250	0.250	0.250	0.250
	0.156	0.156	0.156	0.156	0.156	0.156	0.156	0.156	0.156	0.156	0.156	0.156

MIC values are averages of three replicates per concentration.

Table 4.2 Minimum inhibitory concentrations (MIC; mg ml⁻¹) of fractions A1 to A6 after 48, 60 and 96 h

Fractions/ MRC Strain	MIC mg ml ⁻¹											
	48 h				60 h				96 h			
	<i>F. proliferatum</i>		<i>F. veriticilliodes</i>		<i>F. proliferatum</i>		<i>F. veriticilliodes</i>		<i>F. proliferatum</i>		<i>F. veriticilliodes</i>	
	7140	6908	826	8267	7140	6908	826	8267	7140	6908	826	8267
A1	0.125	0.125	0.125	0.125	0.125*	0.125*	0.250	0.125	NA	NA	NA	NA
A2	0.250	0.500	0.500	0.250	NA	NA	NA	NA	NA	NA	NA	NA
A3	0.250	0.500	0.500	0.250	NA	NA	NA	NA	NA	NA	NA	NA
A4	0.063*	0.063*	0.063*	0.031*	0.125*	0.125*	0.125*	0.063*	NA	NA	NA	NA
A5	0.125	0.250	0.125	0.125	0.500	1.000	0.500	0.500	NA	NA	NA	NA
A6	0.125	0.125	0.125	0.125	0.500	0.500	0.250	0.500	NA	NA	NA	NA
Voriconazole	0.156	0.156	0.156	0.156	0.156	0.156	0.156	0.156	0.156	0.156	0.156	0.156

MIC values are averages of three replicates per concentration.

*- lowest MIC

NA- Not Active

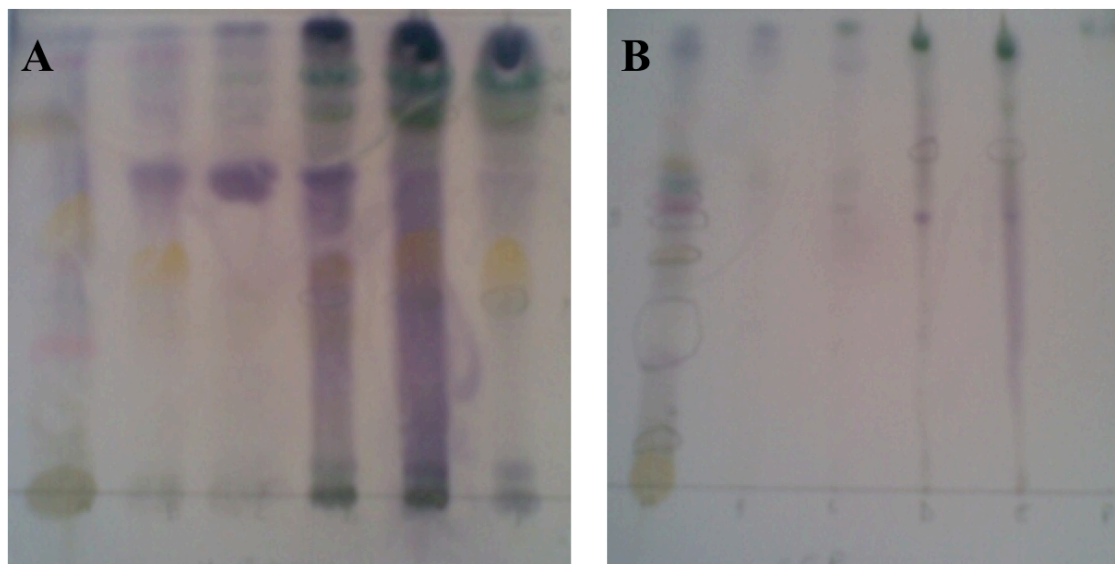


Figure 4.2 Thin layer chromatography plates showing fractions A to F in A: EMW [ethyl acetate (4): methanol (5.4): water (4)] and B: CEF [chloroform (5): ethyl acetate (4): formic acid (1)]

A further six sub-fractions were obtained from Fraction A, named A1 to A6. From the sub-fractions, A4 collected from running with 90:10 of dichloromethane and methanol (collection from the first ten test tubes) showed the best activity. With MIC values of 0.063 mg ml^{-1} for strains MRC 7140, 6908 and 826 while that of 8267 was 0.031 mg ml^{-1} (**Table 4.2**). The observed MIC values were lower than those from fraction A and the whole extract (**Table 4.2**). Fractions A1, A2 and A3 showed no activity after 60 h while A4, A5 and A6 had MIC values ranging from 0.063 mg ml^{-1} as the lowest shown by A4 against MRC 8267 to 1 mg ml^{-1} from A5 against MRC 6908. However, after 96 h none of the fractions showed any activity. Only the control voriconazole (**Table 4.2**) had antifungal activity at 96 h.

Fraction A4 was then further separated using a silica column, producing A4.1-4. Collections from these fractions after being run on LC-MS revealed two major peaks at retention times of 7.94 and 8.99 which were named Stel₁ and Stel₂.

Stel₁ was a white crystalline compound. On LC-MS it had a retention time of 8 mins. The molecular ion showed a peak at 331.2 m/z (M+1) that was also the base peak. This corresponded to $\text{C}_{20}\text{H}_{27}\text{O}_4$. Other prominent peaks were at 303 (M-CO), 285 (M-CO-H₂O). Comparing this with literature and the Chemspider® database, the compound was very similar to carnosol. Based on comparing with a pure compound on LC-MS and preparative TLC it was concluded that Stel₁ was carnosol (**Figure 4.3A**).

This is *o*-diphenolic diterpenes also abundant in *Salvia officinalis* (Bauer et al., 2012; Johnson, 2011). It has been shown to have antioxidant activity, while analgesic and anti-inflammatory activity, have recently been studied by Lucarini et al (2013). Carnosol was first isolated in the North American *Salvia carnososa* (Dougl.) (White and Jenkins, 1942). Identification from a methanol: chloroform *S. chamelaegnea* extract, was reported by Kamatou and others (2007) and this extract was active against *Escherichia coli* (ATCC 8739); *Klebsiella pneumonia* (NTCC 9633); *Bacillus cereus* (ATCC 11778); *Staphylococcus aureus* (ATCC 25823). The same study also showed *Salvia africana-lutea* activity against the same test strains. However, activity against fungi was shown against *Candida albicans* (ATCC 90028) and *C. krusei* (ATCC 6258) (Weckesser et al. 2007). It is the first time it has been shown to have activity against *Fusarium* species.

Stel₂ was a white compound. On LC-MS it had a retention time of 8.98 mins. The molecular mass was 333 m/z (M+1), which corresponded to C₂₀H₂₈O₄, with the other prominent peak 331 (**Figure 4.3B**). Using both the Chemspider database and literature the diterpenoid compound was compared with a pure form of carnosic acid using LC-MS after preparative TLC and was concluded to be carnosic acid. Carnosic acid synonymous with *Rosmarinus officinalis* (Frankel et al., 1996), has been mostly linked with antioxidative effects (Okamura et al., 1994; Zhang et al., 2013), anti-inflammatory and anticarcinogenic properties (Bauer et al., 2012).

Preparative TLC was utilized as well, bands obtained at the same R_f value of pure compounds in an eluent system of hexane: ethyl acetate 7:3 were scrapped off named 1, 2 and 3 then run on LC-MS. These were then run on LC-MS, results that that showed the same retention times and masses when run simultaneously (**Figure 4.3**). The two compounds have been identified in many plant extracts occurring together. Thus it may be loosely inferred their occurrence may subsequently have an impact on the bioactivity of the documented extracts.

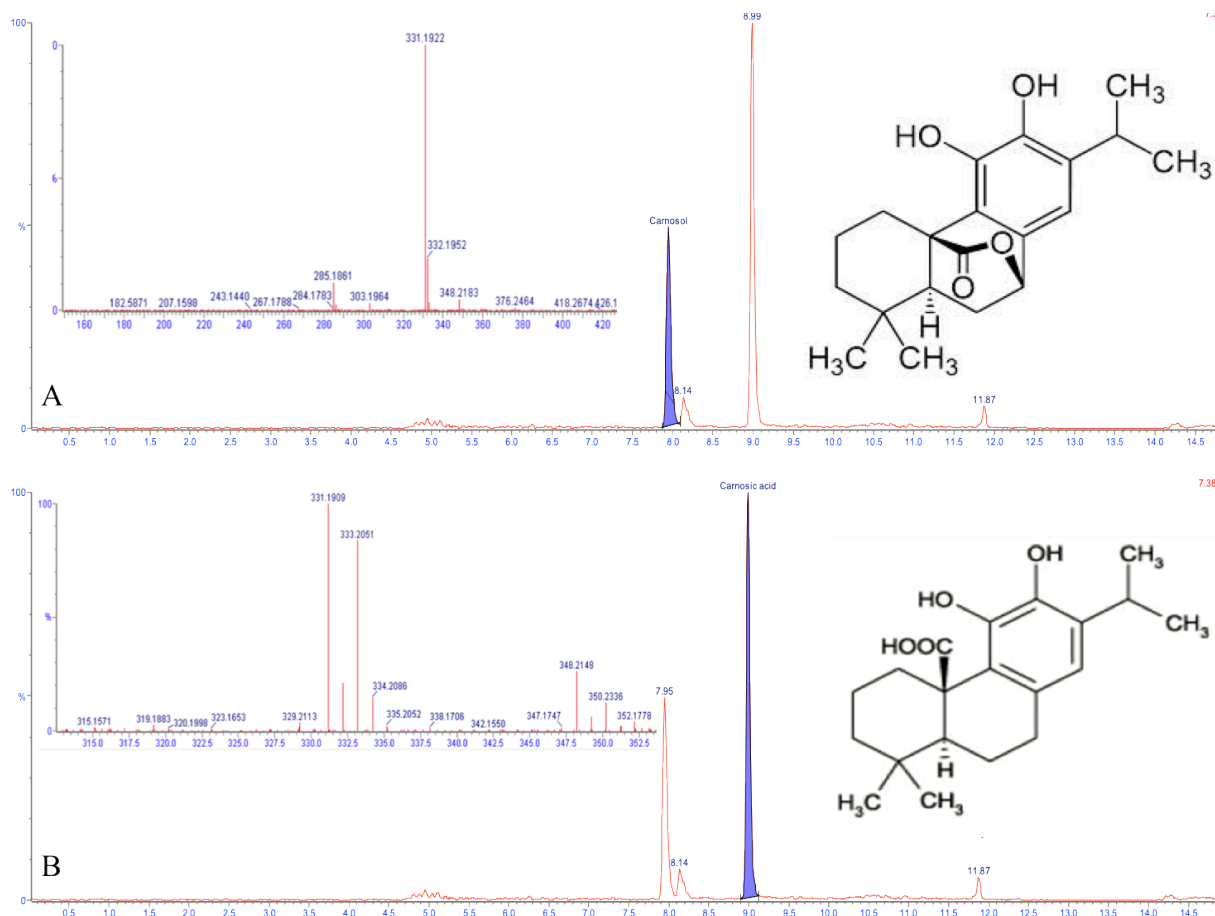


Figure 4.3 LC-MS Chromatograms of one of the sub-fractions collected from preparative TLC, with (A) carnosol and (B) carnosic acid peaks highlighted. Structures and fragmentation patterns are shown as inserts on the total ion chromatograms (TIC)

Relative abundance of the two compounds was also calculated from three sub-fractions. **Tables 4.3** and **4.4** show the variation in quantities, carnosol was present in all three, however, most abundant in 2, while carnosic acid was present in 2 only.

Table 4.3 Relative compound quantities in sub-fractions to carnosol

Fraction/Compound	Standard concentration (ppm)	Retention time	Area	Concentration (ppm)
1		7.94	101.636	191.2
2		7.93	691.589	1572.3
3		7.94	237.955	510.3
Carnosol	50	7.95	237.701	
Carnosol	10	7.95	92.865	
Carnosol	5	7.94	47.782	

This variation thus poses a question to whether ratios of the two compounds may significantly affect bioactivity. In answering this, three combinations of the two compounds carnosol and carnosic acid were tested (1:1, 1:2 and 2:1). Results shown in (Table 4.5).

Table 4.4 Relative compound quantities in sub-fractions to carnosic acid

	<i>F. verticillioides</i>			<i>F. proliferatum</i>
	ATCC 3629	MRC 826	MRC 8267	MRC 7140
Voriconazole	< 16	< 16	< 16	< 16
Carnosol	250	250	250	250
Carnosic acid	125	125	125	125
Carnosol: carnosic acid (1: 1)	250 (3.0)	250 (3.0)	250 (3.0)	250 (3.0)
Carnosol: carnosic acid (1: 2)	125 (1.5)	125 (1.5)	125 (1.5)	125 (1.5)
Carnosol: carnosic acid (2: 1)	250 (3.0)	250 (3.0)	250 (3.0)	250 (3.0)

Table 4.5 Minimum inhibitory concentrations (MIC) ($\mu\text{g ml}^{-1}$) and the sum of fractional inhibitory concentration (ΣFIC) (for the mixtures) of carnosol, carnosic acid and the combinations after 48 h

Fraction/Compound	Standard concentration (ppm)	Retention time	Area	Concentration (ppm)
1				Not detected
2		8.99	691.589	1572.3
3				Not detected
Carnosic acid	100	8.99	896.712	
Carnosic acid	50	8.99	408.955	
Carnosic acid	10	8.99	98.161	
Carnosic acid	5	8.99	31.185	

Of the two compounds carnosic acid showed the best activity with an MIC value of $125 \mu\text{g ml}^{-1}$ while that of carnosol was $250 \mu\text{g ml}^{-1}$. In comparison to crude plant extracts tested earlier Nkomo et al (in press), MIC values were still in a similar activity range (strong inhibitors), as the best activity in crude extracts was 0.031 mg ml^{-1} . This still compares well with tissue cultured hairy roots from the study by Ramogola et al 2009. When tested in combination, in equal volumes an MIC of $250 \mu\text{g ml}^{-1}$ was observed, similar to that were carnosic acid was tested individually. Moreover, it is interesting to note that in fraction 2 these two compounds were observed in this ration (**Tables 4.3** and **4.4**). While the contrary was true, a combination having double the volume of carnosol over carnosic acid showed a lower MIC value. However, fractional inhibitory concentration values reveal that on all the combinations studied can be interpreted as ‘indifferent’ using the range described by Van Vuuren and Viljoen (2011). Fractional inhibitory concentrations ranged between 1.5 and 3.0.

4.4 Conclusion

Fractions from *Salvia africana lutea* extracts obtained via the bioassay guided fractionation in this study containing carnosol and carnosic acid possess fungistatic activities against the five strains of *Fusarium verticillioides* and *F. proliferatum*. While the combination study, revealed that the two compounds are indifferent in their activity. This is of importance as in the development of a fungal control agent(s), exploiting a tissue culture system that produces these two compounds may have other applications.

4.5 Acknowledgements

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Chapter Five

Callus induction from wild *Salvia africana-lutea* L. leaf explants linked to inhibitory activity against two *Fusarium* species.

5.1 Introduction

Plants utilized in alternative and complementary medicine are mostly harvested from the wild. (Calixto, 2000). This is the case in the Western Cape province of South Africa, with regards to *Salvia africana-lutea*. This sage species, distributed along the West Coast forms an integral part of the *materia medica* of the indigenous people of the region.

Cultivation of medicinal plants to curb over-exploitation of wild populations is limited. Thus amongst other medicinal plants with desirable properties *S. africana-lutea* is still gathered from the wild. In spite of laws regulating the harvesting of South African floral biodiversity, harvesting from wild populations still occurs. Along with indiscriminate and unsustainable harvesting practices, wild populations face other aspects that drive population size reduction (Verma et al., 2007). These include the rising demand for housing developments along the coast (Makunga and van Staden, 2008). Secondly this is exacerbated by disturbances in the natural seedbank by a growing number of alien/invasive species on the ecologically fragile fynbos vegetation (Holmes and Cowling, 1997a,b). Such turbulences directly impact negatively on natural succession of fynbos biome plants.

Much work on *Salvia africana-lutea* and other sage species has been undertaken in linking the various biological activities to secondary metabolites (Kamatou et al., 2008; Siwach et al., 2011). This has resulted in secondary metabolites being incorporated into a wide range of both commercial and industrial applications. The most common method is by way of rigorously controlled plant *in vitro* cultures that can generate the same valuable natural products (Park et al., 2008). Plants including these *in vitro* cell cultures have provided bioactive compound sources among other products (Mary, 2005).

The most well studied from *Salvia* species being rosmarinic acid, caffeic acid esters and flavonoids such as quercetin, kaempferol and luteolin to mention a few (Xianget al., 2013).

Utilizing tissue culture methods such as callus induction brings advantages such as production of desirable compounds in controlled conditions thus predictable, simple and may be reliable. Harvesting or isolation of compounds becomes efficient and rapid as compared to interfering compounds (undesirable compounds) in whole plants in the field. Production of compounds is continuous, as they are not affected by weather conditions. Moreover, there is the added advantage of establishing a standard source of phytochemicals in large volumes as they are produced continuously (Park et al., 2008).

In this study explants from Stellenbosch plants were introduced into tissue culture conditions, producing callus that was maintained in solid media and later tested for antifungal activity. In comparison to crude plant extracts callus extracts showed favourable activity, thus no bioactivity was lost due to introduction into tissue culture conditions. This chapter also gives an alternative method of effectively harnessing and manipulating the production of bio-actives identified. Moreover, this chapter evaluates the effect on bioactivity that introduction to tissue culture may or may not have.

5.2 Materials and methods

5.2.1 Plant collection

Arboreal parts of *Salvia africana-lutea* plants were collected from a garden growing plant population at Stellenbosch University (S 33° 55.120' E 18° 51.360') on the 20th of September 2011.

5.2.2 Culture induction

Plant samples were washed with 0.1 M ascorbic acid for 3 min, before being placed in 70% (v/v) ethanol for 7 min with occasional shaking. After rinsing with distilled water samples were placed for 15 min in a 2 % (v/v) fungicide solution of folicur® (active ingredient; tebuconazole 250 g L⁻¹) (Bayer, Namibia) followed by another 15 min soaking period in a diluted solution of chlor guard® (Prime cleaning supplies, South Africa) (active ingredient sodium hypochlorite): distilled water (1:2 v/v). This

was then followed by three 3 min successive rinsing and soaking sessions in sterile water. Samples were then washed in 0.1 M ascorbic acid and placed at 4 °C for 2 h. These were placed onto solid Murashige and Skoog (1962) (MS) medium with 0.1 g L⁻¹ myo-inositol, 30 g L⁻¹ sucrose and 8 g L⁻¹ agar (w/v) (pH of 5.8, adjusted with 1 M sodium hydroxide (NaOH)). Cultures were incubated under a 16/8 h light regime (50 μmol m⁻² s⁻¹) at a temperature of 23 ± 1 °C. After 14 d the callus that generated was sub-cultured to different plant growth regulators (PGR) combinations on MS media. Each petri dish having 5 callus masses ≈ 3-4 mm in diameter. Synthetic PGR combinations between the auxin naphthalene acetic acid (NAA) and cytokinin benzyl adenine (BA), two concentrations of BA utilized individually and in combination with NAA 4.4 and 8.8, while for NAA 0.27, 2.7 and 5.4. Combinations were selected from those described by Makunga and van Staden 2008 as they yielded callus from all different explants used in that study. Namely: 4.4 BA, 8.8 BA, 0.27 NAA: 4.4 BA, 2.7 NAA: 4.4 BA, 5.4 NAA: 4.4 BA, 2.7 NAA: 8.8 BA and 5.4 NAA: 8.8 BA. The experiments were repeated twice. Callus were later pooled together after 16 weeks weighed before and after drying.

5.2.3 Extract preparation

Pooled callus harvested from hormone-free and Murashige and Skoog (MS) solid media Petri dishes was oven dried at 55 °C. This was ground to powder in liquid nitrogen and 5 g dissolved in 20 ml of a 1:1 methanol: dichloromethane (v/v) solvent in a 60 ml glass test tube. This was sonicated for 35 min (Bransonic 220, USA) before filtering with Whatman filter paper number 1. The extraction was repeated twice and pooled extracts were dried using a rotary evaporator (Buchi, Germany) at 55 °C. Extracts were then stored in a desiccator prior to evaluation. Whole plant and callus extracts for LC-MS, were extracted in a similar manner as described above, then dried in a GeneVac[®] EZ 2.3 (Anatech, South Africa) overnight at 45 °C then sent for analysis to the Central Analytical Facility (CAF) at Stellenbosch University.

5.2.4 Antifungal assay

The antifungal assay method used was that described in section 3.2.3.

5.2.5 LC-MS analysis

This work was done as the method described in section 3.2.4.

5.2.6 Data analysis

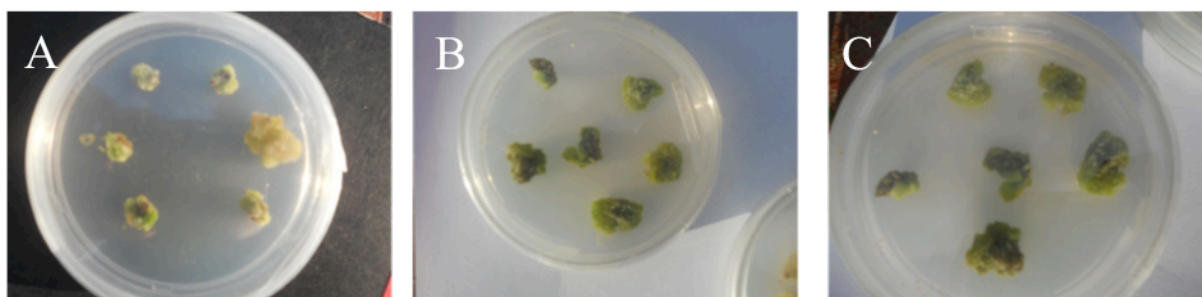
Percentage data was used to assess the effect of different PGR combinations on explants (stems and leaf explants) a nonparametric Kruskal–Wallis ANOVA was utilized using Statistica version 12. The PCA on the LC-MS data was performed using MarkerLynx XS from MassLynx version 4.1. Twenty-four variables were used from 9 sample runs. Data from 4 mins to 20 mins retention time was utilized, with a range of 200 to 1000 molecular masses. Pareto scaling was used for scaling, and four components used for the PCA model. A noise level of 12% was obtained.

5.3 Results and discussion

Plant growth regulator (PGR)-free MS media leaf explants produced callus after 19 to 22 days, while all other PGR combinations showed no callus, shooting nor rooting. These findings show a contrast to the study by Makunga and van Staden 2009, as leaf explants in that study in similar hormonal combinations did produce callus, shoots and roots. However, in that study explants were from plantlets obtained from seed and maintained in tissue culture condition while this study had explants from plants in non-tissue culture conditions. In this study, PGR combinations in the stem explants were observed to have no significance, as there was no growth in all the combinations. A *p*-value of 0.4289 was obtained for the leaf explants, and 1.0000 for stem explants revealing that PGR combinations had no significant effect on the production of neither callus nor rooting (**Table 5.1**). A total of twenty percent of the explants in the hormone-free MS media callused in the first experiment, while upon repeating 34% callused.

Table 5.1 Percentage growth in PGR combinations on stem and leaf explants.

Hormone Combination	Leaf explant callus production (p 0.4289)		Stem explant callus production (p 1.0000)	
	Rep 1	Rep 2	Rep 1	Rep 2
	4.4 BA	4	6	0
8.8 BA	2	0	0	0
0.27 NAA: 4.4 BA	0	0	0	0
2.7 NAA: 4.4 BA	0	0	0	0
5.4 NAA: 4.4 BA	0	2	0	0
2.7 NAA: 8.8 BA	0	0	0	0
5.4 NAA: 8.8 BA	0	0	0	0
PGR-free	20	34	0	0

**Figure 5.1 Callus on Murashige and Skoog media with (A) 4.4 BA (B) 8.8 BA and (C) PGR-free**

The resulting callus obtained from the PGR-free media, was then sub-cultured into the same PGR combinations to evaluate whether they would proliferate these subcultures grew and revealed no relative significant differences in weight (**Table 5.2**).

Table 5.2 The percentage weight loss callus sub-cultured from PGR-free MS media

Hormone Combination	Wet weight (g)	Dry weight (g)
4.4 BA	177.36	12.31
8.8 BA	204.82	13.55
0.27 NAA: 4.4 BA	232.90	14.88
2.7 NAA: 4.4 BA	159.84	11.27
5.4 NAA: 4.4 BA	118.21	10.59
2.7 NAA: 8.8 BA	164.23	11.76
5.4 NAA: 8.8 BA	174.98	12.15

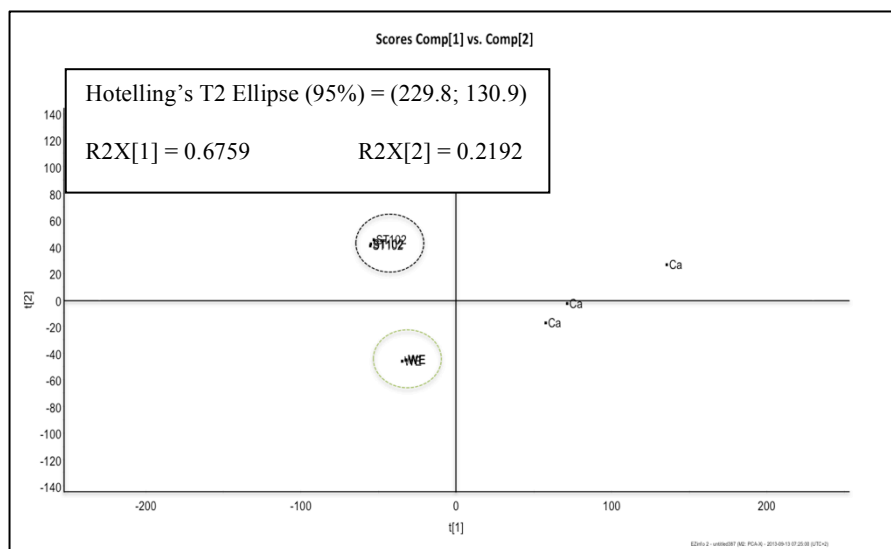
Comparing the minimum inhibitory concentrations (MIC)s obtained against the four *Fusarium* strains, the lowest was 0.125 mg ml⁻¹ against MRC 7140, MRC 8267 and MRC 826, and the highest was 0.25 mg ml⁻¹ after 48 h (**Table 5.3**). Minimum inhibitory concentrations in this range are classified as strong inhibitors as they are below 0.5 mg ml⁻¹ (Souza et al., 2007). These results are in full agreement with those of Ramogola 2009 and Nkomo et al (in press) results on whole wild plant extracts, which had MIC values in the same range (strong inhibitor) at 0.31 mg ml⁻¹. Thus, callus extract tested in this study compared favourably to hairy root extracts and the crude plant extracts, providing an easier alternative to producing the bioactives. As callus is fairly easier to obtain under tissue culture conditions compared to hairy roots, simultaneously a better option than utilizing whole plants this provides the most favourable option to explore further for large-scale production.

The MIC values rose to 0.5 mg ml⁻¹ as was observed after 60 h revealing a fungistatic action; however, from 60 to 92 h the MICs suggest a fungicidal action as there was no change from 0.5 mg ml⁻¹ between the two observation times.

Table 5.3 Minimum inhibitory concentrations (MIC; mg ml⁻¹) after 48, 60 and 92 h

Extract/Isolate		<i>F. proliferatum</i>		<i>F. verticillioides</i>	
		MRC 6908	MRC 7140	MRC 8267	MRC 826
Callus Extract	48 h	0.25	0.125	0.125	0.250
	60 h	0.5	0.500	0.500	0.500
	92 h	0.500	0.500	0.500	0.500
Voriconazole	48 h	0.008	0.008	0.008	0.008
	60 h	0.008	0.008	0.008	0.008
	92 h	0.008	0.008	0.008	0.008

While comparing the metabolite profiles obtained using LC-MS, callus extracts (Ca) were different from the whole plant extract (ST) and the ground whole plant samples (WE). This can be seen clearly on **(Figure 5.2)**, the PCA showing callus samples forming a single grouping much further away from the other two sample groups.

**Figure 5.2 Scores plot of LC-MS data on nine sample runs.**

The discriminating peaks, observed from the loadings plot **(Figure 5.3)** were six; four responsibly for the callus differing from the plant extract (ST) and whole plant samples (WE). While the plant extracts and whole samples had each one discriminating peak from the rest of the samples. With regards to the

four discriminating peaks in the callus, these were observed at retention times; 3.72, 7.12, 9.63 and 11.24. The most discriminating peak in the plant extract was at retention time 9.51, while for the whole plant samples it was at 9.97.

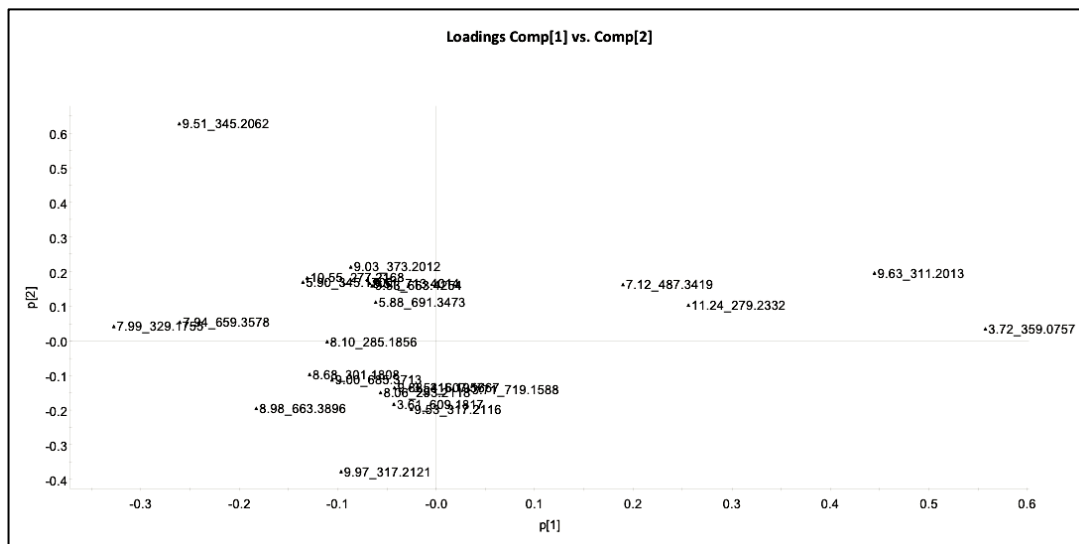


Figure 5.3 Loadings plot of LC-MS data revealing the discriminating peaks

Elemental compositions and peak fragmentation patterns of these discriminating peaks are found in appendix IV.

5.4 Conclusion

The PGR combinations used in this study did not induce shooting nor rooting as was expected, only callus on PGR-free MS media. Antifungal activity of the callus extract was in the same range as the whole plant extracts from which the leaf explants were harvested from, showing no 'loss' of activity after introduction to tissue culture conditions. Metabolite profiles using LC-MS revealed some differences, however, these seem not to influence antifungal activity. This still should suffice, in the event that up-scaling production of the callus to harvest bioactive compounds is pursued. Mass production on callus in a bioreactor system would be much easier in comparison with plantlets. However, the drawback to using solid media cultures is the rate at which callus is produced and also that callus is not genetically stable. In future studies setting up liquid cultures and maintaining them would provide for faster callus production as well as increased quantities.

5.5 References

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Chapter Six

6.1 General Discussion

The aim of this study, which was to evaluate chemotypes that were perceived present in five different *Salvia africana lutea* plant populations as well as to identify the metabolite(s) responsible for the bioactivity, lastly to establish a tissue culture system from the most potent population.

Metabolite profiling and antifungal activity Chapter 3 (Nkomo et al., in press) provided the answers to evaluating the chemotype differences and linking this to bioactivity. Results revealed antifungal activity against two strains of *Fusarium proliferatum* MRC 7140 and 6908 as well as *F. verticillioides* MRC 826 and MRC 8267 were classified as strong inhibitors, as the extract MIC values were all below 0.5 mg ml⁻¹ for all the populations. The most potent was the Stellenbosch site with a low MIC of 0.031 mg ml⁻¹ comparing favourably well to the positive control Voriconazole. This was expected, as the study by Ramogola (2009) on the same genus showed the same inhibitory range (strong inhibitor). Hairy root extracts were used in that study. However, of importance, this study did compare the extracts to Voriconazole, while the latter study used Amphotericin B. As Voriconazole is one of the triazoles considered superior to Amphotericin B (Herbrecht, et al., 2002), findings in this study also provide evidence that the plant extracts compare very well to the most preferred antifungal drugs currently in use.

In evaluating the metabolite profiles, using GC-MS the study revealed chemical differences from the Brackenfell, Koeberg, Silwerstroomstrand and Yzerfontein extracts. Notably Stellenbosch had propanoic acid, rythronic acid, 2-keto-1-gliconic acid and 1,3-dibromobicyclon, which were exclusively detected from extracts from this site. This may be an indication of possible differences accounted for by garden-grown plants and those in the wild. Using NMR and LC-MS peaks distinguishing Stellenbosch where in the region that is mostly for aromatic compounds, which also have been documented to have high antimicrobial activity hence the strong inhibition against the two *Fusarium* test species. Moreover, aromatic regions are usually variable as most secondary metabolites are in this region, further indication of the differences brought about by plant-environment interactions.

Plant-environment interactions are very different comparing wild growing populations to garden growing plants that should experience more perturbations. Bearing this in mind, plants exposed to more disturbances will tend to produce more secondary compounds thus the Stellenbosch site showing chemical and bioactivity differences.

Having identified the most potent plant population, identification of bioactives was done using a bioassay guided fractionation method. Having done a bulk extraction, separation of compounds in the extract was done in two systems namely: through a VersaFlash® and a silica gel column. Fractions showed strong inhibition as MIC values ranged from 0.063 mg ml⁻¹ to 0.25 mg ml⁻¹, still below the 0.5 mg ml⁻¹ (strong inhibitor). Identification was achieved through LC-MS and preparative TLC. Carnosol and carnosic acid were found to be the major compounds in the active fractions. The two compounds have been documented mostly for their antioxidant properties and are known to occur in some *Salvia* species though they are mostly synonymous with *Rosmarinus officinalis* from where the commercially sourced pure compounds are extracted. This study has been the first to explicitly show carnosol and carnosic acid to inhibit *Fusarium verticillioides* and *F. proliferatum*, with MIC values of 250 µg ml⁻¹ for carnosol and 125 µg ml⁻¹ for carnosic acid against all the 4 strains tested. As activity was observed up to 60 h, showing fungistatic property, investigating the mode of action seems more likely to be the next plausible step. Evaluating the mode of action may help in the development of a fungicide, in deciding when best to apply and how to apply it, for example as triazole based fungicides currently in use such as Folicur® have active ingredients that target sterol biosynthesis simultaneously being broad spectrum reducing the chances for fungi developing resistance against them. While other fungicides target fungal cell membrane components and inhibit growth (Marin et al., 2013). Having more than one active ingredient in a fungicide brings the advantage of multiple targeting, reducing the risk of resistance build-up. Thus having carnosol and carnosic acid in a new fungicide may prove invaluable, as they will be targeting different sites than the current fungicides in use.

Synergistic interactions of compounds in plants in providing antimicrobial activity has fast become of importance as shown by Ushimaru and others 2012. Thus several combinations of carnosol and carnosic acid were investigated to observe the best inhibition of *Fusarium*. However, in this study the activity of the compounds in combination showed no interaction as FIC values of 1.5 and 3.0 are regarded as to be indifferent. Though, comparing the MIC values of the pure compounds (ranging between 125 µg ml⁻¹ to 250 µg ml⁻¹), with the callus extract and whole plant extract, all were still in the

same range as strong inhibitors. This is promising as there is no loss in activity while reducing the initial suite of compounds in the whole plant extract. In the event of producing the two compounds in large quantities via tissue culture, manipulating the genes involved in the biosynthetic pathway(s) may also be considered.

In establishing a tissue culture system to maintain the population most potent, the Stellenbosch plant population were introduced in culture and the callus resulting from the introduction was tested for antifungal activity. As with some plant species and forms of tissue culture bioactivity is lost when introduced into *in vitro* conditions, callus produced from leaf explants collected from Stellenbosch inhibited the *Fusarium* strains tested, with MIC values as low as 0.125 mg ml⁻¹. Callus extracts did exhibit the similar activity with which fractions and the whole extract tend to be fungistatic as the MIC values rose to 0.5 mg ml⁻¹ after 92 h. Activity of the callus however, meant that it was no longer necessary to attempt to produce plantlets. Manipulation of callus in bioreactor systems has more advantages than plantlets, thus rooting and shooting hormonal combinations were then not further studied.

On the other hand the study may have provided a better view of the metabolite variation between the populations if genotypic differences were evaluated. This would have also painted a broader view on the population differences.

6.2 Conclusions

Salvia africana lutea plant populations found in the different sites sampled had different chemical profiles and similar activity. Notably the Stellenbosch plant population is in close proximity with human dwellings thus maybe exposed to perturbations significantly more than the other populations in protected areas. Studies have shown that secondary plant metabolites production levels increase directly in response to the surrounding environment. The increased metabolite production may have subsequently influenced the different chemical profile observed in the Stellenbosch population thus also influencing the bioactivity.

In isolating and identifying the bioactives from crude Stellenbosch plant extracts revealed, carnosol and carnosic acid after performing a bioassay guided fractionation. The two compounds have been previously identified in *Salvia* species and *Rosmarinus officinalis*, this study however, is the first to show antifungal activity against *Fusarium verticillioides* and *F. proliferatum*. This is of paramount importance as *Fusarium* is of economical importance to the farming sector, particularly the maize crop and viticulturalists.

Introducing the leaf explants from Stellenbosch plants only produced callus under tissue culture conditions with different hormone combinations, however, still retained bioactivity against the *Fusarium* strains tested. This provides ideal starting stock culture for mass production of *Fusarium* inhibiting callus. Also considering ways of increasing the production of carnosol and carnosic acid in the callus by way of gene manipulation, as future work.

6.3 References

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Appendices

7.1 Appendix I

Nkomo M and Makunga N.P Metabolomic profiling of *Salvia africana-lutea* potent against *Fusarium* fungi.

International Conference on Analytical Sciences. Analitika 2010. Stellenbosch 5 – 9 December 2010

Metabolomic profiling of *Salvia africana-lutea* potent against *Fusarium* fungi

Salvia africana-lutea is a very important medicinal plant in the Western Cape. Amongst all its medicinal uses, recently antifungal activity has been shown against *Fusarium species*. In this study, a total of eight samples from four locations were collected and extracted using a solvent of Dichloromethan: Methanol in the ratio of 1:1. Sample variations were evident with some being more citrus than others. Metabolite profiles of the eight samples were compared using both Diffusion ordered spectroscopy (DOSY) nuclear magnetic resonance (NMR) and GCMS. Differences in the chemical composition across the different populations were evident. This may be related to geographic location, environmental responses and/or genetic hybridization. Antifungal assays were also used to confirm differences in biological activity. Inhibition of fungi by *S. africana-lutea* extracts opens up an avenue for exploitation of this plant for developing new effective fungicides.

Nkomo M and Makunga N.P Metabolomic profiling of *Salvia africana-lutea* L. wild populations in the Western Cape.

IOCD(International Organisation for Chemical Sciences Development) Symposium Cape Town (University of the Western Cape) 10 – 15 January 2011(Poster)

Nkomo M and Makunga N. P

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Salvia africana-lutea is an important medicinal plant in the Western Cape. It has been documented to possess anti-oxidant, anti-inflammatory, cytotoxicity, antituberculosis, antiplasmodial and antimicrobial properties³(Kamatou et al., 2008). Recently, antifungal activity against *Fusarium* has been detected. We thus decided to examine different populations around the Western Cape so as to determine the chemotypes that may exist. In this study, a total of sixteen samples from five locations ranging from the coastal regions to the inland sites in the Cape Wineland district were collected and extracted using dichloromethan and methanol (1:1;v/v). As the five different localities were all in the Cape Floristic Region with some degree of geographical variation, sample variations were evident with some being more citrus than others. Metabolite profiles of the sixteen samples were compared using ¹H NMR, and the data analyzed using ACDC 1D NMR processor and Latentix in producing the PCA. The NMR detected mostly terpenoids, saponins and sugars, in the solvent extracts while GCMS has shown groups being detected ranging from reducing sugars, quinines, alkaloids, diterpenes, triterpenes, phenolics to steroids. These groups contain some of the most important precursors to instrumental compounds utilized in the pharmaceutical industry, suggesting a plethora of compounds that are commercially important.

Nkomo M, Makunga N, Katerere D.R, Vismer H.F, Malet-Martino M. Antifungal activity of Western Cape *Salvia africana-lutea* populations against *Fusarium* species.

³ Kamatou, G.P.P., Makunga, N.P., Ramogola, W.P.N. & Viljoen, A.M. 2008, South African *Salvia* species: A review of biological activities and phytochemistry, Journal of Ethnopharmacology, 119. 664-672

Medical Research Council (MRC) Research Day Bellville 24 – 25 October 2011

Antifungal activity of Western Cape *Salvia africana-lutea* populations against *Fusarium* species.

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³Groupe de RMN Biomédicale, Laboratoire SPCMIB (UMR CNRS 5068), Université Paul Sabatier, 31062 TOULOUSE, France

Salvia africana-lutea L. commonly known as beach or dune sage is a very important medicinal plant in the Western Cape province of South Africa. Secondary metabolites strongly influence the ‘broad spectrum remedy’ property of this species. It has been documented to treat a myriad of ailments ranging from colds, coughs to digestive disorders to mention but a few, suggesting a vast plethora of bioactives. Phytochemical properties include; antibacterial, antiinflammatory, anticancer and antioxidant amongst many others. Importantly recent *S. africana-lutea* antifungal activity against *Fusarium* species has come to the spotlight. *Fusarium* is a plant pathogenic species that causes large agricultural yield losses particularly in the maize crop, one of the staple foods in the African continent. It also produces mycotoxins in infected crop and these chemicals may lead to the onset of cancers when contaminated foods are consumed extensively. Samples were collected from four wild growing *S. africana-lutea* population sites i.e. Yzerfontein, Silwerstroomstrand, Koeberg and Brackenfell and one garden growing site, i.e. Stellenbosch. Metabolite profiles of the samples were studied using GC-MS and NMR to identify potential chemotype differences. An antifungal assay was conducted to correlate the chemistry to the potency against the *Fusarium verticillioides* (MRC 826 & MRC 8267) and *F. proliferatum* (MRC 6908 & MRC 7140) strains. No significant qualitative differences were noted but some quantitative variations were observed. These profiles tended to group by site, an indication of the plant-environment interaction influencing the metabolite composition. Notable antifungal activity was observed and need to be confirmed through standardized methodology. Further research on identifying the bioactives is essential and has great potential for fungal control agents in the agricultural sector, which in turn may lower mycotoxin production.

7.2 Appendix II

Table 7.1 Composition of Murashige and Skoog medium

Medium composition	mg l⁻¹
NH₄NO₃	1650
KNO₃	1900
CaCl₂.2H₂O	440
MgSO₄7H₂O	370
KH₂PO₄	170
KI	830
H₃BO₃	6200
MnSO₄.4H₂O	22300
ZnSO₄.7H₂O	8600
Na₂MoO₄.2H₂O	250
CuSO₄.5H₂O	25
CoCl₂.6H₂O	25
FeSO₄.7H₂O	27850
Na₂EDTA.2H₂O	37250
Myo-inositol	100
Nicotinic acid	500
Pyridoxin-HCl	500
Thiamine-HCl	100
Glycine	2

4

⁴ 1 litre MS media was prepared with:30g (carbon source),4.4g MS salts, 0.1 Myo-inositol, 9g Agar, at a PH of 5.8 at room temperature and pressure

7.3 Appendix III

Table 7.2 Composition of Roswell Park Memorial Institute (RMPI)-1640 medium

Constituent	g L ⁻¹ water	Constituent	g L ⁻¹ water
L-arginine (free base)	0.200	Biotin	0.0002
L-asparagine (anhydrous)	0.050	D-pantothenic	0.00025
L-aspartic acid	0.020	Choline chloride	0.003
L-cystine . 2HCl	0.0652	Folic acid	0.001
L-glutamic acid	0.020	Myo-inositol	0.035
L-glutamine	0.300	Niacinamide	0,001
Glycine	0.010	PABA	0.001
L-histidine (free base)	0,015	Pyridoxine HCl	0.001
L-hydroxyproline	0.020	Riboflavin	0.0002
			0.000005
L-isoleucine	0.050	Thiamine HCl	0.001
L-lysine.HCl	0.040	Calcium nitrate.H ₂ O	0.100
L-methionine	0.015	Potassium chloride	0.400
L-phenylalanine	0.015	Magnesium sulphate (anhydrous)	0.04884
L-proline	0.020	Sodium chloride	6.000
L-serine	0.030	Sodium phosphate, dibasic (anhydrous)	0.800
L-threonine	0.020	D-glucose	2.000
L-tryptophan	0.005	Glutathione, reduced	0.001
L-tyrosine.2Na	0.02883	Phenol red, Na	0.0053
L-valine	0.020		

RMPI-1640 medium was buffered with 0.165 mol/L MOPS, 10.4g powdered RPMI-1640 medium (with glutamine and phenol red, without bicarbonate), 34.53g MOPS (3-[N-morpholino] propanesulfonic acid) buffer.

Dilution series used for the pure compound antifungal assay.

Table 7.3 Voriconazole dilution series.

Voriconazole										
	Conc.		Antifungal	DMSO	Int. Conc	Int. Conc	RPMI	Final Conc	Plate	
Step	ug/ml	Source	Vol ml	Vol ml	ug/ml	Vol ul	Vol ml	ug/ml	Row:	
1	1600	Stock			1600	100	4,9	32	1	
2	1600	Stock	0,5	0,5	800	100	4,9	16	2	
3	1600	Stock	0,5	1,5	400	100	4,9	8	3	
4	1600	Stock	0,5	3,5	200	100	4,9	4	4	
5	200	Step4	0,5	0,5	100	100	4,9	2	5	
6	200	Step4	0,5	1,5	50	100	4,9	1	6	
7	200	Step4	0,5	3,5	25	100	4,9	0,5	7	
8	25	Step 7	0,5	0,5	12,5000	100	4,9	0,2500	8	
9	25	Step 7	0,5	1,5	6,2500	100	4,9	0,1250	9	
10	25	Step 7	0,5	3,5	3,1250	100	4,9	0,0625	10	
			1st set of dilutions			2nd set of dilutions (1:50)				

Table 7.4 Dilution series for carnosol and carnosic acid

Antifungals (carnosol and carnosic acid)									
	Conc.		Antifungal	DMSO	Int. Conc	Int. Conc	RPMI	Final Conc	Plate
Step	ug/ml	Source	Vol ml	Vol ml	ug/ml	Vol ul	Vol ml	1:2 ug/ml	Row:
1	1000	Stock			1000	2500	2.5	500	1
2	1000	Stock	0,5	0,5	500	2500	2.5	250	2
3	1000	Stock	0,5	1,5	250	2500	2.5	125	3
4	1000	Stock	0,5	3,5	125	2500	2.5	62,5	4
5	125	Step4	0,5	0,5	62,5	2500	2.5	31,25	5
6	125	Step4	0,5	1,5	31,25	2500	2.5	15,625	6
7	125	Step4	0,5	3,5	15,625	2500	2.5	7,8125	7
8	15,625	Step 7	0,5	0,5	7,8125	2500	2.5	3,9063	8
9	15,625	Step 7	0,5	1,5	3,9063	2500	2.5	1,9531	9
10	15,625	Step 7	0,5	3,5	1,9531	2500	2.5	0,9766	10
			1st set of dilutions			2nd set of dilutions (1:5)			

Table 7.5 Plate layout

		1	2	3	4	5	6	7	8	9	10	11	12
ATCC 3629	A											DMSO/RPMI	RPMI
ATCC 3629	B											DMSO/RPMI	RPMI
MRC 826	C											DMSO/RPMI	RPMI
MRC 826	D											DMSO/RPMI	RPMI
MRC 8267	E											DMSO/RPMI	RPMI
MRC 8267	F											DMSO/RPMI	RPMI
MRC 7140	G											DMSO/RPMI	RPMI
MRC 7140	H											DMSO/RPMI	RPMI
												Controls	

Well 1-10:100ul of Inoculum: RPMI (1:50) solution
 100ul final concentration of voriconazole: RPMI (1:50) solution
 100ul final concentration of antifungal (compounds): RPMI (1:2) solution

7.4 Appendix IV

Callus discriminating points

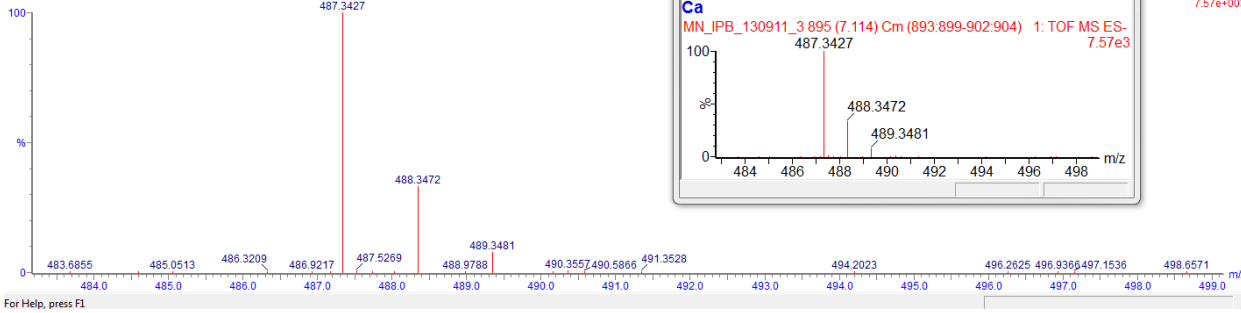
7.12

Single Mass Analysis

Tolerance = 100.0 PPM / DBE: min = -0.5, max = 50.0
 Element prediction: Off
 Number of isotope peaks used for i-FIT = 3
 Monoisotopic Mass, Even Electron Ions
 210 formula(e) evaluated with 19 results within limits (up to 50 closest results for each mass)
 Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O
487.3427	487.3423	0.4	0.8	7.5	C30 H47 O5	35.2	1.325	26.59	30	47	5	
	487.3437	-1.0	-2.1	12.5	C31 H43 N4 O	36.5	2.634	7.18	31	43	4	1
	487.3365	6.2	12.7	16.5	C37 H43	38.6	4.677	0.93	37	43		
	487.3325	10.2	20.9	12.5	C32 H43 N2 O2	36.3	2.407	9.01	32	43	2	2
	487.3536	-10.9	-22.4	7.5	C29 H47 N2 O4	36.2	2.269	10.34	29	47	2	4
	487.3284	14.3	29.3	8.5	C27 H43 N4 O4	37.3	3.372	3.43	27	43	4	4
	487.3576	-14.9	-30.6	11.5	C34 H47 O2	36.9	3.034	4.81	34	47	2	
	487.3717	71.5	44.1	17.5	C33 H42 O2	36.5	2.565	7.69	33	42		2

Ca
 MN_IPB_130911_3 895 (7.114) Cm (893.899-902.904)



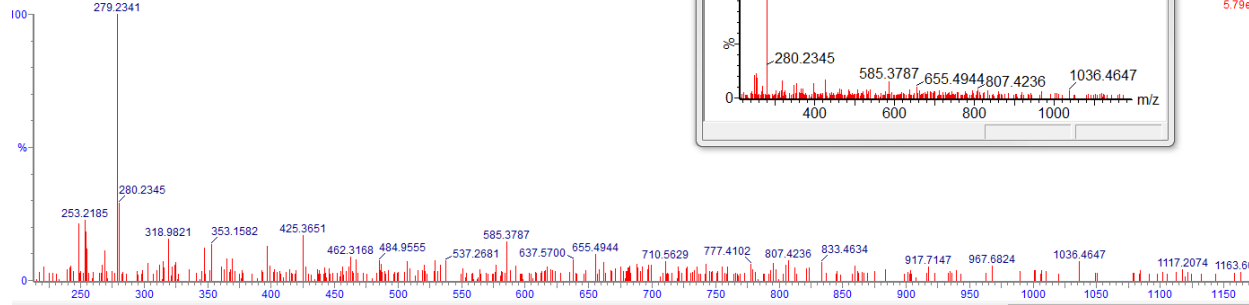
11.24

Single Mass Analysis

Tolerance = 100.0 PPM / DBE: min = -0.5, max = 50.0
 Element prediction: Off
 Number of isotope peaks used for i-FIT = 3
 Monoisotopic Mass, Even Electron Ions
 122 formula(e) evaluated with 9 results within limits (up to 50 closest results for each mass)
 Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O
279.2341	279.2324	1.7	6.1	3.5	C18 H31 O2	17.8	2.649	7.07	18	31		2
	279.2396	-5.5	-19.7	-0.5	C12 H31 N4 O3	16.6	1.428	23.98	12	31	4	3
	279.2284	5.7	20.4	-0.5	C13 H31 N2 O4	17.3	2.165	11.48	13	31	2	4
	279.2436	-9.5	-34.0	3.5	C17 H31 N2 O	17.2	2.110	12.13	17	31	2	1
	279.2185	15.6	55.9	4.5	C15 H27 N4 O	16.9	1.761	17.19	15	27	4	1
	279.2171	17.0	60.9	-0.5	C14 H31 O5	18.3	3.130	4.37	14	31		5
	279.2549	-20.8	-74.5	3.5	C16 H31 N4	17.0	1.884	15.19	16	31	4	
	270.2113	77.8	81.7	8.5	C17 H27	18.5	3.300	3.24	17	27		

Ca
 MN_IPB_130911_3 1416 (11.244) Cm (1416-1417)



9.63

Single Mass Analysis

Tolerance = 100.0 PPM / DBE: min = -0.5, max = 50.0

Element prediction: Off

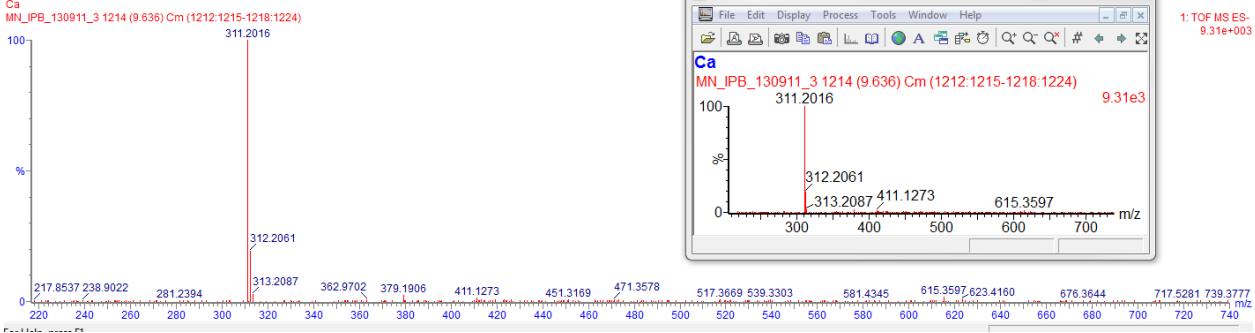
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

135 formula(e) evaluated with 12 results within limits (up to 50 closest results for each mass)

Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O
311.2016	311.2011	0.5	1.6	8.5	C21 H27 O2	17.7	5.658	0.35	21	27	2	2
	311.1971	4.5	14.5	4.5	C16 H27 N2 O4	14.0	2.031	13.12	16	27	2	4
	311.2083	-6.7	-21.5	4.5	C15 H27 N4 O3	16.8	4.835	0.79	15	27	4	3
	311.2123	-10.7	-34.4	8.5	C20 H27 N2 O	18.2	6.241	0.19	20	27	2	1
	311.1872	14.4	46.3	9.5	C18 H23 N4 O	17.4	5.390	0.46	18	23	4	1
	311.1858	15.8	50.8	4.5	C17 H27 O5	12.4	0.429	65.12	17	27	5	5
	311.2222	-20.6	-66.2	3.5	C18 H31 O4	13.6	1.641	19.38	18	31	4	4
	311.1860	17.6	56.4	12.5	C24 H22	20.0	8.887	0.01	24	22		



3.72

Single Mass Analysis

Tolerance = 100.0 PPM / DBE: min = -0.5, max = 50.0

Element prediction: Off

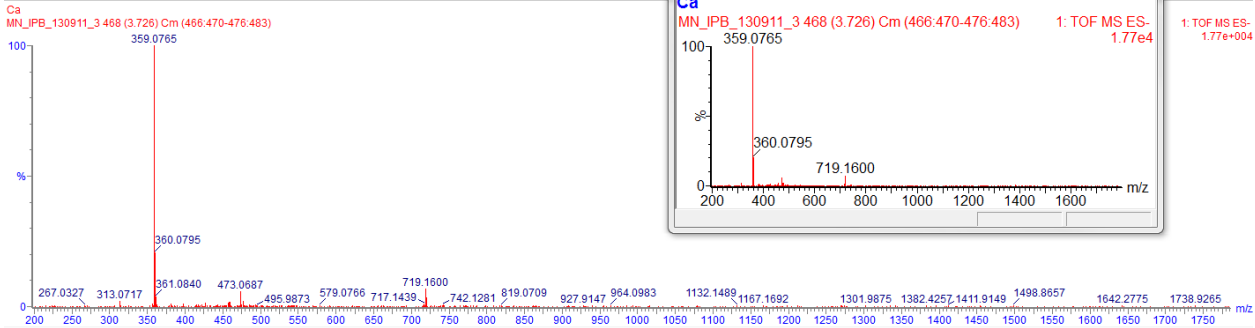
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

154 formula(e) evaluated with 14 results within limits (up to 50 closest results for each mass)

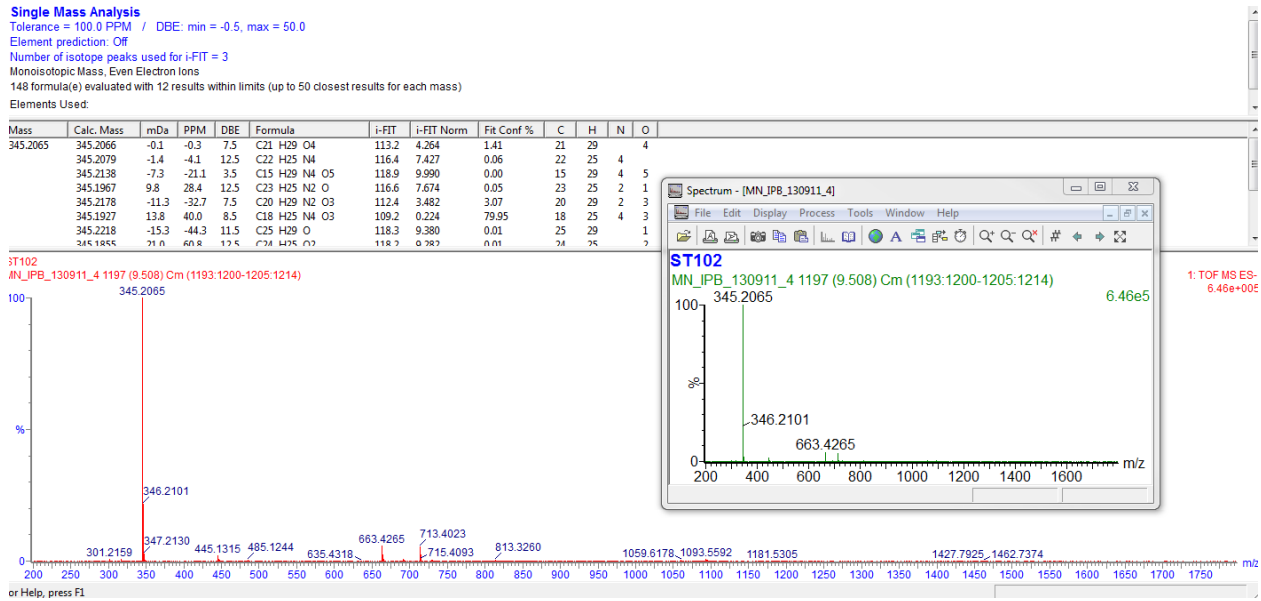
Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O
359.0765	359.0780	-1.5	-4.2	16.5	C19 H11 N4 O4	31.0	1.489	22.56	19	11	4	4
	359.0821	-5.6	-15.6	20.5	C24 H11 N2 O2	33.8	4.273	1.39	24	11	2	2
	359.0708	5.7	15.9	20.5	C25 H11 O3	33.7	4.190	1.52	25	11		3
	359.0861	-9.6	-26.7	24.5	C29 H11	35.0	5.471	0.42	29	11		
	359.0668	9.7	27.0	16.5	C20 H11 N2 O5	30.7	1.233	29.15	20	11	2	5
	359.0919	-15.4	-42.9	15.5	C22 H15 O5	32.5	3.008	4.94	22	15	5	5
	359.0609	15.6	43.4	25.5	C27 H7 N2	35.0	5.508	0.41	27	7	2	2
	359.0833	-16.8	-46.8	20.5	C23 H11 N4 O	34.4	4.088	0.74	23	11	4	1



Stellenbosch plant extract (ST) discriminating peak

9.51



Whole plant ground sample (WE) discriminating peak

9.97

