

**Development of *Siphonochilus aethiopicus* as a treatment for
colds and influenza and gas chromatographic analysis of
volatiles of an insect repellent**

by

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Submitted in partial fulfillment of the requirements for the degree

MAGISTER SCIENTIAE

In the Faculty of Natural & Agricultural Sciences

UNIVERSITY OF PRETORIA

PRETORIA

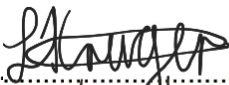
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September 2020

Declaration

I, **LEYLENE KRUGER** declare that the thesis/dissertation, which I hereby submit for the degree of MSc Chemistry at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: 
Date: 29/09/2020

Acknowledgements

I would like to express my gratitude towards my supervisor, Professor V. J. Maharaj, for the time, patience, guidance, support and learning opportunities you have presented me during the course of my work. You have encouraged me to work harder and allowed me to discover my passion for natural products and their endless possibilities. I would also like to thank my co-supervisor, Dr. G. Fouché, for your scientific contribution which has made this work a success.

I would like to give a special thanks to the strong collaboration of Prof. Thomas Klimkait and Ms. Lorena Urda for the training they provided me in the bioassay studies which were performed in a BSL-2 laboratory during my research visit to their facility at the Department of Biomedicine, University of Basel in Switzerland.

This work would not have been possible without the financial support from the National Research Foundation (NRF) and Innovon Technologies. Your support is much appreciated.

To my fellow members of the Bioprospecting group (A. Thakur, B. Tembeni, B. Mzondo, C. Ezeofor, P. Mc Millan, L. Invernizzi, S. Mianda, N.K Khorommbi, S. Muyisa, W. Rudolph and F. Katele) of the Department of Chemistry at the University of Pretoria I would like to thank for your scientific advice, various expertise and assistance. Thank you for the interesting conversations and eventful moments we also shared during our time in the laboratory. To all my friends in the Department of Chemistry for all the support, words of wisdom and laughs we shared.

I would like to thank Prof. W. J. Schoeman for the years of mentorship and guidance during my studies at the University of Pretoria. I would not have been where I am today without your assistance.

I am grateful for the love, kind-heartedness, emotional support and endless encouragement of my parents (Hannes and Hettie Kruger) and my sister (Zinette Kruger). You have always been my biggest supporters and my strongest support system. None of

this would have been possible without your constant motivation and enduring love. Thank you for being so invested in my future. I would also like to thank my grandmother for her continuous support and care.

To my friends whom I keep so close to my heart, each one of you have been with me from the start and never stopped supporting, motivating and caring for me during the course of this time. You encouraged me to follow my dreams and stay strong during the difficult times and filled my life with laughter and memories during the good times. I will forever be grateful for your grace, love and friendship.

And then most importantly I want to thank God for His undefining love and for the endless number of opportunities He has placed on my road to success. He has placed so many people in my life who have played an important role in the success of my work and I will always be grateful for His presence in my life.

***“If you are not willing to learn, no one can help you.
If you are determined to learn, no one can stop you,”***

- Brian Tracy

Abstract

Part A of the study focuses on *Siphonochilus aethiopicus*, also known as African ginger, to determine its antiviral properties and the compounds responsible for the biological activity. African ginger is one of the most well-known medicinal plants in South Africa and is traditionally used to treat asthma, colds, coughs, flu, influenza, headaches and malaria. The biological properties of the plant have been extensively researched, however limited studies are reported on its antiviral activity and the compounds that contribute to this biological activity. Various compounds have been isolated from the plant, the most common being the sesquiterpenoid, siphonochilone, which is reported as the major compound of the plant, and its structurally similar compounds identified as lactones. The aim of this study was to prepare and identify a suitable extract, evaluate extracts for their antiviral properties against the influenza virus and isolate and purify the biologically active compounds.

Chapter 2 describes the different methods and conditions of extraction to quantitatively and qualitatively obtain the most suitable extracts. Fresh rhizomes, freshly dried ground rhizomes and five-year-old dried ground rhizomes were extracted with different polarities of solvents and analysed using GC-MS. It was concluded that the five-year-old dried ground rhizomes were the most suitable plant material to use for extraction since it contained the targeted compounds: siphonochilone and its hydroxylated lactone.

Different purification methods were used for the extracts of the five-year-old dried ground rhizomes for the isolation of the targeted compounds which is described in Chapter 3. In this chapter an automated SPE, liquid handler and HPLC was used to successfully isolate and purify adequate quantities of the hydroxylated lactone and was confirmed using NMR. Siphonochilone crystallized out during steam distillation, and its structure confirmed by NMR.

The isolated compounds along with the essential oil and ethanol extract were used for biological evaluation for antiviral properties against the influenza virus which is described in Chapter 4. The results suggested that the pure compounds, siphonochilone, and its

hydroxylated lactone, showed significant inhibition against an Influenza A-type virus, at concentrations equivalent to the positive control, ribavirin. These results would suggest that siphonochilone and its hydroxylated lactone both could serve as possible natural antiviral drugs.

Part B of the study focuses on the analysis of a natural insect repellent and the longevity of the active ingredients during diffusion. Chapter 5 discusses the analysis of Noot-a-Bug, sweet orange oil blended with a naturally sourced enzyme cocktail, as a possible natural insect repellent. GC-MS analysis was performed to confirm the conversion of nootkatone from valencene in the sweet orange oil. HS-SPME-GC-MS was used to analyse the continuous diffusion of Noot-a-Bug and confirm the presence of nootkatone in the vaporised sample over a period of 24-h in a controlled environment.

Table of Contents

Declaration	2
Acknowledgements	3
Abstract	6
Table of Content.....	8
List of Figures.....	14
List of Tables.....	18
Abbreviations	20
Supplementary Data.....	22
Chapter 1: General Introduction to <i>Siphonochilus aethiopicus</i>.....	23
1.1 Background.....	23
1.1.1 Natural Products.....	23
1.1.2 African Traditional Medicine and their role in health care systems	24
1.1.3 Traditional medicine in South Africa and its commercialization	25
1.1.4 Background to <i>Siphonochilus aethiopicus</i>	27
1.1.4.1 Morphology	28
1.1.4.2 Traditional uses.....	29
1.1.4.3 Conservation	30
1.1.5 Biological evaluation and phytochemical studies of African ginger.....	31
1.1.5.1 Biological evaluation of African ginger	31
1.1.5.2 Chemical composition and extraction of metabolites	33
1.2 Problem statement and justification	37
1.3 Aims and objectives.....	39
1.4 References	39

Chapter 2: Chemical profiling of extracts of African ginger.....	48
2.1 Background.....	48
2.1.1 Secondary metabolites.....	48
2.1.2 Plant material.....	49
2.1.3 Solvents.....	50
2.1.4 Extraction methods.....	52
2.1.4.1 Solid-liquid extraction.....	52
2.1.4.2 Steam distillation.....	53
2.1.5 Extraction of African ginger.....	54
2.2 Methodology.....	55
2.2.1 Chemicals.....	55
2.2.1.1 Analytical grade chemicals.....	55
2.2.1.2 Other chemicals.....	55
2.2.1.3 Standards.....	56
2.2.2 Sourcing and preparation of plant material.....	56
2.2.2.1 Fresh rhizomes for extraction and steam distillation.....	56
2.2.2.2 Dried rhizomes from freshly harvested rhizomes for extraction.....	57
2.2.2.3 Five-year-old rhizomes for extraction.....	57
2.2.3 Organic solvent extraction.....	57
2.2.3.1 Diethyl ether as extractant.....	57
2.2.3.2 Ethanol as extractant.....	57
2.2.3.3 Dichloromethane:methanol as extractant.....	58
2.2.3.4 Dichloromethane:n-hexane as extractant.....	58
2.2.4 Steam distillation.....	59
2.2.5 GC-MS analysis of the various extracts.....	60
2.3 Results and discussion.....	61
2.3.1 Extraction yields.....	61
2.3.2 Qualitative analysis of the extracts.....	64
2.3.2.1 GC-MS analysis of extracts of fresh wet rhizomes.....	64

2.3.2.2	GC-MS analysis of extracts from freshly dried ground rhizomes.....	68
2.3.2.3	GC-MS analysis of extracts from five-year-old dried ground rhizomes	72
2.3.2.4	Comparison of freshly dried ground rhizomes and five-year-old dried ground rhizomes extracted with different organic solvents.....	74
2.4	Conclusion	77
2.5	References.....	79

**Chapter 3: Isolation, purification and characterization of compounds from African
ginger 87**

3.1	Background.....	87
3.1.1	Isolation of compounds from plant extracts and African ginger	87
3.1.2	Modern chromatographic techniques	88
3.1.2.1	Supercritical fluid chromatography (SFC)	89
3.1.2.2	Ultra-performance convergence chromatography (UPC ²).....	90
3.1.2.3	Liquid handler and automated solid-phase extraction (SPE).....	91
3.1.2.4	High-performance liquid chromatography (HPLC)	93
3.2	Methodology	94
3.2.1	Analytical grade solvents.....	94
3.2.2	Other chemicals.....	94
3.2.3	Other equipment and apparatus.....	94
3.2.4	Solvent extraction of plant material.....	95
3.2.5	Standards for analysis	95
3.2.6	Purification using supercritical fluid chromatography (SFC)	95
3.2.7	Ultra-performance convergence chromatography (UPC ²)	97
3.2.8	Purification of fractions using column chromatography	98
3.2.9	Solid-phase extraction (SPE).....	101
3.2.10	High-performance liquid chromatography (HPLC).....	102
3.2.11	Nuclear magnetic resonance spectroscopy (NMR)	105
3.3	Results and discussions	106

3.3.1	SFC purification of the hydroxylated lactone (6) from African ginger extracts and analysis using UPC ²	106
3.3.2	Purification of the combined fractions 7 and 9(FR1) using silica column chromatography	117
3.3.3	Semi-purification using automated SPE of DCM:hexane extract of five-year-old dried ground rhizomes of African ginger.....	118
3.3.4	Preparative HPLC purification of SPE fractions (fraction labelled as FR4).....	119
3.3.5	Structure elucidation of siphonochilone (1) and the hydroxylated lactone (6) using NMR spectroscopy	123
3.3.5.1	Compound and structure confirmation of siphonochilone (1)	123
3.3.5.2	Structure elucidation of the hydroxylated lactone (6)	125
3.4	Conclusion	129
3.5	References	130

Chapter 4: Biological evaluation of extracts and compounds from African ginger against Influenza A-type virus..... 139

4.1	Background.....	139
4.1.1	Influenza virus	139
4.1.2	Prevention of Influenza.....	140
4.1.3	Antiviral drugs.....	140
4.1.3.1	Proton channel inhibitors.....	141
4.1.3.2	Neuraminidase inhibitors.....	141
4.1.3.3	Ribavirin	143
4.1.4	Natural antiviral drugs.....	145
4.1.5	Previous research on the antiviral efficacy of African ginger	145
4.2	Methodology	146
4.2.1	Buffers, chemicals and reagents	147
4.2.2	Apparatus and consumables	147
4.2.3	Plaque Assay.....	147
4.2.3.1	Seeding of MDCK cells	147

4.2.3.2	Infectious media for viral growth.....	148
4.2.3.3	Virus dilution.....	148
4.2.3.4	Read out of viral growth	149
4.2.3.5	Titre calculations	149
4.2.4	Test samples	150
4.2.5	CERA-I bioassaying using the essential oil, ethanol extract and siphonochilone (1) as test samples.....	150
4.2.5.1	Seeding of MDCK cells	151
4.2.5.2	Infectious media for viral growth.....	151
4.2.5.3	Virus dilution.....	151
4.2.5.4	Dilution of test samples	151
4.2.5.5	Dilution of positive control (ribavirin)	152
4.2.5.6	Read out of viral growth	152
4.2.6	CERA-I using siphonochilone (1) and its hydroxylated lactone (6) as test samples	152
4.2.6.1	Seeding of MDCK cells	152
4.2.6.2	Infectious media for viral growth.....	153
4.2.6.3	Virus dilution.....	153
4.2.6.4	Dilution of pure compounds (test samples)	153
4.2.6.5	Dilution of positive control (ribavirin)	153
4.2.6.6	Read out of viral growth	154
4.3	Results and discussions	154
4.3.1	Plaque Assay.....	154
4.3.2	The effect of the essential oil, ethanol extract and siphonochilone (1) in the Cytopathic Effect Reduction Assay against Influenza A (CERA-I).....	157
4.3.3	The effect of siphonochilone (1) and the hydroxylated lactone (6) in the Cytopathic Effect Reduction Assay against Influenza A (CERA-I).....	159
4.4	Conclusion	162
4.5	References	164

Chapter 5: GC-MS and HS-SPME-GC-MS analysis of a natural insect repellent in a formulation	171
5.1 Background.....	171
5.1.1 Insect repellents	171
5.1.2 Natural insect repellents	172
5.1.3 Noot-a-Bug as an insect repellent	173
5.1.3.1 Nootkatone as an insect repellent compound.....	174
5.1.3.2 Sweet orange oil	174
5.1.4 GC-MS and HS-SPME analysis	176
5.2 Problem statement and justification	177
5.3 Goals and objectives.....	177
5.4 Methodology	178
5.4.1 Standards	178
5.4.2 Noot-a-Bug sample.....	178
5.4.3 GC-MS analysis for identification of targeted compounds	178
5.4.4 Diffusing of Noot-a-Bug into the environment.....	179
5.4.5 HS-SPME-GC-MS for identification of targeted compounds in the diffused vapour	179
5.5 Results and discussions	180
5.5.1 GC-MS analysis of insect repellent.....	180
5.5.2 HS-SPME-GC-MS analysis of Noot-a-Bug released as a vapour.....	184
5.6 Conclusion	188
5.7 References	189
Supplementary Data.....	197

List of Figures

Chapter 1: General Introduction to *Siphonochilus aethiopicus*

- Figure 1.1** Bright pink or purple flowers of African ginger that appear at ground level 28
- Figure 1.2** Cone-shaped rhizomes and succulent roots 29
- Figure 1.3** The structures isolated by Holzapfel et al. (2002) of siphonochilone **(1)**, the hydroxylated derivative **(2)** and the acetylated compound **(3)**..... 34
- Figure 1.4** Structure of eucalyptol **(4)** 35
- Figure 1.5** The structures of the lactones isolated by Lategan et al. (2009) 36

Chapter 2: Chemical profiling of extracts of African ginger

- Figure 2.1** Illustration of the steam distillation setup used to isolate essential oil from African ginger 60
- Figure 2.2** Chromatogram from the GC-MS analysis of **(1)** siphonochilone, **(2)** the essential oil and **(3)** the diethyl ether extract from fresh rhizomes 67
- Figure 2.3** Chromatogram from the GC-MS analysis of **(1)** siphonochilone at 1273.60 s and **(2)** the hydroxylated lactone at 1564.20 s..... 69
- Figure 2.4** Chromatogram from the GC-MS analysis of the **(1)** diethyl ether extract, **(2)** ethanol, **(3)** DCM:methanol and **(4)** DCM:hexane extract of freshly dried ground rhizomes 71
- Figure 2.5** Chromatogram from the GC-MS analysis of the **(1)** diethyl ether extract, **(2)** ethanol, **(3)** DCM:methanol and **(4)** DCM:hexane extract of five-year-old dried ground rhizomes 73

Figure 2.6	Graph of the area percentage (%) of the total chromatogram of the different extracts (1 mg/mL) from freshly dried ground rhizomes and five-year-old dried ground rhizomes.	76
Chapter 3:	Isolation, purification and characterization of compounds from African ginger	
Figure 3.1	Illustration of the Investigator SFC system used to fractionate the crude extract.	96
Figure 3.2	Purification of the DCM:methanol (1:1) crude extract using SFC, UPC ² and column chromatography	100
Figure 3.3	Illustration of the liquid handler, automated SPE and single syringe pump used for the solid phase extraction	101
Figure 3.4	Process for the isolation of the hydroxylated lactone (6) using SPE, HPLC and NMR spectroscopy.....	105
Figure 3.5	Chromatogram of (1) the hydroxylated lactone, (2) siphonochilone and (3) the crude extract at 210 – 270 nm from SFC analysis using an analytical column.	107
Figure 3.6	Chromatogram from SFC analysis of the crude extract at 210 – 270 nm with retention times 5.30, 6.05, 7.65 and 10.80 min using a semi-preparative column	108
Figure 3.7	Chromatograms of (1) the hydroxylated lactone, (2) siphonochilone and (3) the crude extract analysed using UPC ² at 215 nm.....	111
Figure 3.8	Chromatogram at 215 nm of (1) fraction 7, (2) fraction 9 pre-fractionated from SFC and (3) crude extract analysed using UPC ²	112
Figure 3.9	Chromatogram at 215 nm of (1) fraction 7 before and (2) fraction 7 after “spiking” with standard hydroxylated lactone	113

Figure 3.10	Chromatograms of (1) the crude extract, (2) fresh rhizomes – and (3) freshly dried ground rhizomes ethanol extracts analysed using UPC ² at 215 nm....	116
Figure 3.11	HPLC chromatograms at 220 nm of (1) the standard hydroxylated lactone and (2) the combined fractions, FR4, obtained from SPE at 220 nm combined fractions (FR4) during the analytical study and method development....	120
Figure 3.12	HPLC chromatogram at 220 nm of FR4 for purification with semi-preparative column	122
Figure 3.13	Structures of siphonochilone (1) and its hydroxylated lactone (2) with numbering of atoms	123
Figure 3.14	Selected NOE interactions of the hydroxylated lactone (6)	128
Chapter 4:	Biological evaluation of extracts and compounds from African ginger against Influenza A-type virus	
Figure 4.1	Structures of amantadine (8) and rimantadine (9)	141
Figure 4.2	Structures of zanamivir (10) , peramivir (11) and oseltamivir (12)	143
Figure 4.3	Structure of ribavirin (13)	144
Figure 4.4	Layout of the methodology for the antiviral screening of test samples ...	147
Figure 4.5	The 24-well plate after 4 h of incubation of the two strains of viruses on the MDCK cells from the plaque assay.	155
Figure 4.6	Percentage inhibition and cytotoxicity (red bars) of the test samples; essential oil, ethanol extract and siphonochilone isolated from African ginger, and the control, ribavirin (orange bars).....	158
Figure 4.7	Percentage inhibition and cytotoxicity (red bars) of pure compounds, siphonochilone and its hydroxylated lactone, and the control, ribavirin (orange bars), against a strain of Influenza.	160

Figure 4.8 IC₅₀ curve of the percentage inhibition and logarithmic concentration of **(1)** siphonochilone, **(2)** the hydroxylated lactone and **(3)** ribavirin 161

Chapter 5: GC-MS and HS-SPME-GC-MS analysis of a natural insect repellent in a formulation

Figure 5.1 Structures of DDT **(1)** and DEET **(2)** 171

Figure 5.2 Commercialized insect repellents containing different essential oils; Wild Basil & Lemongrass mosquito repellent (3), Earthsap Bugs Away roll-on (4), The Apothecary insect repellent spray (5) and Natural Orange House Hold insect repellent (6)..... 173

Figure 5.3 Active compounds with insect repellent properties..... 175

Figure 5.4 Chromatograms from GC-MS analysis of **(1)** valencene; 1015.80 s and **(2)** Nootkatone; 1258.60 s 181

Figure 5.5 Chromatograms from GC-MS analysis of **(1)** Sweet orange oil and **(2)** Noot-a-Bug..... 183

Figure 5.6 Chromatograms from GC-MS of Noot-a-Bug at **(1)** 0 h, **(2)** 1 h, **(3)** 2 h, **(4)** 4 h, **(5)** 6 h and **(6)** 24 h 186

List of Tables

Chapter 2: Chemical profiling of extracts of African ginger

Table 2.1	Mass (grams) of the differently prepared plant material used for extraction with 200 mL different organic solvents	59
Table 2.2	The percentage yield (%) of the differently prepared plant material extracted with different organic solvents	62

Chapter 3: Isolation, purification and characterization of compounds from African ginger

Table 3.1	Elution gradient used for the analysis of fraction FR4 on an X-Bridge C18 analytical column.....	103
Table 3.2	Elution gradient used for the purification of FR4 on a Waters X-Bridge Preparative C18 column.....	104
Table 3.3	Time-based collection of four peaks from FR4 on a Waters X-Bridge Preparative C18 column.....	104
Table 3.4	The retention times and the mass of each fraction collected from the crude extract	109
Table 3.5	The results from the UPC ² analysis of Fraction 7 before and after addition of the standard hydroxylated lactone to confirm the presence of the compound confirmation	114
Table 3.6	The time intervals of fractions collected using HPLC and their collected mass (mg)	121
Table 3.7	¹ H and ¹³ C data of siphonochilone in CD ₂ Cl ₂ compared to published data in CDCl ₃ [15]	125

Table 3.8	^1H , ^{13}C and HMBC data of the hydroxylated lactone compared to literature data ^[18]	127
------------------	--	-----

Chapter 4: Biological evaluation of extracts and compounds from African ginger against Influenza A-type virus

Table 4.1	Plaque assay readout of the two strains (pfu/mL in blue indicate the highest pfu/mL for each strain of virus).....	156
------------------	--	-----

Chapter 5: GC-MS and HS-SPME-GC-MS analysis of a natural insect repellent in a formulation

Table 5.1	The area percentage of the total chromatogram of valencene, nootkatone and other compounds with known repellency properties.....	184
------------------	--	-----

Table 5.2	The retention time and area percentage of the total chromatogram of repellent compounds in Noot-a-Bug diffused over a 24-h period	188
------------------	---	-----

Abbreviations

ABPR	Automated back pressure regulator
ASE	Accelerated solvent extraction
BEH	Ethylene-bridged hybrid
BPR	Back pressure regulator
BSL-2	Biosafety level 2
CC	Convergence chromatography
CD ₂ Cl ₂	Deuterated dichloromethane
CDCl ₃	Deuterated chloroform
cDMEM	Complete Dulbecco's Modified Eagle Medium
CERA-I	Cytopathic Effect Reduction Assay against Influenza A
COX	Cyclooxygenase
CPC	Centrifugal partition chromatography
CPE	Cytopathic effect
CSIR	Council for Scientific and Industrial Research
DCM	Dichloromethane
DDT	Dichlorodiphenyltrichloroethane
DEET	N,N-diethyl-m-toluamide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
FCS	Foetal calf serum
GC-FID	Gas chromatography-flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
GC-TOF-MS	Gas chromatography time-of-flight mass spectrometry
HPLC	High-performance liquid chromatography

HPLC-SPE-NMR	High-performance liquid chromatography-solid phase extraction-nuclear magnetic resonance
HS-SPME	Headspace-solid phase microextraction
HSV	Herpes simplex virus
IC ₅₀	Half maximal inhibitory concentration
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
MDCK	Madin-Darby Canine Kidney epithelial cells
MOI	Multiplicity of infection
NAI	Neuraminidase inhibitors
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered-saline
PDA	Photodiode array
PFU	Plaque-forming units
SDS	Sodium dodecyl sulfate
SFC	Supercritical fluid chromatography
SFE	Supercritical fluid extraction
SPE	Solid phase extraction
TCID ₅₀	Median tissue culture infectious dose
TFA	Trifluoroacetic acid
TLC	Thin-layer chromatography
TPCK	Tosyl phenylalanyl chloromethyl ketone
UAE	Ultrasound-assisted extraction
UHP	Ultra-high purity
UPC ²	Ultra-performance convergence chromatography
UPLC-QTOF-MS	Ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry
VLC	Vacuum liquid chromatography
wp	Well plate

Supplementary Data

Supplementary data 1:	^1H NMR spectrum of siphonochilone	197
Supplementary data 2:	^{13}C NMR spectrum of siphonochilone	198
Supplementary data 3:	^1H - ^1H COSY data of siphonochilone	199
Supplementary data 4:	^1H NMR spectrum of the hydroxylated lactone	200
Supplementary data 5:	^{13}C NMR spectrum of the hydroxylated lactone	201
Supplementary data 6:	^1H - ^1H NOESY data of the hydroxylated lactone	202
Supplementary data 7:	^1H - ^1H COSY data of the hydroxylated lactone	203
Supplementary data 8:	HSQC data of the hydroxylated lactone	204
Supplementary data 9:	HMBC data of the hydroxylated lactone	205

PART A:

Development of *Siphonochilus aethiopicus* as a treatment for colds and influenza

Chapter 1: General Introduction into *Siphonochilus aethiopicus*

1.1 Background

1.1.1 Natural Products

Natural products are compounds derived from natural sources, like plants, animals or micro-organisms, and have been used to treat various ailments for thousands of years.^[1,2]

Fundamental research on living organisms in nature has revealed that they are of utmost importance towards forming new insights into understanding their biological mechanisms, analysing active ingredients and their pharmaceutical possibilities.^[3-5] Society has become aware of the toxicity and side effects of some synthetic- and allopathic drugs and has reverted to natural products.^[1,6] Medicinal products are used by 80% of the world population and there has been an increase in the demand for sustainable plant-based products in cosmeceuticals, household products, pharmaceuticals and wholefoods.^[7,8]

Throughout history, natural products have been used worldwide to treat various diseases and used in the development of medicinal products.^[8,9] Before their biological effects were researched and used in pharmaceuticals, many of these plants were used as traditional medicine to treat various ailments.^[10,12]

Medicinal plants are categorized into pharmaceuticals, which are plants used in pharmaceuticals or Western medicine, and traditional medicine, used by traditional health practitioners.^[13] The competing or complementing tendency between the two has been discussed in many publications in order to integrate them and provide the best possible plant-based pharmaceuticals.^[13] Many believe both can work together to produce better pharmaceuticals, but others believe that producing pharmaceuticals takes away the medicinal belief and cultural importance.^[14,15] For many years traditional medicine was the most common health system in Africa, but this has changed over the years.^[22] Integration of the two has allowed for proper research and commercialization of important pharmaceuticals.^[14,17]

After the discovery of antibiotics from penicillin in the 1920's, a vast amount of research on natural compounds became more typical, resulting in plant-based pharmaceuticals.^[1,8,11,18] Some well-known discoveries include the discovery of quinines from *Cinchona calisaya* and artemisinin from *Artemisia annua* for the treatment of malaria, taxol from *Taxus brevifolia* for the treatment of breast cancer and aspirin from the *Salix spp.* as a pain reliever.^[17,20-22]

African Traditional Medicine plays a big role in the health care system of people living on the African continent and it is important to recognize this as a suitable health care system.^[5]

1.1.2 African Traditional Medicine and their role in health care systems

The use of medicinal plants in African Traditional Medicine is one of the oldest known health care systems in the world.^[23,24] Africa is abundant in plants with different medicinal properties that are used to maintain health, promote healing or treat various illnesses such as cancer, coughs, colds, diabetes, fever, high-blood pressure, skin problems, stomach aches and wounds.^[10,24,25] In South Africa more than 70% of the population makes use of traditional medicine for cultural and health care purposes.^[25] Most people in developing countries or rural areas are limited to using traditional medicine due to

economic and cultural factors as a result of which they are the only source of affordable and accessible healthcare services.^[6,23]

Approximately 10% of the world's plant species can be found in southern Africa with only 10% of African plants being used for traditional medicine. ^[12,26,27] This significantly low number is due to limited available information on the plants' morphology, genetics and chemical characters. With modern technology at our disposal, scientists can now investigate and develop more plant-based products.^[12,26,28]

Until the 1980's, natural product discovery relied only on traditional knowledge. Since the 20th century, active compounds have been isolated, identified and biologically evaluated with the aim of producing better pharmaceuticals.^[13,17] All of this has only been possible due to the cooperation of traditional healers.^[29] They hold the rights to the traditional knowledge of their medicine, but many of them have allowed for sharing of knowledge for the purpose of generating natural products, sharing of profits, and preventing the loss of knowledge.^[15,30] It is important to protect traditional knowledge because it improves the livelihood of the healer and community, and changes peoples outlook of traditional medicine.^[31]

Even with the considerable amount of research already done on natural products, there is still an immense potential for commercialization of so many undiscovered species. Many plants in Africa are/have been researched of which many are found in South Africa.^[12,27]

1.1.3 Traditional medicine in South Africa and its commercialization

More than 700 different plant species are traded in South Africa and approximately 230 are sold at muthi markets.^[32] Indigenous plants are used for the trading of medicinal – and spiritual plants and used to treat various ailments for example *Hypoxis obtuse*, *Siphonochilus aethiopicus* (African ginger), *Drimia sanguinea* (“Transvaal slangkop”), *Helichrysum kraussii* (Curry bush), *Pelargonium sidoides* (geranium) and *Acacia*

xanthophloea (fever tree).^[26,32-34] The overexploitation of these plants can threaten the biodiversity of a country, and for a country like South Africa, which has a rich plant biodiversity, protection of these plants is very important.^[32,34,35] If not protected this can result in price increases and the extinction of certain plants. If this is the case, the plants can no longer be used to treat certain ailments or be researched for commercialization.^[32,34]

The potential for production of natural products from indigenous plants has improved over the years.^[28] Some of the most popular indigenous South African plants that have been used for commercialization include *Aloe ferox* (Cape aloe), *Harpagophytum procumbens* (Devil's claw), *Hoodia gordonii*, *Siphonochilus aethiopicus* (African ginger), *Artemisia afra* (African wormwood), *Athrixia phyllicoides* (Bushman's tea) and *Carpobrotus edulis* (Sour fig).^[12,26,27]

Cape Aloe has been traditionally used for wound healing, treatment of arthritis, herpes, sore throats and ulcers. It has since been identified to have anti-oxidant, anti-inflammatory, antimicrobial and anticancer properties, and has more recently been commercialized into gels to treat leg ulcers, cosmetics to relief sunburn and used as a flavouring agent in beverages and food.^[36,37]

Devil's claw has been traditionally used to treat arthritis, blood diseases, diabetes, fever, pain and ulcers.^[38] It has more recently been researched scientifically and used as an anti-oxidant, anti-inflammatory and antimicrobial.^[38-40] It is also used to treat arthritis, heart diseases, kidney inflammation and tendonitis.^[22,38] It is widely used in herbal products and different teas in countries like Germany, USA and Netherlands, and is the third most popular plant used in Germany and its demand keeps on increasing.^[38]

Hoodia was first used by the San people in South Africa as a source of food and water, and for the treatment of abdominal pain, tuberculosis and diabetes.^[42] Research done on the plant at the Council for Scientific and Industrial Research (CSIR) reported the appetite suppressant properties and has the plant been traded worldwide for commercialization for the management of weight.^[41,42]

One of the most popular indigenous medicinal plants is African ginger (*Siphonochilus aethiopicus*).^[12] It has many traditional uses and has been significantly investigated to scientifically prove its medicinal properties, as discussed later (see section 1.1.5).^[7,43,44] A considerable number of African ginger containing products are available and have been used by consumers: Relief African ginger capsules to relieve allergies, hayfever, colds and coughs; Bioharmony Bio-African Ginger Tablets to relief headaches, colds, flu, sore throats and sinuses; and Phyto-Nova African Ginger Tablets used to treat headaches, influenza and sinuses.^[45-47]

African ginger has many traditional uses, but its active compounds have not been extensively researched.^[27] It has many medicinal properties and it is important to keep on investigating indigenous plants such as African ginger.^[26]

1.1.4 Background to *Siphonochilus aethiopicus*

Siphonochilus aethiopicus (Schweinf.) B.L. Burtt., better known as African ginger, grows naturally in tropical and southern African countries such as Malawi, South Africa, Zimbabwe and Zambia.^[26,43,48] It is part of the ginger family, Zingiberaceae, which consists of 53 plant genera and more than 1200 species.^[49,50]

Two popular ginger species in South Africa are *Siphonochilus aethiopicus* (African ginger) and *Zingiber officinale* (commercial ginger).^[9,51]

The rhizomes of both species contain volatile oils that have very distinct smells.^[4,51,52] There are many physical similarities to the plants, but they differ greatly in chemical composition such as the different types of terpenoids present in both species.^[9,52] It is not only important to understand and investigate the physical characteristics of plants of the same family, but also their physiology and chemical composition.

1.1.4.1 Morphology

African ginger is a bisexual plant with pink, purple or white, short-lived flowers that only appear at ground level (Figure 1.1).^[12,53] They only flower annually in the spring/summer time before the leaves appear (October – December).^[4,27,54]



Figure 1.1 Bright pink or purple flowers of African ginger that appear at ground level. (Taken from Flora of Zimbabwe).^[55]

The large, light green and hairless leaves grow in the spring time and are often used for traditional medicine.^[4,48,52] These flowers and leaves grow from the rhizomes and roots and the plant can reach a height of 0.4 m^[7,27,48] After the growing season the shoots are yellow in April, collapse in May and the plants then go into their dormant stage allowing the rhizomes and roots to grow and be harvested.^[53,54]

The aromatic, cone-shaped rhizomes and succulent roots enlarge during the dormant stage (Figure 1.2).^[9,12] They are more popular for traditional use than leaves, however this leads to overexploitation of the plant.^[7,57] Both the leaves, and rhizomes are harvested but during different seasons; leaves during summer (October – December) and rhizomes and roots during winter (April – September).^[4,54,57] During summer the rhizomes and roots are much smaller in size since water and nutrients are transported to the aerial shoots to assist in leaves, flowers and fruit growth.^[54]

The rhizomes and roots have been researched more extensively and have shown good potential for different biological activities.^[48,56] Traditionally they are more generally used than leaves, but in some cases they show better biological activity, depending on the medicinal use as discussed later in section 1.1.5.1.^[7,56,58] The use of leaf material is more attractive to prevent eradication of the plant, but traditional healers prefer rhizomes and roots and do not want to deviate from traditional usage.^[4,58]



Figure 1.2 Cone-shaped rhizomes and succulent roots (Taken from The Department of Agriculture, Forestry and Fisheries (South Africa)).^[59]

1.1.4.2 Traditional uses

African ginger is one of the most important traditional medicinal plants in South Africa and is generally used by traditional healers to treat various ailments.^[7,9] Most popular traditional uses include chewing or brewing of fresh rhizomes and roots as a tea to treat colds, coughs, influenza, asthma and malaria.^[7,9,21] Another is the hot and cold infusion of rhizomes, steaming and inhalation of the vapours to treat asthma, coughs, colds, malaria and sinus problems.^[7,60]

The plant is also traditionally used for protection against snakes and lightning.^[58] It is also sometimes used to treat hysteria and epilepsy.^[52,61]

All of these traditional uses over the years have resulted in the overexploitation of the plant, resulting in it being placed on the Red Data List of South African plants.^[43,45]

1.1.4.3 Conservation

African ginger is a popular medicinal plant and is one of the most widely used plants in South Africa. Its popularity has caused for an increase in demand by people for its traditional – and scientific use.^[45,46] This has also resulted in low supply and caused for more research into finding suitable solutions to prevent the plant from being completely eradicated.^[27,54,58]

Successful cultivation and propagation has been performed in warmer parts of South Africa provided it is in a well-drained, compost rich soil and warm shady environment.^[27,62] Unfortunately, the lack of cultivation skills or the effect on the genetic diversity can be problematic during cultivation and the cultivated plant may not have the same biological activity as the wild plant.^[4,9,45]

Another investigation relates to the use of different plant parts for different medicinal uses.^[45] Zschocke et al. (2000) investigated the difference in chemical composition and found that the rhizomes and roots consist of a similar chemical composition as the leaves and stems. This was done using thin-layer chromatography (TLC), but is not an adequate method of determining the chemical composition of different plant parts compared to more accurate methods like gas chromatography-mass spectrometry (GC-MS).^[58] The studies revealed that both leaves and stems showed better *in vitro* anti-inflammatory activity, using a cyclooxygenase-1 (COX-1) assay, than rhizomes and roots. These results were also supported by Light et al. (2002) who concluded that depending on the medicinal use, the rhizomes and roots can be substituted with leaves and stems if this is supported by further scientific findings.^[56,58] The traditional healers and patients often refrain from using leaves and stems since they do not believe it to have the same strength as rhizomes and roots.^[59] The use of leaf material will prevent complete eradication and allow the plant to recover and continue growing to be used in the next dormant season.^[9,56,58]

It is also important to harvest the rhizomes during winter when they are larger, hydrated and nutrient-rich.^[9,54] Harvesting the plant during summer, while rhizomes and roots have shrunk, can result in eradication of more plant material and cause over-exploitation.^[54]

Communities and collaborators need to work together to prevent this plant from being over-exploited. Protection of indigenous plants such as African ginger is of the utmost importance, but changing its chemical composition defeats the purpose of cultivation. This makes the plant unusable since its chemical composition is what makes it valuable.

1.1.5 Biological evaluation and phytochemical studies of African ginger

Extensive scientific investigations into the traditional use of African ginger, as mentioned in section 1.1.4.2, have been undertaken with the intention of producing natural medicinal products for use.^[7,43,44] Although the biological properties of the plant have been extensively researched, little has been done on the active ingredients and their mode of action.^[21,61] In some instances, good activity might be due to synergism, but in other cases it might be due to a single active compound.^[74,75] It is important to investigate the biological properties of the plant, but also those of the active ingredients to significantly improve the biological activity and contribute to improved natural ingredients.

1.1.5.1 Biological evaluation of African ginger

Many of the biological properties, i.e. anti-allergic, anti-asthmatic, antibacterial, anti-inflammatory and antifungal properties of the plant have been investigated and coincide with the traditional use.^[21,56,58,62]

The antibacterial and antifungal properties were investigated by Coopoosamy et al. (2000).^[48] For the antibacterial activity, an aqueous, acetone and ethyl acetate extract of the leaves and rhizomes of African ginger were prepared. The extracts were tested against five gram-positive strains (*Bacillus subtilis*, *Micrococcus kristinae*, *Bacillus cereus*, *Staphylococcus aureus* and *Staphylococcus epidermidis*) and four gram-negative strains (*Escherichia coli*, *Proteus vulgaris*, *Enterobacter aerogenes* and *Shigella sonnei*). It was found that the acetone and ethyl acetate extracts showed better inhibition than the aqueous extract against most of the bacteria. The study also showed that the leaves had

less inhibition than the rhizomes, which is possibly due to compounds being stored in the rhizomes and roots and not the leaves.^[9,48,56,61]

Aqueous and ethanol extracts of the bulbs and leaves were prepared and screened for their antifungal activity against *Aspergillus flavus*, *Aspergillus glaucus*, *Candida albicans*, *Candida tropicalis*, *Trichophyton mentagrophytes* and *Trichophyton rubrum*.^[49] It was reported that the ethanol extract had better inhibition than the aqueous extract and that the rhizomes also had better inhibition than the leaves. This could suggest why traditional healers prefer the rhizomes above the leaf material.^[9,48]

The anti-inflammatory properties of the plant were investigated by Light et al. (2002) using a COX-1 and COX-2 assay.^[56] The screening of an aqueous, ethanol and ethyl acetate extract of dried leaves, rhizomes and roots were performed using a COX-1 enzyme from sheep seminal vesicles and COX-2 enzyme from sheep placental cotyledons. It was reported that the aqueous extracts did not show any inhibition, but good inhibition was reported for the ethanol and ethyl acetate extracts.^[56,61]

Another investigation into the anti-inflammatory properties was performed by Stafford, et al. (2005).^[61] They investigated the anti-inflammatory properties of dried rhizomes extracted with water, ethanol and n-hexane and screened against the COX-1 enzyme assay using sheep seminal vesicles.^[63,64] The results showed that the ethanol extract had better inhibition than the water extract even after a one-year storage period, and concluded that different compounds were extracted depending on the polarity of the organic solvents used.^[61,63,64]

The anti-allergic and anti-inflammatory properties of the plant were investigated by Fouche et al. (2011) using mouse models.^[62] Three extracts, diethyl ether, ethanol and water, were prepared using dried rhizomes and roots. The diethyl ether extract was purified by flash chromatography on silica gel for the isolation of siphonochilone (**1**). Screening for anti-asthmatic properties was done by screening the extracts and siphonochilone in a glucocorticoid and histamine H1 binding assay and a phosphodiesterase IV enzyme inhibition assay.^[62] The *in vivo* efficacy of the extracts and

siphonochilone was tested against ovalbumin (OVA)-induced allergic airway disease in BALB/c mice. The study revealed that the diethyl ether extract and siphonochilone had the best anti-inflammatory activity *in vitro* and *in vivo*.^[62]

The plant is also traditionally known to treat influenza, coughs and colds, prompting investigation into the antiviral properties of the plant.^[9,43] The activity of an aqueous extract was investigated against the herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and the Influenza-A virus (strain Panama) by Light et al. (2002). However, no significant activity against HSV-1 and HSV-2 and the Influenza-A virus were observed.^[56]

1.1.5.2 Chemical composition and extraction of metabolites

Very little was known about the chemical composition of the plant and has initiated more investigations into the isolation and identification of active ingredients.^[9,43] Initially the active ingredients were isolated as essential oils, but were later extracted using organic solvents.^[43,65] The major compound is usually a terpene or terpenoid, which is responsible for the biological activity of the plant.^[28,66] Holzapfel et al. (2002) identified the major compound of African ginger to be a sesquiterpenoid, siphonochilone (**1**), and was also confirmed by Viljoen et al. (2002) to be the major compound since it contributes 20 – 30% of the essential oil.^[43,44]

Holzapfel was the first to investigate the chemical compounds by isolating and identifying pure compounds from the essential oil using flash chromatography with silica gel and 2% triethylamine and nuclear magnetic resonance spectroscopy (NMR).^[43] He identified monoterpenoids and isolated and identified three sesquiterpenoids (Figure 1.3): siphonochilone (**1**), 4 α H-3,5 α ,8 α β -trimethyl-4,4a,8a,9-tetrahydronaphtho[2,3b]-furan-8-one (C₁₅H₁₈O₂, *m/z* = 230.13), the hydroxylated derivative of siphonochilone (**2**), 2-hydroxy-4 α H-3,5 α ,8 α β -trimethyl-4,4a,8a,9-tetrahydronaphtho-[2,3b]-furan-8(5H)-one (C₁₅H₁₈O₃, *m/z* = 246.30) and 2-acetoxy-4 α H-3,5 α ,8 α β -trimethyl-4,4a,8a,9-tetrahydronaphtho-[2,3b]-furan-8-one (**3**) (C₁₆H₂₀O₄, *m/z* = 276.33). Compounds **1** and **2**

were considered naturally occurring in the plant while compound **3** was obtained by reacting compound **2** with acetic anhydride and triethylamine.^[43]

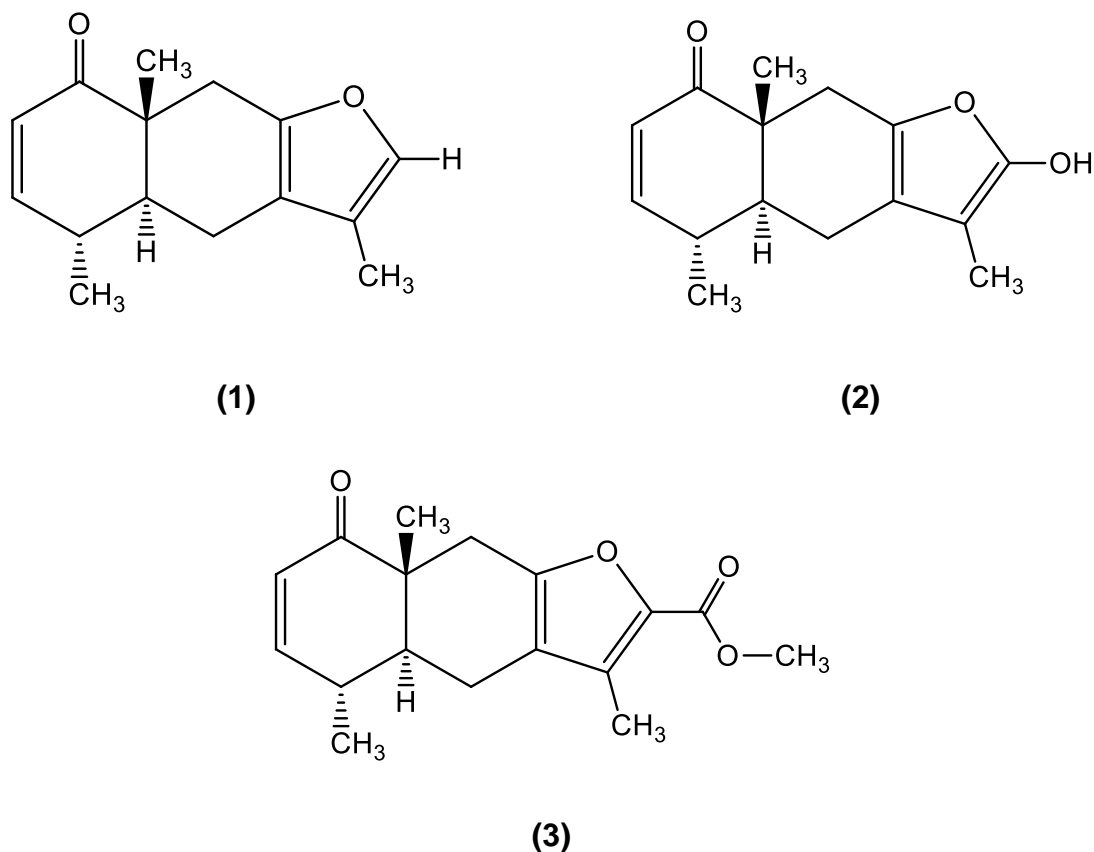
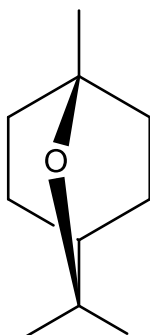


Figure 1.3 The structures isolated by Holzapfel et al. (2002) of siphonochilone **(1)**, the hydroxylated derivative **(2)** and the acetylated compound **(3)**.

Viljoen et al. (2002) investigated the chemical composition of the essential oil using a gas chromatography-flame ionization detector (GC-FID) where he identified the presence of 1,8-cineole, (*E*)- β -ocimene, cis-allocimene and siphonochilone.^[44] Eucalyptol, or 1,8-cineole **(4)** is a naturally occurring, aromatic, monoterpenoid found in many essential oils, e.g. eucalyptus, and has been traditionally used to treat bronchitis, sinuses, coughs and colds.^[67-69] It is the major ingredient in “Vicks” which is one of the most popularly branded used products to treat coughs, colds and flu.^[70,71] It has more recently also been formulated as a decongestant or used for aromatherapy for sick patients.^[69,72]

Eucalyptol was more recently confirmed to be present in the vapour phase of the hot infusion of rhizomes and roots, coinciding with the traditional use.^[7] The vapours from fresh rhizomes and roots were analysed through headspace-solid phase microextraction (HS-SPME) and gas chromatography time-of-flight mass spectrometry (GC-TOF-MS). Eucalyptol was identified as a major compound that can contribute to its traditional use as a decongestant.^[60]



(4)

Figure 1.4 Structure of eucalyptol (4).^[60]

Lategan et al. (2009) isolated and identified three other sesquiterpenoids using solid phase extraction (SPE), liquid-liquid extraction, high-performance liquid chromatography (HPLC) and NMR spectroscopy from dried, powdered rhizomes extracted with ethyl acetate. The three sesquiterpenoid lactones (Figure 1.5) were; 4 α H-3,5 α ,8 β -trimethyl-4,4a,8a,9-tetrahydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one (5) (C₁₅H₁₈O₃, *m/z* = 247.13), 9 α -hydroxy 4 α H-3,5 α ,8 β -trimethyl-4,4a,8a,9-tetrahydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one (6) (C₁₅H₁₈O₄, *m/z* = 263.13) and 4 α H-3,5 α ,8 β -trimethyl-4,4a,8a-trihydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one (7) (C₁₅H₁₆O₃, *m/z* = 245.12).^[9]

Zongwe et al. (2016) studied the decomposition of siphonochilone (1) into the sesquiterpenoid lactones previously reported by Holzapfel and Lategan using ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-

QTOF-MS ES⁺).^[65] Fresh rhizomes and dried powdered rhizomes stored for nine months were extracted with (dichloromethane) DCM:hexane (1:1) and observed for any changes in the profiles. It was concluded that auto-oxidation of siphonochilone (**1**) from stored dried, powdered rhizomes took place and ultimately led to the formation of the lactones reported by Lategan et al. (2009) (**5 – 7**). The lactones are more stable and better to use than siphonochilone.^[9]

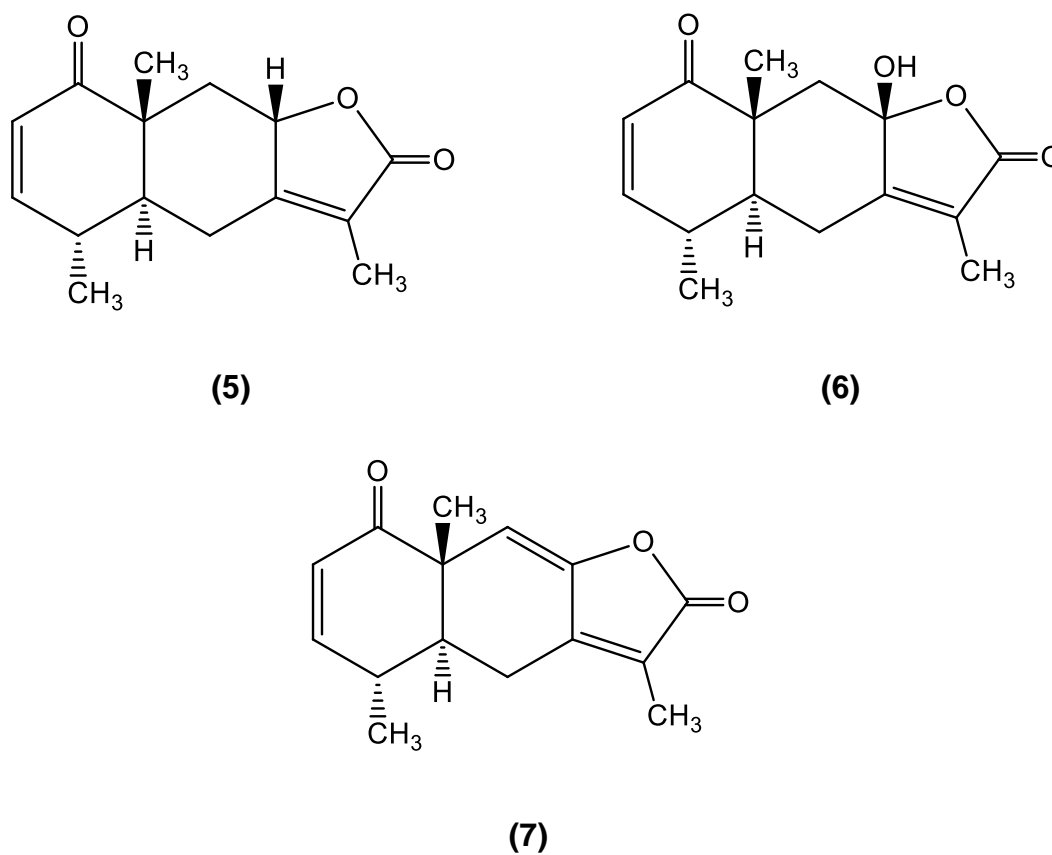


Figure 1.5 The structures of the lactones isolated by Lategan et al. (2009).^[9]

These structural changes to siphonochilone can be due to environmental factors (pH, temperature and light) that can cause a chemical change in certain plant material resulting in decomposition and affect the biological activity of the plant.^[73]

1.2 Problem statement and justification

Due to the overburden of harvesting of medicinal plants from the wild, many plants are now being threatened in their natural environment and this leads to their extinction. Many harvesting practices use the roots and stems of the plants and the destructive harvesting is even more of a burden to the survival of the plants. One such plant is *Siphonochilus aethiopicus* that is close to extinction due to the use of the underground rhizomes and roots for traditional practices. The cultivation of medicinal plants offers a solution to wild harvesting. As part of the cultivation, it is essential to determine the chemical profiles of the plants and compare this to wild plant material. Often in traditional healing, traditional health practitioners do not use cultivated material since they believe these do not have the same “strength” as wild material.^[58] Chemical analysis to show equivalence between wild and cultivated is one option since the pharmacological and toxicological properties are dependent on the phytochemicals in the plants. It is also well-known that the photochemistry of plants can vary from season to season and also with the geographical location.

Studies on the cultivation of the plant has suggested that it is easy to cultivate and propagate provided it is in a well-drained, compost-rich soil and warm shady environment.^[4,26,27] No studies have been undertaken to determine the differences or similarities in phytochemicals between different seasons as this is very important to determine the correct season or time for harvesting.

There are numerous reasons for the importance of determining the harvesting period in plant-based drug development. The first is to prevent extinction of the plant, depending on the plant part used for drug development. In the case of African ginger, the plant's rhizomes and roots are very popular in traditional medicine and hence would be better to harvest during the winter when the rhizomes and roots have grown.^[54] In the second instance harvesting during summer will result in a lower yield of plant material since the rhizomes and roots shrink during this season. The demand for more plant material during summer will result in a higher risk of eradication of the plant. Finally, as certain chemicals

are transported to the aerial parts of the plant during different seasons, there can be a change in the chemical composition of the rhizomes and roots.

Majority of the studies done have been on the aqueous and organic solvent extracts and essential oils of the plant. A few monoterpenes, monoterpenoids and sesquiterpenoids have been isolated from the plant but their pharmacological properties have not been confirmed. Siphonochilone, the major compound from fresh plant material, and various other sesquiterpenoids were previously isolated from the plant but not extensively tested for any biological activity.^[9,43] These sesquiterpenoid constituents were originally believed to be naturally occurring, but recent studies have suggested otherwise. It was reported that only siphonochilone occurs naturally in the plant and that the other sesquiterpenoids are lactones of siphonochilone that form due to auto-oxidation.^[66] This also brought to light that storage time and other physical factors including light and oxygen exposure can change the chemical composition of the plant material. The question that arises is whether siphonochilone or one of its lactones are responsible for the pharmacological properties reported from extracts, or whether it is synergism that is responsible for the biological activity.

African ginger holds many medicinal values and has been extensively researched to determine its pharmacological properties e.g. anti-asthmatic, antimalarial, antibacterial, antifungal and anti-allergic. However, limited research has been reported to determine its biological efficacy against influenza viruses that lead to colds, coughs and fever and to determine which chemical compounds are responsible for these pharmacological properties of the plant. Previous research on the antiviral activity has shown that the aqueous extracts of the plant does not show antiviral activity at the doses tested. The active ingredient for the pharmacological effect against the influenza virus has not been reported while cognizance of the auto-oxidation process has to be taken into account as well as synergistic affects.^[56] The antiviral activity of certain extracts and pure compounds of the plant has not yet been investigated and it is necessary to find and evaluate its potential as suitable natural antiviral drug leads.

This study undertakes to investigate the impact various methods of extraction, solvents or solvent-systems and plant material plays on the chemical composition of the extracts of rhizomes from African ginger and the antiviral properties of extracts and pure compounds isolated from the plant.

1.3 Aims and objectives

The aim of the study was to investigate the differences and/or similarities in chemical composition of extracts of fresh, freshly dried ground and five-year-old dried ground rhizomes of African ginger; and to evaluate the antiviral effects of different extracts of the plant against the influenza virus including the isolation and identification of the active ingredient responsible for the activity.

The overall objectives of the study are:

- Extraction of fresh, freshly dried ground and five-year-old dried ground rhizomes using different methods of extraction and organic solvents;
- Chemical analysis using GC-MS of different extracts and identify differences and/or similarities in chemical composition through comparison of the chromatograms;
- Fractionation and isolation of active compounds, confirmation of their structures using NMR and antiviral bio-assaying of pure compounds;
- Antiviral screening of extracts of African ginger against a strain of the Influenza A-type virus (108 617).

1.4 References

- [1] Baker, D.D., Chu, M., Oza, U. and Rajgarhia, V., 2007, The value of natural products to future pharmaceutical discovery, *Natural Product Reports* 24(6), pp.1225-1244.

- [2] Ji, H.F., Li, X.J. and Zhang, H.Y., 2009, Natural products and drug discovery: can thousands of years of ancient medical knowledge lead us to new and powerful drug combinations in the fight against cancer and dementia?, *EMBO Reports* 10(3), pp.194-200.
- [3] Bohlin, L., Göransson, U., Alsmark, C., Wedén, C. and Backlund, A., 2010, Natural products in modern life science, *Phytochemistry Reviews* 9(2), pp.279-301.
- [4] Manzini, T.Z., 2005, Production of Wild ginger (*Siphonochilus aethiopicus*) under protection and indigenous knowledge of the plant from traditional healers, PhD Thesis, Pretoria, University of Pretoria, South Africa.
- [5] Sheng-Ji, P., 2001, Ethnobotanical approaches of traditional medicine studies: some experiences from Asia, *Pharmaceutical Biology* 39(sup1), pp.74-79.
- [6] Romero-Daza, N., 2002, Traditional medicine in Africa, *The Annals of The American Academy of Political and Social Science* 583(1), pp.173-176.
- [7] Fouche, G., Nieuwenhuizen, N., Maharaj, V., van Rooyen, S., Harding, N., Nthambeleni, R., Jayakumar, J., Kirstein, F., Emedi, B. and Meoni, P., 2011, Investigation of *in vitro* and *in vivo* anti-asthmatic properties of *Siphonochilus aethiopicus*, *Journal of Ethnopharmacology* 133(2), pp.843-849.
- [8] Verma, S. & Singh, S.P. 2008, Current and future status of herbal medicines, *Veterinary World* 1(11), pp. 347-350.
- [9] Lategan, C.A., Campbell, W.E., Seaman, T. and Smith, P.J., 2009, The bioactivity of novel furanoterpenoids isolated from *Siphonochilus aethiopicus*, *Journal of Ethnopharmacology* 121(1), pp.92-97.
- [10] Davids, D., Gibson, D. and Johnson, Q., 2016, Ethnobotanical survey of medicinal plants used to manage high blood pressure and type 2 diabetes mellitus in Bitterfontein, Western Cape Province, South Africa, *Journal of Ethnopharmacology* 194, pp.755-766.
- [11] Dias, D.A., Urban, S. & Roessner, U., 2012, A Historical Overview of Natural Products in Drug Discovery, *Metabolites* 2(2), pp. 303-336.

- [12] Van Wyk, B.E., 2011, The potential of South African plants in the development of new medicinal products, *South African Journal of Botany* 77(4), pp.812-829.
- [13] Pouliot, M., 2011, Relying on nature's pharmacy in rural Burkina Faso: Empirical evidence of the determinants of traditional medicine consumption, *Social Science & Medicine* 73(10), pp.1498-1507.
- [14] Byg, A., Salick, J. and Law, W., 2010, Medicinal plant knowledge among lay people in five eastern Tibet villages, *Human Ecology* 38(2), pp.177-191.
- [15] Etkin, N.L., Ross, P.J. and Muazzamu, I., 1990, The indigenization of pharmaceuticals: therapeutic transitions in rural Hausaland, *Social Science & Medicine* 30(8), pp.919-928.
- [16] Muela, S.H., Ribera, J.M., Mushi, A.K. and Tanner, M., 2002, Medical syncretism with reference to malaria in a Tanzanian community, *Social Science & Medicine* 55(3), pp.403-413.
- [17] David, B., Wolfender, J.L. and Dias, D.A., 2015, The pharmaceutical industry and natural products: historical status and new trends, *Phytochemistry Reviews* 14(2), pp.299-315.
- [18] Bush, K., 2010, The coming of age of antibiotics: discovery and therapeutic value, *Annals of the New York Academy of Sciences* 1213(1), pp.1-4.
- [19] Andrade-Neto, V.F., Brandão, M.G.L., Stehmann, J.R., Oliveira, L.A. and Krettli, A.U., 2003, Antimalarial activity of Cinchona-like plants used to treat fever and malaria in Brazil, *Journal of Ethnopharmacology* 87(2-3), pp.253-256.
- [20] Goodman, J. and Walsh, V., 2001, The story of taxol: nature and politics in the pursuit of an anti-cancer drug, *Cambridge University Press*, pp.1-2.
- [21] Lindsey, K., Jäger, A.K., Raidoo, D.M. and van Staden, J., 1998, Screening of plants used by Southern African traditional healers in the treatment of dysmenorrhoea for prostaglandin-synthesis inhibitors and uterine relaxing activity, *Journal of Ethnopharmacology* 64(1), pp.9-14.
- [22] Watt, J.M. and Breyer-Brandwijk, M.G., 1962, *The Medicinal and Poisonous Plants of Southern and Eastern Africa being an Account of their Medicinal and other Uses*,

- Chemical Composition, *Pharmacological Effects and Toxicology in Man and Animal*, 2nd ed., E. & S. Livingstone Ltd, Teviot Place, Edinburgh.
- [23] Abdullahi, A.A., 2011, Trends and challenges of traditional medicine in Africa, *African Journal of Traditional, Complementary and Alternative Medicines* 8(S), pp. 115-123.
- [24] Mahomoodally, M.F., 2013, Traditional medicines in Africa: an appraisal of ten potent African medicinal plants, *Evidence-Based Complementary and Alternative Medicine*, 2013, viewed on 29 April 2020, from <http://dx.doi.org/10.1155/2013/617459>
- [25] Van Staden, J., 2008, Ethnobotany in South Africa, *Journal of Ethnopharmacology* 119(3), pp. 329-330.
- [26] Street, R.A. and Prinsloo, G., 2012, Commercially important medicinal plants of South Africa: a review, *Journal of Chemistry* 2013, viewed on 29 April 2020, from <http://dx.doi.org/10.1155/2013/205048>
- [27] Van Wyk, B.E., 2008, A broad review of commercially important southern African medicinal plants, *Journal of Ethnopharmacology* 119(3), pp.342-355.
- [28] Svoboda, K.P. and Hampson, J.B., 1999, Bioactivity of essential oils of selected temperate aromatic plants: antibacterial, antioxidant, anti-inflammatory and other related pharmacological activities, *Plant Biology Department, SAC Auchincruive, Ayr, Scotland, UK., KA6 5HW*, 16, pp.1-7.
- [29] Kale, R., 1995, South Africa's Health: Traditional healers in South Africa: a parallel health care system, *British Medicinal Journal* 310(6988), pp.1182-1185.
- [30] Korany, M.A., Mahgoub, H., Haggag, R.S., Ragab, M.A. and Elmallah, O.A., 2017, Green chemistry: Analytical and chromatography, *Journal of Liquid Chromatography & Related Technologies* 40(16), pp.839-852.
- [31] Twarog, S. and Kapoor, P., 2004, *Protecting and promoting traditional knowledge: systems, national experiences and international dimensions*, United Nations Conference on Trade and Development.

- [32] Moeng, E.T. and Potgieter, M.J., 2011, The trade of medicinal plants by muthi shops and street vendors in the Limpopo Province, South Africa, *Journal of Medicinal Plants Research* 5(4), pp.558-564.
- [33] Dalton, B.P.A., 2007, The coastal grasslands of Maputaland, South Africa: Effects of fire and grazing on vegetation structure, diversity, and composition, PhD Thesis, Pietermaritzburg, University of KwaZulu-Natal, South Africa.
- [34] Ndawonde, B.G., Zobolo, A.M., Dlamini, E.T. and Siebert, S.J., 2007, A survey of plants sold by traders at Zululand muthi markets, with a view to selecting popular plant species for propagation in communal gardens, *African Journal of Range and Forage Science* 24(2), pp.103-107.
- [35] Dold, A.P. and Cocks, M.L., 2002, The trade in medicinal plants in the Eastern Cape Province, South Africa, *South African Journal of Science* 98(11), pp.589-597.
- [36] Chen, W., Van Wyk, B.E., Vermaak, I. and Viljoen, A.M., 2012, Cape aloes—A review of the phytochemistry, pharmacology and commercialisation of *Aloe ferox*, *Phytochemistry Letters* 5(1), pp.1-12.
- [37] Yagi, A. and Takeo, S., 2003, Anti-inflammatory constituents, aloesin and aloemannan in Aloe species and effects of tanshinon VI in *Salvia miltiorrhiza* on heart, *Yakugaku zasshi: Journal of the Pharmaceutical Society of Japan* 123(7), pp.517-532.
- [38] Mncwangi, N., Chen, W., Vermaak, I., Viljoen, A.M. and Gericke, N., 2012, Devil's Claw—A review of the ethnobotany, phytochemistry and biological activity of *Harpagophytum procumbens*, *Journal of Ethnopharmacology* 143(3), pp.755-771.
- [39] Betancor-Fernández, A., Pérez-Gálvez, A., Sies, H. and Stahl, W., 2003, Screening pharmaceutical preparations containing extracts of turmeric rhizome, artichoke leaf, devil's claw root and garlic or salmon oil for antioxidant capacity, *Journal of Pharmacy and Pharmacology* 55(7), pp.981-986.
- [40] Weckesser, S., Engel, K., Simon-Haarhaus, B., Wittmer, A., Pelz, K. and Schempp, C.Á., 2007, Screening of plant extracts for antimicrobial activity against

- bacteria and yeasts with dermatological relevance, *Phytomedicine* 14(7-8), pp.508-516.
- [41] Van Heerden, F.R., 2008, *Hoodia gordonii*: a natural appetite suppressant, *Journal of Ethnopharmacology* 119(3), pp.434-437.
- [42] Lee, R.A. and Balick, M.J., 2007, Indigenous use of *Hoodia gordonii* and appetite suppression, *Explore* 3(4), pp.404-406.
- [43] Holzapfel, C.W., Marais, W., Wessels, P.L. and Van Wyk, B.E., 2002, Furanoterpenoids from *Siphonochilus aethiopicus*, *Phytochemistry* 59(4), pp.405-407.
- [44] Viljoen, A.M., Demirci, B., Baser, K.H.C., Van Wyk, B.E. and Jäger, A.K., 2002, The essential oil composition of the roots and rhizomes of *Siphonochilus aethiopicus*, *South African Journal of Botany* 68(1), pp.115-116.
- [45] Bergh, Z., 2016, Chemical characterisation and *in vitro* permeation of *Siphonochilus aethiopicus* extracts, PhD Thesis, Potchefstroom Campus, North-West University, South Africa.
- [46] Erasmus, M., Du Plessis, L.H. and Viljoen, J.M., 2019, *In vitro* cytotoxicity of various *Siphonochilus aethiopicus* (Schweinf.) BL Burt extracts in combination with selected tableting excipients, *Journal of Pharmacy and Pharmacology* 71(11), pp.1714-1724.
- [47] Hartzell, J.F., 2011, Response of the Endangered Medicinal Plant: *Siphonochilus Aethiopicus* (Schweif) BL Burt. to Agronomic Practices, PhD Thesis, Pietermaritzburg, University of KwaZulu-Natal, South Africa.
- [48] Coopoosamy, R.M., Naidoo, K.K., Buwa, L. and Mayekiso, B., 2010, Screening of *Siphonochilus aetiopicus* (Schweinf.) BL Burt for antibacterial and antifungal properties, *Journal of Medicinal Plants Research* 4(12), pp.1228-1231.
- [49] Kress, W.J., Prince, L.M. and Williams, K.J., 2002, The phylogeny and a new classification of the gingers (Zingiberaceae): evidence from molecular data. *American Journal of Botany* 89(10), pp.1682-1696.

- [50] Leistner, O.A., 2000. *Seed plants of southern Africa: families and genera*. National Botanical Institute, Pretoria, South Africa.
- [51] Gatabazi, A., Marais, D., Steyn, M.J., Araya, H.T., Mofokeng, M.M. and Mokgehle, S.N., 2019, Evaluating growth, yield, and water use efficiency of African and commercial ginger species in South Africa, *Water* 11(3), p.548.
- [52] Van Wyk, B., Gericke, N. and van Oudtshoorn, B., 2001, *Medicinal Plants of South Africa*, 2nd ed. Briza Publishers, South Africa.
- [53] Gordon-Gray, K.D., Cunningham, A.B. and Nichols, G.R., 1989, *Siphonochilus aethiopicus* (Zingiberaceae): observations on floral and reproductive biology, *South African Journal of Botany* 55(3), pp.281-287.
- [54] Nichols, G., 1989, Some notes on the cultivation of Natal ginger *Siphonochilus aethiopicus*, *Veld & Flora* 75(3), pp.92-3.
- [55] Flora of Zimbabwe n.d., *Siphonochilus aethiopicus*, viewed 29 April 2020, from https://www.zimbabweflora.co.zw/speciesdata/image-display.php?species_id=116150&image_id=1.
- [56] Light, M.E., McGaw, L.J., Rabe, T., Sparg, S.G., Taylor, M.B., Erasmus, D.G., Jäger, A.K., Van Staden, J. and Eloff, J.N., 2002, Investigation of the biological activities of *Siphonochilus aethiopicus* and the effect of seasonal senescence, *South African Journal of Botany* 68(1), pp.55-61.
- [57] Igoli, N.P. and Obanu, Z., 2011, The volatile components of wild ginger (*Siphonochilus aethiopicus* (Schweinf) Bl Burt), *African Journal of Food Science* 5(9), pp.541-549.
- [58] Zschocke, S., Rabe, T., Taylor, J.L., Jäger, A.K. and Van Staden, J., 2000, Plant part substitution—a way to conserve endangered medicinal plants?, *Journal of Ethnopharmacology* 71(1-2), pp.281-292.
- [59] Department of Agriculture, Forestry and Fisheries (South Africa) n.d., *Wild ginger production guidelines*, Viewed 10 March 2020, from www.daff.gov.za.
- [60] Naudé, Y., Makuwa, R. and Maharaj, V., 2016, Investigating volatile compounds in the vapour phase of (1) a hot water infusion of rhizomes, and of (2) rhizomes of

- Siphonochilus aethiopicus* using head space solid phase microextraction and gas chromatography with time of flight mass spectrometry, *South African Journal of Botany* 106, pp.144-148.
- [61] Stafford, G.I., Pedersen, M.E., van Staden, J. and Jäger, A.K., 2008, Review on plants with CNS-effects used in traditional South African medicine against mental diseases, *Journal of Ethnopharmacology* 119(3), pp.513-537.
- [62] Fouche, G., Nieuwenhuizen, N., Maharaj, V., van Rooyen, S., Harding, N., Nthambeleni, R., Jayakumar, J., Kirstein, F., Emedi, B. and Meoni, P., 2011, Investigation of *in vitro* and *in vivo* anti-asthmatic properties of *Siphonochilus aethiopicus*, *Journal of Ethnopharmacology* 133(2), pp.843-849.
- [63] Jäger, A.K., Hutchings, A., van Staden, J., 1996, Screening of Zulu medicinal plants for prostaglandin-synthesis inhibitors, *Journal of Ethnopharmacology* 52(2), pp.95-100.
- [64] White, H.L. and Glassman, A.T., 1974, A simple radiochemical assay for prostaglandin synthetase, *Prostaglandins* 7(2), pp.123-129.
- [65] Zongwe, F.K., Muya, J.T., Mutimana, R., Virima, M., Mayaliwa, M., Chung, H. and Maharaj, V., 2018, Auto-oxidation of Siphonochilone in Processed Rhizomes and Stored Powders of *Siphonochilus aethiopicus* (Schweinf.) BL Burt., *ChemistrySelect* 3(30), pp.8569-8574.
- [66] Bakkali, F., Averbeck, S., Averbeck, D. and Idaomar, M., 2008, Biological effects of essential oils—a review, *Food and Chemical Toxicology* 46(2), pp.446-475.
- [67] Bhowal, M. and Gopal, M., 2015, Eucalyptol: safety and pharmacological profile, *Journal of Pharmaceutical Sciences* 5(4), pp.125-131.
- [68] De Vincenzi, M., Silano, M., De Vincenzi, A., Maialetti, F. and Scazzocchio, B., 2002, Constituents of aromatic plants: eucalyptol, *Fitoterapia* 73(3), pp.269-275.
- [69] Santos, F.A. and Rao, V.S.N., 2000, Anti-inflammatory and antinociceptive effects of 1, 8-cineole a terpenoid oxide present in many plant essential oils, *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives* 14(4), pp.240-244.

- [70] Bailey, T., Barnes, G., Hicks, J., Saleh, M., Stephens, E. and Wilson, S., 2020, 'Vicks Vapourub Review: Does Vicks Vapourub Really Work?' in *The Health Aisle*, viewed 28 April 2020, from <https://www.thehealthaisle.com/products/vicks-vapourub-review/>.
- [71] Cal, K. and Sopala, M., 2008, Ex vivo skin absorption of terpenes from Vicks Vapourub ointment, *Medical Science Monitor*, 14(8), pp.PI19-PI23, viewed on 28 April 2020, from <https://www.medscimonit.com/download/index/idArt/865799> .
- [72] Juergens, U.R., Dethlefsen, U., Steinkamp, G., Gillissen, A., Reppes, R. and Vetter, H., 2003, Anti-inflammatory activity of 1.8-cineol (eucalyptol) in bronchial asthma: a double-blind placebo-controlled trial, *Respiratory Medicine* 97(3), pp.250-256.
- [73] Fennell, C.W., Light, M.E., Sparg, S.G., Stafford, G.I. and Van Staden, J., 2004, Assessing African medicinal plants for efficacy and safety: agricultural and storage practices, *Journal of Ethnopharmacology* 95(2-3), pp.113-121.
- [74] Rasmann, S. and Agrawal, A.A., 2009, Plant defense against herbivory: progress in identifying synergism, redundancy, and antagonism between resistance traits, *Current Opinion in Plant Biology* 12(4), pp.473-478.
- [75] Handa, S.S., 2008, *An overview of extraction techniques for medicinal and aromatic plants*, *Extraction technologies for medicinal and aromatic plants*, International Centre for Science and High Technology.

Chapter 2: Chemical profiling of extracts of African ginger

2.1 Background

2.1.1 Secondary metabolites

Plants have been around before humans and animals have roamed the Earth.^[1] Throughout history, humans have been dependent on plants, not only for consumption, but also for medicinal use.^[2] These plants are still being used worldwide especially in developing countries as a main source of medicinal care.^[3, 4] It is only in the last century that developed countries have studied traditional medicine and used or synthesized these active compounds for pharmaceutical purposes.^[2,3,5] The demand for plant-based pharmaceuticals have increased over the last few decades and these active compounds have extensively been used by the drug industry to enhance the quality of life.^[3,4,6]

These active compounds are known as the secondary metabolites produced by the plants as a result of the plant evolving and changing its internal chemistry for its own survival.^[3,4,7]

All plants contain primary and secondary metabolites, each responsible for different functions in the survival of the plant.^[8,9] Primary metabolites are responsible for the basic life functions of the plant like growth, storage, development and reproduction, and was long thought to make up majority of the compounds of a plant.^[9] It was only in the mid-20th century that researchers discovered that secondary metabolites are more abundant and just as crucial to the survival of the plant.^[8-11]

Secondary metabolites are chemical and biological components of a plant responsible for the survival and protection against natural enemies (insects and predators) and environmental factors (growing site, precipitation and seasonal changes).^[4,8,10] These metabolites have been proven to be responsible for various biological activities and has extensively been researched for drug development.^[9,10] This research has resulted in the discovery of many well-known secondary metabolites for example alkaloids, flavonoids,

phenolics, tannins, terpenes and saponins.^[4,9,11,12] These well-known groups are usually the major compounds in plants with structurally similar compounds, or derivatives, as minor compounds.^[4,13,14]

It is important to keep in mind that all plant species contain many different secondary metabolites at different concentrations.^[4,15-17] The biological activity in a plant depends on the active compound(s) in the plant and may work synergistically or antagonistically to protect the plant.^[18,19] This research is done by first extracting the main compound(s) from the plant through different methods of extraction, followed by isolation, purification and biological evaluation.^[19,20] The biological evaluation will determine whether an extract, fraction or pure compound is responsible for the biological activity, and will therefore express the importance of the extraction process for the qualitative and quantitative analysis of the plant.^[19,21]

There are various factors to consider for the efficacy of the extraction: plant material, solvent type and method of extraction.^[5,12,19] These factors all depend on the information one can collect from literature and traditional health practitioners to make an informed decision on the extraction process.^[12]

2.1.2 Plant material

The first thing to consider before any extraction is the preparation of the plant to ensure adequate qualitative and quantitative analysis of the targeted compound(s).^[1,5,12,22] The preparation of the plant includes the use of different plant parts (leaves, stems, bark, roots, flowers and fruit) as fresh or dried plant material in different forms (cut, ground or powder).^[5,12,23] These different preparations and conditions have resulted in many studies confirming that various yields of extracts are obtained with different concentrations of biologically active compounds.^[4,12,22,24]

The chemical composition and the concentrations of certain compounds differ in different plant parts as it contributes to the survival of the plant.^[24-26] This can greatly affect the outcome of an extract when a specific biologically active compound is being targeted.

Fresh and dry plant material also have an effect on the outcome of the extract. If the targeted compounds are volatiles, or thermally sensitive, it will be sensible to rather use fresh plant material.^[5,12,15] Fresh plant material however, can hold the risk of compound degeneration and contamination by microbial organisms if it is stored and not used immediately.^[5,12] Dried plant material is therefore preferred since the preparation of experiments can be time-consuming resulting in the loss of many valuable compounds when using fresh plant material.^[5] It also has a much lower water content, is easier to store and has a longer shelf life.^[15,27] It is however advised to rather keep plant material stored for a short time to prevent the decomposition of compounds.^[12,28]

The particle size of dried plant material also has a quantitative effect on the extract.^[5,19,29] Plant material can be cut into smaller pieces, ground or powdered, but the smaller the particle size the more surface area is available for solvent penetration.^[5,12,15] This will allow the solvents more access to targeted compounds resulting in higher yields of the extract.^[5,29]

The preparation of plant material is important to ensure that biologically active compounds are still intact at reasonable concentrations.^[5,24] This information is necessary before harvesting and preparation of plant material.^[5] After the appropriate preparation has been selected, then the solvent for extraction needs to be considered.

2.1.3 Solvents

Solvent extraction has been used in many plant-based drug developmental studies for the removal of targeted compounds from plant material using different organic solvents.^[12,15] The first ever extractions performed were aqueous extracts where plant material was traditionally boiled in water.^[19,22] This method of extraction was especially

used for bark and roots, that was more challenging to release biologically active compounds. It was only later that alcohol and other organic solvents were used for extraction.^[12,22]

If the chemical composition or chemical nature of the plant is unknown, it is necessary to perform different extractions to obtain a sufficient solvent or solvent-system.^[30] There are many factors to consider when choosing a solvent or solvent-system to qualitatively and quantitatively produce the best extract.^[16,24,31]

A targeted compound might not be soluble in the chosen solvent due to its polarity and it is therefore important to know the characteristics and polarities of these compounds.^[12,16,24] Different solvents have different polarities (polar, medium polar and non-polar) and a polar solvent cannot be used to extract non-polar compounds.^[19,32] The popular phrase 'like dissolves like' is important to keep in mind when selecting a solvent-system.^[21,24,33] It is sometimes found that a solvent-system, of more than one solvent, with different polarities, can improve the solubility of the compounds and improve the extract quality, and can be used instead of a single solvent.^[12,29]

The duration and temperature of extraction is also an important factor to consider since this can improve the solubility.^[12,29,34] It is important to consider the thermal stability of the targeted compounds since high temperatures can decompose the biologically active compounds.^[19] It is also important to consider the boiling point of the solvent for later removal of the solvent from the crude extract. This evaporation should not disrupt the integrity of the targeted biologically active compound.^[12,24]

The solvent of choice will ultimately be responsible for the quantity (yield) and quality (presence of biologically active compounds) of the crude extract and is this the most important factor to consider before extraction.^[5,12,21]

It is a well-known fact that several extractions using small volumes of solvent are more efficient than one large volume (Azwanida et al. (2015)).^[5] There has been an increase in demand for more environmentally friendly solvents like water, ethanol and carbon dioxide in modern extractions due to the toxic and damaging effect of organic solvents to the

environment and humans.^[35] It is clear that in the future green-chemistry will become more popular and the use of organic solvents will drastically decline in order to protect the environment.^[36]

2.1.4 Extraction methods

Extraction is the separation of biologically active compounds from plant material using a preferred solvent or solvent-system to obtain a sufficient amount of the crude extract.^[5,19]

A few commonly known methods of extraction include maceration, infusion, Soxhlet extraction, ultrasound-assisted extraction (UAE), accelerated solvent extraction (ASE), supercritical fluid extraction (SFE), steam distillation, water distillation, liquid-liquid extraction and solid-liquid extraction.^[5,12,19,31] Many of these methods require the use of large volumes of solvent and energy, are time-consuming or too expensive to use.^[19] The method of extraction depends mainly on the nature of the targeted compounds and their characteristics and all factors should be taken into account to obtain the highest quality and quantity of crude extracts.^[12]

2.1.4.1 Solid-liquid extraction

Solid-liquid extraction is one of the oldest, most popular and well-known, and easiest methods of extraction.^[12,37] It allows for a mass transfer of biologically active compounds by extracting them from the plant material to the solvent of choice (aqueous or organic solvent).^[5,19] The method is used to remove targeted compounds from contaminants in the plant and can take several steps of extraction to achieve this.^[12] Solvents are then removed from the crude extract by increasing the temperature, resulting in an oily, sticky extract that can be used for further isolation.^[19]

Other factors to consider for solid-liquid extraction is stirring, extraction time and temperature.^[19] Increasing the temperature, provided none of the targeted compounds

are thermally sensitive, will result in the increase in concentration of compounds and accelerated mass transfer.^[19]

The method has been reported to be the most popular means of extraction and is also used for industrial purposes.^[5,37] There is however the chance of working with volatile compound where hydrodistillation, i.e. steam distillation, is a more effective method of extraction.^[12,19] This method is also more environmentally friendly since only water is used, making this an efficient substitution for solid-liquid extraction.^[19]

2.1.4.2 Steam distillation

Steam distillation is a popular method of extraction of volatile biologically active compounds from fresh plant material by producing essential oils.^[15,19] These oils are traditionally used to treat various ailments and is also used in food, cosmetics, pharmaceuticals, toiletries and perfumes.^[19,23,38]

During steam distillation, water is boiled, releasing steam that allows the opening of the plant's glands, thereby releasing the oils.^[39] The oil and water are then collected after being condensed and form an immiscible layer making it easy to separate the oil.^[12] Unfortunately, low yields of essential oils are produced upon steam distillation and can produce a problem for industrial purposes.^[19]

Essential oils are complex mixtures which consist of 20 – 60 (or more depending on the plant) compounds which include terpenes, terpenoids, phenylpropanoids and aromatic compounds, with one or two compounds being the highly concentrated major components.^[12,38,40] Some of the compounds in the oils are thermally sensitive, which can result in the decomposition of some targeted compounds, depending on the structure of the compound.^[19,38,40] This can result in a decline in the quality and quantity of the essential oil.^[19,40] Depending on the preparation of the plant and how the oils are handled, it is possible that steam distillation may not be an effective method of extraction since it can decompose the targeted biologically active compound.^[19,23,40] It is then necessary to

look at other methods including solid-liquid extraction which is better for the extraction of thermally sensitive compounds.^[5]

All steps in the preparation of an extract, from the plant material to the method of extraction, will have an effect on the chemical composition of the extract, ultimately affecting the qualitative and quantitative analysis of the extract.^[12,19] There is no correct way of extraction but only to keep in mind all factors before conducting an extraction.^[5] Even though all methods of extractions produce adequate amounts of crude extract, it is clear that these extracts still need isolation and purification before obtaining the targeted biologically active compounds.^[5] Only once a method of extraction has been proven successful in extracting the targeted biologically active compound and adequate amounts of the crude extract, will it be possible for large-scale extraction.^[12]

2.1.5 Extraction of African ginger

African ginger is well-known to contain monoterpenes and sesquiterpenoids, with a sesquiterpenoid as the major compound, siphonochilone (**1**).^[41-43] The essential oil contains 20 – 30% of siphonochilone but it is unclear what the constitution of siphonochilone is in the different crude extracts since so many factors can come into play.^[43] Holzapfel et al. (2002) reported two naturally occurring sesquiterpenoids in the plant extract, but was later proven differently by Zongwe et al. (2018). They reported that all structurally similar compounds of siphonochilone are formed due to auto-oxidation of the major compound.^[28] These findings confirmed that sesquiterpenoids are sensitive to light and high temperatures, resulting in the decomposition of the compounds.^[19,28] Derivatives of siphonochilone are discussed in Chapter 1, section 1.1.5.2, and have been researched and shown an increase in concentration the longer the plant is stored.^[28,44]

Other major compounds reported in the plant extracts of African ginger were 1,8-cineole, cis- β -ocimene and cis-alloocimene.^[17,43,45] 1,8-Cineole, better known as eucalyptol, (**4**) is also present in the plant.^[45] Eucalyptol has been traditionally used to treat bronchitis, sinusitis and colds, and more recently formulated as a decongestant or used for

aromatherapy.^[46,47] Eucalyptol can work synergistically with other compounds in the essential oil to improve the biological activity of the oils or extracts.^[45]

Many compounds are present in the essential oil and crude extracts from African ginger and there are many possibilities of further research into the biological activity of specific compounds, and not just the essential oil or extracts.

The aim of the chapter is to focus on the extraction of African ginger plant material under different conditions using different organic solvents and methods of extractions to perform a qualitative study to determine the most suitable crude extract for further purification and biological evaluation.

2.2 Methodology

2.2.1 Chemicals

2.2.1.1 Analytical grade chemicals

n-Hexane (C_6H_{14} ; 86.18 g/mol) and diethyl ether anhydrous ($(C_2H_5)_2O$; 74.12 g/mol) were purchased from Radchem Laboratory Suppliers (Pty) Ltd. Dichloromethane (DCM) (CH_2Cl_2 ; 84.93 g/mol) was purchased from Sigma-Aldrich (Pty) Ltd. Methanol (CH_3OH ; 32.04 g/mol) (ROMIL-SpS™ Super Purity Solvent) was purchased from ROMIL Ltd. (ROMIL-SpS™ Super Purity Solvent).

2.2.1.2 Other chemicals

99% Ethanol (C_2H_6O ; 46.07 g/mol) and sodium sulfate (Na_2SO_4 ; 142.04 g/mol) were purchased from Radchem Laboratory Suppliers (Pty) Ltd.

2.2.1.3 Standards

Siphonochilone (**1**) crystals were obtained during steam distillation (section 2.2.4) and their identities confirmed through NMR (Chapter 3, section 3.3.5.1). The crystals were used as standards for siphonochilone.

A standard of the hydroxylated lactone (**6**) was previously isolated, purified and identified by Mr. Félix Junior Katele Zongwe as part of his MSc Thesis in the Department of Chemistry at the University of Pretoria and was available in pure form in the laboratory.^[28]

2.2.2 Sourcing and preparation of plant material

The rhizomes used for the purpose of this research was sourced from Muthi Futhi. Muthi Futhi is a community business based in Dakeni, KwaZulu-Natal and is responsible for cultivation and processing of indigenous medicinal plants. Their market focuses mainly on nutrition, cosmeceuticals, pharmaceuticals and fragrances.

A portion of fresh rhizomes were used for extraction with various organic solvents and steam distillation. The remaining portion of the plant material was dried and used for extraction with various organic solvents.

Five-year-old dried ground rhizomes of African ginger was available in the laboratory in the Department of Chemistry at the University of Pretoria and was prepared by Mr. Félix Junior Katele Zongwe and used for extraction with various organic solvents.

2.2.2.1 Fresh rhizomes for extraction and steam distillation

Fresh rhizomes were kept in a cold room at arrival at the University of Pretoria and used immediately after storage. Rhizomes were cut into 2 – 4 mm slices and used for organic solvent extraction, steam distillation and labelled as fresh rhizomes.

2.2.2.2 Dried rhizomes from freshly harvested rhizomes for extraction

Fresh rhizomes were cut into 2 – 4 mm slices and dried in a Prolab oven at 60°C for 4 days and followed by grinding using a Kinematic™ Polymix® PX-MFC 90 D grinder. An average of 75% moisture loss was recorded after 4 days in the oven. The fine powder of dried rhizomes was stored at room temperature at the University of Pretoria and labelled as freshly dried ground rhizomes.

2.2.2.3 Five-year-old rhizomes for extraction

Fresh plant material was cut and dried in an oven at 60°C for 11 days and ground into a fine powder with a coffee grinder in 2015 at the University of Pretoria. The powder was stored at room temperature in closed packaging to limit oxidation and labelled as five-year-old dried ground rhizomes.

2.2.3 Organic solvent extraction

All three sets of plant material: fresh rhizomes, freshly dried ground rhizomes and five-year-old dried ground rhizomes were extracted with four different solvents or solvent-systems.

2.2.3.1 Diethyl ether as extractant

The differently prepared plant materials (masses provided in Table 2.1) were extracted with 200 mL diethyl ether and stirred for 3 hours at room temperature (25°C). The extract was filtered through 110 mm Ø Whatman filter paper and the residual plant material was extracted twice more with diethyl ether. The combined diethyl ether extracts were dried with Na₂SO₄ and was filtered using Whatman filter paper to remove any residual plant material and Na₂SO₄. The diethyl ether was evaporated with a BUCHI rotary evaporator R-124 (Switzerland) at 30°C and weighed.

2.2.3.2 Ethanol as extractant

The differently prepared plant materials (masses provided in Table 2.1) were extracted with 200 mL ethanol and stirred for 3 hours at room temperature (25°C). The extract was filtered through 110 mm Ø Whatman filter paper and the residual plant material was extracted twice more with ethanol. The combined ethanol extracts were dried with Na₂SO₄ and was filtered using Whatman filter paper to remove any residual plant material and Na₂SO₄. The ethanol was evaporated with a BUCHI rotary evaporator R-124 (Switzerland) at 40°C and weighed.

2.2.3.3 Dichloromethane:methanol as extractant

The differently prepared plant materials (masses provided in Table 2.1) were extracted with 200 mL DCM:methanol (1:1) and stirred for 3 hours at room temperature (25°C). The extract was filtered through 110 mm Ø Whatman filter paper and the residual plant material was extracted twice more with DCM:methanol. The combined DCM:methanol extract was dried with Na₂SO₄ and was filtered using Whatman filter paper to remove any residual plant material and Na₂SO₄. The DCM:methanol was evaporated with a BUCHI rotary evaporator R-124 (Switzerland) at 30°C and weighed.

2.2.3.4 Dichloromethane:n-Hexane as extractant

The differently prepared plant materials (masses provided in Table 2.1) were extracted with 200 mL DCM:hexane (1:1) and stirred for 3 hours at room temperature (25°C). The extract was filtered through 110 mm Ø Whatman filter paper and the residual plant material was extracted twice more with DCM:hexane. The combined DCM:hexane extract was dried with Na₂SO₄ and was filtered using Whatman filter paper to remove any residual plant material and Na₂SO₄. The DCM:hexane was evaporated with a BUCHI rotary evaporator R-124 (Switzerland) at 30°C and weighed.

Table 2.1 Masses (grams) of the differently prepared plant material used for extraction with 200 mL of different organic solvents or solvent-systems.

Plant material	Mass of plant material used for organic solvent extraction (g)			
	Diethyl ether	Ethanol	DCM:methanol	DCM:hexane
Fresh rhizomes	30.14	30.04	-	-
Freshly dried ground rhizomes	30.13	30.10	15.84	15.69
Five-year-old dried ground rhizomes	15.07	15.43	35.16	29.97

2.2.4 Steam distillation

A steam distillation setup, purchased from GlassChem, was used to extract volatiles from the fresh rhizomes of African ginger.

Fresh rhizomes were cut into 2 – 4 mm slices and 6.43 kg was loaded into the storage glass container. Distilled water in the distilling flask was heated and boiled at 100°C for 5 hours. A polyurethane coated aluminum exterior and a high-quality knitted heating mantle, with glass fiber underlay for uniform distribution of heat, was used for heating (Glassco) (Figure 2.1). As the water boils, steam moves through the plant material in the storage container, opening microscopic protective glands and releasing the oils.^[39] The steam and oil move through the still head through to the condenser where they are cooled down and collected in the separator (Figure 2.1). The separator allows for liquid-liquid extraction to partition oils and water based on their densities. The crystals that formed in the condenser after cooling were removed by washing with n-hexane. The n-hexane was separately air dried to give remaining crystals (2.04 g; 0.03% w/w). These crystals were believed to be siphonochilone, the major compound of the essential oil, and was later confirmed as described in Chapter 3, Section 3.3.5.1.^[43] The crystals were used as standards for siphonochilone (section 2.2.1.3)

The essential oil and water are immiscible and were easily separated. The water and oil were both separated through draining *via* the tap of the separator. Due to the limited quantity of the oil, it was dissolved in 8.00 mL hexane and dried with anhydrous Na₂SO₄. The solution was filtered with 110 mm Ø Whatman filter paper to remove all the Na₂SO₄. All hexane was evaporated off with a BUCHI Rotavapour at 40°C and the oil was weighed, using the analytical balance.

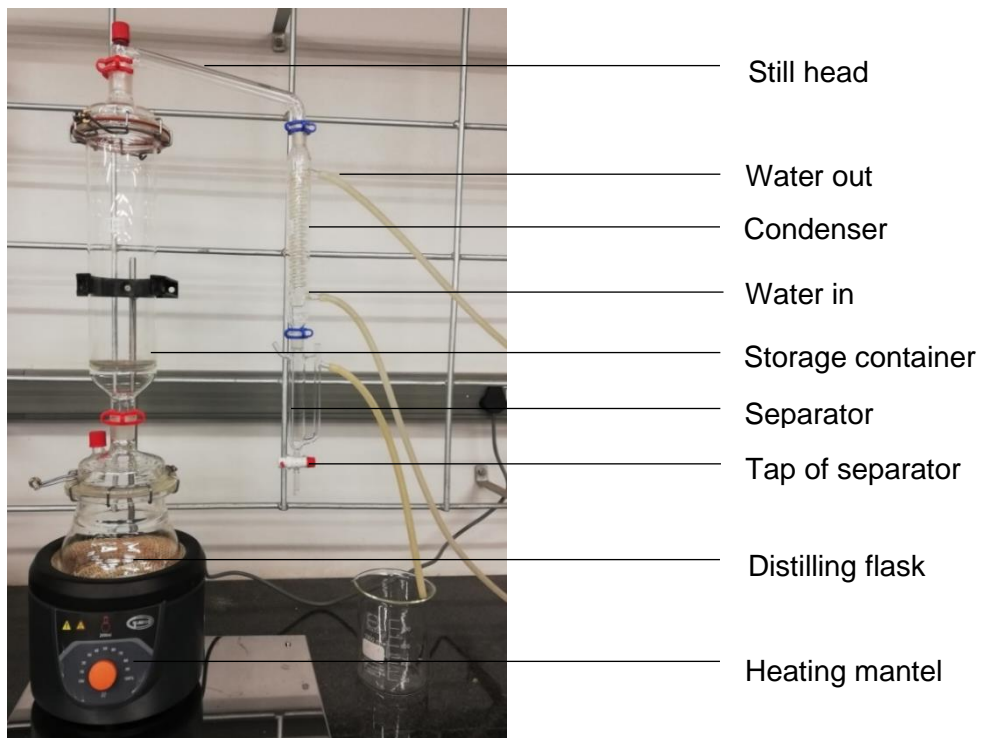


Figure 2.1 Illustration of the steam distillation setup used to isolate essential oil from African ginger.

2.2.5 GC-MS analysis of the various extracts

Analyte separation was done using a LECO Pegasus 4D GC–TOFMS including an Agilent 7890 GC (LECO Africa (Pty) Ltd., Kempton Park, South Africa) on an apolar Rxi-5Sil MS 30 m×0.25 mm ID×0.25 µm df (Restek, Bellefonte, PA, USA) capillary column. The carrier gas, helium, was of ultra-high purity (UHP) grade (Afrox, Gauteng, South

Africa) and was set at a flow rate of 1.00 mL/min in the constant flow mode. An injection volume of 1.00 μ L of sample was injected into the GC inlet set at 250°C. The GC inlet was operated in the split mode (50:1 split ratio for the standards and 10:1 split ratio for the extracts). The initial GC oven temperature programme was increased to 40°C (3 min) at 10°C/min to 300°C (5 min). The GC run time was 35.9 min. The MS transfer line temperature was set at 280°C and the ion source temperature was set at 230°C. The electron energy was 70 eV in the electron impact ionization mode (EI+), the data acquisition rate was 10 spectra/s, the mass acquisition range was 40–500 Daltons, and the detector voltage was set at 1750 V. The sample preparation before injection included 0.50 mg of standards dissolved in 1.00 mL methanol and 1.00 mg essential oil and organic solvent extracts dissolved in 1.00 mL methanol. A NIST 14 Mass Spectral Library version 2.2 was used to identify compounds detected based on their molecular ions and % similarity.

2.3 Results and discussion

Different factors need to be considered for the extraction process before isolation, purification and identification of targeted biologically active compounds can take place. These factors are considered to ensure the best quality and quantity of extracts: extract yield, plant material condition and type, solvent or solvent-system type and method of extraction.

2.3.1 Extraction yields

The comparison of the extraction yields was obtained by comparing the yields of the crude extracts with each other. After evaporation of the solvents, all the extracts had an orange to brown colour and sticky texture and were weighed on an analytical balance. Table 2.2 shows the percentage yield (% w/w) of the crude extracts obtained from the differently prepared plant material using different solvents or solvent-systems for extraction.

There was an increase in the crude extract yields recovered from solid-liquid extraction of freshly dried ground rhizomes compared to the fresh rhizomes for the diethyl ether and ethanol extracts (Table 2.2). The diethyl ether extracts of the fresh rhizomes had a 0.95% yield whereas freshly dried ground rhizomes gave a 2.99% yield. The ethanol extracts gave a 1.83% yield for the fresh rhizomes and 3.49% yield for the freshly dried ground rhizomes. These differences are not surprising since dried plant material are known to produce higher extraction yields than fresh plant material since fresh plant material contains water in the rhizomes. Therefore making freshly dried plant material the preferred plant material for extraction.^[5,12] The main reason for this is that drying fresh plant material results in desiccation and damaging of intact cells of the plant, allowing for the release and greater extractability of more secondary metabolites.^[48-52] The results of higher crude extract yields are no different for African ginger.^[5,19,48]

Table 2.2 The percentage yield (%) of the differently prepared plant material extracted with different organic solvents.

Percentage yield of crude extract of different organic solvent extracts (% w/w)				
Plant material	Diethyl ether	Ethanol	DCM:methanol	DCM:hexane
Fresh rhizomes	0.95	1.83	-	-
Freshly dried ground rhizomes	2.99	3.49	4.20	2.74
Five-year-old dried ground rhizomes	4.65	10.9	20.3	7.18

It has been reported that the chemical composition of African ginger consists mainly of monoterpenes and sesquiterpenoids.^[17,41,43,44] Drying of the fresh rhizomes allows for the release of sesquiterpenoids from the laticifers of the plant material, resulting in the extraction of more secondary metabolites.^[48,53] Depending on the polarity of the solvent used for the extraction, this can result in a higher yield as was seen with the diethyl ether

and ethanol extracts in Table 2.2.^[54,55] It has been suggested that dried plant material be used to extract biologically active secondary metabolites to ensure better quality and quantity of extracts.^[56]

There was also a significant difference in the crude extract yields recovered from freshly dried ground rhizomes compared to five-year-old dried ground rhizomes (Table 2.2). The data showed that for all solid-liquid extractions, there was a higher yield for the five-year-old dried ground rhizomes than that of the freshly dried ground rhizomes. The probable reason for this is that the storage of dried plant material over a period of time can cause the formation of new oxidized compounds and more secondary metabolites trapped in the cells of freshly dried plant material might be released, resulting in higher yields of the extracts.^[57-60]

There was a significant increase in the extract yields using the more polar solvents, ethanol and DCM:methanol, as compared to the diethyl ether and DCM:hexane. The ethanol extracts from the five-year-old dried ground rhizomes had a 10.9% yield compared to that from the freshly dried ground rhizomes, which had a 3.49% yield. For the DCM:methanol extracts, the freshly dried ground rhizomes had a 4.20% yield whereas the five-year-old dried ground rhizomes had a significantly higher yield of 20.3%. This significant increase was attributed to certain compounds being insoluble in different polarities of organic solvents and are therefore not easily extractable.^[61,62] More polar or medium polar compounds are usually extracted with polar solvents such as methanol, thereby increasing the extract yields. It is also possible that more polar or medium polar compounds oxidized during storage resulting in the extraction of additional compounds, resulting in the significantly higher yield.^[58,59]

Another method of extraction, steam distillation, was also performed using fresh rhizomes. During steam distillation of 6.43 kg of fresh rhizomes, a light-yellow oil was collected in the separator and removed. The light-yellow oil was dissolved in hexane since the quantity was too small to separate all the water droplets. The hexane containing the essential oil solution was dried with Na₂SO₄ to remove all the water. After drying, the

solution was filtered and evaporated using a BUCHI rotary evaporator R-124 (Switzerland) at 30°C and the oil weighed 7.59 g (0.12% w/w).

In addition, during steam distillation, clear white crystals formed in the condenser of the apparatus and were removed by washing these with hexane. After air drying of the hexane, 2.04 g of crystalline material (0.03% w/w) was obtained and later confirmed to be the major compound, siphonochilone (Chapter 3, section 3.3.5.1). The pure siphonochilone crystals were used as a standard for the qualitative analysis of the various extracts.

Based on the yields of crude extracts and essential oil, it was determined that the best material and conditions for extraction was the five-year-old dried ground rhizomes extracted with DCM:methanol and ethanol.

The chemical composition analysis of the crude extracts was performed using GC-MS as a measure to determine any relation to the extraction yields.

2.3.2 Qualitative analysis of the extracts

The qualitative analysis using GC-MS of the different extracts was performed to determine mainly the presence of two targeted compounds, siphonochilone (**1**) and its hydroxylated lactone (**6**). The analysis was also undertaken to determine if there was a change in the chemical composition due to different factors, including plant material condition and type, solvent or solvent-system type and method of extraction.

2.3.2.1 GC-MS analysis of extracts of fresh wet rhizomes

African ginger is known to contain volatile compounds and has been extensively researched to confirm its various traditional uses using different organic solvent extracts and essential oils.^[41,43,44] For the qualitative analysis, the essential oil and diethyl ether extracts of fresh rhizomes were analysed using GC-MS for comparison of their chemical profiles. Selected compounds were identified and confirmed using standards and others

were based on mass spectral comparison with the NIST 14 Mass Spectral Library version 2.2.

In the first instance, the chromatograms of the crystals obtained from steam distillation, the essential oil and diethyl ether extracts, which were all produced from fresh rhizomes, were compared to each other focusing on the major peaks.

For the GC-MS analysis 0.50 mg of the crystals were dissolved in 1.00 mL methanol and injected into a LECO Pegasus 4D GC-TOF-MS including an Agilent 7890 GC. Figure 2.2.(1) shows the chromatogram of the crystals with an intense peak at 849.90 s. Since only one intense peak is observed, it would suggest that the crystals are a pure compound. Based on the mass spectral data from the NIST library, the peak had a molecular ion (m/z) at 230 which corresponded to a molecular formula of $C_{15}H_{18}O_2$. The molecular formula of siphonochilone was reported by Holzapfel et al. (2002) to be $C_{15}H_{18}O_2$ and the calculated molecular mass for siphonochilone is 230.30 g/mol. The m/z at 230 suggested the intense peak to be siphonochilone and was confirmed through 1-D and 2-D NMR as described in section 3.3.5.1. The crystals were further used as a siphonochilone standard for comparison purposes based on its retention times and confirmation of its presence in the extracts.

Figure 2.2.(2) shows the chromatogram of the essential oil prepared from the fresh rhizomes. The peak at 839.28 s had a m/z of 230 corresponding to siphonochilone in the essential oil. Three other intense peaks at 143.80, 144.60 and 235.05 s were also observed. Based on the NIST library match (89.0%), the compound at 144.60 s was identified as eucalyptol and its presence in the essential oil was also confirmed by previous studies.^[43,45] The other compounds at 143.80 and 235.05 s could not be identified since they did not show any acceptable mass spectral matches with any compounds from the NIST library. The intensities of the peaks in the chromatogram suggested that siphonochilone is not the major compound in the essential oil, which could have been attributed to a significant portion of the compound crystalizing in the condenser during the distillation process. The presence of eucalyptol is highly significant as this

compound is well-known for its uses in management of respiratory diseases and could through a synergistic effect with siphonochilone, which has also been shown to have similar effects, have much more significant pharmacological effects.^[43,45, 63,64]

The chromatogram of the diethyl ether extract from fresh rhizomes (Figure 2.2.(3)) had an intense peak at 841.80 s with a m/z of 230 corresponding to siphonochilone (Figure 2.2.(3)). The intensity of the peak suggested that siphonochilone is the major compound in the extract while the peak at 144.03 s corresponded to eucalyptol based on the NIST library match (83.6%). The results also showed that the peak corresponding to eucalyptol is significantly smaller in intensity compared to the siphonochilone peak indicating that eucalyptol is relatively low in the diethyl ether extract compared to that in the essential oil. It is also probable that the more volatile compounds are released with steam distillation than with organic solvent extraction, in this case, diethyl ether as solvent.^[15,19,39]

In the comparison of the chromatograms of the essential oil (Figure 2.2.(2)) and the diethyl ether extract (Figure 2.2.(3)), there were additional differences in the chromatograms as more distinct intense peaks were observed for the essential oil which was due to more volatile compounds being extracted with steam distillation since it allowed for the release of more volatiles than organic solvent extraction.^[15,19,39]

The use of fresh rhizomes showed that the targeted compound, siphonochilone, is present in both the essential oil and diethyl ether extract. It is also more feasible to use steam distillation to obtain the siphonochilone crystals since these crystallize out easily as opposed to isolating and purifying the diethyl ether extract to obtain the pure compound.

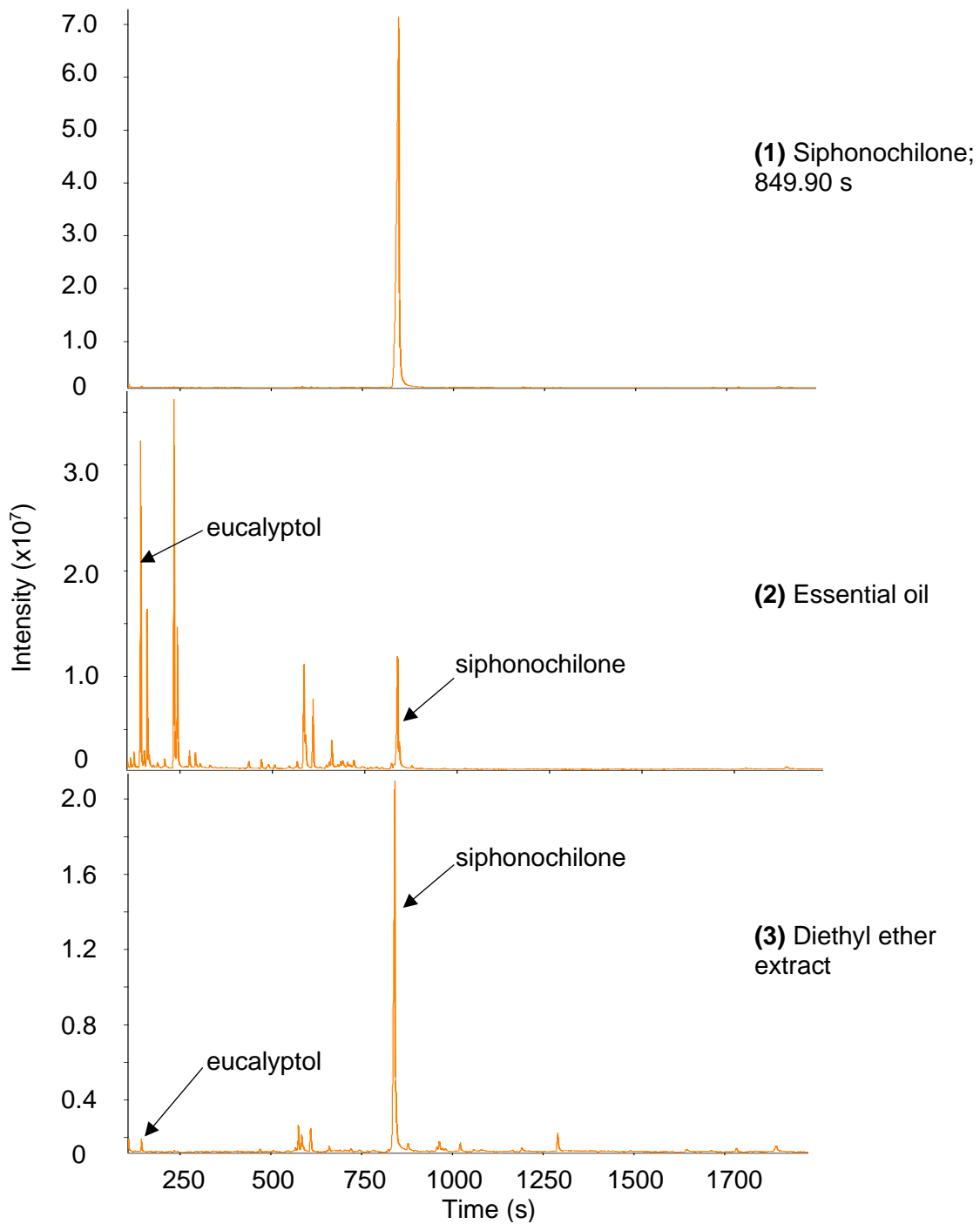


Figure 2.2 Chromatogram from the GC-MS analysis of **(1)** siphonochilone, **(2)** the essential oil and **(3)** the diethyl ether extract from fresh rhizomes.

2.3.2.2 GC-MS analysis of extracts from freshly dried ground rhizomes

All the extracts prepared from the fresh dried ground rhizomes and standards, siphonochilone (**1**) and its hydroxylated lactone (**6**), were analysed by GC-MS for comparative purposes and confirmation of the presence of the two compounds. During this analysis, maintenance was performed on the GC column by having to trim the column before, which resulted in a change in the retention time of the siphonochilone (**1**) as compared to that reported in section 2.3.2.1 (foregoing section).

The standards, siphonochilone (**1**) and its hydroxylated lactone (**6**), were injected into the GC-MS (0.50 mg in 1.00 mL methanol) and the retention times were used for comparative purposes to confirm their presence in the various extracts. Figure 2.3.(1) shows the chromatogram of siphonochilone with a retention time of 1273.60 s and m/z of 230. Figure 2.3.(2) shows the chromatogram of the hydroxylated lactone with a minor peak at retention time 1495.30 s and a major peak at 1564.20 s with a m/z of 262, corresponding with the molecular formula of $C_{15}H_{18}O_4$. Since the structure of the compound was previously confirmed by Zongwe et al. (2018) using NMR it was used for comparative purposes and to confirm its presence in the extracts.^[44]

Figure 2.4 shows the chromatograms of freshly dried ground rhizomes extracted with four different organic solvents: diethyl ether (Figure 2.4.(1)), ethanol (Figure 2.4.(2)), DCM:methanol (Figure 2.4.(3)) and DCM:hexane (Figure 2.4.(4)). The four different solvents and solvent-systems were used to determine whether there would be a difference in the chemical profiles of the extracts and to confirm the presence of siphonochilone and its hydroxylated lactone in the freshly dried ground rhizomes.

The extracts (1.00 mg) were dissolved in 1.00 mL methanol and injected into the GC-MS. An intense peak was detected in all the extracts (1276.40 – 1278.90 s) with a m/z of 230. This confirmed the presence of siphonochilone in all the extracts based on the retention time of the standard and also being the major peak based on its intensity.

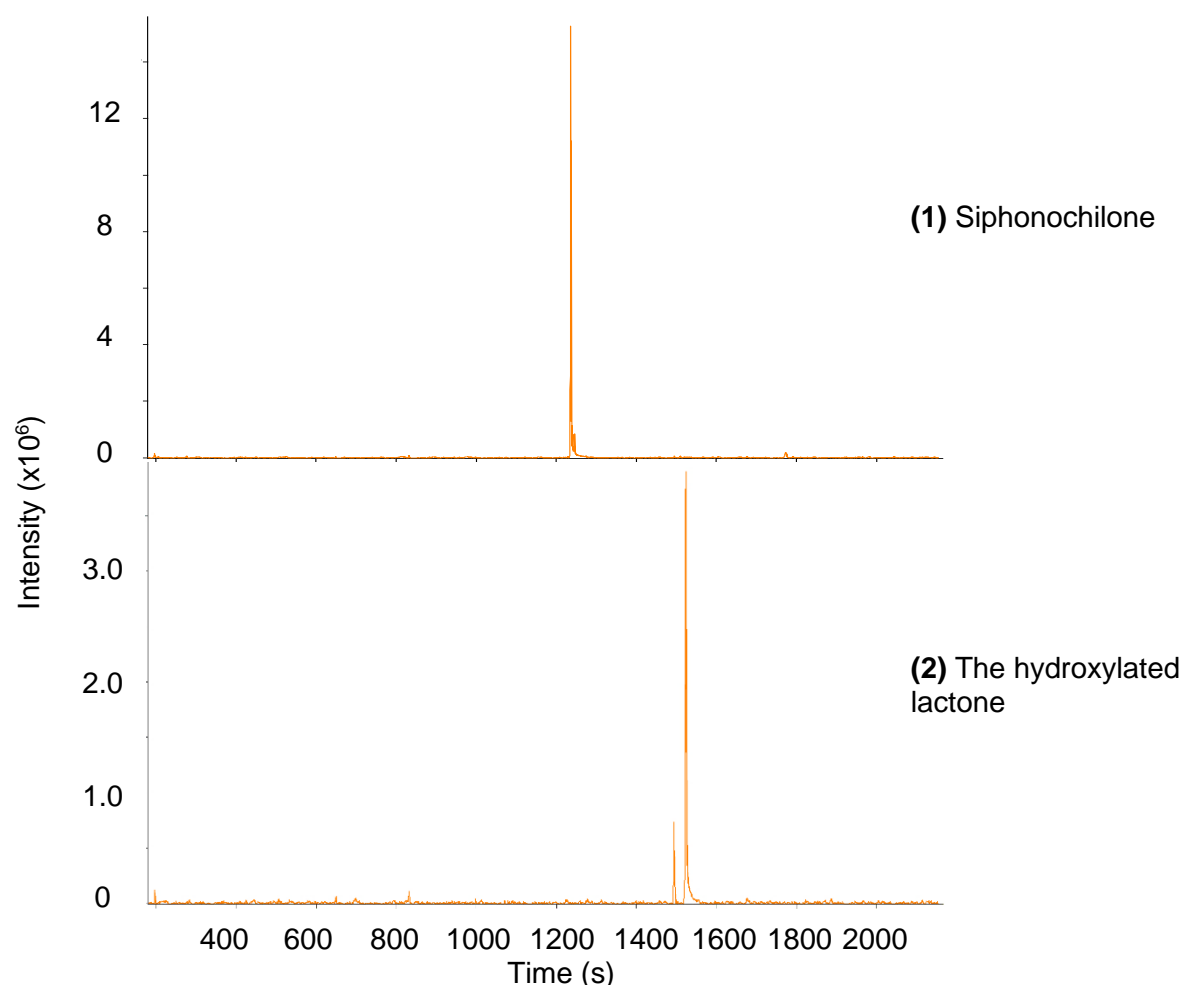


Figure 2.3 Chromatogram from the GC-MS analysis of **(1)** siphonochilone at 1273.60 s and **(2)** the hydroxylated lactone at 1564.20 s.

The presence of the hydroxylated lactone was confirmed by comparison of the retention time of the standard (1564.20 s) and its m/z of 262 to that in the extracts (retention time 1563.80 – 1565.40 s). Although the presence was confirmed, the peaks corresponding to the compounds were of low intensities suggesting that the compound is a minor compound in the extracts.

The ethanol, DCM:methanol and DCM:hexane extracts (Figures 2.4.(2) – (4)) showed a higher peak intensity for the hydroxylated lactone compared to the diethyl ether extract (Figure 2.4.(1)) which showed a very minor peak indicating trace quantities of the compound.

There were a few minor compounds in all four extracts, with most of them unidentified due to their low percentage match using the NIST library. Eucalyptol, an important compound to substantiate the pharmacological properties of the plant, which was confirmed to be present mainly in the essential oil of the fresh plant material, was not detected at any significant levels in any of the extracts prepared after drying the plants.^[45] This indicated that eucalyptol is more likely to be released through the steam distillation process as was also seen in low concentrations in the diethyl ether extract of fresh plants (non-dried). This was confirmed by Naude et al. (2016) who suggested that eucalyptol is present in a higher concentration in the essential oil than in the dried plant material.

Overall, the chromatograms of all four extracts were qualitatively the same, except for the diethyl ether extract with lower intensity peaks in the retention time region from 1400 to 1500 s. As more polar compounds occur in this region, such as the hydroxylated lactone, the more polar solvents such as DCM and ethanol would have extracted such compounds in higher concentrations than diethyl ether.

These results confirm that siphonochilone is the major compound in the extracts of freshly dried ground rhizomes. The lower concentration of the hydroxylated lactone, confirms that minimal auto-oxidation occurs in the fresh plant material as reported previously by Zongwe et al. (2018). The almost negligible concentrations of eucalyptol in the extracts does indicate that the extracts from the non-dried fresh plants, e.g., essential oil, would be expected to perform better in the biological assays. These results are discussed in Chapter 4, section 4.3.2.

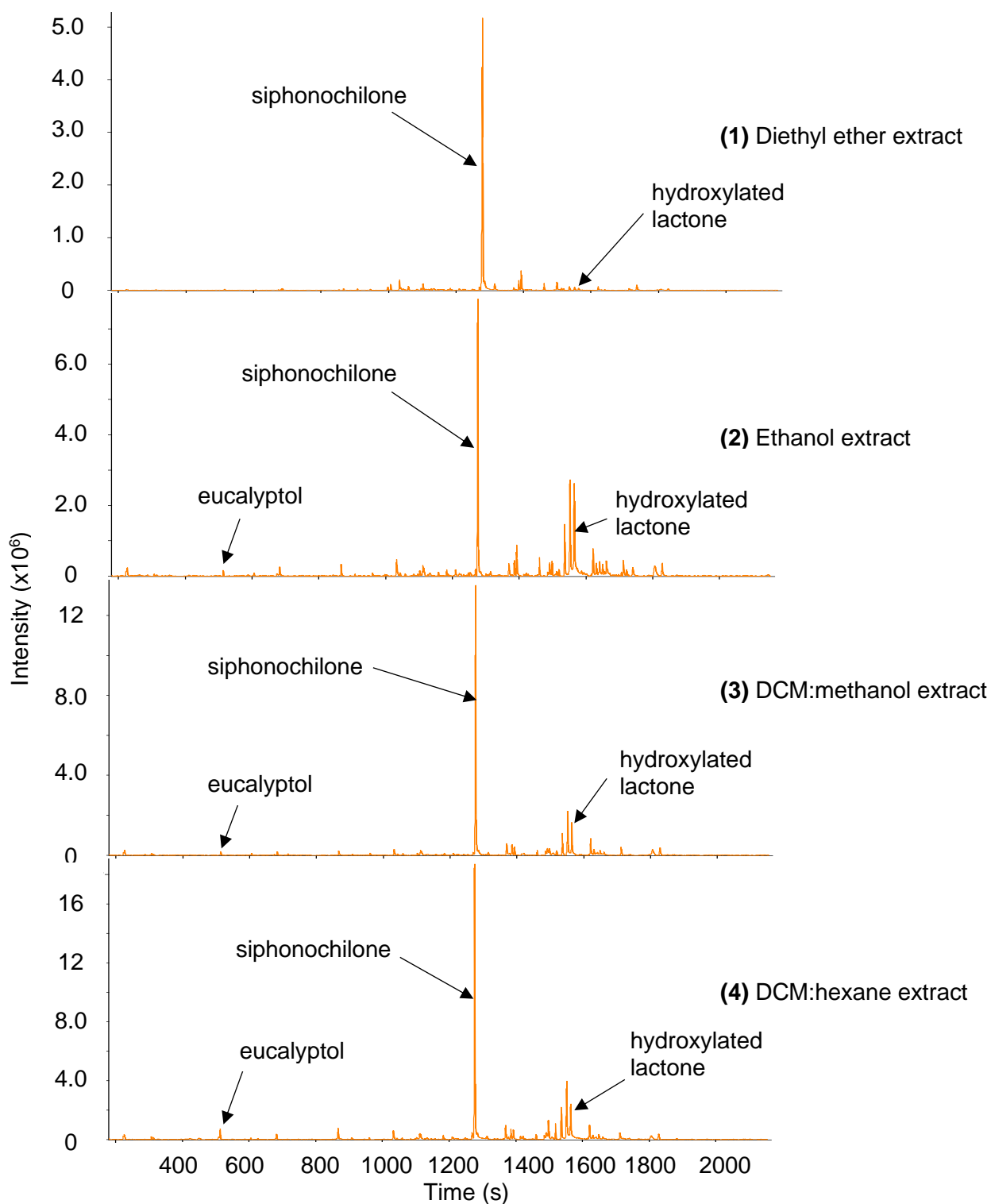


Figure 2.4 Chromatogram from the GC-MS analysis of the **(1)** diethyl ether extract, **(2)** ethanol, **(3)** DCM:methanol and **(4)** DCM:hexane extract of freshly dried ground rhizomes.

2.3.2.3 GC-MS analysis of extracts from five-year-old dried ground rhizomes

The chemical profile of five-year-old dried ground rhizomes extracted with four different organic solvents or solvent-systems were investigated to confirm the presence of the two targeted compounds, siphonochilone (**1**) and its hydroxylated lactone (**6**), and to compare their profiles. The chemical profiles were based on the chromatograms generated from the GC-MS and comparison to known compounds in the NIST library.

All samples were prepared by dissolving 1.00 mg of each extract in 1.00 mL methanol and injecting into the GC-MS. Figure 2.5 shows the chromatograms of the four different extracts. Siphonochilone was present in all four extracts and was confirmed through its retention time (1275.90 – 1277.50 s), which compared favourably to the retention time of the standard. It was clear from the chromatograms that siphonochilone was not the major peak in the diethyl ether and ethanol extracts, based on the peak intensities (Figures 2.5.(1) and Figure 2.5.(2)) since there were other more intense peaks present in the extracts. The DCM:methanol and DMC:hexane extracts (Figures 2.5.(3) and Figure 2.5.(4)) however showed siphonochilone to be the most intense peak.

Irrespective of the intensity of the peak, it was confirmed that siphonochilone is still present in the five-year-old dried ground rhizomes when extracted with different solvents or solvent-systems. The results indicated that the use of DCM is more conducive or suitable to the extraction of siphonochilone.

In all four extracts, the presence of the hydroxylated lactone was confirmed through the retention time (1565.30 – 1568.10 s) as it compared favourably with the retention time of the standard compound in the GC-MS chromatogram. The intensity of the peak for the hydroxylated lactone in the diethyl ether and ethanol extracts (Figures 2.5.(1) and Figure 2.5.(2)) showed this to be of higher intensity than that for siphonochilone and being the major peak in the chromatogram. However, it has a lower intensity peak in the DCM:methanol and DCM:hexane extracts (Figures 2.5.(3) and 2.5.(4)). The possible reason for this is that the common use of DCM may have resulted in more of the siphonochilone being extracted from the plants.

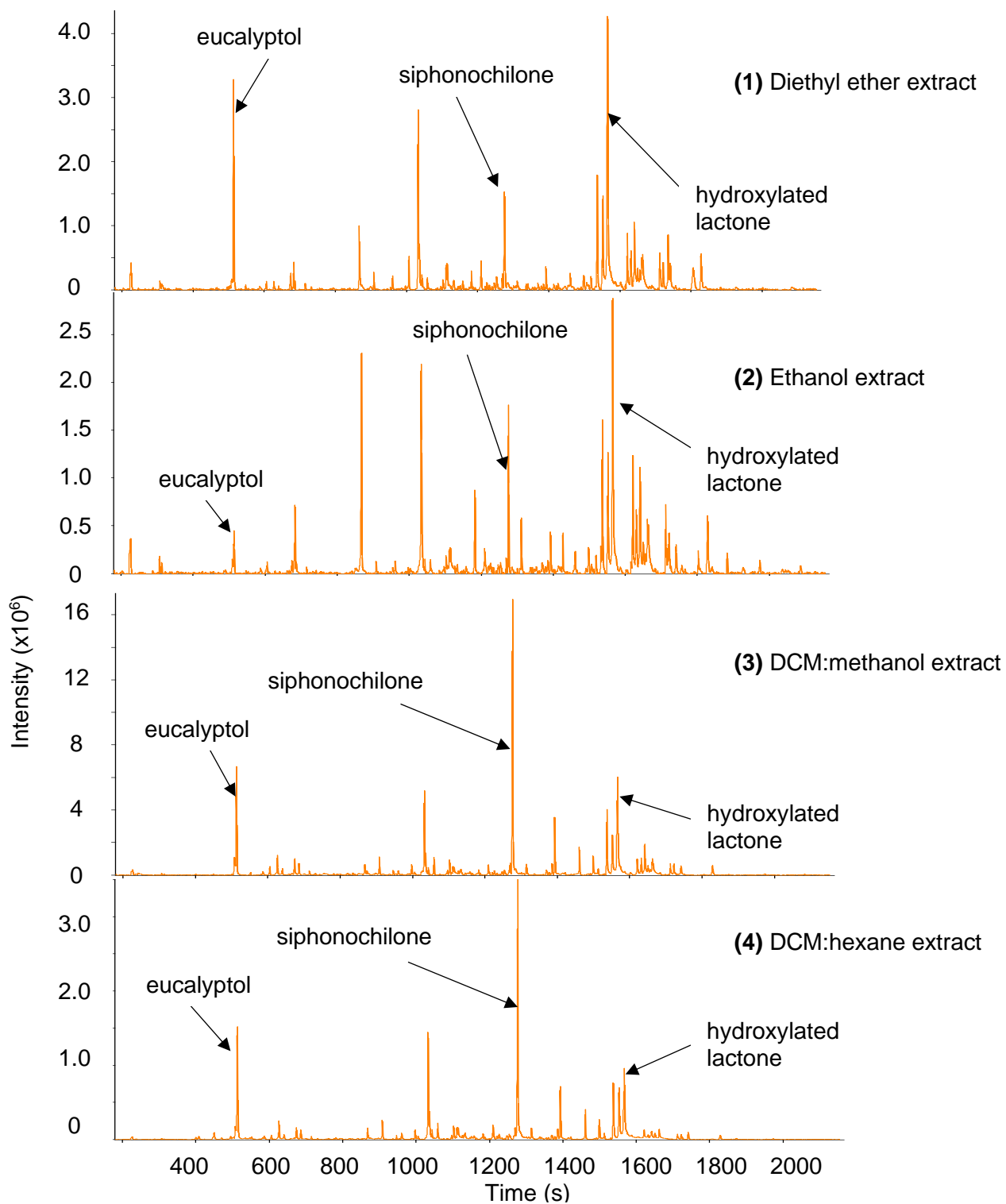


Figure 2.5 Chromatogram from the GC-MS analysis of the **(1)** diethyl ether extract, **(2)** ethanol, **(3)** DCM:methanol and **(4)** DCM:hexane extract of five-year-old dried ground rhizomes.

The NIST library match based on the mass spectra also confirmed the presence of eucalyptol in all extracts (513.40 – 513.90 s), all with >87% match. This was surprising as the results indicated that eucalyptol was also present in the five-year-old dried ground rhizomes and still intact after being stored over a long period. This also indicated that the storage of the plants over the five-year period has the same effect as steam distillation in the extraction of the volatile compounds.

A qualitative comparison (chemical profile) of the chromatograms indicated that the diethyl ether and ethanol extracts were substantially similar to each other, while the DCM:methanol and DCM:hexane extracts were similar to each other. The possible reason for the difference seen between the two pairs of chromatograms could possibly be due to the use of DCM which resulted in more selected extraction of compounds or a higher extraction efficiency of siphonochilone.

2.3.2.4 Comparison of freshly dried ground rhizomes and five-year-old dried ground rhizomes extracted with different organic solvents

Fresh plant material is seldom the sample of choice for quantitative analysis, but is considered for the qualitative analysis if the targeted compounds are thermally sensitive.^[5,19] Dried plant material is preferred over fresh plant material since it holds a lower risk of contamination, produces a higher yield and can be stored for a short period before usage.^[5,19,48] It is also reported that plant material stored over a long period of time might cause decomposition of compounds resulting in the change in the chemical composition of the plant material.^[12,28]

Zongwe et al. (2018) investigated the auto-oxidation of siphonochilone in stored dried ground rhizomes of African ginger and proved that siphonochilone oxidizes to its hydroxylated lactone and other structurally similar lactones (Chapter 1, section 1.1.5.2) if stored for a long period of time.

In this section, the extracts of freshly dried ground rhizomes (Figure 2.4) and five-year-old dried ground rhizomes (Figure 2.5) were compared to each other to identify changes or to recognize any changes in their chemical composition. This was done by comparing the area percentage (%) of the peaks of the total chromatogram for each of the compounds, siphonochilone, the hydroxylated lactone and eucalyptol in the extracts prepared from the freshly dried ground rhizomes and five-year-old dried ground rhizomes.

Figure 2.6 shows the difference in area percentage of the total chromatogram of the freshly dried ground rhizomes and five-year-old dried ground rhizomes extracted with four different solvents or solvent-systems. The graph shows the different area percentages of the total chromatogram for siphonochilone, its hydroxylated lactone and eucalyptol.

The diethyl ether extracts showed that siphonochilone has a significantly higher area percentage of the total chromatogram in freshly dried ground rhizomes. The hydroxylated lactone and eucalyptol only appeared to be present after storage, with the hydroxylated lactone being significantly higher in the five-year-old dried ground rhizomes.

The ethanol extracts showed that siphonochilone concentration is higher in the freshly dried ground rhizomes than in the five-year-old dried ground rhizomes. The hydroxylated lactone showed a similar area percentage in the freshly dried ground rhizomes and five-year-old dried ground rhizomes. This is possibly due to the oxidation of siphonochilone during extraction and drying and not just during storage of the plant material. The decrease in siphonochilone in the five-year-old dried ground rhizomes would suggest oxidation of siphonochilone to the hydroxylated lactone as reported by Zongwe et al. (2018).

The DCM:methanol and DCM:hexane extracts both showed a high area percentage for siphonochilone for the freshly dried ground rhizomes, but decreased in the five-year-old dried ground rhizomes. Both extracts showed low quantities of the hydroxylated lactone in the freshly dried ground rhizomes which is possibly due to oxidation taking place during extraction and drying of extracts. The five-year-old dried ground rhizomes extracts showed an increase in the area percentage for the hydroxylated lactone which confirmed

that oxidation takes place upon storage of plant material as reported by Zongwe et al. (2018).

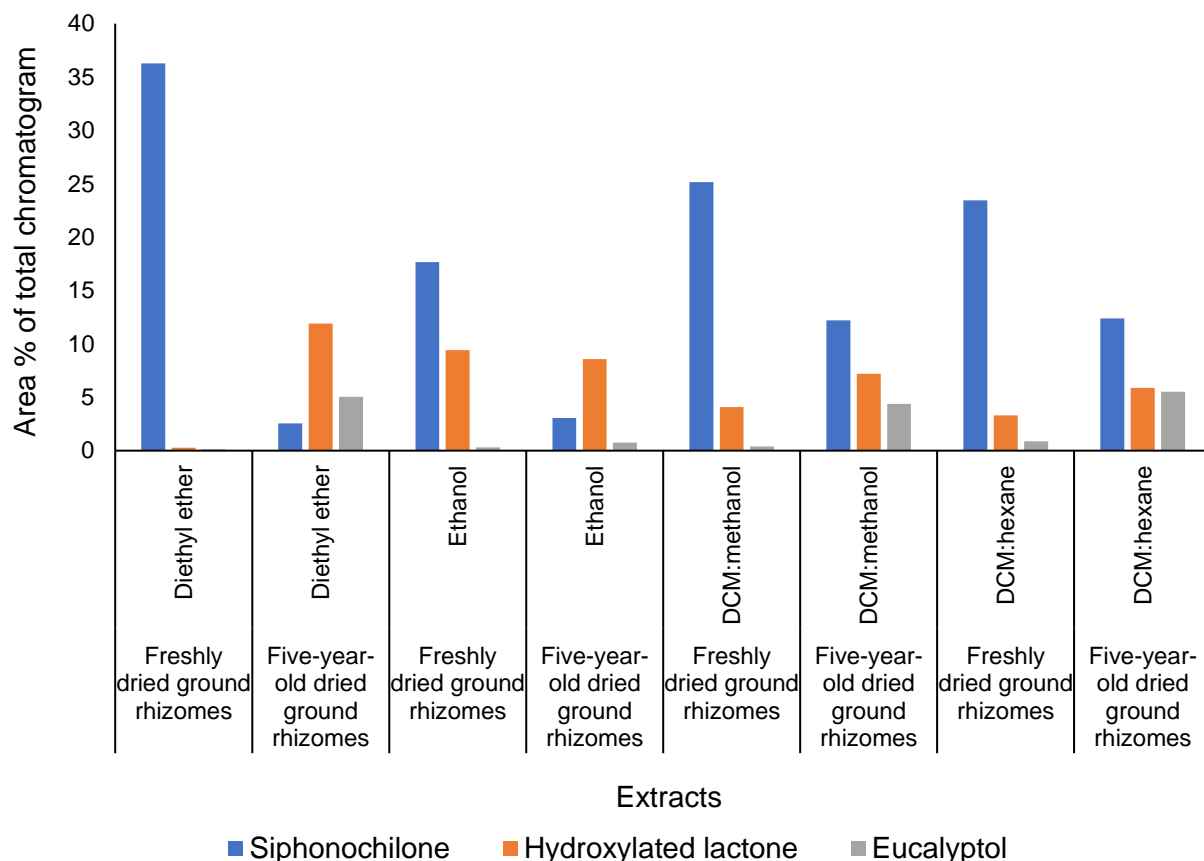


Figure 2.6 Graph of the area percentage (%) of the total chromatogram of the different extracts (1 mg/mL) from freshly dried ground rhizomes and five-year-old dried ground rhizomes.

Both eucalyptol and the hydroxylated lactone showed an increase in their area percentage of the total chromatogram from the freshly dried ground rhizomes to the five-year-old dried ground rhizomes extracts.

Seeing as the different solvents or solvent-systems can extract more medium polar compounds, this can result in the dilution of other compounds and the intensity of the peaks cannot be an accurate representative of the concentration of the compound in the

extract. It can therefore not be used for quantitative analysis and only qualitative analysis to determine the presence of the compounds in the extract. The intensity is only a representation of how concentrated the compound might possibly be in the extract and if there was an increase in its intensity, meaning there is more of the compound present as with the hydroxylated lactone in the five-year-old dried ground rhizomes.

2.4 Conclusion

The extraction of plant material is one of the most important steps in the purification process of biologically active compounds. There are many factors to consider before quantitatively and qualitatively obtaining an extract.

African ginger is well-known for its many traditional uses in treating many ailments like influenza. The study focused on the scientific verification of these claims by biological screening of extracts and isolated pure compounds. The reported major compound, siphonochilone, is the first compound to consider since it is reportedly easy to isolate. The second is the structurally similar compound of siphonochilone, its hydroxylated lactone, which was isolated by Zongwe et al (2018). The aim of this chapter was to compare different extracts from differently prepared plant material and solvents for extraction to quantitatively and qualitatively obtain the best extract for further isolation and purification (Chapter 3) and biological evaluation (Chapter 4).

Different factors were investigated and discussed. From the extracted yields it was found that dried plant material provided a higher yield of extracts than fresh plant material, and the five-year-old dried ground rhizomes had the highest overall yield. All four extracts of the five-year-old dried ground rhizomes showed the highest yields: 4.65% for diethyl ether, 7.18% for DCM:hexane, 10.9% for ethanol and 20.3% for DCM:methanol. The two most polar solvent-systems, ethanol and DCM:methanol, had the highest yields with DCM:hexane also with an acceptable yield. It was expected that more polar solvents would deliver a higher yield since it extracts medium and more polar compounds. Based

on the extractable yields, it was confirmed that five-year-old dried ground rhizomes are the best condition of plant material to use.

Fresh rhizomes were used for steam distillation and solid-liquid extraction to produce the essential oil and a diethyl ether extract. The results concluded that siphonochilone was present in both extracts, but of lower intensity in the GC-MS chromatogram for the essential oil due to some of the compound crystallizing out during steam distillation. Since one of the targeted compounds is siphonochilone, the best way of obtaining it is through steam distillation. The essential oil also included other compounds not detected in the diethyl ether extract which are most likely more volatile compounds only extracted through steam distillation.

Both the freshly dried ground rhizomes and five-year-old dried ground rhizomes contained siphonochilone in their respective organic solvent extracts. The hydroxylated lactone was also present in all extracts from dried plant material but of different peak intensities in the GC-MS chromatograms. In the freshly dried ground rhizomes, the siphonochilone peak was more intense than the hydroxylated lactone since there were only trace amounts of the lactone in the GC-MS data. However, the intensity of the hydroxylated lactone peak increased in the five-year-old dried ground rhizomes and decreased for the siphonochilone. This would confirm Zongwe et al. (2018) that auto-oxidation of siphonochilone occurs, resulting in the formation of its hydroxylated lactone and possibly other lactones in stored plant material and will this be considered when targeting the hydroxylated lactone.

Based on the qualitative and quantitative analysis of the different extracts, it was concluded that the five-year-old dried ground rhizomes can be used for extraction with any of the four solvents or solvent-systems. Selection of the solvent or solvent-system predominantly depends on the safety of the solvents used and an ethanol extract would be the most suitable for further commercialization. After selection of the appropriate extraction of targeted compounds, can isolation and purification be undertaken.

2.5 References

- [1] Wood, D., 2019, How Daily & Seasonal Cycles Affect Plants, viewed 5 August 2020, from <https://study.com/academy/lesson/how-daily-seasonal-cycles-affect-plants.html>
- [2] Halberstein, R.A., 2005, Medicinal plants: historical and cross-cultural usage patterns, *Annals of Epidemiology* 15(9), pp.686-699.
- [3] Alam, M.T., Karim, M.M. and Khan, S.N., 2009, Antibacterial activity of different organic extracts of *Achyranthes aspera* and *Cassia alata*, *Journal of Scientific Research* 1(2), pp.393-398.
- [4] Gololo, S.S., Shai, L.J., Agyei, N.M. and Mogale, M.A., 2016, Effect of seasonal changes on the quantity of phytochemicals in the leaves of three medicinal plants from Limpopo province, South Africa, *Journal of Pharmacognosy and Phytotherapy* 8(9), pp.168-172.
- [5] Azwanida, N.N., 2015, A review on the extraction methods use in medicinal plants, principle, strength and limitation, *Medicinal & Aromatic Plants* 4(196), pp.2167-0412.
- [6] Craig, W.J., 1999, Health-promoting properties of common herbs, *The American Journal of Clinical Nutrition* 70(3), pp.491s-499s.
- [7] Fennell, C.W., Light, M.E., Sparg, S.G., Stafford, G.I. and Van Staden, J., 2004, Assessing African medicinal plants for efficacy and safety: agricultural and storage practices, *Journal of Ethnopharmacology* 95(2-3), pp.113-121.
- [8] Croteau, R., Kutchan, T.M. and Lewis, N.G., 2000, Chapter 24: Natural Products (Secondary Metabolites), Buchanan, B.B., Grisseem, W. and Jones, R.L., *Biochemistry and Molecular Biology of Plants*, 2nd ed., American Society of Plant Biologists, pp.1250-1319.
- [9] Bourgaud, F., Gravot, A., Milesi, S. and Gontier, E., 2001, Production of plant secondary metabolites: a historical perspective, *Plant Science* 161(5), pp.839-851.

- [10] Figueiredo, A.C., Barroso, J.G., Pedro, L.G. and Scheffer, J.J., 2008, Factors affecting secondary metabolite production in plants: volatile components and essential oils, *Flavour and Fragrance Journal* 23(4), pp.213-226.
- [11] Xego, S., Kambizi, L. and Nchu, F., 2017, Effects of different hydroponic substrate combinations and watering regimes on physiological and anti-fungal properties of *Siphonochilus aethiopicus*, *African Journal of Traditional, Complementary and Alternative Medicines* 14(3), pp.89-104.
- [12] Sarker, S.D., Latif, Z. and Gray, A.I., 2006, *Natural Product Isolation: An Overview*, 2nd ed., Humana Press, Totowa, New Jersey.
- [13] Müller-Riebau, F.J., Berger, B.M., Yegen, O. and Cakir, C., 1997, Seasonal variations in the chemical compositions of essential oils of selected aromatic plants growing wild in Turkey, *Journal of Agricultural and Food Chemistry* 45(12), pp.4821-4825.
- [14] Richards, L.A., Dyer, L.A., Forister, M.L., Smilanich, A.M., Dodson, C.D., Leonard, M.D. and Jeffrey, C.S., 2015, Phytochemical diversity drives plant–insect community diversity, *Proceedings of the National Academy of Sciences* 112(35), pp.10973-10978.
- [15] Romanik, G., Gilgenast, E., Przyjazny, A. and Kamiński, M., 2007, Techniques of preparing plant material for chromatographic separation and analysis, *Journal of Biochemical and Biophysical Methods* 70(2), pp.253-261.
- [16] Johansen, H.N., Glitsø, V. and Bach Knudsen, K.E., 1996, Influence of extraction solvent and temperature on the quantitative determination of oligosaccharides from plant materials by high-performance liquid chromatography, *Journal of Agricultural and Food Chemistry* 44(6), pp.1470-1474.
- [17] Igoli, N.P. and Obanu, Z., 2011, The volatile components of wild ginger (*Siphonochilus aethiopicus* (Schweinf) Bl Burt), *African Journal of Food Science* 5(9), pp.541-549.

- [18] Rasmann, S. and Agrawal, A.A., 2009, Plant defense against herbivory: progress in identifying synergism, redundancy, and antagonism between resistance traits, *Current Opinion in Plant Biology* 12(4), pp.473-478.
- [19] Handa, S.S., 2008, *An overview of extraction techniques for medicinal and aromatic plants*, *Extraction technologies for medicinal and aromatic plants*, International Centre for Science and High Technology.
- [20] Poole, S.K., Dean, T.A., Oudsema, J.W. and Poole, C.F., 1990, Sample preparation for chromatographic separations: an overview, *Analytica Chimica Acta* 236, pp.3-42.
- [21] Bucić-Kojić, A., Planinić, M., Tomas, S., Bilić, M. and Velić, D., 2007, Study of solid–liquid extraction kinetics of total polyphenols from grape seeds, *Journal of Food Engineering* 81(1), pp.236-242.
- [22] Azmir, J., Zaidul, I.S.M., Rahman, M.M., Sharif, K.M., Mohamed, A., Sahena, F., Jahurul, M.H.A., Ghafoor, K., Norulaini, N.A.N. and Omar, A.K.M., 2013, Techniques for extraction of bioactive compounds from plant materials: A review, *Journal of Food Engineering* 117(4), pp.426-436.
- [23] Charles, D.J. and Simon, J.E., 1990, Comparison of extraction methods for the rapid determination of essential oil content and composition of basil, *Journal of The American Society for Horticultural Science* 115(3), pp.458-462.
- [24] Sultana, B., Anwar, F. and Ashraf, M., 2009, Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts, *Molecules* 14(6), pp.2167-2180, from <https://doi.org/10.3390/molecules14062167>
- [25] Mejri, J., Abderrabba, M. and Mejri, M., 2010, Chemical composition of the essential oil of *Ruta chalepensis* L: Influence of drying, hydro-distillation duration and plant parts, *Industrial Crops and Products* 32(3), pp.671-673.
- [26] Sadras, V.O. and Denison, R.F., 2009, Do plant parts compete for resources? An evolutionary viewpoint, *New Phytologist* 183(3), pp.565-574.
- [27] Phrompittayarat, W., Putalun, W., Tanaka, H., Jetiyanon, K., Wittaya-areekul, S. and Ingkaninan, K., 2013, Comparison of various extraction methods of Bacopa

- monnieri, *Naresuan University Journal: Science and Technology (NUJST)* 15(1), pp.29-34.
- [28] Zongwe, F.K., Muya, J.T., Mutimana, R., Virima, M., Mayaliwa, M., Chung, H. and Maharaj, V., 2018, Auto-oxidation of Siphonochilone in Processed Rhizomes and Stored Powders of *Siphonochilus aethiopicus* (Schweinf.) BL Burtt., *ChemistrySelect* 3(30), pp.8569-8574.
- [29] Spigno, G., Tramelli, L. and De Faveri, D.M., 2007, Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics, *Journal of Food Engineering* 81(1), pp.200-208.
- [30] Chavan, U.D., Shahidi, F. and Naczki, M., 2001, Extraction of condensed tannins from beach pea (*Lathyrus maritimus* L.) as affected by different solvents, *Food Chemistry* 75(4), pp.509-512.
- [31] Wang, L. and Weller, C.L., 2006, Recent advances in extraction of nutraceuticals from plants, *Trends in Food Science & Technology* 17(6), pp.300-312.
- [32] Widyawati, P.S., Budianta, T.D.W., Kusuma, F.A. and Wijaya, E.L., 2014, Difference of solvent polarity to phytochemical content and antioxidant activity of *Pluchea indica* less leaves extracts, *International Journal of Pharmacognosy and Phytochemical Research* 6(4), pp.850-855.
- [33] Lapornik, B., Prošek, M. and Wondra, A.G., 2005, Comparison of extracts prepared from plant by-products using different solvents and extraction time, *Journal of Food Engineering* 71(2), pp.214-222.
- [34] Ju, Z.Y. and Howard, L.R., 2003, Effects of solvent and temperature on pressurized liquid extraction of anthocyanins and total phenolics from dried red grape skin, *Journal of Agricultural and Food Chemistry* 51(18), pp.5207-5213.
- [35] Bubalo, M.C., Vidović, S., Redovniković, I.R. and Jokić, S., 2018, New perspective in extraction of plant biologically active compounds by green solvents, *Food and Bioproducts Processing* 109, pp.52-73.

- [36] Płotka-Wasyłka, J., Rutkowska, M., Owczarek, K., Tobiszewski, M. and Namieśnik, J., 2017, Extraction with environmentally friendly solvents, *Trends in Analytical Chemistry* 91, pp.12-25.
- [37] Romdhane, M. and Gourdon, C., 2002, Investigation in solid–liquid extraction: influence of ultrasound, *Chemical Engineering Journal* 87(1), pp.11-19.
- [38] Bakkali, F., Averbeck, S., Averbeck, D. and Idaomar, M., 2008, Biological effects of essential oils—a review, *Food and Chemical Toxicology* 46(2), pp.446-475.
- [39] Baser, K.H.C. and Buchbauer, G. eds., 2015, *Handbook of essential oils: science, technology, and applications*, 2nd ed., CRC press, Boca Raton, Florida.
- [40] Turek, C. and Stintzing, F.C., 2013, Stability of essential oils: a review, *Comprehensive Reviews in Food Science and Food Safety* 12(1), pp.40-53.
- [41] Holzapfel, C.W., Marais, W., Wessels, P.L. and Van Wyk, B.E., 2002, Furanoterpenoids from *Siphonochilus aethiopicus*, *Phytochemistry* 59(4), pp.405-407.
- [42] Noudogbessi, J.P.A., Tchobo, P.F., Alitonou, G.A., Avlessi, F., Soumanou, M., Chalard, P., Figueredo, G., Chalchat, J.C. and Sohounhloue, D.C.K., 2013, Chemical study of extracts of *Siphonochilus aethiopicus* (Schweinf.) BL Burt (Zingiberaceae) from Benin, *Asian Journal of Chemistry* 25(15), pp.8489-8492.
- [43] Viljoen, A.M., Demirci, B., Baser, K.H.C., Van Wyk, B.E. and Jäger, A.K., 2002, The essential oil composition of the roots and rhizomes of *Siphonochilus aethiopicus*, *South African Journal of Botany*, 68(1), pp.115-116.
- [44] Lategan, C.A., Campbell, W.E., Seaman, T. and Smith, P.J., 2009, The bioactivity of novel furanoterpenoids isolated from *Siphonochilus aethiopicus*, *Journal of Ethnopharmacology* 121(1), pp.92-97.
- [45] Naudé, Y., Makuwa, R. and Maharaj, V., 2016, Investigating volatile compounds in the vapour phase of (1) a hot water infusion of rhizomes, and of (2) rhizomes of *Siphonochilus aethiopicus* using head space solid phase microextraction and gas chromatography with time of flight mass spectrometry, *South African Journal of Botany* 106, pp.144-148.

- [46] Juergens, U.R., Dethlefsen, U., Steinkamp, G., Gillissen, A., Repges, R. and Vetter, H., 2003, Anti-inflammatory activity of 1,8-cineol (eucalyptol) in bronchial asthma: a double-blind placebo-controlled trial, *Respiratory Medicine* 97(3), pp.250-256.
- [47] Santos, F.A. and Rao, V.S.N., 2000, Anti-inflammatory and antinociceptive effects of 1,8-cineole a terpenoid oxide present in many plant essential oils, *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives* 14(4), pp.240-244.
- [48] Soysal, Y. and Öztekin, S., 1999, Equilibrium moisture content equations for some medicinal and aromatic plants, *Journal of Agricultural Engineering Research* 74(3), pp.317-324.
- [49] Díaz-Maroto, M.C., Pérez-Coello, M.S., Gonzalez Vinas, M.A. and Cabezudo, M.D., 2003, Influence of drying on the flavor quality of spearmint (*Mentha spicata* L.), *Journal of Agricultural and Food Chemistry* 51(5), pp.1265-1269.
- [50] Asekun, O.T., Grierson, D.S. and Afolayan, A.J., 2007, Characterization of essential oils from *Helichrysum odoratissimum* using different drying methods, *Journal of Applied Science* 7(7), pp. 1005-1008.
- [51] Lewicki, P.P., 1998, Effect of pre-drying treatment, drying and rehydration on plant tissue properties: A review, *International Journal of Food Properties* 1(1), pp.1-22.
- [52] Starmans, D.A. and Nijhuis, H.H., 1996, Extraction of secondary metabolites from plant material: a review, *Trends in Food Science & Technology* 7(6), pp.191-197.
- [53] Chadwick, M., Trewin, H., Gawthrop, F. and Wagstaff, C., 2013, Sesquiterpenoids lactones: benefits to plants and people, *International Journal of Molecular Sciences* 14(6), pp.12780-12805.
- [54] Iloki-Assanga, S.B., Lewis-Luján, L.M., Lara-Espinoza, C.L., Gil-Salido, A.A., Fernandez-Angulo, D., Rubio-Pino, J.L. and Haines, D.D., 2015, Solvent effects on phytochemical constituent profiles and antioxidant activities, using four different extraction formulations for analysis of *Bucida buceras* L. and *Phoradendron californicum*, *BioMed-Central Research Notes* 8(1), p.396.

- [55] Thouri, A., Chahdoura, H., El Arem, A., Hichri, A.O., Hassin, R.B. and Achour, L., 2017, Effect of solvents extraction on phytochemical components and biological activities of Tunisian date seeds (var. Korkobbi and Arechti), *BMC Complementary and Alternative Medicine* 17(1), p.248.
- [56] Altemimi, A., Lakhssassi, N., Baharlouei, A., Watson, D.G. and Lightfoot, D.A., 2017, Phytochemicals: Extraction, isolation, and identification of bioactive compounds from plant extracts, *Plants* 6(4), p.42.
- [57] Fernández-Sandoval, M.T., Ortiz-Garcia, M., Galindo, E. and Serrano-Carreón, L., 2012, Cellular damage during drying and storage of *Trichoderma harzianum* spores, *Process Biochemistry* 47(2), pp.186-194.
- [58] Thakur, L., Ghodasra, U., Patel, N. and Dabhi, M., 2011, Novel approaches for stability improvement in natural medicines, *Pharmacognosy Reviews* 5(9), p.48-54.
- [59] França, M.B., Panek, A.D. and Eleutherio, E.C.A., 2007, Oxidative stress and its effects during dehydration, *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 146(4), pp.621-631.
- [60] Mediani, A., Abas, F., Tan, C.P. and Khatib, A., 2014, Effects of different drying methods and storage time on free radical scavenging activity and total phenolic content of *Cosmos caudatus*, *Antioxidants* 3(2), pp.358-370.
- [61] Do, Q.D., Angkawijaya, A.E., Tran-Nguyen, P.L., Huynh, L.H., Soetaredjo, F.E., Ismadji, S. and Ju, Y.H., 2014, Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*, *Journal of Food and Drug Analysis* 22(3), pp.296-302.
- [62] Nawaz, H., Shad, M.A., Rehman, N., Andaleeb, H. and Ullah, N., 2020, Effect of solvent polarity on extraction yield and antioxidant properties of phytochemicals from bean (*Phaseolus vulgaris*) seeds, *Brazilian Journal of Pharmaceutical Sciences*, 56, e17129 [Online]. Available from: <https://doi.org/10.1590/s2175-97902019000417129> . [Accessed 21 August 2020].

- [63] Seol, G.H. and Kim, K.Y., 2016, 'Eucalyptol and its role in chronic diseases', *In Drug Discovery from Mother Nature*, Switzerland: Springer, Cham, pp. 389-398.
- [64] Shao, J., Yin, Z., Wang, Y., Yang, Y., Tang, Q., Zhang, M., Jiao, J., Liu, C., Yang, M., Zhen, L. and Hassouna, A., 2020, Effects of Different Doses of Eucalyptus Oil From *Eucalyptus globulus* Labill on Respiratory Tract Immunity and Immune Function in Healthy Rats, *Frontiers in Pharmacology* 11, p.1287.

Chapter 3: Isolation, purification and characterization of compounds from African ginger

3.1 Background

3.1.1 Isolation of compounds from plant extracts and African ginger

For years people have shown interest in the medicinal properties of plant-based traditional medicine for the production of natural products.^[1,2] These plants are used to produce standardized extracts or pure compounds with endless possibilities for commercialization.^[2,3] Standardized extracts may contain many active compounds which can work synergistically or antagonistically towards the treatment of certain ailments.^[4-6] It is important to identify, characterize, isolate and purify compounds for structure elucidation and biochemical characterization (toxicity assays, *in vivo* testing and clinical studies).^[2,3]

Some well-known biologically active compounds isolated from plants include artemisinin from *Artemisia annua* as an antimalarial, morphine from *Papaver somniferum* to treat Parkinson's disease, cannabidoil from *Cannabis sativa* as a pain reliever and forskolin from *Coleus forskohlii* to treat obesity.^[7] These compounds have been isolated through different means of chromatographic techniques including high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), column chromatography, centrifugal partition chromatography (CPC) and gas chromatography (GC).^[2,8-10]

There is a growing demand for novel compounds derived from plants to treat various diseases based on their traditional use.^[11,12] African ginger is no different; it has many popular traditional uses and little research has been done on the biological assaying of the active compounds from the plant (Chapter 1, section 1.1.5).^[13,14]

The major compounds, siphonochilone (**1**), and structurally similar compounds (**2**, **3**, **5 – 7**) (see Chapter 1, section 1.1.5.2) were isolated using techniques such as vacuum liquid

chromatography (VLC), flash and column chromatography, HPLC-SPE-NMR and preparative TLC.^[13,17-20] Some of the techniques used for the purification can be time consuming and make use of hazardous solvents.^[15,18,19] The excessive use of solvents is unfavourable for the environment and hazardous to human life.^[20,21]

In present times the search and use of healthy, safe, biodegradable and renewable resources are of the utmost importance to replace less environmentally friendly techniques, such as column chromatography, with cleaner or greener technologies.^[22,23] These also aid in the prevention of the formation of artefacts which can form using harsher chromatographic techniques.^[19,24] It has also been shown that some of the compounds previously identified from extracts of African ginger, are formed through auto-oxidation which could also occur during harsh chromatographic steps.^[21]

3.1.2 Modern chromatographic techniques

Natural products are known to potentially have active ingredients, making them very popular.^[25-27] This popularity has however declined due to the time-consuming process of isolation and purification and has resulted in the demand for high-throughput automated technology and its revolutionizing way of fractionation, isolation and purification of active ingredients.^[26-28] High-throughput technology is faster, more economical and greener.^[11,29,30]

One of the principles for green chemistry is limiting the usage of hazardous chemicals and replacing them with greener options.^[20,31] Organic solvents are one of the biggest sources of waste and need to be replaced with gas and liquid chromatography.^[30-32] This can be done by replacing the organic solvents with supercritical fluids such as CO₂, which is often used alone without modifiers or with small quantities of organic solvents (ethanol or methanol).^[32-34] These solvents assist in separation, making it a safer, more efficient and quicker method of purification.^[31,33]

Using automated high-throughput systems or integrating them can assist in efficient use of energy and result in safer chemistry. [21,30,35] This can be done by combining techniques such as liquid handlers, SPE and HPLC to reduce the use of solvents and be more time efficient. [35] The rising demand for safe and environmentally friendly products and techniques have forced pharmaceutical companies and researchers to think innovatively on how to implement this in the production of natural products. [21,31,34]

3.1.2.1 Supercritical fluid chromatography (SFC)

Supercritical Fluid Chromatography (SFC) is a normal-phase chromatographic technique that allows solvents to perform under supercritical conditions to perform better than GC or HPLC. [26,36] The objective of the technique is to allow compounds to elute at subcritical temperature (35°C), without decomposing, and by introducing high pressures. [25,37,38]

The technique was developed in the 1960's, but was facetious since it was not reliable or sustainable because the instrument could not handle the supercritical conditions and CO₂ as a mobile phase. [38-40] It was only since the 1980's that the instrument was compatible with the mobile phase and since 2012 the instrument was improved with better features. [38,40]

SFC uses CO₂ as the major component of the mobile phase instead of an organic solvent. [23,38,41] The mobile phase carries the substance through the stationary phase until it is collected. [22] Only a small quantity of organic solvent (ethanol or methanol) is used as a co-solvent to improve elution of polar compounds. [38] This reduces the use of hazardous solvents, decreases retention time and allows for more injections. [22,40,42] The stationary phase consists of a pre-packed column of different packaging; BEH 2-ethylpyridine, BEH (Ethylene-Bridged Hybrid), CSH Fluoro-phenyl, silica and silica 2-ethylpyridine (Viridis®), and can be used depending on the type of compounds that need to be separated. [43,44]

This instrument can be used on an analytical or preparative scale. [22,45] Analytical scale is used for qualitative purposes for method development and must be transferable to

preparative scale.^[22,33] A few parameters can affect the sensitivity and separation during analytical development; changing the co-solvent type, co-solvent percentage, flow rate and injection volume.^[22,42,46] Once a method is developed, the user switches to preparative scale for quantitative analysis. This is used in the pharmaceutical, environmental, petroleum and polymer industry and used for fractionation of non-polar compounds.^[22,23,47]

The technique is more advantageous than normal GC and LC (liquid chromatography) for its low viscosity, high diffusivity, high density, low use of hazardous solvent waste, high separation efficiency, short analysis time and collection of more concentrated fractions.^[22,34,40,46]

This separation technique has not been used as frequently as GC or HPLC, but it has separated compounds for certain plant species and offers better separation and sensitivity for non-polar compounds^[23,46,48] It is only recently that the instrument has been used on natural products and has resulted in the isolation of lipids, monosaccharides, saponins and flavonoids.^[22,39,48] It is imperative to promote the use of greener chemistry such as SFC to reduce the use of hazardous solvents.

3.1.2.2 Ultra-performance convergence chromatography (UPC²)

Convergence chromatography (CC) refers to the combination of GC and LC into one system.^[41,49] It is used to separate structurally similar chiral and achiral compounds and has improved on the resolution and selectivity of SFC.^[23,50,51] It is a normal phase chromatographic technique which uses a polar co-solvent such as methanol instead of water, has lower retention times, which are prompted by a change in pressure and it retains polar compounds for a longer time.^[36,49,51]

Convergence chromatography uses a mixture of CO₂ and mostly methanol as the mobile phase.^[23,46] The former needs to be liquified/compressed to improve reliability and reproducibility by using an automated back pressure regulator (ABPR).^[42,44,52] This allows

for elution of polar compounds and better separation and sensitivity.^[23,49] CC is more sensitive than SFC and allows for more reliable and reproducible work.^[42,49] This technique is perfect for the analysis of complex compounds from natural extracts, however the system is only available for analysis and not yet for fractionation or isolation of compounds.^[37,45]

3.1.2.3 Liquid handler and automated solid-phase extraction (SPE)

Integrating different high-throughput components such as a liquid handler, automated SPE, HPLC and NMR, can improve productivity and reduce the time of fractionation, isolation and purification of plant extracts.^[21,53-55]

SPE has been widely used in natural product research and has shown good results in extraction of compounds.^[56-58] It has been a very popular green technique used for fractionation of a sample to obtain a more concentrated fraction before being analysed, isolated and purified by more sensitive analytical methods such as GC or HPLC.^[21,57,59] Over the years the technique has been easily automated to deliver high-throughput systems to aid in accuracy, consistency and reproducibility.^[21,27,28,57]

SPE was originally designed to replace liquid-liquid extraction by being more cost effective, less time-consuming, have a lower solvent consumption, fewer fractions and fewer errors.^[21,59,60] The system uses SPE disposable column-like cartridges for fractionation and the type depends on the type of extract or SPE system that is being used.^[21,54,58] The mobile phase passes through the cartridge by gravitational force or positive pressure (air or syringes) to wash the stationary phase and allow for fractionation.^[54]

This has however resulted in a lower recovery of analytes due to the low selectivity sorption between the samples and matrices.^[59,61] Other disadvantages include the method development for the fractionation procedure that can take a longer time to

develop and the reproducibility of fractionation can take longer if the system is not automated.^[61,62]

These disadvantages have resulted in the manufacturing of automated SPE's that has made it less time-consuming, safer in management of solvents and more consistent.^[28] By integrating a liquid handler with an automated SPE has improved productivity since it is more user friendly, cost effective, quicker, and resulted in higher sample recovery and less solvent consumption.^[34,54,63] The liquid handler aids in the movement of liquid (eluent) from one location to another.^[63] It can have different numbers of robotic arms, pipetting heads/syringes, accessories and functionalities of the probes and is very important in improving the consistency and reproducibility of fractionation of plant extracts.^[21,53,63]

The integration of an automated SPE system and liquid handler was investigated by Thornburg et al. (2018) where they addressed and improved some concerns regarding the use of an automated robotic SPE system:

- The sample mass and accurate dispensing of an aqueous:organic (95:5) solvent-system is very important to prevent disrupting the reproducibility and to prevent low selectivity,
- the sample is dissolved in an organic solvent-system and loaded onto cotton and dried to allow for cotton-absorbed samples for fractionation to prevent clogging of the SPE frit and matrix, and
- the elution of the sample using polar solvents like water, methanol and acetonitrile for normal-phase fractionation to get a solvent system that had adequate elution strength for fractionation.^[21,53,64-66]

The integration of different techniques has been embraced by the pharmaceutical, cosmeceutical, food, pesticide and environmental industries for various uses and is the future of modern-day chemistry.^[59,60]

3.1.2.4 High-performance liquid chromatography (HPLC)

HPLC is very popular in the identification, quantification and purification of compounds from plant extracts.^[67] It is used with the purpose of isolating compounds for structure elucidation through NMR with the hope of identifying a compound(s) with significant biological activity.^[68,69]

HPLC is a mainly reversed-phase chromatographic technique used to qualitatively and quantitatively resolve mixtures of compounds and separate and purify them based on their variable interaction with the stationary phase.^[26,66,70,71] Using high pressure, the mobile phase containing the sample is pushed through an immobile stationary phase, resulting in different elution times.^[66,70,72] The mobile phase is responsible for the separation of compounds using polar solvents like water, acetonitrile and methanol.^[66,72] Separation is therefore possible due to the interactions between the analyte, mobile phase and stationary phase. The polar mobile phase and non-polar stationary phase favours the elution of polar compounds, therefor making it a reversed-phase chromatographic technique.^[70]

The use of HPLC for purification of plant extracts can either include using a crude extract or a fraction from a crude extract to do analytical development for qualitative analysis and preparative development for quantitative analysis as discussed with SFC.^[73,74] The importance of analytical development is to confirm purity, develop an appropriate method and identify the targeted compound(s).^[71,74] The method is then used on preparative scale for the collection of pure compound(s).^[26,34,71,72] It has been reported to be successful in the isolation of secondary metabolites such as flavonoids, phenolic acids and steroids.^[66,67,71,75,76]

It is preferred over other high-throughput systems like SFC due to its consistency and robustness.^[26] Automated high-throughput systems are becoming more popular and integrating them with different components such as HPLC and LC-MS improves its value.^[25,28,53,65] It improves the fractionation, isolation and purification of active compounds by doing so much quicker and being more environmentally friendly.^[35] It is

very clear that this technique has become very popular amongst pharmaceutical companies for drug discovery and development, and is most certainly the future of natural product drug development.^[28,65,66,70-72]

3.2 Methodology

3.2.1 Analytical grade solvents

Acetone ($(\text{CH}_3)_2\text{CO}$; 58.08 g/mol), ethyl acetate ($\text{CH}_3\text{COOC}_2\text{H}_5$; 88.11 g/mol) and n-hexane (C_6H_{14} ; 86.18 g/mol) were purchased from Merck. Dichloromethane (DCM) (CH_2Cl_2 ; 84.93 g/mol) was purchased from Sigma-Aldrich. Methanol (CH_3OH ; 32.04 g/mol) and acetonitrile ($\text{C}_2\text{H}_3\text{N}$; 41.05 g/mol) were purchased from ROMIL (ROMIL-SpS™ Super Purity Solvent). Analytical grade H_2O purchased from Microsep (ROMIL). Diethyl ether anhydrous ($(\text{C}_2\text{H}_5)_2\text{O}$; 74.12 g/mol) was purchased from Radchem Laboratory Suppliers (Pty) Ltd.

3.2.2 Other chemicals

Acetone, DCM, 98% sulfuric acid (H_2SO_4 ; 98.08 g/mol), vanillin ($\text{C}_8\text{H}_8\text{O}_3$; 152.15 g/mol) and trifluoroacetic acid HPLC (TFA) ($\text{C}_2\text{HF}_3\text{O}_2$; 114.02 g/mol) were purchased from Radchem Laboratory Suppliers (Pty) Ltd. Silica gel 60 GF254 TLC plates from Merck. Silica gel MN-60 from Macherey-Nagel GmbH & Co. KG. Iodine resublimed from SAARCHEM.

3.2.3 Other equipment and apparatus

Glass polytops (26 mL) No. 6 was purchased from Listco Glassblowers, Johannesburg. An AB265-S/FACT DualRange analytical balance was purchased from Mettler Toledo. An EZ-2 Plus Genevac centrifugal evaporator was purchased from SP Scientific and a Spectroline ENF-240C/FE Handheld 4W Inspection UV Lamp (254/365 nm) from Lasec.

3.2.4 Solvent extraction of plant material

The DCM:methanol (1:1) and DCM:hexane (1:1) extracts of five-year-old dried ground rhizomes were prepared as described in Chapter 2, section 2.2.3 and were used for the isolation and purification of compounds. The extracts were stored in the cold room (6 – 8°C) prior to further analysis. The fresh and freshly dried ground rhizomes extracted with ethanol (Chapter 2, section 2.2.3.2) were used for UPC² analysis as described in Section 3.2.7.

3.2.5 Standards for analysis

The two standards, siphonochilone (**1**) and its hydroxylated lactone (**6**), were used to identify their presence in different extracts.

Clear crystals of siphonochilone that crystallized during steam distillation, as described in Chapter 2, section 2.2.4, was used as a standard for siphonochilone (**1**). The hydroxylated lactone (**6**) was previously isolated, purified and identified by Mr. Félix Junior Katele Zongwe as discussed in Chapter 2, section 2.2.1.3.

3.2.6 Purification using supercritical fluid chromatography (SFC)

The analytical scale method development and semi-preparative purification Investigator SFC system from Waters was used for the fractionation of a DCM:methanol (1:1) crude extract. The system comprises of a 3.00x100 mm Viridis® BEH column, 130Å 1.70 µm analytical column (Ireland, Waters) for method development. A 19x150 mm Viridis® BEH Prep 2-EP OBD™ 5.00 µm semi-preparative column (Waters, Ireland) was used for fractionation and placed inside a thermally controlled oven. A temperature and pressure controlled back pressure regulator (BPR) was used to compensate for cooling during depressurization and pumps through the CO₂ (Foodfresh by AFROZ) and co-solvent, methanol. The sample was manually injected at the Sample Manager, which has two well plates and holds 96 HPLC vials (1.50 mL each).

The system is coupled to a 2998 Photodiode Array (PDA) detector.^[20] The collection system comprises twelve 500 mL collector bottles with 26 mL glass polytops. Figure 3.1 shows a scheme of the Investigator SFC system from Waters used with all its components. The elution gradient (solvent A, CO₂; solvent B, methanol) was constant, started at 1% of solvent B and ended at 1% solvent B after 15 min, with a constant flow of 7.00 mL/min. The back pressure was set at 140 bar and temperature at 40°C. Detection of UV radiation absorbance of the crude was done at 210 – 270 nm.



Figure 3.1 Illustration of the Investigator SFC system used to fractionate the crude extract.

The presence of the targeted compounds, siphonochilone (**1**) and its hydroxylated lactone (**6**), was determined by separately injecting their standards (see section 3.2.5) into the SFC and comparing their retention times to the peaks of the DCM:methanol crude extract (see section 3.3.1). For both standards and the crude extract, 1.00 mg was dissolved in 1.00 mL methanol and 7.00 μ L was injected.

For method development a sample was prepared using an analytical balance to weigh 1.00 mg of the DCM:methanol crude extract and dissolved in 1.00 mL of methanol and 7.00 μ L was injected per run. For semi-preparative purification, a sample was prepared

with 40 mg of the DMC:methanol crude extract dissolved in 1.00 mL of methanol and 20 μ L was injected per run.

Successive injections were accomplished until 438 mg of the crude extract dissolved in methanol was injected onto the column of the SFC system. Eleven fractions were collected, based on the peaks detected on UV radiation, in 26 mL glass polytops placed in the collector bottles (Figure 3.2). Due to the rather large volumes of fractions collected, they were first evaporated using the Genevac at low boiling point (30°C) for 2 hours. The mass of each fraction was recovered and weighed, using the analytical balance, before further analysis using of TLC and the UPC².

All 11 fractions and the two standards were dissolved in hexane and spotted on TLC plates to determine which fraction contained the targeted compound, the hydroxylated lactone (**6**). The TLC plates were developed in ethyl acetate:hexane (2:3) and observed under UV radiation at 254 nm. One plate was stained with a vanillin stain (0.20 g vanillin, 30 mL methanol and 1.00 ml H₂SO₄) and heated, and the other in an iodine chamber.

The fractions were dried using the centrifugal evaporator at low boiling point (30°C) for 2 hours and used for analysis on the UPC².

3.2.7 Ultra-performance convergence chromatography (UPC²)

After evaporation of the eleven fractions collected from SFC, only fractions 7 and 9 contained sufficient quantities of the fractions and were the only fractions used for further analysis (Figure 3.2). TLC analysis showed fractions 7 and 9 to also have the most intense spots corresponding to siphonochilone (**1**) and the hydroxylated lactone (**6**) under UV light at 254 nm, and from the vanillin and iodine stain. From the two fractions, 1.00 mg of each were dissolved separately in 1.00 mL of methanol and 1.00 μ L of each fraction was injected onto a UPC² system.

The UPC² system and chromatographic conditions of The Waters ACQUITY UPC² system was equipped with a binary solvent manager, fixed loop sample manager,

convergence manager with an ABPR, column manager and PDA detector. The qualitative analysis was performed at 50°C using a 30×100 mm ACQUITY UPC²™ CSH Fluoro-Phenyl 1.70 µm column (Waters, USA).

The elution gradient (solvent A, CO₂; solvent B, methanol) was constant starting at 2% of solvent B and ended with 2% of solvent B after 8.00 min, with a constant flow of 0.60 mL/min. The back pressure was set at 1500 psi and peaks were detected at 215 nm.

The presence of the targeted compounds, siphonochilone (**1**) and its hydroxylated lactone (**6**), was determined by two experiments (section 3.3.1). Firstly, by separately injecting their standards onto the UPC² and comparing their retention times to the major peaks of the crude extract and fractions. Secondly, by confirmation of the presence of the standards through a “spiking” experiment, i.e., the addition of 200 µL of the standard hydroxylated lactone sample (1.00 mg in 1.00 mL methanol) to the fraction. Then re-analysing the chromatograms by comparing the retention times and the area percentage of the total chromatogram of the peak of the chromatogram before and after the “spiking” experiment.

Ethanol extracts of fresh (wet) and freshly dried ground rhizomes and roots were also analysed using UPC² and compared to the crude extract to see if there was a change in the chromatograms and intensity of the siphonochilone (**1**) and its hydroxylated lactone (**6**) peaks. The samples were prepared by dissolving 1.00 mg of each extract in 1.00 mL of methanol and injecting 1.00 µL of each extract onto the UPC² system. All samples that were analysed using UPC² had the same concentration of 1.00 mg/mL.

3.2.8 Purification of fractions using column chromatography

Fractions 7 and 9 were combined and labelled as FR1 (48.96 mg, 11.17% w/w) and the methanol was evaporated using a centrifugal evaporator at low boiling point (30°C) for 1 h.

Column chromatographic separation of the combined fractions were performed with a 300 mm internal diameter, 1000 mL glass column packed with silica gel. The column was prepared by pouring slurry of 185 g of silica gel in acetone:DCM (1:9) into the column. The combined fractions, FR1, were dissolved in acetone:DCM (1:9) (Figure 3.2) and loaded onto the silica gel. Elution was performed gradient-wise with acetone and DCM mixtures starting with 5% and increasing to 30% acetone in DCM. Fractions were collected until the targeted hydroxylated lactone eluted with 47 fractions being collected (Figure 3.2).

The purification sample (FR1), two standards, siphonochilone (**1**) and the hydroxylated lactone (**6**), and the collected fractions were analysed by TLC and observed under UV radiation at 254 and 365 nm and then placed in an iodine chamber. The iodine stain revealed a distinct yellow spot for the hydroxylated lactone. Siphonochilone was detected under the UV light at 254 nm and by staining it with a vanillin stain. The hydroxylated lactone was detected with an iodine stain. After examining the column fractions using TLC with the various stains, fractions 6 – 10 was found to contain the siphonochilone, and fractions 12 – 16 the hydroxylated lactone. Fractions 12 – 16 were combined, evaporated and weighed to give 2.90 mg of the subfractions and labelled as FR2 and used for further purification (Figure 3.2).

A second column was packed to isolate the hydroxylated lactone from fraction FR2 using a 300 mm internal diameter, 1000 mL glass column. Silica (55 g) was slurry-packed in an acetone:ethyl acetate:hexane (2:3:5) solvent-system and poured into the column. FR2 (2.90 mg), dissolved in hexane, was loaded on the bed of the silica gel. Elution was through a gradient-wise solvent mixture of acetone:ethyl acetate:hexane starting with 2:3:5 and ending with 1:4:5, resulting in the collection of 53 fractions. The fractions were analysed by TLC and stained in an iodine chamber, which revealed that the hydroxylated lactone was present in fractions 28 – 37 (Figure 3.2). The fractions were combined, evaporated using a rotavapour at 30°C and labelled as FR3. A limited quantity (0.17 mg, 0.05% w/w) of sample was obtained and analysed by ¹H NMR spectroscopy. Due to the impurity and limited quantity, further purification could not be done.

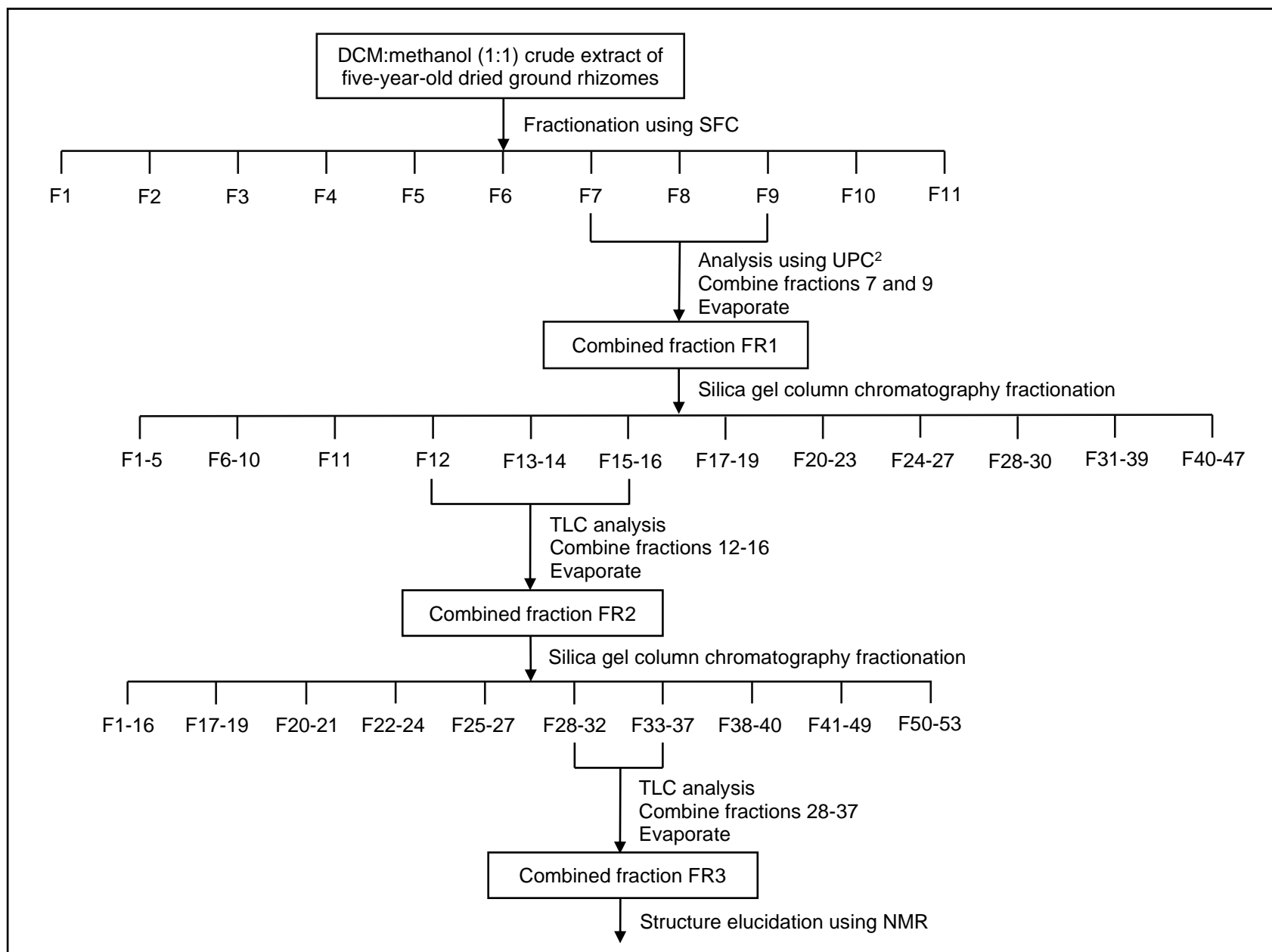


Figure 3.2 Purification of the DCM:methanol (1:1) crude extract using SFC, UPC² and column chromatography.

3.2.9 Solid-phase extraction (SPE)

Figure 3.3 is an illustration of a VERITY GX-241 Aspec Liquid Handler and 4060 Single syringe pump (Gilson) programmed by Trilution LH v4.0 used for the solid phase extraction using an empty SPE cartridge (Phenomenex), SPE tube adapter (Lasec) and 2.00 g HyperSep SPE cartridge (octyl, nonend-capped: 50 μm , 60 \AA) (Thermo Scientific) cartridge for pre-fractionation.

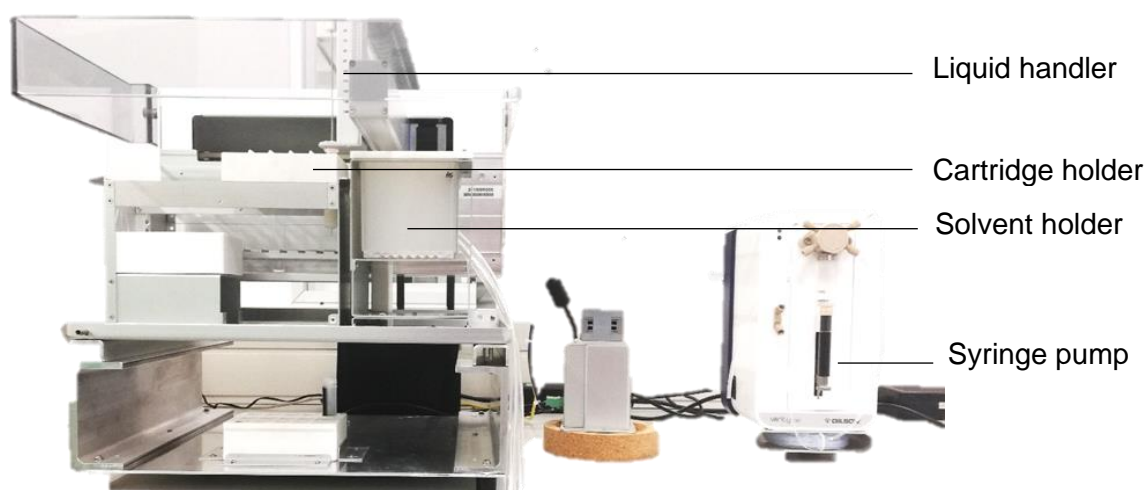


Figure 3.3 Illustration of the Liquid Handler, automated SPE and single syringe pump used for the solid phase extraction.

The DCM:hexane (1:1) extract of five-year-old dried ground rhizomes of African ginger (290 mg and 276 mg) was separately dissolved in each of 4.50 mL methanol:ethyl acetate (3:2). The dissolved samples were adsorbed separately onto two cotton wools and dried in the centrifugal evaporator at low boiling point (30°C) for 20 min before being transferred to an empty SPE cartridge.

The cotton-adsorbed, lyophilized SPE sample cartridge was stacked above an adsorbent containing Hypersep SPE cartridge using a SPE tube adapter for individualized fractionation. At pre-fractionation, the HyperSep SPE cartridge was washed with 18 mL of 100% methanol, followed by equilibration with 18 mL of H₂O:methanol (9:1), at a controlled rate of elution of 6.00 mL/min.

Each of the dried extracts were separately pre-fractionated using the method of Thornburg et al. (2018) with modifications to give a better and improved separation. An elution gradient (solvent A, H₂O; solvent B, methanol) started at 9:1 and ended at 1:4, resulting in the collection of 14 fractions, which were collected in 20 mL beakers and transferred to 26 mL glass polytops (Figure 3.4). The fractions were evaporated using a centrifugal evaporator at HPLC setting (51°C) for 8 h and weighed. After solvent removal the mass of each fraction was recorded, dissolved in 0.50 mL methanol and analysed using TLC.

All 14 fractions were spotted on two TLC plates (acetone:ethyl acetate:hexane (1:4:5)) and viewed under UV light (254 nm). One plate was stained with vanillin and the other with iodine. The iodine stain showed the presence of the hydroxylated lactone in fractions 3 – 5. The fractions were combined (labelled as FR4) and evaporated using a centrifugal evaporator at low boiling point (30°C) for 1 h to reveal a dark yellow oily substance (146 mg, 25.8% w/w) (Figure 3.4). FR4 was used for further purification using HPLC.

3.2.10 High-performance liquid chromatography (HPLC)

A HPLC-MS (Waters) chromatographic system apparatus consisted of a system fluidics organizer (Waters), binary gradient module (2545) coupled to a Waters PDA (2998). It was equipped with a Waters 2767 injector (20 µL/1000 µL sample loop) interfaced to a PC running MassLynx V4.1™ software (Waters, USA).

The combined fractions from the SPE purification, FR4 (146 mg), was dissolved in 1.50 mL methanol. Method development was done using an X-Bridge C18 column (150x4.6 mm, i.d. 3.50 µm particle size) (Waters) operating at room temperature with a flow rate of 1.00 mL/min. Elution was done using a gradient system (Table 3.1) of solvent A (H₂O + 0.1% TFA) and solvent B (Acetonitrile + 0.1% TFA). FR4 dissolved in methanol was injected onto the column (5.00 µL) with a run time of 18 min and a post-run of 1 min.

The standard hydroxylated lactone (**6**), obtained as described in section 3.2.5, was used to determine the retention time of the hydroxylated lactone on HPLC and was compared to the chromatogram of FR4 to identify the peak representing the lactone based on its

retention time. This was done by dissolving 1.00 mg of the standard hydroxylated lactone **(6)** in 1.00 mL of methanol and analysing the solution using the same method applied for the analysis of FR4.

Table 3.1 Elution gradient used for the analysis of fraction FR4 on an X-Bridge C18 analytical column.

Time (min)	0	10	13	15	16	17
Flow rate (mL/min)	1	1	1	1	1	1
Solvent A (%)	90	0	0	90	90	90
Solvent B (%)	10	100	100	10	10	10

The chromatogram of the hydroxylated lactone presented a well-resolved peak at 6.65 min and corresponded to the major peak at 6.61 min for FR4 (see section 3.3.4). This confirmed the presence of the hydroxylated lactone **(6)** in FR4 based on its retention time.

The gradient system for method development was adjusted to accommodate the purification on a semi-preparatory scale using a Preparative OBD columns calculator by Waters. Semi-preparatory purification was performed on a reversed-phase Waters X-Bridge Preparative C18 column (19x250 mm, i.d. 5 μ m particle size) (Waters) operating at room temperature with a flow rate of 17.06 mL/min. Detection was carried out from 220 to 254 nm. Elution was done using a gradient system (Table 3.2) of solvent A (H₂O + 0.1% TFA) and solvent B (Acetonitrile + 0.1% TFA). FR4 (146 mg) dissolved in 1.50 mL methanol was repeatedly injected (200 μ L) onto the semi-preparative column with a flow rate of 17.06 mL/min and a run time of 29 min and a 0.47 min post-run time.

Table 3.2 Elution gradient used for the purification of FR4 on a Waters X-Bridge Preparative C18 column.

Time (min)	0	16.67	21.67	25.00	26.67	28.53
Flow rate (mL/min)	17.06	17.06	17.06	17.06	17.06	17.06
Solvent A (%)	90	0	0	90	90	90
Solvent B (%)	10	100	100	10	10	10

The retention times changed with the adjustments made for the semi-preparatory purification and the lactone eluted at 9.12 min after the injection of the pure standard using the same method as per Table 3.2. This retention time was used as a guide to collect four peaks based on a time-based collection as shown in Table 3.3. and the lactone was collected at 9.00 – 9.30 min.

Table 3.3 Time-based collection of four peaks from FR4 on a Waters X-Bridge Preparative C18 column.

Peak number	Time interval (min)
1	7.61 – 7.84
2	8.10 – 8.35
3	9.00 – 9.30
4	10.27 – 10.50

After repeated injections, all collections corresponding to peak number 3 were combined (labelled as FR5) (Figure 3.4) and the solvent was removed using the centrifugal evaporator using the HPLC setting (51°C) for 8 hours. The fraction FR5 was weighed (23 mg, 4.1% w/w) and dissolved in hexane for TLC analysis. FR5 was spotted on a TLC plate (acetone:ethyl acetate:hexane (1:4:5)) and viewed under UV light (254 nm), stained

with vanillin and iodine. The iodine stain showed the presence of the hydroxylated lactone for FR5 and the fraction was evaporated using a centrifugal evaporator at low boiling point (30°C) for 1 h to give a light-yellow oily substance. FR5 was analysed by NMR spectroscopy to confirm its structure.

3.2.11 Nuclear magnetic resonance spectroscopy analysis (NMR)

Compound confirmation and structure elucidation was done using a Bruker Avance III 400 MHz magnetic operating at 400.21 MHz for ^1H and 100.64 MHz for ^{13}C , ^1H , ^{13}C and COSY NMR experiments were performed for compound confirmation of siphonochilone (**1**) and ^1H , ^{13}C , HSQC, HMBC, COSY and NOESY NMR experiments were performed using the Bruker Avance III 400M Hz for structure elucidation of the fraction (FR5) containing the hydroxylated lactone (**6**).

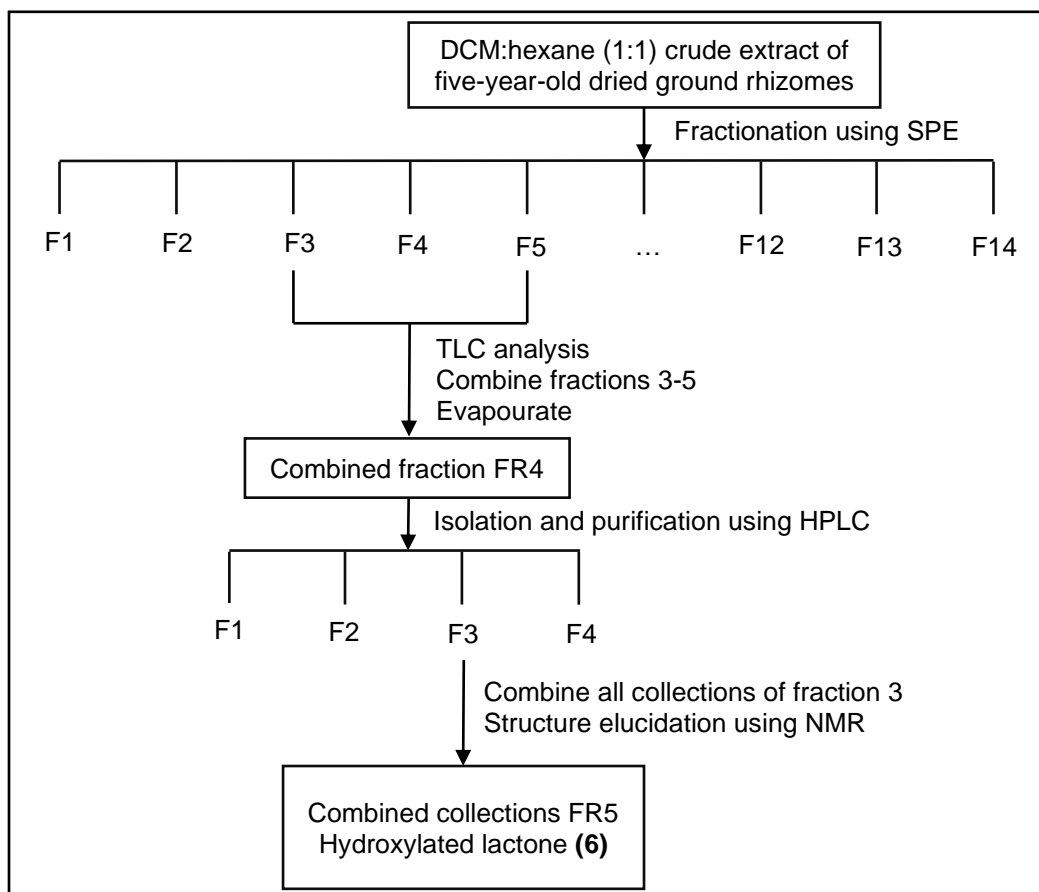


Figure 3.4 Process for the isolation of the hydroxylated lactone (**6**) using SPE, HPLC and NMR spectroscopy.

3.3 Results and discussion

3.3.1 SFC purification of the hydroxylated lactone (6) from African ginger extracts and analysis using UPC²

Siphonochilone (**1**) and the hydroxylated lactone (**6**) are two important compounds in the chemical composition of the plant and have been extensively reported by other researchers.^[15,18,19] Zongwe et al. (2018) reported the presence of both compounds in the crude extract and purified both using silica gel column chromatography. In order to investigate the purification and isolation to determine that auto-oxidation is in fact due to the storage in the plant as reported by Zongwe et al. (2018), a milder purification technique was investigated. The presence of the two compounds in a DCM:methanol extract from five-year-old dried ground rhizomes was confirmed by GC-MS analysis (Chapter 2, Section 2.3.2.3, Figure 2.5.(3)).

The purification of the DCM:methanol extract using the SFC was first evaluated through the development of a purification method on analytical scale using a 3.00×100 mm Viridis® BEH column, 130Å 1.70 µm analytical column. The standards of the two compounds, siphonochilone (**1**) and the hydroxylated lactone (**6**), were first injected and their retention times determined. The crude extract was then injected onto the SFC system using the same method and concentration (1.00 mg/mL in methanol) and compared to the chromatograms of the standards.

Figures 3.5.(1) and Figure 3.5.(2) show the chromatograms of the standard hydroxylated lactone (3.93 min) and siphonochilone (2.60 min) with each showing one major peak. Siphonochilone (Figure 3.5.(2)) showed minor peaks which can be due to background noise or decomposition of the compound already occurring. The DCM:methanol crude extract of five-year-old dried ground rhizomes (Figure 3.5.(3)) revealed one major peak at 3.90 min, which corresponded to the hydroxylated lactone, and two minor peaks at 2.61 and 5.41 min (Figure 3.5.(3)). The minor peak at 2.61 min corresponded to the siphonochilone (Figure 3.5.(1)). Overall, the chromatograms of the crude extract using the analytical method showed good separation of the two targeted compounds.

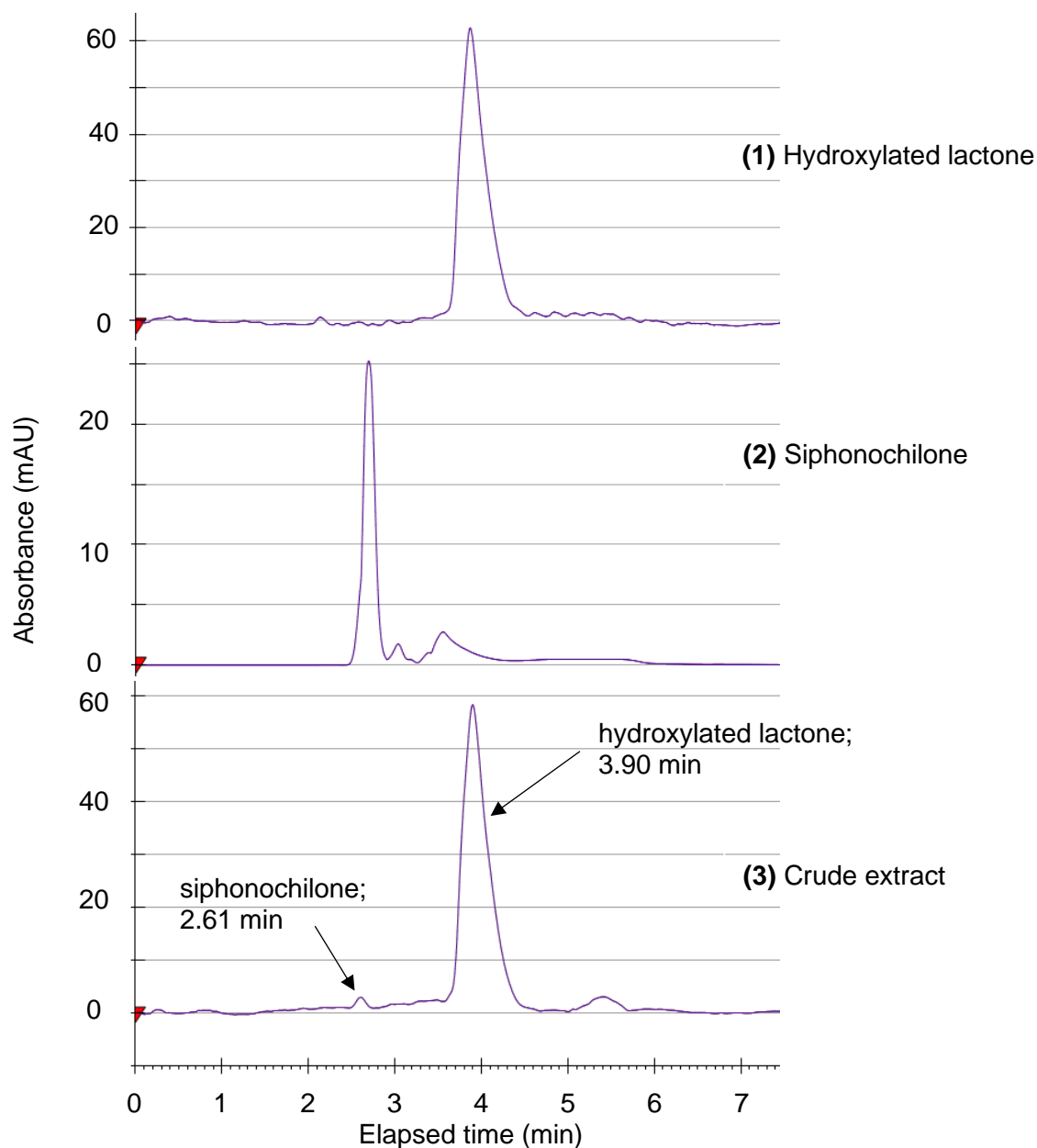


Figure 3.5 Chromatogram of **(1)** the hydroxylated lactone, **(2)** siphonochilone and **(3)** the crude extract at 210 – 270 nm from SFC analysis using an analytical column.

Based on the method used on the analytical column, the crude extract was fractionated using the semi-preparatory method using a 19×150 mm Viridis® BEH Prep 2-EP OBD™ 5.00 µm semi-preparative column (Waters, Ireland). The fraction collector was set to collect targeted peaks based on retention times (Table 3.4).

The chromatogram of the crude DCM:methanol extract is shown in Figure 3.6 and is similar to that of the analytical method indicating that the semi-preparatory method developed was suitable for the scale-up repeated purification of a larger quantity of the extract. Figure 3.6 showed a major peak at 7.65 min and three minor peaks at 5.30, 6.05 and 10.80 min which were collected using time-based intervals (Table 3.4). The peak at 7.65 min was the hydroxylated lactone and 5.30 min the siphonochilone. There was however a change in the retention times of the targeted compounds between the chromatograms from the analytical analysis (Figure 3.5.(3)) and semi-preparative columns (Figure 3.6). This was due to the differences in particle sizes of the stationary phases of the different columns and did this not affect the chromatogram, but only the retention times. [80]

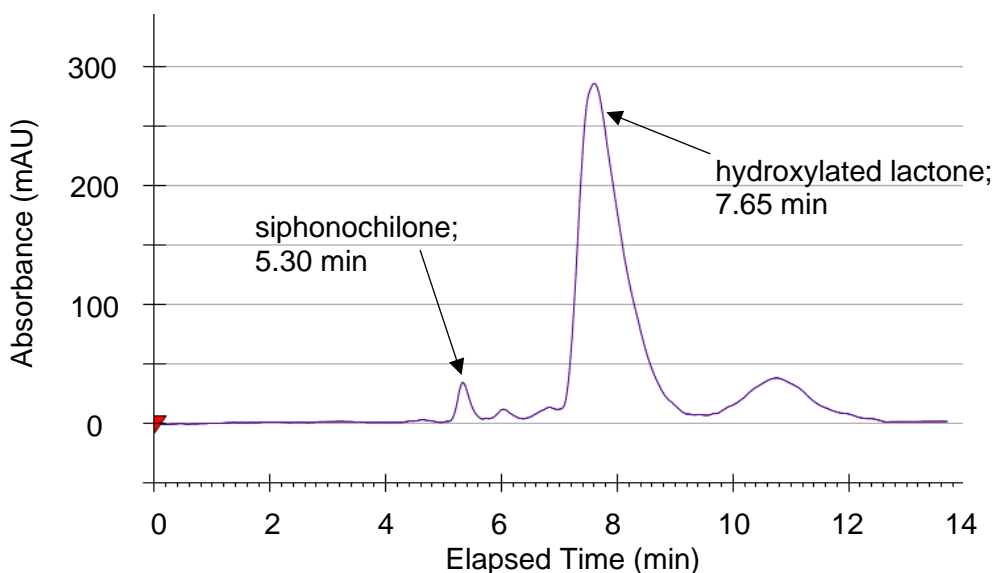


Figure 3.6 Chromatogram from SFC analysis of the crude extract at 210 – 270 nm with retention times 5.30, 6.05, 7.65 and 10.80 min using a semi-preparative column.

After the successive injections of a total of 438 mg of the crude extract, 11 fractions were collected for each run and evaporated to dryness using the centrifugal evaporator at low boiling point (30°C) for 2 h and a total of 60 mg of the fractions was recovered (Table 3.4). This suggested that 86% of the sample was lost during the purification in fractions that

were not collected, which were possibly fatty acids and oils that did not have a UV radiation absorbance in the 210 – 270 nm range.

Table 3.4 The retention times and the mass of each fraction collected from the crude extract.

Fraction number	Collection start time (min)	Collection end time (min)	Mass of fraction (mg)
1	5.10	5.59	3.73
2	5.60	5.89	0.38
3	5.90	6.20	0.82
4	6.21	6.58	0.23
5	6.59	7.00	0.91
6	7.01	7.07	0.23
7	7.08	8.00	28.15
8	8.01	8.20	1.36
9	8.21	9.20	20.81
10	9.10	10.00	1.56
11	10.01	11.6	2.47
Total			60.65

The purity of the fractions was investigated by means of TLC with ethyl acetate:hexane (2:3) as the mobile phase. The fractions were compared to the two standards (siphonochilone; $R_f = 0.72$, and its hydroxylated lactone; $R_f = 0.39$). TLC plates were observed under UV light (254 nm) and stained with vanillin and iodine. It was found that fractions 1 – 11 were impure and minimal significant differences with the presence of more than one compound in each fraction. The TLC analysis suggested that fractions 1 – 9 contained the siphonochilone ($R_f = 0.72$) and fractions 7 – 11 the hydroxylated lactone ($R_f = 0.39$) and small traces of siphonochilone. These results suggested that SFC does not appear to separate structurally similar classes of compounds on semi-preparatory

scale even though the chromatograms appear to do so. As SFC is also a mild technique with limited quantities of methanol used, the presence of both compounds in these fractions was likely due to auto-oxidation of siphonochilone to its hydroxylated form as reported by Zongwe et al. (2008).^[19,77] SFC is more commonly used to separate different classes of compounds than structurally similar compounds.^[78,79] Based on the yields, only fractions 7 and 9 (Table 3.4) were in sufficient quantities to continue with further analysis.

In order to further confirm the presence of the two compounds in the crude extract and fractions, these were analysed using the UPC² instrument, at 215 nm. Due to the high level of sensitivity of the instrument, it provided some semi-quantitative information on the two compounds.

The two standards and the DCM:methanol crude extract, used earlier for pre-fractionation with SFC, were dissolved in methanol (1.00 mg/mL) and separately injected directly onto the UPC². Figure 3.7.(1) shows the chromatogram of the standard hydroxylated lactone with a major peak at retention time 2.35 min, and siphonochilone with a major peak at 1.23 min (Figure 3.7.(2)).

There was a difference in the retention times of the hydroxylated lactone between the chromatograms of the SFC and the UPC². As described earlier, the column packaging, column length and particle size all play a role in the change in the chromatograms and retention times of targeted compounds. The method developed for the UPC² can still be used on the SFC, however the changes to the column need to be taken into account.^[80]

The crude extract was then injected using the same method and concentration as the standards (1.00 mg/mL) and two major peaks were identified at 1.23 and 2.20 min (Figure 3.7.(3)). The peak at 0.77 min was identified as the methanol solvent peak and was present in all the chromatograms from the UPC² analysis. The peaks with retention times 1.23 and 2.20 min (Figure 3.7.(1) and Figure 3.7.(2)) correlated to the standards, siphonochilone and its hydroxylated lactone, respectively, and confirmed the presence of the two compounds in the crude extract.

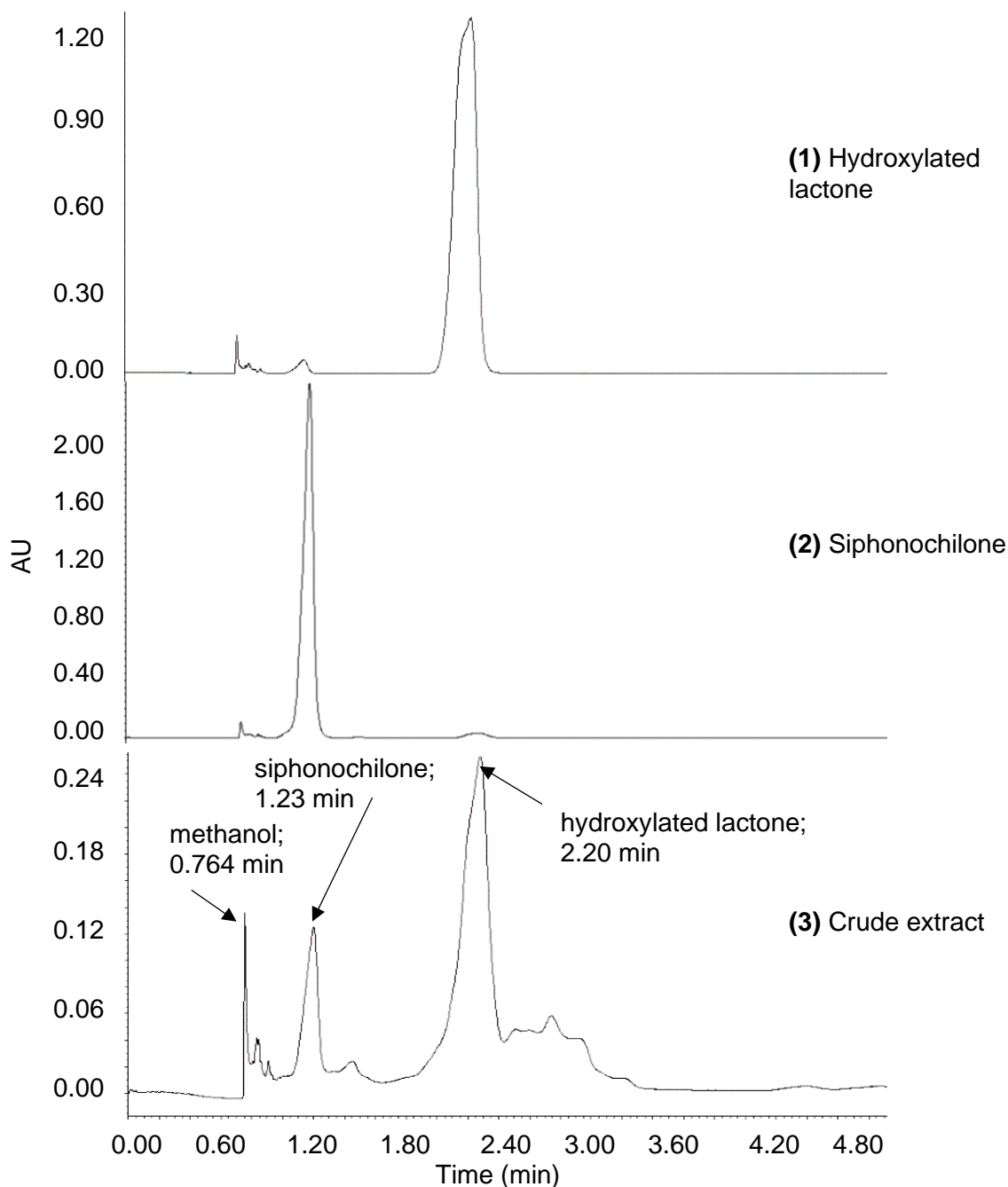


Figure 3.7 Chromatograms of **(1)** the hydroxylated lactone, **(2)** siphonochilone and **(3)** the crude extract analysed using UPC² at 215 nm.

The pre-fractionated fractions 7 and 9 generated from the SFC were separately analysed by UPC² to establish their levels of purity compared to the crude extract (Figure 3.8.(3)). This further confirmed the presence of the two terpenoid compounds. Figures 3.8.(1) and Figure 3.8.(2) shows the chromatograms of fraction 7 and 9 with two major peaks at 1.22

and 2.34 min and confirmed the presence of siphonochilone (1.22 min) and its hydroxylated lactone (2.34 min) in both these fractions. The results confirmed the earlier TLC analysis performed on these fractions.

The chromatograms for the two fractions also showed a significant decrease in the broad peaks present in the crude extract eluting after 2.34 min (Figure 3.8.(3)). Even though the SFC did not separate the compounds into single chemical entities, it was successful in separating unwanted compounds from those that were targeted.

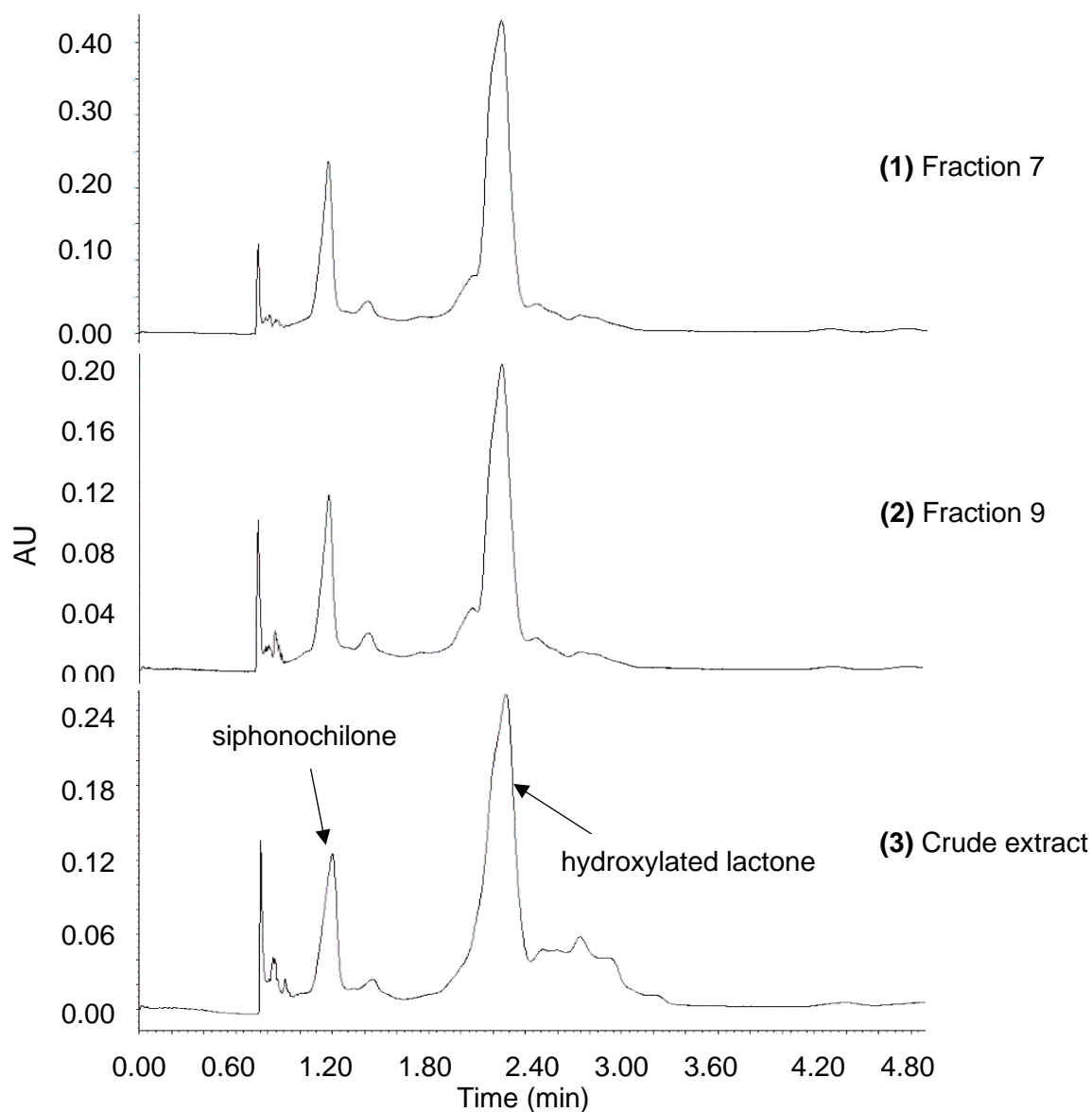


Figure 3.8 Chromatogram at 215 nm of (1) fraction 7, (2) fraction 9 pre-fractionated from SFC and (3) crude extract analysed using UPC².

Further confirmation of the presence of the hydroxylated lactone in Fraction 7 was undertaken through a “spiking” experiment. The “spike” was performed by adding 200 μL of the standard hydroxylated lactone (1.00 mg/mL) to fraction 7 and comparing the chromatograms and retention times of the peak that is believed to be the hydroxylated lactone. From the chromatograms in Figure 3.9, it was seen that there is a major peak at 2.34 min (before “spiking” – Figure 3.9.(1)) and 2.35 min (after “spiking” – Figure 3.9.(2)). Both chromatograms showed a peak at the same retention time indicating that the peak at 2.34 min (Figure 3.8.(1)) was the, targeted, hydroxylated lactone **(6)**.

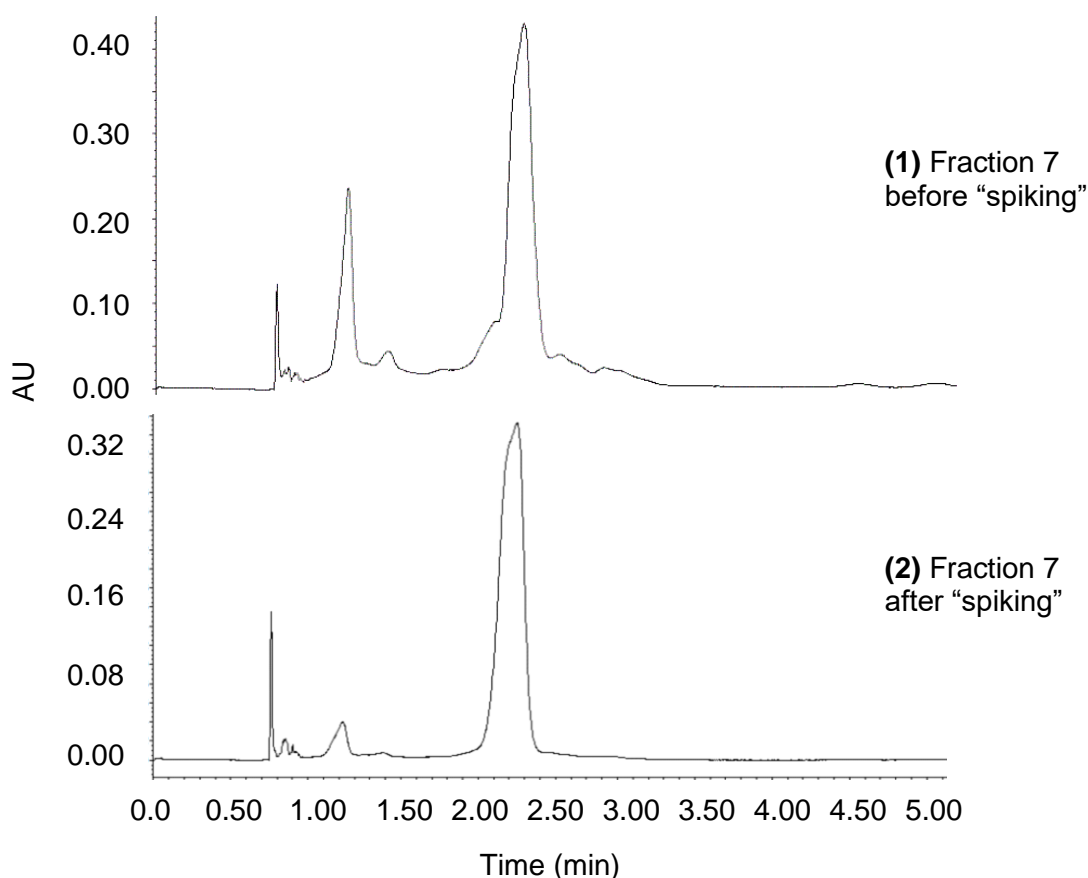


Figure 3.9 Chromatogram at 215 nm of **(1)** fraction 7 before and **(2)** fraction 7 after “spiking” with standard hydroxylated lactone.

The major peak at 2.34 min in Figure 3.9.(1) was already more intense than the rest of the peaks in the chromatogram. After “spiking” fraction 7 increased further compared to the other peaks. There was also an increase in area under the peak as a percentage of the total chromatogram after the “spiking” experiment (Table 3.5).

Table 3.5 The results from the UPC² analysis of Fraction 7 before and after addition of the standard hydroxylated lactone to confirm the presence of the compound confirmation.

Targeted compound	Retention time (min) before spiking	Area under the peak as a percentage of the total chromatogram before the spike	Retention time (min) after spiking	Area under the peak as a percentage of the total chromatogram after the spike
Siphonochilone	1.22	14.3	1.23	5.03
Hydroxylated lactone	2.34	48.1	2.35	79.8

The results from the “spiking” experiment confirmed and concluded that the targeted hydroxylated lactone is indeed the peak at 2.34 min and is present in fraction 7. Since fraction 9 had the same or similar chromatogram of fraction 7, both were combined to give combined fraction labelled as FR1 (48.96 mg) and was used for further purification using silica column chromatography.

Based on the excellent chromatographic separation capabilities of the UPC², especially to separate closely related compounds (in this case sesquiterpenoids), analysis of the ethanol extracts prepared from fresh (wet) and freshly dried ground rhizomes and roots was also performed. The chromatograms of the extracts were compared to the crude extract prepared by DCM:methanol extraction of five-year-old dried ground rhizomes.

The chromatograms at 215 nm for the three extracts are shown in Figure 3.10. The crude extract from the stored rhizomes (Figure 3.10.(1)) showed a far more intense peak for the hydroxylated lactone, at 2.20 min, compared to the ethanol extracts from the fresh

rhizomes (Figure 3.10.(2); 1.20 min) and freshly dried ground rhizomes (Figure 3.10.(3); 1.22 min) when analysed at the same concentrations (1.00 mg/mL in methanol).

The chromatogram of the fresh (Figure 3.10.(2)) and freshly dried ground rhizomes (Figure 3.10.(3)) showed the opposite to the crude extract, with a more intense peak at 1.20 and 1.22 min, respectively. From Figure 3.7 it was concluded that siphonochilone elutes first, followed by the hydroxylated lactone. This concluded that the peaks at 1.20 min (Figure 3.10.(2)) and 1.22 min (Figure 3.10.(3)) was siphonochilone and the peaks at 2.29 min (Figure 3.10.(2)) and 2.34 min (Figure 3.10.(3)) was the hydroxylated lactone. These chromatograms (Figure 3.10.(2) and Figure 3.10.(3)) showed that the peak corresponding to siphonochilone is the most intense peak, which suggested that there was more siphonochilone present in the fresh rhizomes. This was also confirmed in Chapter 2, section 2.3.2.4.

These results showed that in fresh plant material, siphonochilone is the major compound with traces of the hydroxylated lactone already present in the extract (Figure 3.10.(2) and Figure 3.10.(3)). This does however differ for the plant material stored over a period of time. Once more, these studies confirmed the findings of Zongwe et al. (2018) that siphonochilone decomposes or oxidizes into the structurally related compounds reported by Holzapfel et al. (2002) and Lategan et al. (2008).^[15,18,19] These compounds (Chapter 1, section 1.1.5.2) **(2,3 and 5 – 7)** in all likelihood, auto-oxidize during storage and do not occur naturally in the plant as previously reported and were confirmed by the UPC² analysis.^[19]

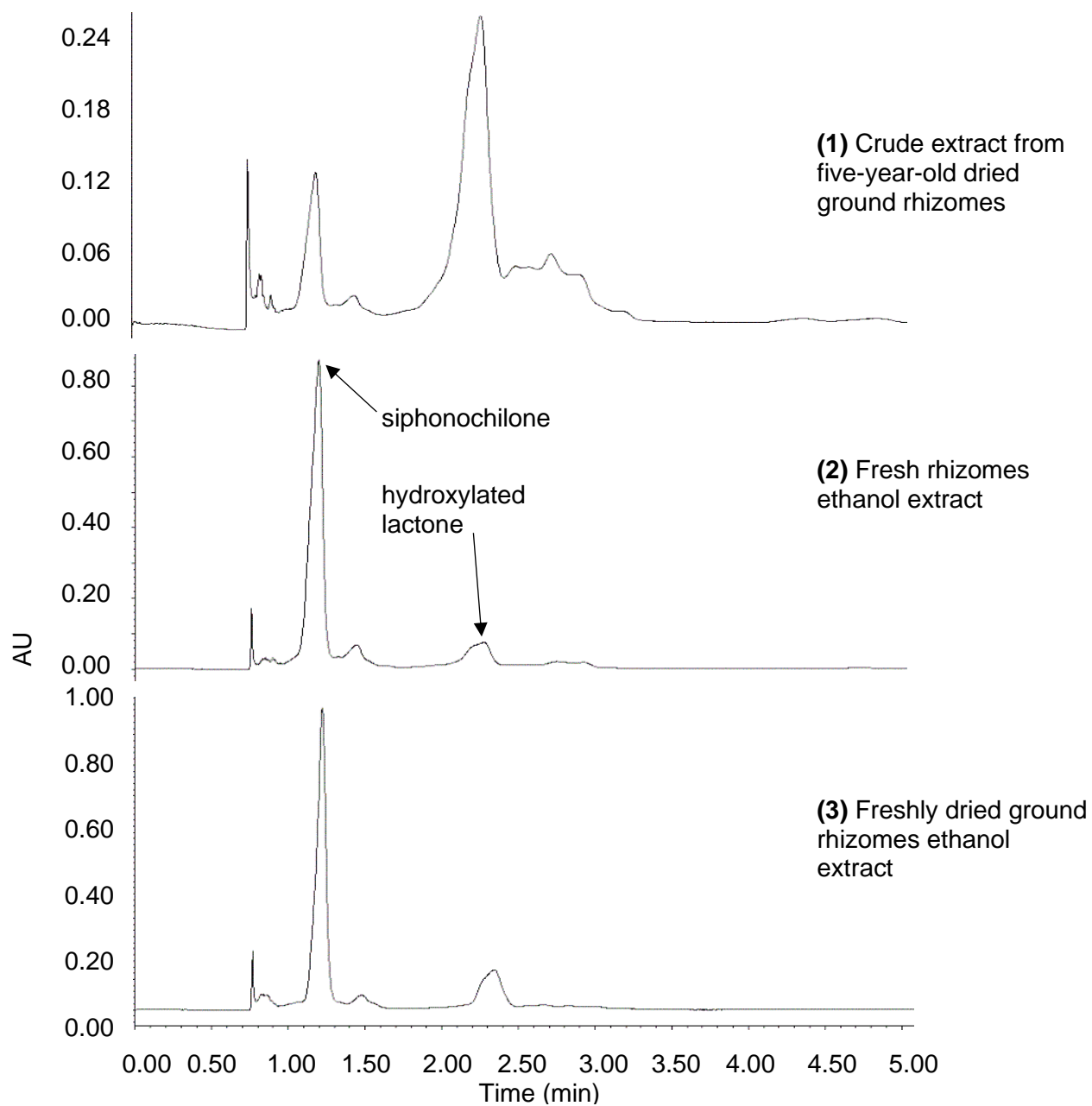


Figure 3.10 Chromatograms of **(1)** the crude extract, **(2)** fresh rhizomes – and **(3)** freshly dried ground rhizomes ethanol extract analysed using UPC² at 215 nm.

Further purification of the fractions was performed in an attempt to purify the hydroxylated lactone and confirm its presence in the stored plant material.

3.3.2 Purification of the combined fractions 7 and 9 (FR1) using silica column chromatography

The isolation and purification of the hydroxylated lactone (**6**) was attempted using the milder and greener technique, SFC. Since the pre-fractionation using SFC did not give good separation into pure compounds, silica column chromatography was then attempted for the fractionation and isolation of the pure compound. The same methods used by Zongwe et al. (2018) was used for fractionation and isolation.

Combined sample FR1 (48.96 mg) was fractionated by silica column chromatography using acetone:DCM and eluting gradient-wise from 5% to 30% acetone in DCM and 47 fractions were collected. Each fraction was concentrated by evaporating the fractions in the centrifugal evaporator at low boiling point (30°) for 2 h and dissolving in small volumes of hexane. The fractions were spotted on TLC plates and developed in acetone:DCM (1:9). The two standards of siphonochilone (**1**) and the hydroxylated lactone (**6**) were used to identify the targeted compounds in the fractions collected.

The TLC plates were observed under the UV light (254 nm) and stained using vanillin to identify siphonochilone. The standard siphonochilone had an R_f value of 0.73 and when compared to all fractions, showed its presence in fractions 1 – 7.

TLC plates spotted with all fractions and the standard hydroxylated lactone were placed in an iodine chamber to identify the hydroxylated lactone containing fractions. This was done by comparing the standard of the hydroxylated lactone ($R_f = 0.43$) to all the fractions and revealed fractions 12 – 16 ($R_f = 0.43$) to contain the hydroxylated lactone. Fractions 12 – 16 was not pure as there were other spots also visible on TLC and further purification was required. Fractions 12 – 16 were combined and evaporated into the subfraction FR2 (2.90 mg).

FR2 was dissolved in small volumes of hexane, and was spotted with the standard hydroxylated lactone (**6**) on a TLC plate and developed in acetone:ethyl acetate:hexane (1:4:5). The iodine stained TLC revealed three spots for subfraction FR2 with R_f values of 0.43 (most intense), 0.58 and 0.73 (minor spots) and one spot for the standard with R_f value of 0.43. This suggested that the hydroxylated lactone was the major compound in subfraction FR2 and however needed further purification.

Subfraction FR2 was purified by silica column chromatography using acetone:ethyl acetate:hexane, eluting gradient-wise from 2:3:5 to 1:4:5, resulting in the collection of 53 fractions. Each fraction was concentrated by evaporation and dissolving in small volumes of hexane, and spotted on TLC plates and developed in acetone:ethyl acetate:hexane (1:4:5). The subfractions were compared with the standard hydroxylated lactone (**6**) to identify which subfractions contain the hydroxylated lactone. The plates were stained with iodine, overall revealing three spots with R_f values of 0.44, 0.58 and 0.73. Fractions 28 – 37 had a distinct spot with an R_f value of 0.44, corresponding with the standard hydroxylated lactone ($R_f = 0.44$), confirming fractions 28 – 37 as the hydroxylated lactone containing subfractions. Subfractions 28 – 37 were combined and labelled FR3 (0.17 mg). FR3 was dissolved in a small volume of hexane to spot on a TLC and developed in acetone:ethyl acetate:hexane (1:4:5). The plate was stained with iodine and revealed one distinct spot with an R_f value of 0.44 which confirms the hydroxylated lactone. However, there were some impurities in FR3 as detected by TLC and due to the limited quantities (0.17 mg) no further purification was attempted as insufficient material was obtained for NMR analysis.

Significantly low yields of fractions were collected during pre-fractionation after injecting 480 mg of crude extract onto the SFC and recovery of only 60.65 mg of fractions. This can be attributed to compounds still being retained on the column or compounds discarded as waste due to the poor separation of the extract on the SFC.^[81] Recovery of low yields continued during purification using column chromatography, which was due to compounds being retained on the column and not eluting. It can be concluded that these methods of fractionation and purification were not completely successful.^[81]

3.3.3 Semi-purification using automated SPE of DCM:hexane extract of five-year-old dried ground rhizomes of African ginger

The use of SFC was ineffective in the fractionation, isolation and purification of sufficient quantities of the hydroxylated lactone (**6**) from the crude extract. The use of silica gel column chromatography resulted in the use of excess of organic solvents and did not support the purpose of substituting less greener methods in combination with SFC. In

order to avoid repeated chromatography using silica gel and organic solvents as it became clear that the pure compounds could only be obtained through this method, an alternative automated SPE system and a liquid handler was attempted. This resulted in lower consumption of organic solvents and semi-purifying over a shorter time period.

Two separate samples of five-year-old dried ground rhizomes extracted with DCM:hexane (1:1), with a final quantity of 566 mg, were absorbed onto cotton wool after drying in the centrifugal evaporator and placed into an empty SPE cartridge. The cartridge was stacked above a Hypersep SPE cartridge and used for fractionation with H₂O:methanol from 9:1 to 1:4 resulting in 14 fractions. Each fraction was dried using the Genevac and weighted to have a total recovery of 502.48 mg (88.64%) which indicated a much-improved recovery of the fractionation compared to that of SFC.

The 14 fractions, dissolved in small volumes of hexane, along with the standard hydroxylated lactone (**6**), were spotted on TLC plates using acetone:ethyl acetate:hexane (1:4:5) as the mobile phase. One TLC plate was stained with iodine and the other with vanillin. The different fractions were compared to the standard hydroxylated lactone ($R_f = 0.44$) and the iodine stained TLC confirmed that the lactone was present in fractions 3 – 5 (labelled FR4).

Fractions 3 – 5 were combined and spotted on a TLC plate with the same mobile phase and revealed three spots at $R_f = 0.31$, 0.44 and 0.62. The intense spot at $R_f = 0.44$ corresponded to the standard hydroxylated lactone ($R_f = 0.44$), which further confirmed the presence of the hydroxylated lactone in the combined fractions. The combined fractions (labelled FR4) were evaporated using the centrifugal evaporator, weighed (146.49 mg), and was used for further purification using HPLC.

3.3.4 Preparative HPLC purification of SPE fractions (fraction labelled as FR4)

HPLC was used for the final purification of the SPE combined fraction (FR4) since column chromatography resulted in high losses of sample (see section 3.3.2).

A method was first developed using an analytical method which consisted mainly of H₂O and acetonitrile using an X-Bridge C18 column to ensure good separation. The

chromatograms of the pure standard of the hydroxylated lactone (Figure 3.11.(1)) was initially used to determine its retention time followed by identifying the corresponding peak in the labelled FR4 fraction (Figure 3.11.(2)).

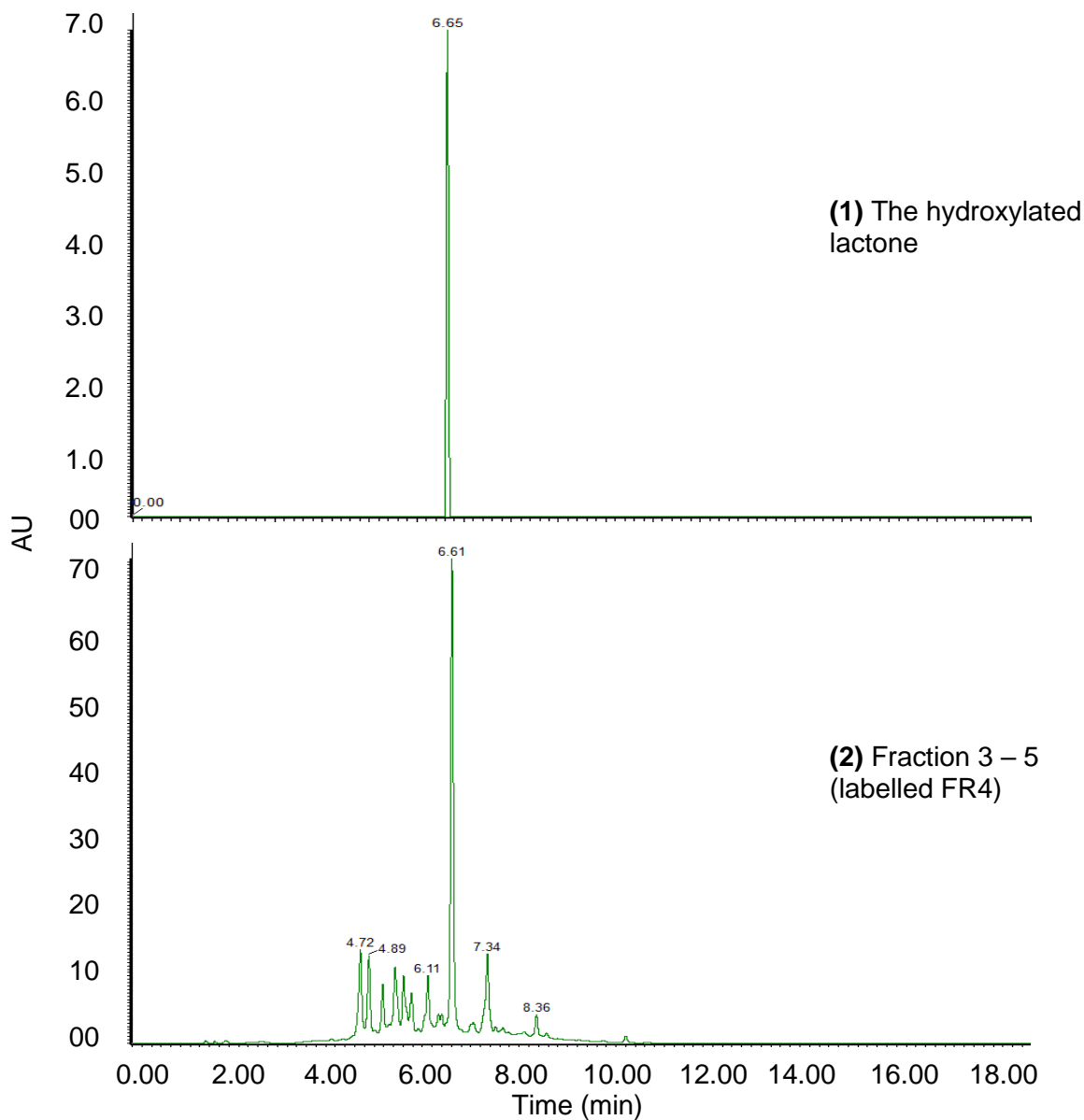


Figure 3.11 HPLC chromatograms at 220 nm of (1) the standard hydroxylated lactone and (2) the combined fractions, FR4, obtained from SPE at 220 nm combined fractions (FR4) during the analytical study and method development.

The results showed that the standard eluted at 6.65 min and the major peak in the FR4 fraction corresponded to this at 6.61 min. Based on this separation a semi-preparatory method was then developed using the instrument automated scale up functionality.

The use of the semi-preparatory HPLC method consisted mainly of H₂O and acetonitrile using a reversed-phase Waters X-Bridge Preparative C18 column to ensure good separation. FR4 (146.49 mg) was dissolved in 1.50 mL methanol and 200 μ L of the sample was injected to give the chromatogram shown in Figure 3.12. The sample was then repeatedly injected 7 times. A gradient wise system (Table 3.3; Section 3.2.10) was used to isolate and purify FR4 with a run time of 29 min and 0.47 min post-run time.

A major peak was observed at 9.21 min and from the method development it was confirmed that the major peak is the hydroxylated lactone (Figure 3.12). Four fractions were collected based on time-based intervals (Table 3.6).

Table 3.6 The time intervals of fractions collected using HPLC and their collected mass (mg).

Fraction number	Time intervals (min)	Mass (mg)
1	7.61 – 7.84	3.92
2	8.10 – 8.35	3.71
3	9.00 – 9.30	23.15
4	10.27 – 10.50	3.77
Total		34.55

The fractions were evaporated and weighed before being analysed by TLC (Table 3.6). Small portions of the four fractions (dissolved in hexane) and the standard hydroxylated lactone (**6**) were spotted on a TLC plate and developed in acetone:ethyl acetate:hexane (1:4:5) and stained with iodine. Fraction 3 showed only one spot at $R_f = 0.30$ and

compared to the standard hydroxylated lactone ($R_f = 0.30$). No impurities were detected by TLC and this confirmed that fraction 3 was the hydroxylated lactone.

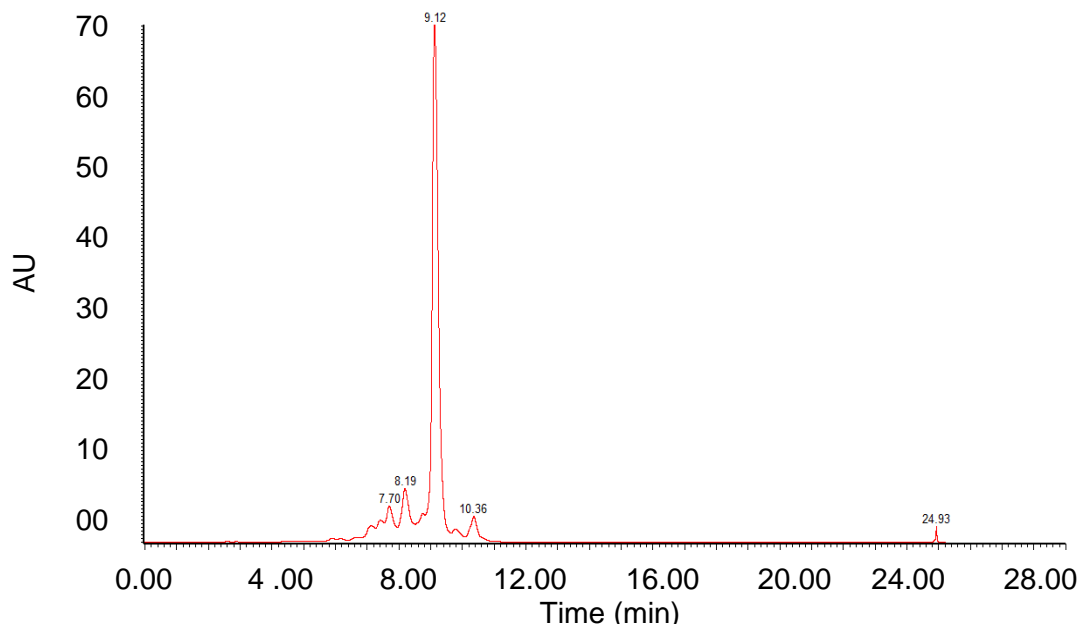


Figure 3.12 HPLC chromatogram at 220 nm of combined fractions, FR4, for purification with semi-preparative column.

Fraction 3 (labelled FR5), which was a light-yellow, oily compound and weighed 23.15 mg (4.08% w/w), and the siphonochilone, which were clear crystals that crystallized out during steam distillation (see Chapter 2, section 2.2.4), were analysed by NMR for final confirmation of their structures.

SPE and HPLC were more efficient in fractionation, isolation and purification of the hydroxylated lactone than SFC and column chromatography. The SPE was much faster in the pre-fractionation step than SFC, it had a better recovery yield of the sample injected and was easier to use. The final compound isolated and purified using SPE and HPLC was purer than the final compound obtained using the SFC and column chromatography approach, and it had a much higher yield of the possible hydroxylated lactone. Even though SFC is reported to be a greener method of isolation of compounds, it resulted in the use of more organic solvents, took much longer and resulted in a lower yield of

targeted compound, the hydroxylated lactone, (0.04%) compared to that collected using SPE and HPLC (4.08%).

3.3.5 Structure elucidation of siphonochilone (1) and the hydroxylated lactone (6) using NMR spectroscopy

The NMR structure elucidation of both siphonochilone (1) and the hydroxylated lactone (6) have been previously reported and was used to confirm the structure of the two compounds.^[15,18,19]

The structures of both compounds are given in Figure 3.13 with their atom numbers to use in the assignment of protons and carbons. The molecular mass and – formula of both compounds were confirmed in Chapter 2, Section 2.3.2.2, using GC-MS from the NIST library.

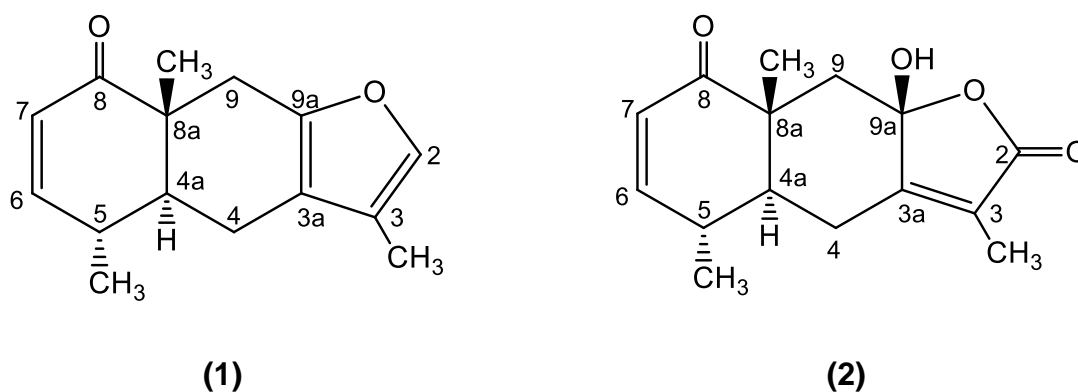


Figure 3.13 Structures of (1) siphonochilone and (2) its hydroxylated lactone with numbering of atoms.

3.3.5.1 Compound and structure confirmation of siphonochilone (1)

The clear crystals of siphonochilone (1) (Figure 3.13.(1)) were easily obtained through crystallization during steam distillation as described in Chapter 2, section 2.2.4. Based on the mass spectral data the compound showed a m/z at 230 based on the data from the GC-MS (Chapter 2, section 2.3.2.2, Figure 2.3.(1)) indicating a molecular formula of

C₁₅H₁₈O₂. Only the ¹H NMR, ¹³C NMR and COSY experiments were performed and compared to literature data by Holzapfel et al. (2002) to confirm the structure of the compound. The formula was further confirmed by the number of protons in the ¹H NMR and of carbon atoms in the ¹³C NMR spectrum.

Siphonochilone (10 mg) was dissolved in CD₂Cl₂, as CDCl₃ was known to decompose the compounds, and the NMR data was acquired from the Bruker Avance III 400 MHz magnetic operating at 400.21 MHz. Furthermore, the ¹H NMR spectrum suggested that the compound was a sesquiterpenoid and the following features were observed: a singlet, which was assigned to the proton on position H-2 (δ_H 6.98, 1H) and a double doublets for each of H-6 (δ_H 6.63, 1H, *J* = 10.07, 2.02) and H-7 (δ_H 5.83 1H, *J* = 10.09, 2.81), all three showing distinct olefinic hydrogens based on the chemical shifts; a double doublet of doublets for H-4a (δ_H 1.76, 1H, *J* = 10.49, 10.37, 5.37) and a multiplet for H-5 (δ_H 2.36); two doublets each integrating for three protons (δ_H 1.86 and 1.15) characteristic of 3-Me and 5-Me and a singlet at δ_H 0.96 integrating for three protons characteristic of 8a-Me, all three showing distinct methyl hydrogens at their respective positions.

The ¹³C NMR revealed 15 signals with different features and were compared to the literature data to assign signals:^[15] a ketone group on C-8 (δ_C 203.16), a methyl group on C-3, C-5 and C-8a (δ_C 5.60, 16.53 and 14.49), a methylene group at C-4 and C-9 (δ_C 20.76 and 30.49), tertiary carbons at C-4a and C-5 (δ_C 43.66 and 32.92), a methine group at C-2, C-6 and C-7 (δ_C 136.25, 154.18 and 124.44) and quaternary carbons at C-3, C-3a, C-8a and C-9a (δ_C 117.77, 113.59, 43.51 and 147.55) .

The assignments for the proton and carbon atoms were compared to that published by Holzapfel et al. (2002) and compared favourably (Table 3.7) to conclude that the crystals that crystallized out during steam distillation is indeed siphonochilone as expected.

Table 3.7 ^1H and ^{13}C data of siphonochilone in CD_2Cl_2 compared to published data in CDCl_3 .^[15]

^1H and ^{13}C data of siphonochilone				^1H and ^{13}C literature data ^[15]	
Position	^1H (ppm, J in Hz)	^{13}C (ppm)	COSY	^1H (ppm, J in Hz)	^{13}C (ppm)
2	6.98 (1H, s)	136.25		7.02 (<i>br m</i>)	137.50
3		117.77			119.00
3a		113.59			114.60
4 _{eq}	2.04 (<i>dddd</i> , $J = 15.84$, 11.02, 3.00, 1.70)	20.76	H-4a, H-4 _{ax}	2.12 (<i>dddd</i> , $J = 15.70$, 10.80, 3.00, 1.40)	22.50
4 _{ax}	2.64 (<i>ddd</i> , $J = 15.15$, 7.21, 1.56)	20.76	H-4a, H-4 _{eq}	2.68 (<i>ddd</i> , $J = 15.70$, 5.40, 1.60)	22.50
4a	1.76 (1H, <i>ddd</i> , $J = 10.49$, 10.37, 5.37)	43.66	H-4 _{ax} , H-4 _{eq} , H-4a, H-5	1.81 (<i>ddd</i> , $J = 10.80$, 10.20, 5.40)	45.00
5	2.36 (<i>m</i>)	32.92	5-Me	2.40 (<i>ddqd</i> , $J = 10.20$, 7.10, 2.70, 2.10)	34.20
6	6.63 (1H, <i>dd</i> , $J = 10.07$, 2.02)	154.18	H-7	6.66 (<i>dd</i> , $J = 10.10$, 2.10)	154.20
7	5.83 (1H, <i>dd</i> , $J = 10.09$, 2.81)	124.44	H-6	5.91 (<i>dd</i> , $J = 10.10$, 2.70)	126.60
8		203.16			204.00
8a		43.51			44.90
9 _{ax}	2.62 (<i>d</i> , $J = 16.72$)	30.49	H-9 _{eq}	2.64 (<i>br d</i> , $J = 16.70$)	31.90
9 _{eq}	2.65 (<i>dd</i> , $J = 15.85$, 1.57)	30.49	H-9 _{ax}	2.73 (<i>dd</i> , $J = 16.70$, 1.40)	31.90
9a		147.55			149.30
3-Me	1.86 (<i>d</i> , $J = 1.22$)	5.60		1.90 (<i>d</i> , $J = 1.30$)	8.10
5-Me	1.15 (<i>d</i> , $J = 7.18$)	16.53	H-5	1.21 (<i>d</i> , $J = 7.10$)	18.70
8a-Me	0.96 (<i>s</i>)	14.49		1.02 (<i>s</i>)	16.60

3.3.5.2 Structure elucidation of the hydroxylated lactone (6)

The compound was a light-yellow oil obtained through SPE and preparative HPLC purification and was identified as the hydroxylated lactone (**6**) (Figure 3.13.(2)) by comparing the compound with the standard on TLC. The final confirmation was done by NMR spectroscopy using 1-D and various 2-D NMR experiments i.e. ^1H , ^{13}C , HSQC,

HMBC, COSY and NOESY NMR experiments. Based on the mass spectrometry data obtained from the GC-MS (Chapter 2, section 2.3.2.2), the compound showed a m/z at 262 which indicated a molecular formula of $C_{15}H_{18}O_4$. The molecular formula was also supported by the number of protons on the 1H NMR and carbon atoms on the ^{13}C NMR.

The compound (23.15 mg) was dissolved in CD_2Cl_2 and the NMR data were acquired. The 1H NMR spectrum suggested that the compound was a sesquiterpenoid (supplementary data 1, p. 197) and the following features were observed: a double doublet of doublets at H-4a (δ_H 1.52, 1H, $J = 13.43, 9.80, 3.67$) and a multiplet at H-5 (δ_H 2.44), a doublet integrating for three protons (δ_H 1.18) characteristic of a methyl group at 5-Me, a singlet integrating for three protons (δ_H 1.29) characteristic of a methyl group at 8a-Me and a doublet integrating for three protons (δ_H 1.75) characteristic of methyl group for 3-Me. All three showing distinct methyl hydrogens at their respective positions. Two double doublets to H-6 (δ_H 6.59, $J = 10.07, 1.95$) and H-7 (δ_H 5.81, $J = 10.08, 2.74$), both showed olefinic hydrogens at their respective positions.

The ^{13}C NMR revealed 15 signals with different features: one oxygenated carbon C-9a (δ_C 103.49), two ketones C-2 and C-8 (δ_C 172.08 and 202.89, respectively), methylene groups C-4 and C-9 (δ_C 24.15 and 43.73), methine groups C-6 and C-7 (δ_C 153.61 and 126.12), tertiary carbons C-4a and C-5 (δ_C 49.95 and 33.84), methyl group on positions C-3, C-5 and C-8a (δ_C 8.23, 18.16 and 16.70) and three quaternary carbons C-8a, C-3a and C-3 (δ_C 44.91, 158.47 and 122.41).

The carbons bearing protons are confirmed through HSQC, long range correlation between protons and carbon atoms confirmed by HMBC. The assignments for the proton and carbon atoms compared favorably to that published by Lategan et al. (2009) as shown in Table 3.8.

Table 3.8 ^1H , ^{13}C and HMBC data of the hydroxylated lactone in CD_2Cl_2 compared to published data in CD_3CN .^[18]

^1H and ^{13}C data of the hydroxylated lactone				^1H and ^{13}C literature data ^[18]	
Position	^1H (ppm)	^{13}C (ppm)	HMBC	^1H (Lit.) (ppm) ^[18]	^{13}C (Lit.) (ppm) ^[18]
2		172.08			174.20
3		122.41			119.70
3a		158.47			160.10
4 _{ax}	2.33 (<i>m</i>)	24.15	C-3, C-3a, C-4a	2.36 (<i>dd</i> , <i>J</i> = 13.50, 13.30)	25.30
4 _{eq}	2.77 (<i>dd</i> , <i>J</i> = 13.55, 3.70)	24.15	C-3, C-3a, C-4a, C-9a	2.90 (<i>dd</i> , <i>J</i> = 13.30, 3.60)	25.30
4a	1.52 (<i>ddd</i> , <i>J</i> = 13.43, 9.80, 3.67)	49.95	C-8a, 8a-Me	1.62 (<i>ddd</i> , <i>J</i> = 9.00, 9.00, 3.60)	51.10
5	2.44 (<i>m</i>)	33.84	C-8a	2.53 (<i>tq</i> , <i>J</i> = 7.20, 2.40)	35.30
6	6.59 (<i>dd</i> , <i>J</i> = 10.07, 1.95)	153.61	C-4a, C-5, 5-Me, C-8	6.74 (<i>dd</i> , <i>J</i> = 10.20, 1.80)	156.00
7	5.81 (<i>dd</i> , <i>J</i> = 10.08, 2.74)	126.12	C5, C8a	5.81 (<i>dd</i> , <i>J</i> = 10.20, 3.00)	126.80
8		202.89			204.30
8a		44.91			46.40
9 _{eq}	1.65 (<i>d</i> , <i>J</i> = 14.54)	43.73	C-8, C-8a, 8a-Me, C-9a	1.60 (<i>d</i> , <i>J</i> = 14.10)	44.80
9 _{ax}	2.60 (<i>d</i> , <i>J</i> = 14.48)	43.73	C-3a, C-4a, C-8a, 8-Me, C-9a	2.49 (<i>d</i> , <i>J</i> = 14.10)	44.80
9a		103.49			104.50
3-Me	1.75 (<i>d</i> , <i>J</i> = 1.36)	8.23	C-2, C-3, C-3a	1.78 (<i>d</i> , <i>J</i> = 1.2)	8.90
5-Me	1.18 (<i>d</i> , <i>J</i> = 7.18)	18.16	C-4a, C-5, C-6	1.22 (<i>d</i> , <i>J</i> = 7.20)	18.90
8a-Me	1.29 (<i>s</i>)	16.70	C-4a, C-8, C-8a, C-9	1.30 (<i>s</i>)	17.80
9a-OH	3.42 (<i>s</i>)			4.84 (<i>br s</i>)	

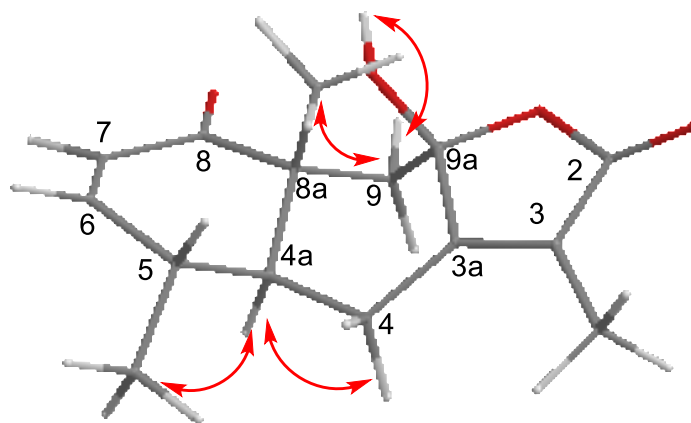


Figure 3.14 Selected NOESY interactions of the hydroxylated lactone (**6**).

Figure 3.14 shows the NOESY correlations between selected hydrogen atoms to assist in assigning stereochemistry. A NOESY correlation between the protons on the methyl group on C-5 and the H-4a was seen, suggesting both these are in the same plane. The assignment of the two diastereotopic protons at H-4 was based on their NOESY interaction with H-4a. As a correlation was seen between H-4a and the proton at 2.77 ppm, this proton was on the same plane as H-4a and the methyl group at C-5. By default, the proton at 2.33 ppm will be on the opposite plane.

As no correlation was seen in the NOESY between H-4a and the methyl group at C-8a, implied that these two are on the opposite plane.

Another two diastereotopic protons are present at C-9 which were assigned based on their NOESY interactions. As a correlation was seen between 8a-Me and the doublet at 2.60 ppm, this proton was assigned as being on the same plane as Me-8a allowing its assignment. By default, the proton at 1.65 ppm will be on the opposite plane. The spatial relationship between the methyl groups on C-5 (δ_c 18.16) and C-8a (δ_c 16.70) are based on the HMBC and NOESY correlations. ^1H – and ^{13}C NMR and HMBC correlations are recorded in Table 3.8.

Based on the assigned protons and carbons, and the literature, fraction 3, labelled FR5, can be confirmed to be the hydroxylated lactone (**6**).

3.4 Conclusion

Different methods of fractionation, isolation and purification were used to obtain siphonochilone (**1**) and its hydroxylated lactone (**6**). Siphonochilone was easily obtainable since it crystallized out during steam distillation as described in Chapter 2, section 2.2.4. The hydroxylated lactone was however more difficult to isolate, but various greener methods of fractionation and isolation were used that resulted in the successful isolation of the lactone.

The first method included the use of SFC for fractionation and UPC² for analysis of a DCM:methanol extract of five-year-old dried ground rhizomes. SFC however did not successfully separate the structurally similar compounds and confirmed that SFC is not sustainable in the separation of structurally similar compounds, but more useful in separating different classes of compounds. SFC also gave a low recovery yield from the crude extract of the sample that was purified (60.65 mg; 13.84%). The UPC² analysis confirmed that no separation occurred and that only some impurities, possibly fatty acids, were separated and discarded as waste. Fractionation using SFC took much longer, used more methanol than expected and had a low recovery yield. Repeated silica gel column chromatography still had to be used to isolate the compound and only resulted in a 0.04% recovery of the possible hydroxylated lactone. Due to the low quantities recovered of the fractions possibly containing the hydroxylated lactone, no NMR was performed for structure elucidation.

Using SFC as a possible greener technique of fractionation and column chromatography for isolation was not suitable for the specific extract and did not efficiently separate the structurally similar compounds.

The method of using a liquid handler, automated SPE and HPLC for fractionation, isolation and purification was much more suitable for the extract of African ginger. The SPE was used for pre-fractionation and had a much higher recovery yield of 88.64% based on the crude extract. The hydroxylated lactone containing fractions were identified using TLC and were further isolated and purified using HPLC.

The HPLC chromatogram had one distinct major peak which was identified to be the hydroxylated lactone. Using a time-based collection method, the peak was isolated and

23.15 mg of the hydroxylated lactone was obtained. This provided sufficient material for NMR analysis compared to the SFC technique. Various NMR experiments were performed on the pure compound obtained by preparative HPLC purification and on the crystals, as described in Chapter 2, section 2.2.4, to complete the compound confirmation and structure elucidation. The NMR data also confirmed that both compounds were semi-pure and that the crystals were indeed siphonochilone and the fraction isolated from HPLC was the hydroxylated lactone.

This SPE and HPLC method had a much higher yield of the pure hydroxylated lactone (4.08%) compared to the SFC (0.04%) approach. The technique had a higher recovery rate, higher yield, use of lower volumes of organic solvents and faster method of purification of active compounds from plant extracts.

3.5 References

- [1] Kinghorn, A.D., 1987, Biologically active compounds from plants with reputed medicinal and sweetening properties, *Journal of Natural Products* 50(6), pp.1009-1024.
- [2] Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K.M. and Latha, L.Y., 2011, Extraction, isolation and characterization of bioactive compounds from plants' extracts, *African Journal of Traditional, Complementary and Alternative Medicines* 8(1), pp.1-10.
- [3] Balunas, M.J. and Kinghorn, A.D., 2005, Drug discovery from medicinal plants, *Life Sciences* 78(5), pp.431-441.
- [4] Altemimi, A., Lakhssassi, N., Baharlouei, A., Watson, D.G. and Lightfoot, D.A., 2017, Phytochemicals: Extraction, isolation, and identification of bioactive compounds from plant extracts, *Plants* 6(4), p.42.
- [5] Nciki, S., Vuuren, S., van Eyk, A. and de Wet, H., 2016, Plants used to treat skin diseases in northern Maputaland, South Africa: antimicrobial activity and in vitro permeability studies, *Pharmaceutical Biology* 54(11), pp.2420-2436.
- [6] Savelev, S., Okello, E., Perry, N.S.L., Wilkins, R.M. and Perry, E.K., 2003, Synergistic and antagonistic interactions of anticholinesterase terpenoids in *Salvia*

- lavandulaefolia* essential oil, *Pharmacology Biochemistry and Behavior* 75(3), pp.661-668.
- [7] Veeresham, C., 2012, Natural products derived from plants as a source of drugs, *Journal of Advanced Pharmaceutical Technology & Research* 3(4), pp.200-201, viewed 27 March 2020, from www.japtr.org/article.asp?issn=2231-4040;year=2012;volume=3;issue=4;spage=200;epage=201;auiast=Veeresham.
- [8] Briars, R. and Paniwnyk, L., 2012, Examining the extraction of artemisinin from *Artemisia annua* using ultrasound, *In AIP Conference Proceedings, American Institute of Physics* 1433(1), pp. 581-585.
- [9] Hazekamp, A., Simons, R., Peltenburg-Looman, A., Sengers, M., van Zweden, R. and Verpoorte, R., 2004, Preparative isolation of cannabinoids from *Cannabis sativa* by centrifugal partition chromatography. *Journal of Liquid Chromatography & Related Technologies* 27(15), pp.2421-2439.
- [10] Ingle, K.P., Deshmukh, A.G., Padole, D.A., Dudhare, M.S., Moharil, M.P. and Khelurkar, V.C., 2017, Phytochemicals: Extraction methods, identification and detection of bioactive compounds from plant extracts, *Journal of Pharmacognosy and Phytochemistry* 6(1), pp.32-36.
- [11] Lahlou, M., 2013, The success of natural products in drug discovery, *Pharmacology & Pharmacy* 4, pp.17-31.
- [12] Cragg, G.M. and Newman, D.J., 2001, Natural product drug discovery in the next millennium, *Pharmaceutical Biology* 39(sup1), pp.8-17.
- [13] Fouche, G., Nieuwenhuizen, N., Maharaj, V., van Rooyen, S., Harding, N., Nthambeleni, R., Jayakumar, J., Kirstein, F., Emedi, B. and Meoni, P., 2011, Investigation of in vitro and in vivo anti-asthmatic properties of *Siphonochilus aethiopicus*, *Journal of Ethnopharmacology* 133(2), pp.843-849.
- [14] Lindsey, K., Jäger, A.K., Raidoo, D.M. and van Staden, J., 1998, Screening of plants used by Southern African traditional healers in the treatment of dysmenorrhoea for prostaglandin-synthesis inhibitors and uterine relaxing activity, *Journal of Ethnopharmacology* 64(1), pp.9-14.
- [15] Holzapfel, C.W., Marais, W., Wessels, P.L. and Van Wyk, B.E., 2002, Furanoterpenoids from *Siphonochilus aethiopicus*, *Phytochemistry* 59(4), pp.405-

407.

- [16] Light, M.E., McGaw, L.J., Rabe, T., Sparg, S.G., Taylor, M.B., Erasmus, D.G., Jäger, A.K., Van Staden, J. and Eloff, J.N., 2002, Investigation of the biological activities of *Siphonochilus aethiopicus* and the effect of seasonal senescence, *South African Journal of Botany* 68(1), pp.55-61.
- [17] Igoli, N.P. and Obanu, Z., 2011, The volatile components of wild ginger (*Siphonochilus aethiopicus* (Schweinf) Bl Burttt), *African Journal of Food Science* 5(9), pp.541-549
- [18] Lategan, C.A., Campbell, W.E., Seaman, T. and Smith, P.J., 2009, The bioactivity of novel furanoterpenoids isolated from *Siphonochilus aethiopicus*, *Journal of Ethnopharmacology* 121(1), pp.92-97.
- [19] Zongwe, F.K., Muya, J.T., Mutimana, R., Virima, M., Mayaliwa, M., Chung, H. and Maharaj, V., 2018, Auto-oxidation of Siphonochilone in Processed Rhizomes and Stored Powders of *Siphonochilus aethiopicus* (Schweinf.) Bl Burttt., *ChemistrySelect* 3(30), pp.8569-8574.
- [20] Korany, M.A., Mahgoub, H., Haggag, R.S., Ragab, M.A. and Elmallah, O.A., 2017, Green chemistry: Analytical and chromatography, *Journal of Liquid Chromatography & Related Technologies* 40(16), pp.839-852.
- [21] Thornburg, C.C., Britt, J.R., Evans, J.R., Akee, R.K., Whitt, J.A., Trinh, S.K., Harris, M.J., Thompson, J.R., Ewing, T.L., Shipley, S.M. and Grothaus, P.G., 2018, NCI program for natural product discovery: a publicly-accessible library of natural product fractions for high-throughput screening, *American Chemical Society Chemical Biology* 13(9), pp.2484-2497.
- [22] Lesellier, E. and West, C., 2015, The many faces of packed column supercritical fluid chromatography—A critical review, *Journal of Chromatography A* 1382, pp.2-46.
- [23] Rao, G.V.N., Gnanadev, G., Ravi, B., Dhananjaya, D., Manoj, P., Indu, B. and Nadh, R.V., 2013, Supercritical fluid (carbon dioxide) based ultra performance convergence chromatography for the separation and determination of fulvestrant diastereomers, *Analytical Methods* 5(18), pp.4832-4837.
- [24] Sandron, S., Rojas, A., Wilson, R., Davies, N.W., Haddad, P.R., Shellie, R.A.,

- Nesterenko, P.N., Kelleher, B.P. and Paull, B., 2015, Chromatographic methods for the isolation, separation and characterisation of dissolved organic matter, *Environmental Science: Processes & Impacts* 17(9), pp.1531-1567.
- [25] Berger, T.A., Fogleman, K., Staats, T., Bente, P., Crocket, I., Farrell, W. and Osonubi, M., 2000, The development of a semi-preparatory scale supercritical-fluid chromatograph for high-throughput purification of 'combi-chem' libraries, *Journal of Biochemical and Biophysical Methods* 43(1-3), pp.87-111.
- [26] Searle, P.A., Glass, K.A. and Hochlowski, J.E., 2004, Comparison of preparative HPLC/MS and preparative SFC techniques for the high-throughput purification of compound libraries, *Journal of Combinatorial Chemistry* 6(2), pp.175-180.
- [27] Tu, Y., Jeffries, C., Ruan, H., Nelson, C., Smithson, D., Shelat, A.A., Brown, K.M., Li, X.C., Hester, J.P., Smillie, T. and Khan, I.A., 2010, Automated high-throughput system to fractionate plant natural products for drug discovery, *Journal of Natural Products* 73(4), pp.751-754.
- [28] Rossi, D.T. and Zhang, N., 2000, Automating solid-phase extraction: current aspects and future prospects, *Journal of Chromatography A* 885(1-2), pp.97-113.
- [29] Kolukisaoglu, Ü. and Thurow, K., 2010, Future and frontiers of automated screening in plant sciences, *Plant Science* 178(6), pp.476-484.
- [30] Van der Vorst, G., Van Langenhove, H., De Paep, F., Aeltermann, W., Dingenen, J. and Dewulf, J., 2009, Energetic life cycle analysis for the selection of chromatographic separation processes in the pharmaceutical industry: preparative HPLC versus preparative SFC, *Green Chemistry* 11(7), pp.1007-1012.
- [31] Tobiszewski, M. and Namieśnik, J., 2014, Developments in green chromatography, *LC-GC EUROPE* 27(8), pp.405-408.
- [32] Poliakoff, M. and Licence, P., 2007, Green chemistry, *Nature* 450(7171), pp.810-812.
- [33] Dispas, A., Lebrun, P., Andri, B., Rozet, E. and Hubert, P., 2014, Robust method optimization strategy—a useful tool for method transfer: the case of SFC, *Journal of Pharmaceutical and Biomedical Analysis* 88, pp.519-524.
- [34] Płotka, J.M., Biziuk, M., Morrison, C. and Namieśnik, J., 2014, Pharmaceutical and forensic drug applications of chiral supercritical fluid chromatography, *Trends in*

- Analytical Chemistry* 56, pp.74-89.
- [35] Koel, M. and Kaljurand, M., 2006, Application of the principles of green chemistry in analytical chemistry, *Pure and Applied Chemistry* 78(11), pp.1993-2002.
- [36] Jones, J.W., Carter, C.L., Li, F., Yu, J., Pierzchalski, K., Jackson, I.L., Vujaskovic, Z. and Kane, M.A., 2017, Ultrapformance convergence chromatography-high resolution tandem mass spectrometry for lipid biomarker profiling and identification, *Biomedical Chromatography* 31(3), pp.1-23.
- [37] Hudalla, C.J. and McDonald, P.D., 2012, A new separation tool for a broad range of analytical challenges: ultrapformance convergence chromatography, *Chromatography Today* 5, pp.18-20.
- [38] Taylor, L.T., 2009, Supercritical fluid chromatography for the 21st century, *The Journal of Supercritical Fluids* 47(3), pp.566-573.
- [39] Desfontaine, V., Guillaume, D., Francotte, E. and Nováková, L., 2015, Supercritical fluid chromatography in pharmaceutical analysis, *Journal of Pharmaceutical and Biomedical Analysis* 113, pp.56-71.
- [40] Losacco, G.L., Veuthey, J.L. and Guillaume, D., 2019, Supercritical fluid chromatography–Mass spectrometry: Recent evolution and current trends,' *Trends in Analytical Chemistry* 118, pp.731-738.
- [41] Zhou, Q., Gao, B., Zhang, X., Xu, Y., Shi, H. and Yu, L.L., 2014, Chemical profiling of triacylglycerols and diacylglycerols in cow milk fat by ultra-performance convergence chromatography combined with a quadrupole time-of-flight mass spectrometry, *Food Chemistry* 143, pp.199-204.
- [42] Qi, N., Gong, X., Feng, C., Wang, X., Xu, Y. and Lin, L., 2016, Simultaneous analysis of eight vitamin E isomers in *Moringa oleifera* Lam. leaves by ultra performance convergence chromatography, *Food Chemistry* 207, pp.157-161.
- [43] Gere, D.R., Board, R. and McManigill, D., 1982, Supercritical fluid chromatography with small particle diameter packed columns, *Analytical Chemistry* 54(4), pp.736-740.
- [44] Waters n.d., *SFC columns*, Waters Corporation, viewed 13 November 2019, from https://www.waters.com/waters/en_US/SFC-Columns---Trefoil%2C-Torus%2C-Viridis-/nav.htm?cid=134696052&locale=en_US .

- [45] Gao, B., Luo, Y., Lu, W., Liu, J., Zhang, Y. and Yu, L.L., 2017, Triacylglycerol compositions of sunflower, corn and soybean oils examined with supercritical CO₂ ultra-performance convergence chromatography combined with quadrupole time-of-flight mass spectrometry, *Food Chemistry* 218, pp.569-574.
- [46] Gee, P.T., Liew, C.Y., Thong, M.C. and Gay, M.C., 2016, Vitamin E analysis by ultra-performance convergence chromatography and structural elucidation of novel α -tocodienol by high-resolution mass spectrometry, *Food Chemistry* 196, pp.367-373.
- [47] Combs, M.T., Ashraf-Khorassani, M. and Taylor, L.T., 1997, Packed column supercritical fluid chromatography-mass spectroscopy: A review, *Journal of Chromatography A* 785(1-2), pp.85-100.
- [48] García-Risco, M.R., Vicente, G., Reglero, G. and Fornari, T., 2011, Fractionation of thyme (*Thymus vulgaris* L.) by supercritical fluid extraction and chromatography, *The Journal of Supercritical Fluids* 55(3), pp.949-954.
- [49] Gong, X., Qi, N., Wang, X., Lin, L. and Li, J., 2014, Ultra-performance convergence chromatography (UPC²) method for the analysis of biogenic amines in fermented foods, *Food Chemistry* 162, pp.172-175.
- [50] Chen, Z., Dong, F., Xu, J., Liu, X., Cheng, Y., Liu, N., Tao, Y., Pan, X. and Zheng, Y., 2014, Stereoselective separation and pharmacokinetic dissipation of the chiral neonicotinoid sulfoxaflor in soil by ultraperformance convergence chromatography/tandem mass spectrometry, *Analytical and Bioanalytical Chemistry* 406(26), pp.6677-6690.
- [51] Mullin, L., Burgess, J.A., Jogsten, I.E., Geng, D., Aubin, A. and van Bavel, B., 2015, Rapid separation of hexabromocyclododecane diastereomers using a novel method combining convergence chromatography and tandem mass spectrometry, *Analytical Methods* 7(7), pp.2950-2958.
- [52] Hou, J.J., Cao, C.M., Xu, Y.W., Yao, S., Cai, L.Y., Long, H.L., Bi, Q.R., Zhen, Y.Y., Wu, W.Y. and Guo, D.A., 2018, Exploring lipid markers of the quality of coix seeds with different geographical origins using supercritical fluid chromatography mass spectrometry and chemometrics, *Phytomedicine* 45, pp.1-7.
- [53] Kong, F., Yuan, L., Zheng, Y.F. and Chen, W., 2012, Automatic liquid handling for

- life science: a critical review of the current state of the art, *Journal of Laboratory Automation* 17(3), pp.169-185.
- [54] Płotka-Wasyłka, J., Szczepańska, N., de la Guardia, M. and Namieśnik, J., 2016, Modern trends in solid phase extraction: new sorbent media, *Trends in Analytical Chemistry* 77, pp.23-43.
- [55] Tweed, J.A., Gu, Z., Xu, H., Zhang, G., Nouri, P., Li, M. and Steenwyk, R., 2010, Automated sample preparation for regulated bioanalysis: an integrated multiple assay extraction platform using robotic liquid handling, *Bioanalysis* 2(6), pp.1023-1040.
- [56] Andrade-Eiroa, A., Canle, M., Leroy-Cancellieri, V. and Cerdà, V., 2016, Solid-phase extraction of organic compounds: a critical review. Part II, *Trends in Analytical Chemistry* 80, pp.655-667.
- [57] Żwir-Ferenc, A. and Biziuk, M., 2006, Solid Phase Extraction Technique-Trends, Opportunities and Applications, *Polish Journal of Environmental Studies* 15(5), pp.677-690
- [58] Agnolet, S., Wiese, S., Verpoorte, R. and Staerk, D., 2012, Comprehensive analysis of commercial willow bark extracts by new technology platform: Combined use of metabolomics, high-performance liquid chromatography–solid-phase extraction–nuclear magnetic resonance spectroscopy and high-resolution radical scavenging assay, *Journal of Chromatography A* 1262, pp.130-137.
- [59] Poole, C.F., 2003, New trends in solid-phase extraction, *Trends in Analytical Chemistry* 22(6), pp.362-373.
- [60] Ötles, S. and Kartal, C., 2016, Solid-Phase Extraction (SPE): Principles and applications in food samples, *Acta Scientiarum Polonorum Technologia Alimentaria* 15(1), pp.5-15.
- [61] Hennion, M.C., 1999, Solid-phase extraction: method development, sorbents, and coupling with liquid chromatography, *Journal of Chromatography A* 856(1-2), pp.3-54.
- [62] Guo, X. and Kristal, B.S., 2012, The use of underloaded C18 solid-phase extraction plates increases reproducibility of analysis of tryptic peptides from unfractionated human plasma, *Analytical Biochemistry* 426(1), pp.86-90.

- [63] Lorenz, M.G., 2004, Liquid-handling robotic workstations for functional genomics, *Journal of the Association for Laboratory Automation* 9(4), pp.262-267.
- [64] Gaisford, W., 2012, Robotic liquid handling and automation in epigenetics, *Journal of Laboratory Automation* 17(5), pp.327-329.
- [65] Bladergroen, M.R., Derks, R.J., Nicolardi, S., de Visser, B., van Berloo, S., van der Burgt, Y.E. and Deelder, A.M., 2012, Standardized and automated solid-phase extraction procedures for high-throughput proteomics of body fluids, *Journal of Proteomics* 77, pp.144-153.
- [66] Ahuja, S. and Dong, M. (eds.), 2005, *Handbook of pharmaceutical analysis by HPLC*, 1st ed., Elsevier Incorporated, United Kingdom.
- [67] Boligon, A.A. and Athayde, M.L., 2014, Importance of HPLC in analysis of plants extracts, *Austin Chromatogr* 1(3), p.2.
- [68] Clarkson, C., Stærk, D., Hansen, S.H., Smith, P.J. and Jaroszewski, J.W., 2006, Discovering New Natural Products Directly from Crude Extracts by HPLC-SPE-NMR: Chinane Diterpenes in *Harpagophytum procumbens*, *Journal of Natural Products* 69(4), pp.527-530.
- [69] Wolfender, J.L., 2009, HPLC in natural product analysis: the detection issue, *Planta Medica* 75(07), pp.719-734.
- [70] Meyer, V.R., 2013, *Practical high-performance liquid chromatography*, 5th ed. John Wiley & Sons Publications, United Kingdom.
- [71] Moldoveanu, S.C. and David, V., 2012, *Essentials in modern HPLC separations*, Elsevier Incorporated, United States of America.
- [72] Thammana, M., 2016, A review on high performance liquid chromatography (HPLC), *Research & Reviews: Journal of Pharmaceutical Analysis* 5(2), pp.22-28.
- [73] Dong, M.W., 2006, *Modern HPLC for practicing scientists*. John Wiley & Sons Publications, Hoboken, New Jersey.
- [74] Kazakevich, Y.V. and Lobrutto, R., 2007, *HPLC for pharmaceutical scientists*. John Wiley & Sons Publications, Hoboken, New Jersey.
- [75] Boligon, A.A., Agertt, V., Janovik, V., Cruz, R.C., Campos, M., Guillaume, D., Athayde, M.L. and dos Santos, A.R., 2012, Antimycobacterial activity of the fractions and compounds from *Scutia buxifolia*, *Brazilian Society of*

- Pharmacognosy* 22(1), pp.45-52.
- [76] Barbosa Filho, V.M., Waczuk, E.P., Kamdem, J.P., Abolaji, A.O., Lacerda, S.R., da Costa, J.G.M., de Menezes, I.R.A., Boligon, A.A., Athayde, M.L., da Rocha, J.B.T. and Posser, T., 2014, Phytochemical constituents, antioxidant activity, cytotoxicity and osmotic fragility effects of Caju (*Anacardium microcarpum*), *Industrial Crops and Products* 55, pp.280-28.
- [77] Gamache, P.H., 2017, *Charged aerosol detection for liquid chromatography and related separation techniques*, John Wiley & Sons Publications, Hoboken, New Jersey.
- [78] Runco, J. and Chen, R., 'Encompassing Method Development, Small Scale Purification, and Post-Purification Analysis with the Investigator SFC System,' Waters Corporation.
- [79] Acharjee, A., Pullancheri, D., Gopinadh, B., Kurup, A., Wagh, P., Bharathi, D.V., Sharma, H.K and Meganathan, S.K., 'The use of AQUITY UPC² and the Waters SFC Investigator System for the Analysis, Separation, and Isolation of Canagliflozin and Two Isomeric Impurities,' Waters Corporation.
- [80] Hudalla, C.J., Tarafder, A., Jablonski, J.M. and Fountain, K.J., *UPC² Strategy for Scaling from Analytical to Preparative SFC Separations*, Waters, viewed 19 June 2020, from <https://www.waters.com/nextgen/xg/en/library/application-notes/2013/upc2-strategy-for-scaling-from-analytical-to-preparative-sfc-separations.html> .
- [81] Romanik, G., Gilgenast, E., Przyjazny, A. and Kamiński, M., 2007, Techniques of preparing plant material for chromatographic separation and analysis, *Journal of Biochemical and Biophysical Methods* 70(2), pp.253-261.

Chapter 4: Biological evaluation of extracts and compounds from African ginger against Influenza A-type virus

4.1 Background

4.1.1 Influenza virus

The influenza virus has been around since Hippocrates, 2 400 years ago, with numerous outbreaks throughout history.^[1] It is a serious global health challenge causing 1 billion cases, resulting in 290 000 – 650 000 fatalities annually. It is a respiratory infection that is most common amongst old people (>65 years), children (<2 years) and pregnant women and if not treated on time, it may cause bacterial pneumonia, sinus infections and cardiovascular diseases, eventually leading to death.^[2,3] It is important for these patients to get treated/vaccinated annually to prevent the risk of infection.^[4-6]

There are four types of influenza viruses; three known to affect humans (Influenza A, B and C) and only one known to affect animals (Influenza D).^[4,7] Influenza A and B are members of the Orthomyxoviridae family and is responsible for the seasonal spread of flu amongst humans.^[4,8]

Type A is the most common of the four and is an enveloped single negative-strand RNA virus and can only survive by reproducing in a living cell and infecting it.^[9,10] It causes an infectious disease, better known as flu, resulting in high fever, sore throat, headaches, cough, sneezing, tiredness, and muscle pain.^[4,10,11]

Many influenza viruses are seasonal with new strains surfacing annually in the winter time and can spread easily through coughing and sneezing in a crowded place.^[4,12,13] The identification of these new strains is important in order for it to be included into the production of vaccines for the upcoming influenza season.^[14] With the growing increase in influenza viruses, it is important to try and prevent an epidemic through any means necessary.

4.1.2 Prevention of Influenza

It is important to prevent infection since these viruses form an immunity towards vaccines as they mutate. Prevention is most effective in the form of vaccination and has been used for more than 60 years. This proves as protection against infection of circulating viruses and it is important for high risk patients to get vaccinated annually.^[4,5,13] It strengthens the immune system and protects the human body usually against three different seasonal viruses.^[6,14]

To prevent spreading of the virus the patient must stay home, get plenty of rest and fluids, and take a drug to relieve the fever and sinus pains (e.g.. paracetamol).^[13,15] Antibiotics are ineffective since they are only useful against bacterial infections and failure to treat the virus can result in high replication efficiency leading to organ failure or pneumonia, and ultimately death of the patient.^[4,11,16]

Antiviral drugs can also be used since they cover a wider range of the viruses, whereas vaccines are only effective against three or four viruses.^[17] In the case of a pandemic outbreak, various measures can be made to prevent spread such as wearing a facemask and gloves and closing of public places. This might not be effective and a vaccine may take too long to be developed and approved, therefore presenting antiviral drugs as a first line of defence.^[2,18]

4.1.3 Antiviral drugs

Antiviral drugs are administered to unvaccinated, high-risk, or already infected patients by limiting the infection in a certain timeframe.^[16,19] An antiviral drug is the most appropriate to use in pandemic situations and its production and formulation is quicker than vaccines.^[20,21]

Viruses use their host cells to replicate, making it difficult for antiviral drugs to target only the virus and not damage the cell.^[10,22] This can be done by targeting only the viral protein, overlooking other proteins and preventing side effects when using the drugs.^[16] Currently two types of synthetic antiviral drugs are used: proton channel inhibitors and neuraminidase inhibitors.^[16]

4.1.3.1 Proton channel inhibitors

M2 proton channel inhibitors are known as first generation antiviral drugs which include amantadine **(8)** and rimantadine **(9)**.^[23,24] They inhibit the functioning of M2 proteins by blocking acidification of the M2 channel and interrupting replication of the virus.^[23,25] M2 channels are only available in Influenza A-type viruses, therefore only making the drugs effective against Influenza A-type viruses.^[2,16,23]

Amantadine (Symmetrel™) was previously considered one of the best antiviral drugs, but toxicity in patients has refrained people from using it.^[23,26] Rimantadine is more popular since it shows fewer side effects such as nervousness, nausea, insomnia, dizziness and anxiety.^[27,28]



Figure 4.1 Structures of **(8)** amantadine and **(9)** rimantadine.^[29]

4.1.3.2 Neuraminidase inhibitors

Neuraminidase inhibitors (NAI's) are known as second generation antiviral drugs which attempt to prevent the activation and initiation of the influenza virus.^[24,30] It has proven effective against Influenza A – and B-type viruses and include drugs like zanamivir (Relenza) **(10)**, peramivir **(11)** and oseltamivir (Tamiflu) **(12)**.^[18,30,31] These drugs have showed only minor side effects including dizziness, nausea and headaches and have less viral resistance than proton channel inhibitors.^[16,25,30]

The virus can easily replicate and it is important to focus on the development of selective inhibitors of the viral neuraminidase enzyme of the virus.^[2] The enzyme attaches to the

sialic acid of the cell membrane, removing it and releasing the virus to penetrate the mucus layer of the respiratory tract.^[32] The drug binds to the active site of the cell where the neuraminidase is attached, inhibits it from infecting other cells, weakening the virus and then inhibiting replication.^[16,25,30] Inhibiting viral replication is the main function of these drugs and it is important to focus on the development of inhibitor enzymes by using effective drugs.

Zanamivir (Relenza) is a potent inhibitor that is inhaled and used to treat patients with Influenza A – and B-type viral infections.^[33,34] It has fewer side effects, reduces the time of symptoms and have a quicker recovery rate than other NAI's.^[33]

Oseltamivir, available as Tamiflu, is one of the most well-known and widely used antiviral drugs on the market.^[25,35] It has a fast recovery rate and is taken orally to treat patients with bronchitis and pneumonia.^[35,36] This makes it more popular than zanamivir, which needs to be inhaled to be effective, increasing the demand for Tamiflu.^[36] The drug has been around for many years, but unfortunately viral resistance has started to occur in the past decades due to mutation or use of higher concentrations.^[37] This viral resistance has urged users to use alternative NAI's or anti-influenza drugs.^[35,37]

Another well-known NAI is peramivir. It is a potent influenza inhibitor and has shown significantly better antiviral inhibition in animal – and human studies compared to zanamivir and oseltamivir.^[30,31,38] It has however shown to take longer to relieve the patient of symptoms and has shown low blood levels. Regardless of this, the drug still has potential to act as an antiviral drug that can replace zanamivir and oseltamivir if resistance occurs.^[30,39]

NAI's have been the more popular antiviral drugs with a broader spectrum of activity, less resistance and side effects, and higher potency.^[16,18,33] Unfortunately, after the H1N1 Pandemic (H1N1pdm09 virus) in 2009, these drugs, especially Tamiflu, have become less effective due to the growing resistance and mutation of the virus.^[40,41]

The demand for more effective antiviral drugs has increased, and since 2009 there has been an emergence of new strains of Influenza A-type viruses. These have shown natural resistance towards known proton inhibitor channels and NAI's.^[41] This makes all known antiviral drugs ineffective and is there still a high demand for effective antiviral drugs.^[18,42]

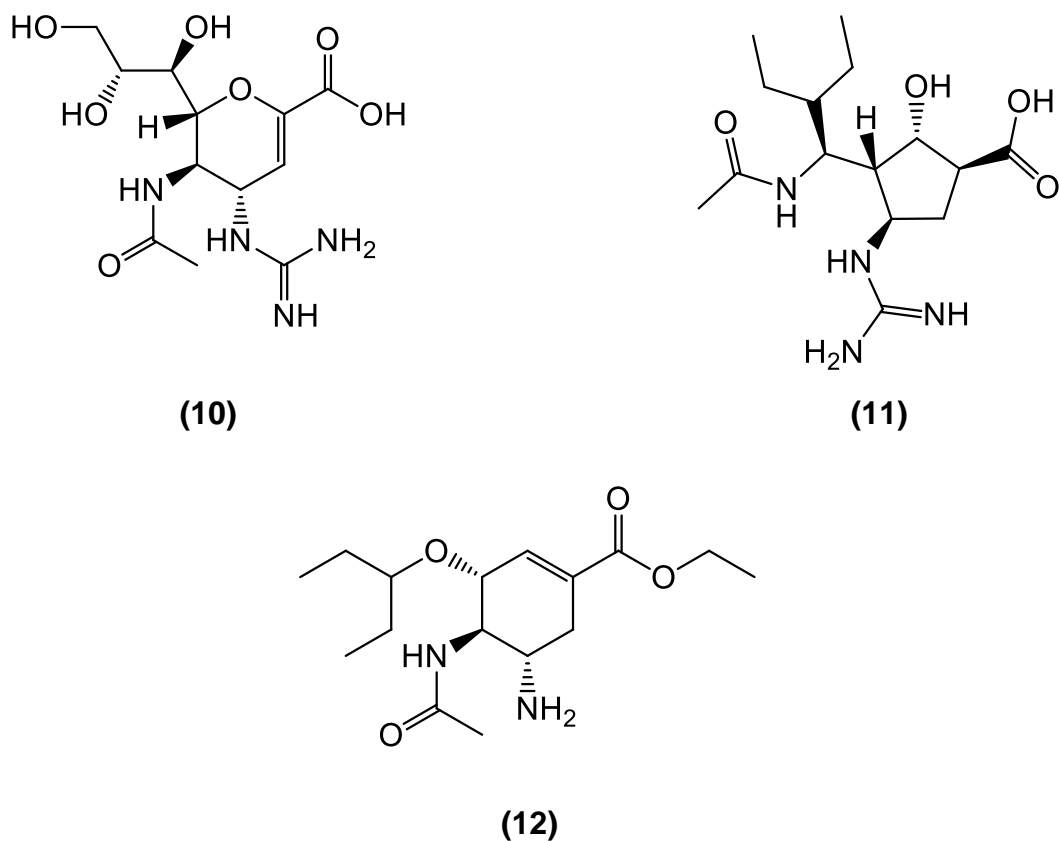
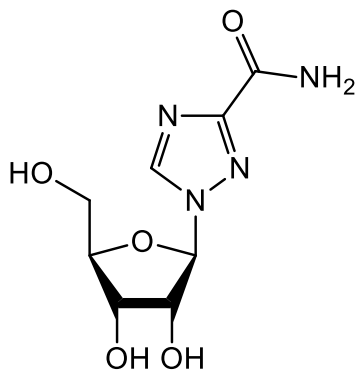


Figure 4.2 Structures of zanamivir (10), peramivir (11) and oseltamivir (12).^[29]

4.1.3.3 Ribavirin

Ribavirin (13) is a synthetic nucleoside analogue and antiviral drug effective against most DNA and RNA viruses.^[43-45] It is also a polymerase inhibitor that ceases the synthesis and capping of viral RNA and mRNA.^[18,44,46] It was synthesized in 1972 and has been used to treat Influenza A – and B-type viruses.^[44,47,48] It is more effective, shows little drug-resistance and crosses the blood-barrier easier than other known drugs.^[44,49] It is also a safer and less resistant antiviral drug, which comes in the form of an oral – or aerosol formulation.^[43,47] The aerosols showed better and faster recovery than drugs taken orally.^[44]

Ribavirin has a broad spectrum of antiviral activity, with clinical studies showing limited resistance for certain cell lines of hepatitis C, polio and other respiratory syncytial viruses.^[43,44,47,50] Different strains of influenza viruses and other viruses were tested on animal models (Influenza A – and B type viruses, and parainfluenza in mice and Influenza A-type and Lassa fever virus in monkeys) and showed effective, dose-dependent treatment of the virus, making it to clinical trials.^[44,49,51]



(13)

Figure 4.3 Structure of ribavirin (13).^[44]

Ribavirin is however a much stronger drug when combined with other drugs like Symmetrel and Tamiflu, suggesting that the synergistic interaction between these drugs show better *in vitro* antiviral activity.^[26,51-53] Tamiflu is a very popular and well researched antiviral drug but ribavirin has proven better. The former is not as good because it is less stable and has a higher recurrence of drug-resistance.^[37,51]

Ribavirin is effective against most NAI resistant viruses, making it the better antiviral drug and most effective as a control for this study.^[18,38] The growing resistance from different strains of influenza viruses toward synthetic antiviral drugs have resulted in the demand for plant-based pharmaceuticals.^[24] They have fewer side effects, are less toxic, better for the ecosystem and easily available and affordable, making them the next generation of antiviral drugs.^[24,54,55]

4.1.4 Natural antiviral drugs

Natural antiviral drugs are known as the third generation of antiviral drugs, an alternative to synthetic drugs.^[24] The production of antiviral drugs can be very complex because they should invade the cell, prevent viral reproduction and be non-toxic to the host cell and adjacent cells.^[10,22] The drugs should also either directly restrain the virus or indirectly inhibit it by strengthening the immune system.^[10]

The antiviral properties of various South African plants have been researched with the purpose of producing natural antiviral drugs.^[55,57] These plants include: *Aspalathus linearis*, *Burkea africana*, *Clerodendrum glabrum*, *Cussonia spicata*, *Pelargonium sidoides*, *Pittosporum viridiflorum*, *Psidium guajava*, *Rapanea melanophloeos*, *Tabernaemontana ventricosa* and *Siphonochilus aethiopicus*.^[55,58,59] In most cases only the extracts or essential oils of the plant was biologically screened, but active compounds were not identified.

Identification of active ingredients are important for pharmacological purposes. These can be obtained through various methods of fractionation, isolation, purification and structure elucidation of the plant extracts or oils resulting in novel antiviral compounds.^[24] The most common compounds responsible for antiviral activity are alkaloids, anthocyanin, flavonoids, glycosides, phenols, saponins, tannins and terpenoids.^[60-62]

4.1.5 Previous research on the antiviral efficacy of African ginger

Siphonochilus aethiopicus, African ginger, is one of the most popular medicinal plants and is traditionally used to treat coughs, colds, flu and influenza.^[63-65] Little research has been done on the traditional use of African ginger for its antiviral activity.^[58]

Light et al. (2002) investigated the antiviral properties of the aqueous extracts of the fresh leaves, rhizomes and roots by performing a cytotoxicity and Influenza A-type (strain Panama) antiviral assay. Vervet monkey kidney (VK) cells were used for antiviral testing and seeding in 96-well microtitre plates with 200 μ L of 10⁵ cells/mL cell suspension.

The aqueous extract showed high levels of cytotoxicity at concentrations 3.90 – 1000 μ g/mL which increased as the days passed. The aqueous extract also showed no

noteworthy antiviral activity against the Influenza A-type virus at concentrations 3.90 – 500 µg/mL. [58]

As compounds often work synergistically to have good antiviral activity, it is possible that pure compounds can work better antagonistically. [66, 68] The antiviral activity of the essential oil, ethanol extracts and pure compounds; siphonochilone **(1)** and its hydroxylated lactone **(6)**, have not yet been screened and is the essence of this chapter.

4.2 Methodology

The bioassaying was made possible with the support of the Department of Biomedicine, University of Basel, Switzerland. Special thanks to the strong collaboration of Prof. Thomas Klimkait and Ms. Lorena Urda for the training they provided in the bioassay studies which were performed in a BSL–2 laboratory during my research visit to their facility.

The *in vitro* screening of selected samples against a strain of the Influenza A-type virus was performed followed by evaluation of their toxicity (Figure 4.4). A plaque assay was first developed to determine the viral titre as plaque-forming units per mL (pfu/mL) of two strains of Influenza A-type viruses (108 617 and 61332 065) (Figure 4.4). This was then followed by a Cytopathic Effect Reduction Assay against Influenza A (CERA-I) to determine the inhibition and toxicity of the test samples against the selected strain from the plaque assay.

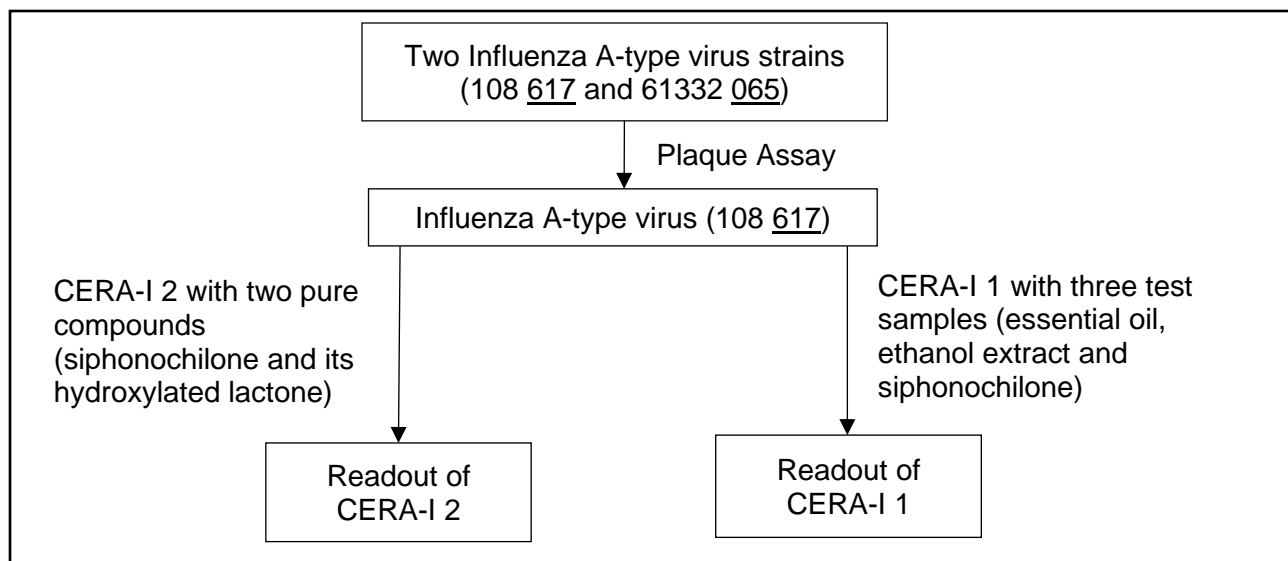


Figure 4.4 Layout of the methodology for the antiviral screening of test samples.

4.2.1 Buffers, chemicals and reagents

Dulbecco's Modified Eagle Medium (DMEM) High Glucose with stable Glutamine, FBS (Fetal Bovine Serum), Dulbecco's PBS (Phosphate buffered-saline) without Ca^{++} / Mg^{++} and TPCK-treated trypsin was purchased from Sigma. Agarose was purchased from Biorad. Penicillin-Streptomycin was purchased from BioConcept.

4.2.2 Apparatus and consumables

Neubauer chamber and Light Microscope from Leika. ABgene storage 24 well-plates (24-wp) from Sarstedt and ABgene storage 96 well-plates (96-wp) from Lubio Science.

4.2.3 Plaque Assay

4.2.3.1 Seeding of MDCK cells

Madin-Darby Canine Kidney epithelial cells (MDCK) were seeded in a T75 flask for 24 hours before being counted. The cells were seeded in Complete Dulbecco's Modified Eagle Medium (cDMEM) (complete DMEM with 10% FBS, 1% Penicillin-Streptomycin)

and 1.00×10^6 cells/mL were counted using a Neubauer chamber (number/mL) and light microscope. A total of 1.6×10^7 MDCK cells in 16 mL of cDMEM were seeded after the 24 h.

After the cells were counted, they were washed with 500 μ L PBS and 500 μ L of the cells were suspended to each well in a 24-well plate (wp). A 24-wp was preferred over a 96-wp for the plaque assay to ensure more precise screening at higher volumes.

Different seeding conditions were used to determine which conditions allow cells to be confluent in the negative control when the plaques are counted. These conditions included the suspension of seeds to a 24-wp, 24 h and 48 h before infection. The cells suspended 48 h before infections only had 2.00×10^5 cells/well and cells suspended 24 h before infections were assumed to have doubled in the 24 h (4.00×10^5 cells/well).

Both seeding densities showed confluency at the different conditions so either one could have been used. For the plaque assay, cells were seeded for 24 h and suspended to a 24-wp, 24 h before infection (4.00×10^5 cells/well).

4.2.3.2 Infectious media for viral growth

An AgDMEM-T solution (0.30% Agarose/DMEM/1 μ g/mL DMPK treated with trypsin) was prepared for a 24-wp (20 mL) to keep cells stable and limit virus spread:

2.00 mL of 3% Agarose in DMEM was autoclaved and 18 mL of DMEM (no FCS (foetal calf serum)) was heated in a microwave to 45°C for 20 – 30 minutes. The agarose and DMEM was combined. Cells were washed once with 500 μ L PBS and 400 μ L of TPCK-treated trypsin (final concentration of 1.00 μ g/mL) was added to the heated DMEM. 700 μ L of the media was pipetted into each well of the 24-wp.

4.2.3.3 Virus dilution

Two strains of the Influenza A-type virus (108 617 and 61332 065), both available at the Department of Biomedicine at the University of Basel, Switzerland, were used for the plaque assay to determine which has the highest titre and is most suitable for the

Cytopathic Effect Reduction Assay against Influenza A (CERA-I). Well numbers 1-12 were infected with strain 108 617 and well numbers 13 – 24 with strain 61332 065. A viral dilution of each strain was prepared with 10 µL of the virus stock and 990 µL DMEM, starting with a 1:100 dilution and followed by a 1:5 serial dilution in 500 µL DMEM (without FBS). The plate was left to incubate at 37°C with 5% CO₂ for 4 days.

4.2.3.4 Read out of viral growth

Growth and cytopathic effect (CPE) formation was observed regularly. Cell confluency as monolayer in the negative control wells and clear CPE with plaque formation (30 – 60%) in the infected wells were verified under the microscope.

After 4 days, the agarose was removed by turning the plate over a liquid waste container with pre-added 1% Korsolex for virus deactivation. The cells were fixed by adding 250 µL of 3.60% formaldehyde (1:10 dilution from 37% in PBS) to each well and incubated for 45 min at room temperature. The formaldehyde was removed and the cells were stained with 0.50% crystal violet and incubated for 5 min at room temperature. Excess dye was removed and washed with distilled H₂O and the blue stained plaques were counted.

4.2.3.5 Titre calculations

The titre of a virus stock was reported as plaque-forming units (PFU) per mL:

Count plaques in highest dilution at which plaque count is reliable (10 – 100 plaques).

Calculations:

PFU/well = counted plaques x highest dilution

PFU/mL = PFU/well x factor (1.00 mL/well volume in mL)

The TCID₅₀/mL (Median Tissue Culture Infectious Dose) was calculated using the Spearman Karber Formula.^[67]

4.2.4 Test samples

The essential oil from fresh rhizomes and ethanol extract prepared from freshly dried ground rhizomes, and siphonochilone (**1**), were first screened for antiviral activity using a CERA-I assay. In a follow-up, both pure compounds identified as described in Chapter 3, siphonochilone (**1**) and the hydroxylated lactone (**6**), were separately tested in the same assay.

4.2.5 CERA-I bioassaying using the essential oil, ethanol extract and siphonochilone (1) as test samples

CERA-I was used to study the inhibition and cytotoxicity of the test samples against a strain of an Influenza A-type virus (108 617) as determined by the plaque assay in Section 4.2.3 (Figure 4.4).

For the CERA-I a 96-wp was used instead of a 24-wp as with the plaque assay. This does not affect the outcome of the results since the titre calculations in the plaque assay were expressed as per mL and with the appropriate factor per mL the results would have been the same with a 96-wp for the plaque assay. The volume and values can be adjusted to a 96-wp by using the correct factor and calculations.

For each CERA-I, a 96-wp was prepared for the inhibition study containing the MDCK cells, virus, infectious media and the test samples. The following controls were included: virus control (cells that were only infected with the virus, but not treated with the test samples), cell control (cells that were not infected with the virus or treated with the test samples), solvent control (cells that were only treated with 20 μ L of 0.50% DMSO), and the positive controls (virus-infected cells treated with ribavirin).

A separate 96-wp was prepared for the cytotoxicity study containing the MDCK cells, virus, infectious media and the test samples. The following controls were included: cell control, solvent control (20 μ L of 10% DMSO) and test samples.

4.2.5.1 Seeding of MDCK cells

MDCK cells were seeded in a T75 flask and incubated with 5% CO₂ at 37°C for 48 h before infection. A Neubauer chamber (number/mL) was used to count the seeds: 3.00×10⁶ cells in 10 mL DMEM for one 96-wp. To have enough cells for all the well plates to perform the inhibition and cytotoxicity studies, the MDCK cells were resuspended in 20 mL DMEM and 6.00×10⁶ cells were available.

100% of the cells were confluent and 19.80 mL were suspended with DMEM (200 µL) to make up 20 mL for all the well plates. The cells were thoroughly washed twice with PBS and 100 µL were suspended to each well (3.00×10⁴ cells/well) of a 96-wp.

4.2.5.2 Infectious media for viral growth

The infectious media (DMEM-T) was prepared for all plates by adding 49 mL of DMEM (without serum) with 1.00 mL of 0.10% TPCK treated trypsin (final concentration of 1.00 µg/mL). The media (100 µL) was dispensed to each well of the 96-wp.

4.2.5.3 Virus dilution

From the plaque assay, Influenza A-type virus strain 108 617 was selected and used for the virus dilution. The TCID₅₀ of the virus was determined during the plaque assay and 10 mL of virus stock was prepared per 96-wp, MOI 0.001 (multiplicity of infection). Each well was infected with 190 µL of the viral stock, except for six wells, which include the cell – and solvent controls.

4.2.5.4 Dilution of test samples

Test samples were resuspended in 0.50% DMSO and the dilution was performed starting at 50.0 µg/mL, and were prepared as 10-fold concentrations with a serial dilution of 1:4, up to 0.05 µg/mL. For each plate 1.00 mL of each dilution was prepared and 10 µL was dispensed to each well. Each test sample dilution was done in duplicate for reproducibility.

4.2.5.5 Dilution of positive control (Ribavirin)

Ribavirin was used as a positive control (10 mg/mL in H₂O) starting at 100 µg/mL and ending at 0.025 µg/mL. The control was diluted with a 3-step serial dilution of 1:10. Each dilution of the control was done in duplicate.

The plate was left to incubate at 37°C with 5% CO₂ for 2 days.

4.2.5.6 Read out of viral growth

A cell titre blue protocol (Thermo Fisher) was used for the readout of viral growth and 20 µL was used to stain the cells. The well-plate was incubated for 1 h in the BSL-2 incubator and measured using a TecanSaphire II spectrometer with excitation at 545 nm and emission at 590 nm, both with a bandwidth of 20 nm, integrating for 40 min. Measurements were again taking 4 – 6 h after incubation. The reaction was stopped and stabilized with 3% SDS and again measured 24 hours later.

4.2.6 CERA-I using siphonochilone (1) and its hydroxylated lactone (6) as test samples

Siphonochilone and the hydroxylated lactone were further studied for their antiviral activity at different concentrations based on the results obtained from the first CERA-I (Figure 4.4).

4.2.6.1 Seeding of MDCK cells

MDCK cells were seeded in a T75 flask and incubated with 5% CO₂ at 37°C for 48 hours before infection. A Neubauer chamber was used to count the seeds: 3.00×10⁶ cells in 10 mL for one 96-wp. To have enough cells for two well plates to perform the inhibition and cytotoxicity studies, the MDCK cells were resuspended in 10 mL and 6.0×10⁶ cells were available.

All cells were confluent (100%) and 7.90 mL were suspended with DMEM (2.10 mL) to make up 10 mL per well plate. The cells were thoroughly washed once with PBS and 100 μ L were suspended to each well (3.00×10^4 cells/well) of a 96-wp.

4.2.6.2 Infectious media for viral growth

The infectious media (DMEM-T) was prepared for all plates by adding 49 mL of DMEM (without serum) with 1.00 mL of 0.10% TPCK treated trypsin (final concentration of 1.00 μ g/mL) and dispensing 100 μ L of media to each well of the 96-wp.

4.2.6.3 Virus dilution

Influenza A-type virus strain 108 617 was selected and used for the virus dilution. The TCID₅₀ of the virus was determined during the plaque assay and 10 mL of virus stock was prepared per 96-wp, MOI 0.001. Each well was infected with 190 μ L of the viral stock, except for six wells that represent the cell – and solvent controls.

4.2.6.4 Dilution of pure compounds (test samples)

Siphonochilone and its hydroxylated lactone were resuspended in 0.5% DMSO before dilution. Twelve dilutions were made, starting at 50, 40 and 30 μ g/mL, and were prepared as 10-fold concentrations with a 3-step serial dilution of 1:2. For each plate 1.00 mL of each dilution as prepared and 10 μ L was dispensed to each well.

Each compound dilution was done in triplicate to produce reproducibility.

4.2.6.5 Dilution of positive control (Ribavirin)

Ribavirin was used as a positive control (10 mg/mL in H₂O) starting at 100 μ g/mL and ending at 0.025 μ g/mL. The control was diluted with a 3-step serial dilution of 1:10. Each dilution of the control was done in triplicate.

The plate was left to incubate at 37°C with 5% CO₂ for 3 days.

4.2.6.6 Read out of viral growth

A cell titre blue protocol was used for the readout of viral growth and 20 μ L of titre blue was used to stain the cells. The plates were incubated 1 h in the BSL-2 incubator and the solution's absorbance in each well was measured using a TecanSaphire II spectrometer with excitation at 545 nm and emission at 590 nm, both with a bandwidth of 20 nm. The plates were again measured after 4 – 6 h. The reaction was stopped and stabilized with 3% SDS and again measured 24 h later.

4.3 Results and discussions

The activity of a direct inhibiting virus was tested through *in vitro* testing by examining the survival of MDCK cells against a strain of an Influenza A-type virus (108 617) after introducing essential oil, ethanol extracts and siphonochilone from *S. aethiopicus*.^[45]

Before the inhibiting effects of test samples were evaluated, a plaque assay was done to determine the strain and concentration of the virus that was needed. This assay was then followed by two CERA-I studies using the influenza strain and the virus concentration was calculated in the plaque assay. The aim of the first CERA-I was to study the inhibition and cytotoxicity of the essential oil, ethanol extract and isolated siphonochilone from *S. aethiopicus* against the Influenza A-type virus (108 617) selected from the plaque assay. The second study was done to determine the effect of the pure compounds, siphonochilone (**1**) and its hydroxylated lactone (**6**), for comparative purposes. The dose-response relationship of these test samples was investigated and the biological activities are discussed.

4.3.1 Plaque Assay

The plaque assay was performed to determine which strain of an Influenza A-type virus had the highest titre based on the virus concentration and the number of pfu/mL. This information was needed for the *in vitro* testing of the samples.

After 72 h of incubation, dead cells were observed at the lower dilutions and after 96 h the plaques were counted and the titre calculations were performed (Table 4.1).

Figure 4.5 shows the readout of the 24-wp that was stained with a blue dye and incubated for 4 h. There was a noticeable change in plaques forming in the less diluted wells (well numbers 1 – 4, 13 and 14), which was the focus point of determining the virus concentration.

Table 4.1 gives the plaque assay readout of the two strains including the final dilution factor in 500 μ L, the plaques counted for each well and the plaques forming units per millilitre.

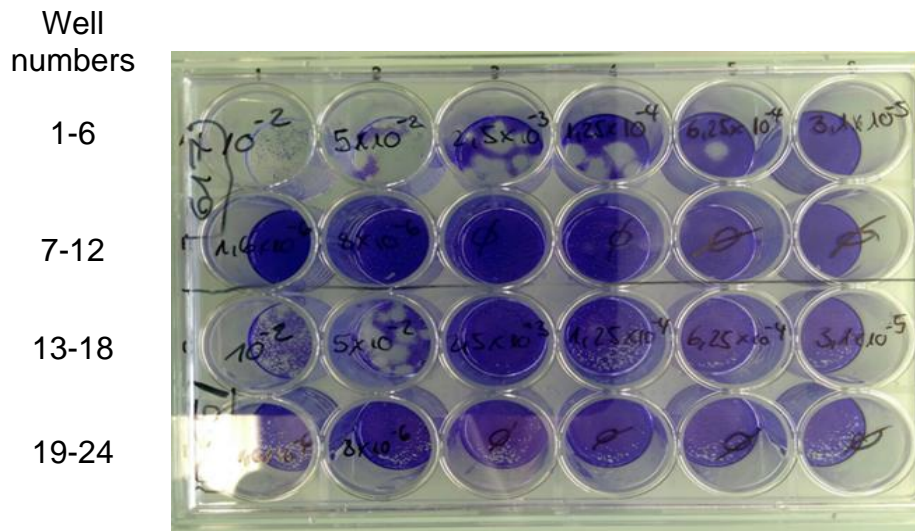


Figure 4.5 The 24-well plate after 4 h of incubation the two strains of viruses on the MDCK cells from the plaque assay.

Table 4.1 shows the titre calculations and best titre for each strain based on the highest dilution at which the plaques counted can still be reliable. It was observed that wells 1 – 5, which contained strain 108 617, had more plaques forming in the higher concentrations (100, 70, 6, 4 and 1 plaques) than strain 61332 065 (50 and 11 plaques). At all concentrations, strain 108 617 had a higher pfu/mL (9.92×10^4 pfu/mL, 1:13 000 dilution factor) than the highest pfu/mL for strain 61332 065 (1.10×10^4 pfu/mL, 1:500 dilution factor). Even though well 5 had a higher pfu/mL (1.25×10^5 pfu/mL, 1:62 500 dilution factor) than well 4, it only had one plaque forming compared to the 4 plaques that formed in well 4. This made the pfu/mL for well 5 unreliable and was the dilution from well 4 used for further bioassaying (Figure 4.5).

Table 4.1 Plaque assay readout of the two strains (pfu/mL in blue indicate the highest pfu/mL for each strain of virus).

Strain: 108 617						
Well number	1	2	3	4	5	6
Final dilution factor (500 μ L)	100	500	2500	1.3×10^4	6.25×10^4	3.13×10^5
Counted plaques	100	70	6	4	1	0
Plaque forming units per mL (pfu/mL)	2.00×10^4	7.00×10^4	3.00×10^4	9.92×10^4	1.25×10^5	0
Well number	7	8	9	10	11	12
Final dilution factor (500 μ L)	1.56×10^6	7.81×10^6	3.91×10^7	2.0×10^8	9.77×10^8	4.88×10^9
Counted plaques	0	0	0	0	0	0
Plaque forming units per mL (pfu/mL)	0	0	0	0	0	0
Strain: 61332 065						
Well number	13	14	15	16	17	18
Final dilution factor (500 μ L)	100	500	2500	1.3×10^4	6.25×10^4	3.13×10^5
Counted plaques	50	11	0	0	0	0
Plaque forming units per mL (pfu/mL)	1.00×10^4	1.10×10^4	0	0	0	0
Well number	19	20	21	22	23	24
Final dilution factor (500 μ L)	1.56×10^6	7.81×10^6	3.91×10^7	2.0×10^8	9.77×10^8	4.88×10^9
Counted plaques	0	0	0	0	0	0
Plaque forming units per mL (pfu/mL)	0	0	0	0	0	0

At 9.92×10^4 pfu/mL, with a 1:13 000 dilution factor, strain 108 617 showed a MOI of 0.001 and was therefore used for further CERA-I studies of the test samples.

The volume of the virus dilution for CERA was calculated by Ms. Lorena Urda to be 90 μ L/well. These calculations are based on the calculations made by the Department of Biomedicine (University of Basel, Switzerland) and the Spearman Karber Formula.^[67]

4.3.2 The effect of the essential oil, ethanol extract and siphonochilone (1) in the Cytopathic Effect Reduction Assay against Influenza A (CERA-I)

The first CERA-I assay was conducted to study the inhibition and toxicity of different test samples from African ginger, against a strain of Influenza A-type virus (108 617). The seeded MDCK cells used for the assay were 100% confluent before infection started and were treated in a dose-response manner at different concentrations (0.05, 0.20, 0.80, 3.10, 12.5 and 50.0 μ g/mL) of the test samples. The aim of this assay was to determine the best antiviral activity of these test samples at different test concentrations.

Figure 4.6 presents the results of the inhibition and cytotoxicity study of the three test samples and the control, ribavirin, against the strain of influenza using the CERA-I. Essential oil (Figure 4.6 – blue bars) showed no/minimal activity, with <20% inhibition of the virus at 50.0 μ g/mL and <5% or no inhibition at the lower test concentrations, some of them even having negative inhibition, which can suggest viral replication. The cytotoxicity assay showed toxicity was minimal, but higher than the viral inhibition at all concentrations. Compared to the positive control, ribavirin (Figure 4.6 – orange bars), the essential oil showed no/minimal activity with significantly higher toxicity (Figure 4.6).

The ethanol extract (Figure 4.6 – pink bars) showed no inhibition at any of the test concentrations, however it showed cytotoxicity (<35%) at all test concentrations which would have had an effect on the antiviral properties. The zero inhibition was consistent with the findings by Light et al. (2002) who reported that an aqueous extract showed little to no inhibition.^[58] Compared to ribavirin, the ethanol extract showed no activity with significantly high toxicity. Both the essential oil and ethanol extract showed higher toxicity

and no/minimal viral inhibition thereby not substantiating its use as an anti-influenza treatment.

Siphonochilone (Figure 4.6 – green bars) only showed inhibition at 12.5 µg/mL with >100% inhibition and no inhibition at the rest of the test concentrations. This compared well to ribavirin, at an equivalent test concentration. Ribavirin showed >100% inhibition of the virus at test concentrations of 100, 50 and 25 µg/mL (Figure 4.6 – orange bars), however at 10.0 µg/mL it had <20% inhibition as compared to siphonochilone which had >100% inhibition at 12.5 µg/mL. Based on this, the data suggests that siphonochilone performed the same as ribavirin at similar test concentrations.

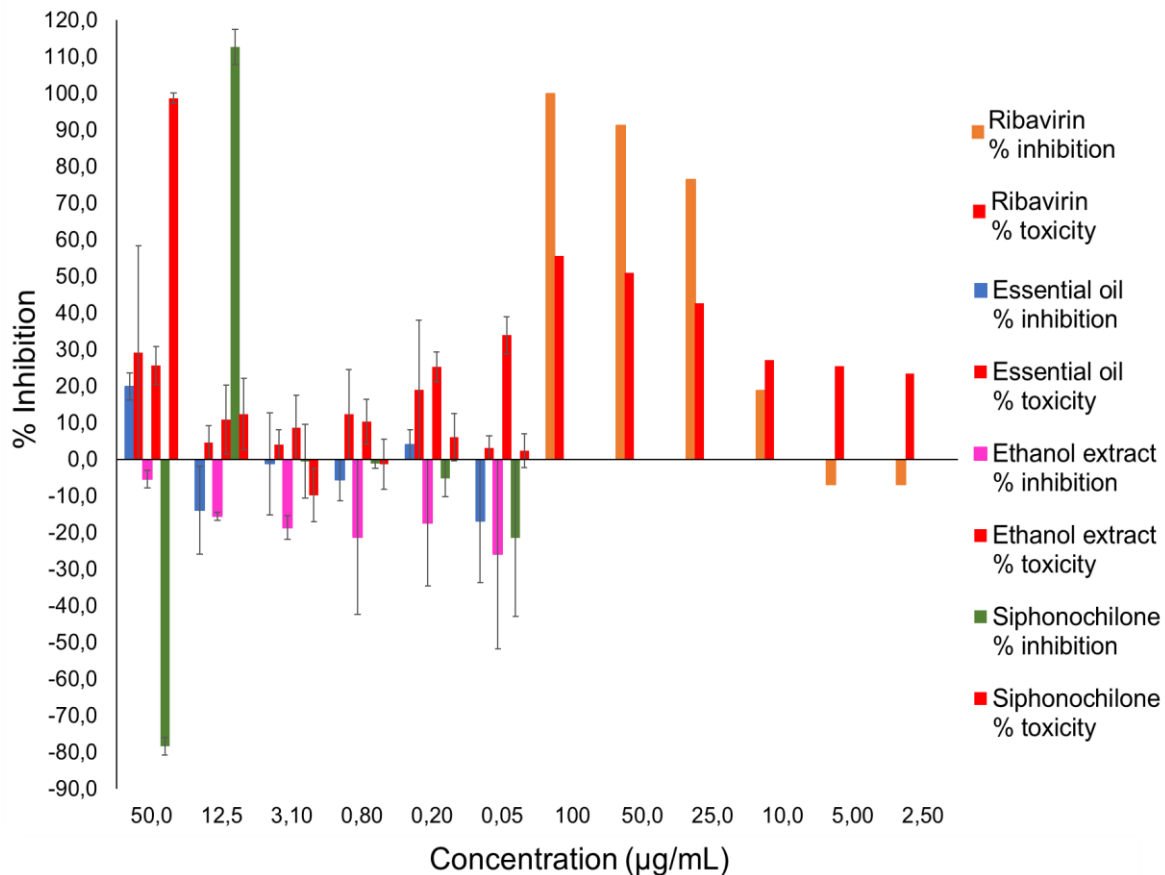


Figure 4.6 Percentage inhibition and cytotoxicity (red bars) of the test samples; essential oil, ethanol extract and siphonochilone isolated from African ginger, and the control, ribavirin (orange bars).

The cytotoxicity study for siphonochilone only showed high toxicity at 50.0 µg/mL, but minimal at the rest of the test concentrations. The lack of antiviral activity at the highest test concentration of 50.0 µg/mL was attributed to the high cytotoxicity of the compound at this test concentration. It was concluded that siphonochilone only had significant antiviral activity at 12.5 µg/mL with minimal toxicity and should be tested in another CERA-I assay at test concentrations closer to 12.5 µg/mL range.

Ordinarily extracts from different plant species show better antiviral activity since several compounds act synergistically to inhibit viral growth, but in some occurrences it is possible that only a single/pure compound is responsible for the antiviral activity.^[66,68] This seems to be the case for *S. aethiopicus* since the essential oil and ethanol extract showed no/minimal inhibition of the influenza virus. Even though the essential oil (Figure 2.2.(2); Chapter 2, Section 2.3.2.1) and ethanol extract (Figure 2.4.(2); Chapter 2, Section 2.3.2.2) contained siphonochilone, it is possible that it was of low concentration in the oil and extract. This is possible since a significant quantity of the siphonochilone crystallized out during steam distillation (Chapter 2, section 2.2.4), leaving only small quantities remaining in the oil for efficacy. For the ethanol extract, the low concentrations might have been due to several other polar compounds being extracted resulting in low concentrations of the compound (dilution effect). Another possibility could be the poor solubility of the essential oil and ethanol extract in the DMSO and these test samples may not have fully dissolved during the dilutions, resulting in no efficacy.

4.3.3 The effect of siphonochilone (1) and the hydroxylated lactone (6) in the Cytopathic Effect Reduction Assay against Influenza A (CERA-I)

As siphonochilone and its hydroxylated lactone are compounds of similar structural types, with the main difference being the ketone on C-2 and the hydroxyl group on C-9a of the hydroxylated lactone (6), they were both evaluated for their antiviral activity at the same test concentrations using the CERA-I assay for comparative purposes.

Figure 4.7 shows the antiviral efficacy of siphonochilone and its hydroxylated lactone in MDCK cells infected with Influenza A-type virus (strain 108 617). Treatment was at

different concentrations with 3 steps of serial dilution of 1:2 at a dose-response manner from 50.0 to 3.75 µg/mL.

Siphonochilone (Figure 4.7 – green bars) showed inhibition at concentrations from 50.0 to 10.0 µg/mL with >100% and >20% at 10.0 µg/mL, respectively, with no/minimal toxicity. The significant inhibition of >115% at 50.0 µg/mL compared very well to ribavirin with >105% inhibition at equivalent test concentrations.

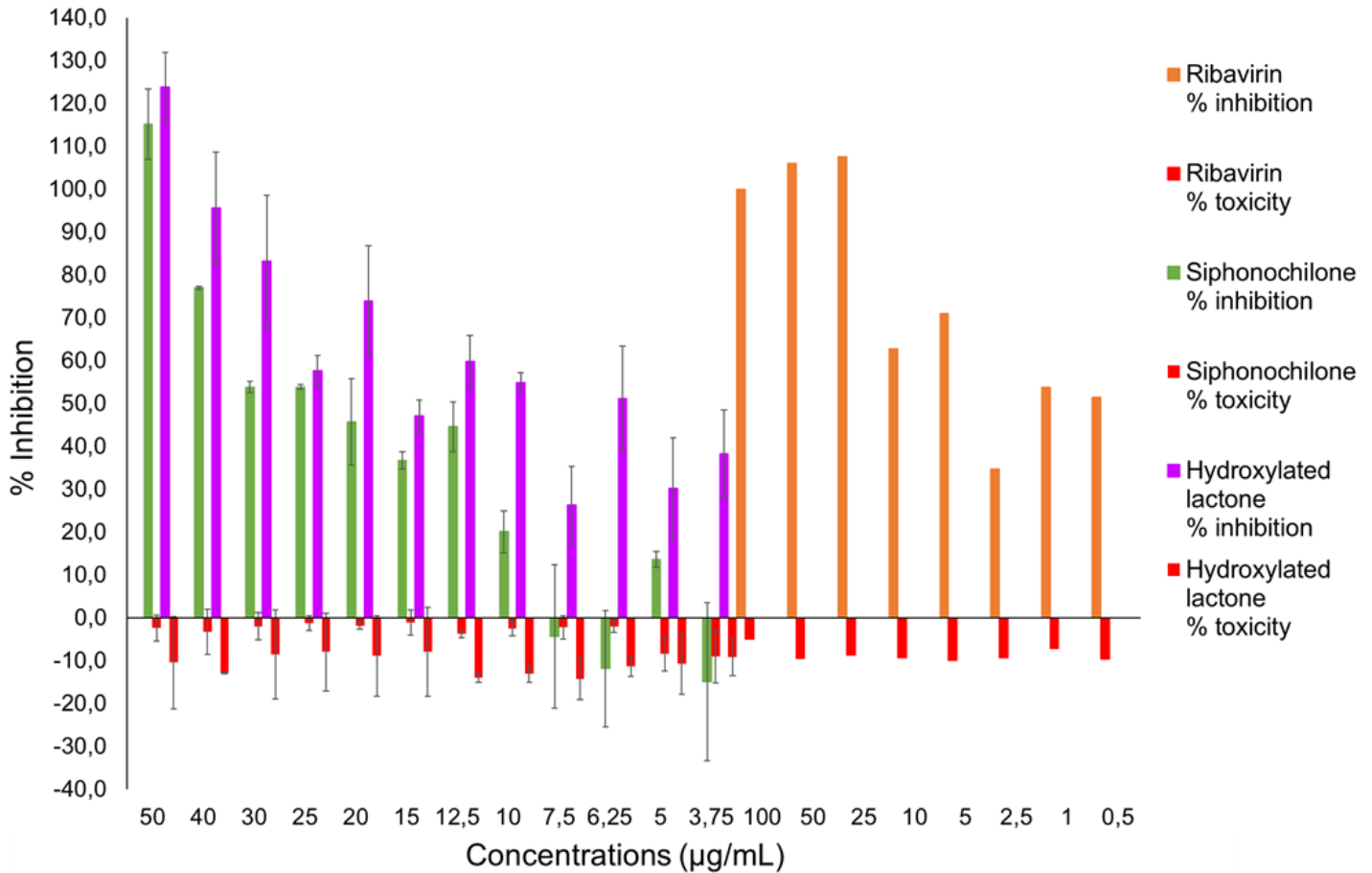


Figure 4.7 Percentage inhibition and cytotoxicity (red bars) of pure compounds, siphonochilone and its hydroxylated lactone, and the control, ribavirin (orange bars), against a strain of Influenza.

The hydroxylated lactone (Figure 4.7 – purple bars) exhibited excellent antiviral activity at all test concentrations with >100% and >30% inhibition at 50.0 µg/mL and 3.75 µg/mL,

respectively, with no toxicity. It also showed better inhibition than siphonochilone at all test concentrations. At 50.0 $\mu\text{g}/\text{mL}$ the lactone showed >123% inhibition which compared well with ribavirin, >105% inhibition, at the equivalent test concentration (Figure 4.7).

Both pure compounds reported significantly good activity against the Influenza A-type virus and showed good antiviral activity compared to the ribavirin. As with the two compounds, and ribavirin, there was a gradual decrease in the inhibition as the test concentrations decreased. Using the test concentrations and percentage inhibition of each of these compounds and ribavirin, the IC_{50} was calculated using GraphPad Prism. This resulted in the IC_{50} results of siphonochilone as 21.0 $\mu\text{g}/\text{mL}$ ($R^2 = 0.82$) (Figure 4.8.(1)), the hydroxylated lactone as 9.20 $\mu\text{g}/\text{mL}$ ($R^2 = 0.71$) (Figure 4.8.(2)) and ribavirin as 2.16 $\mu\text{g}/\text{mL}$ ($R^2 = 0.79$) (Figure 4.8.(3)).

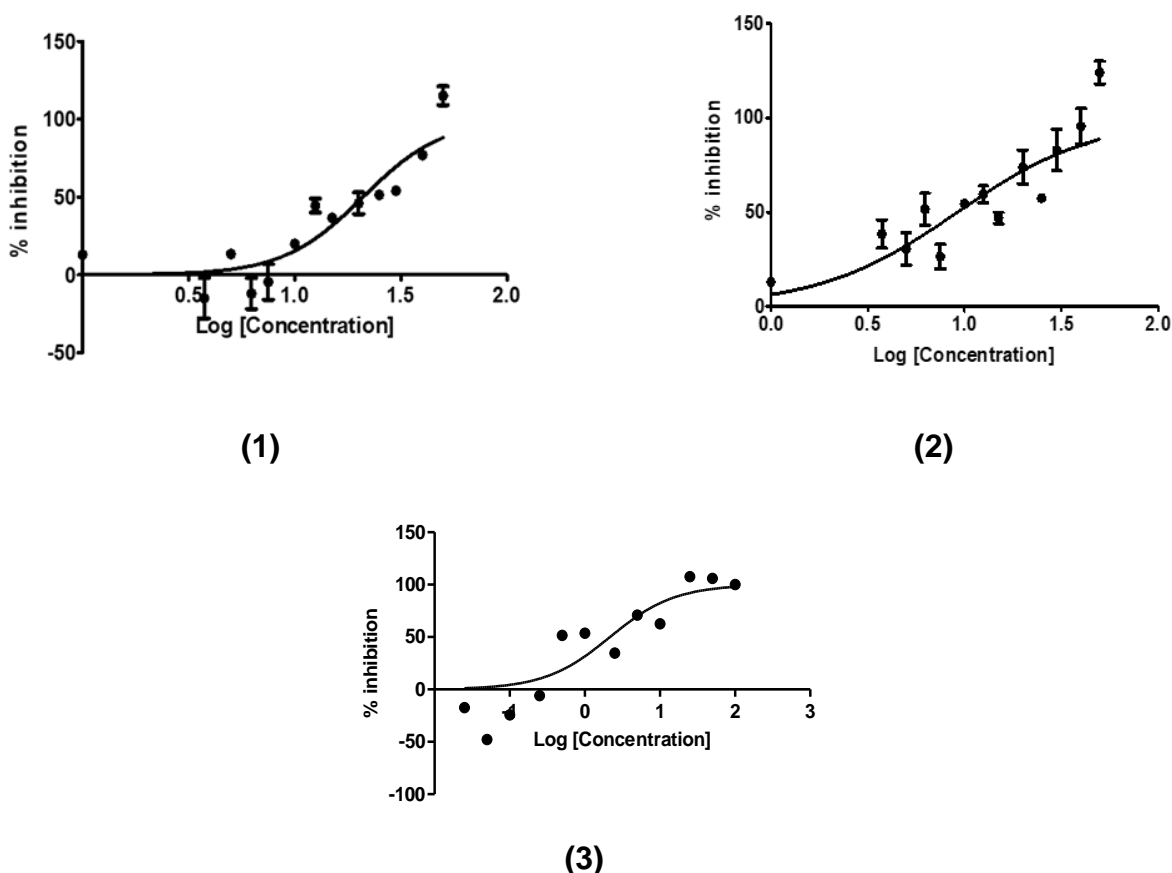


Figure 4.8 IC_{50} curve of the percentage inhibition and logarithmic concentration of (1) siphonochilone, (2) the hydroxylated lactone and (3) ribavirin.

These results suggested that siphonochilone has a higher IC₅₀ (21.0 µg/mL) than the hydroxylated lactone and ribavirin. The hydroxylated lactone had a lower IC₅₀ of 9.20 µg/mL than siphonochilone and performed better than siphonochilone. Both pure compounds have a higher IC₅₀ than ribavirin (2.16 µg/mL), but are in the same order of magnitude as ribavirin, with no cytotoxicity.

The results suggested both compounds have possible antiviral activity and that further investigation such as medicinal chemistry approaches should follow to research the antiviral properties of the pure compounds from African ginger.

It was demonstrated that the hydroxylated lactone is present in stored dried plant material and limited in fresh plant material (Chapter 2, section 2.3.2.4). It is known that the plant is commonly traded in muthi markets which often may be as stored dried plants. This implies that the hydroxylated lactone would be present in the plants used by traditional health practitioners and is in fact the main active ingredient for the treatment of influenza virus.

4.4 Conclusion

The use of plants in ethnomedicine provide valuable lead to their pharmacological properties. One of the most popular traditional uses of *S. aethiopicus* is its use against the influenza virus and credible scientific data is needed to support its use. Its traditional use includes the hot and cold infusion of the rhizomes and roots or steaming and inhalation of the vapours. This has previously been investigated and showed that an aqueous extract was screened and showed no significant activity against a strain of influenza virus. Various factors can affect the medicinal properties of a plant, including type of extract, solvents used for extraction, harvesting time, concentration of extracts and exposure time. Different extracts and isolated compounds of the plant were tested for their antiviral properties and its inhibition and cytotoxicity was determined through a CERA-I assay.

The essential oil, ethanol extract and pure siphonochilone was screened against a strain of Influenza A-type virus to determine its antiviral activity. The essential oil and ethanol

extract showed no/minimal inhibition towards the virus and significant toxicity towards the cells. These results are in agreement with those of Light et al. (2002) where they reported that an aqueous extract had no/minimal inhibition.

The pure siphonochilone showed more interesting results with significant inhibition at 12.5 µg/mL (>100% inhibition), compared to the control ribavirin with <20% inhibition at 10 µg/mL. At the concentrations higher and lower than 12.5 µg/mL there was no/minimal inhibition and was it necessary to investigate the activity of the compound at concentrations closer to 12.5 µg/mL.

The results from the first CERA-I concluded that the traditional use of the plant in the form of various extracts, did not show significant antiviral activity. It is widely known that synergism between various compounds of a plant extract is often responsible for its biological activity. In the case of African ginger extracts, it is possibly acting antagonistically since the pure compounds showed activity and not the extracts.

From a second CERA-I assay, both test samples, siphonochilone (**1**) and its hydroxylated lactone (**6**), showed significantly good activity and no/minimal toxicity. At 50 µg/mL siphonochilone showed >115% inhibition and its lactone >123% inhibition. Both compared well against the control, ribavirin, which had a 100% inhibition at the same test concentration and had no/minimal toxicity. Both compounds showed a gradual decrease in inhibition with a decrease in concentration, however they still had good IC₅₀ values; 20.97 µg/mL for siphonochilone and 9.20 µg/mL for the hydroxylated lactone.

The hydroxylated lactone has a better inhibition than siphonochilone at the higher concentrations and it had a lower IC₅₀ value than siphonochilone, which made it a better and more suitable compound for further development as an antiviral.

Since the hydroxylated lactone was mainly present in stored plant material, and limited in fresh plants, this has to be taken into consideration for its further development. This data also provided some evidence on the use of the plants in the muthi market since these are often dried and stored plant material.

Collectively, the data provides evidence substantiating for the continued investigation of the *in vitro* testing of pure compounds from African ginger in search of new efficacious

and safe antiviral drugs. The assays alone cannot be reliable to predict dose requirements and animal testing or clinical trials necessary for further investigation.

4.5 References

- [1] Martin, P.M. and Martin-Granel, E., 2006, 2,500-year evolution of the term epidemic, *Emerging Infectious Diseases* 12(6), p.976.
- [2] Bantia, S., Arnold, C.S., Parker, C.D., Upshaw, R. and Chand, P., 2006, Anti-influenza virus activity of peramivir in mice with single intramuscular injection, *Antiviral Research* 69(1), pp.39-45.
- [3] Rothberg, M.B., Haessler, S.D. and Brown, R.B., 2008, Complications of viral Influenza, *The American Journal of Medicine* 121(4), pp.258-264.
- [4] Kiani, M.A., Ghazanfarpour, M. and Saeidi, M., 2019, Influenza: A Unique Disease, *International Journal of Pediatrics* 7(4), pp.9349-9354.
- [5] Maitre, D.A., 2013, An Evidence-Based Intervention to Improve Vaccination Rates for Seasonal Influenza Among Registered Nurses, PhD Thesis, Regis University, Denver, Colorado, United States.
- [6] World Health Organization. 2019, Types of seasonal Influenza vaccine, viewed 24 September 2019, from <http://www.euro.who.int/en/health-topics/communicable-diseases/Influenza/vaccination/types-of-seasonal-Influenza-vaccine> .
- [7] Centres for Disease Control and Prevention (CDC), 2019, Understanding Influenza Viruses, viewed 3 September 2019, from <https://www.cdc.gov/flu/about/viruses/index.htm>.
- [8] Bouvier, N.M. and Palese, P., 2008, The biology of Influenza viruses, *Vaccine* 26, pp.D49-D53.
- [9] Schmidtke, M., Schnittler, U., Jahn, B., Dahse, H.M. and Stelzner, A., 2001, A rapid assay for evaluation of antiviral activity against coxsackie virus B3, Influenza virus A, and herpes simplex virus type 1, *Journal of Virological Methods* 95(1-2), pp.133-143.
- [10] Wang, X., Jia, W., Zhao, A. and Wang, X., 2006, Anti-Influenza agents from plants and traditional Chinese medicine, *Phytotherapy Research: An International*

- Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives* 20(5), pp.335-341.
- [11] Wiselka, M., 1994, Influenza: diagnosis, management, and prophylaxis, *British Medicinal Journal* 308(6940), pp.1341-1345.
- [12] Procop, G. W. and Pritt, B. S. (eds.), 2015, *Pathology of infectious diseases*. Philadelphia, PA: Elsevier, Saunders (Foundations in Diagnostic Pathology), viewed 2 September 2019, from <https://www-clinicalkey-com.uplib.idm.oclc.org/#!/browse/book/3-s2.0-C20090389564>.
- [13] World Health Organization, 2019, *Influenza (Seasonal)*, viewed 24 September 2019, from [https://www.who.int/news-room/fact-sheets/detail/Influenza-\(seasonal\)](https://www.who.int/news-room/fact-sheets/detail/Influenza-(seasonal)).
- [14] Ahmed, M., 2018, Investigating the association of receipt of seasonal Influenza vaccine with occurrence of anesthesia/paresthesia, headaches and generalized convulsive seizures for all ages, Canada 2012/13-2016/17, PhD Thesis, University of British Columbia.
- [15] Eccles, R., 2006, Efficacy and safety of over-the-counter analgesics in the treatment of common cold and flu, *Journal of Clinical Pharmacy and Therapeutics* 31(4), pp.309-319.
- [16] Stiver, G., 2003, The treatment of Influenza with antiviral drugs, *Canadian Medical Association Journal* 168(1), pp.49-57.
- [17] Rossignol, J.F., 2014, Nitazoxanide: a first-in-class broad-spectrum antiviral agent, *Antiviral Research* 110, pp.94-103.
- [18] Bi, Y., Wong, G., Liu, Y., Liu, L., Gao, G.F. and Shi, Y., 2016, Ribavirin is effective against drug-resistant H7N9 Influenza virus infections, *Protein & Cell* 7(8), pp.611-614.
- [19] European Scientific Working group of Influenza (ESWI), 2019, Difference between vaccines, antivirals and antibiotics, viewed 24 September 2019, from <https://eswi.org/knowledge-center/difference-between-vaccines-antivirals-and-antibiotics/>.
- [20] Monto, A.S. and Arden, N.H., 1992, Implications of viral resistance to amantadine in control of Influenza A, *Clinical Infectious Diseases* 15(2), pp.362-367.

- [21] Rubinstein, H., Marcu, A., Yardley, L. and Michie, S., 2015, Public preferences for vaccination and antiviral medicines under different pandemic flu outbreak scenarios, *BioMed Central Public Health* 15(1), p.190.
- [22] Abonyi, D.O., Abonyi, M.U., Esimone, C.O. and Ibezim, E.C., 2009, Plants as sources of antiviral agents, *African Journal of Biotechnology* 8(17), pp. 3989-3994.
- [23] Oxford, J.S., Bossuyt, S., Balasingam, S., Mann, A., Novelli, P. and Lambkin, R., 2003, Treatment of epidemic and pandemic Influenza with neuraminidase and M2 proton channel inhibitors, *Clinical Microbiology and Infection* 9(1), pp.1-14.
- [24] Rajasekaran, D., Palombo, E.A., Yeo, T.C., Ley, D.L.S., Tu, C.L., Malherbe, F. and Grollo, L., 2013, Identification of traditional medicinal plant extracts with novel anti-influenza activity, *Public Library of Science One* 8(11), p.e79293.
- [25] Shie, J.J., Fang, J.M., Wang, S.Y., Tsai, K.C., Cheng, Y.S.E., Yang, A.S., Hsiao, S.C., Su, C.Y. and Wong, C.H., 2007, Synthesis of Tamiflu and its phosphonate congeners possessing potent anti-influenza activity, *Journal of the American Chemical Society* 129(39), pp.11892-11893.
- [26] Morrison, D., Roy, S., Rayner, C., Amer, A., Howard, D., Smith, J.R. and Evans, T.G., 2007, A randomized, crossover study to evaluate the pharmacokinetics of amantadine and oseltamivir administered alone and in combination, *Public Library of Science One* 2(12), p.e1305.
- [27] Bryson, Y.J., Monahan, C., Pollack, M. and Shields, W.D., 1980, A prospective double-blind study of side effects associated with the administration of amantadine for Influenza A virus prophylaxis, *Journal of Infectious Diseases* 141(5), pp.543-547.
- [28] Monto, A.S., 2006, Vaccines and antiviral drugs in pandemic preparedness, *Emerging Infectious Diseases* 12(1), p.55.
- [29] Lagoja, I.M. and De Clercq, E., 2008, Anti-Influenza virus agents: Synthesis and mode of action, *Medicinal Research Reviews* 28(1), pp.1-38.
- [30] Hayden, F.G., 2006, Antivirals for Influenza: historical perspectives and lessons learned, *Antiviral Research* 71(2-3), pp.372-378.

- [31] McLaughlin, M.M., Skoglund, E.W. and Ison, M.G., 2015, Peramivir: an intravenous neuraminidase inhibitor, *Expert Opinion on Pharmacotherapy* 16(12), pp.1889-1900.
- [32] Cox, R.J., Mykkeltvedt, E., Sjursen, H. and Haaheim, L.R., 2001, The effect of zanamivir treatment on the early immune response to Influenza vaccination, *Vaccine* 19(32), pp.4743-4749.
- [33] Cheer, S.M. and Wagstaff, A.J., 2002, Zanamivir, *Drugs* 62(1), pp.71-106.
- [34] Hayden, F.G., Gubareva, L.V., Monto, A.S., Klein, T.C., Elliott, M.J., Hammond, J.M., Sharp, S.J. and Ossi, M.J., 2000, Inhaled zanamivir for the prevention of Influenza in families, *New England Journal of Medicine* 343(18), pp.1282-1289.
- [35] Ward, P., Small, I., Smith, J., Suter, P. and Dutkowski, R., 2005, Oseltamivir (Tamiflu®) and its potential for use in the event of an Influenza pandemic, *Journal of Antimicrobial Chemotherapy* 55(suppl_1), pp.i5-i21.
- [36] Hurt, A.C. and Kelly, H., 2016, Debate regarding oseltamivir use for seasonal and pandemic Influenza, *Emerging Infectious Diseases* 22(6), p.949.
- [37] Smith, J.R., Rayner, C.R., Donner, B., Wollenhaupt, M., Klumpp, K. and Dutkowski, R., 2011, Oseltamivir in seasonal, pandemic, and avian Influenza: a comprehensive review of 10-years clinical experience, *Advances in Therapy* 28(11), pp.927-959.
- [38] Smee, D.F., Wong, M.H., Bailey, K.W. and Sidwell, R.W., 2006, Activities of oseltamivir and ribavirin used alone and in combination against infections in mice with recent isolates of Influenza A (H1N1) and B viruses, *Antiviral Chemistry and Chemotherapy* 17(4), pp.185-192.
- [39] Kohno, S., Kida, H., Mizuguchi, M. and Shimada, J., 2010, Efficacy and safety of intravenous peramivir for treatment of seasonal Influenza virus infection, *Antimicrobial Agents and Chemotherapy* 54(11), pp.4568-4574.
- [40] Butler, D., 2014, Tamiflu report comes under fire: conclusions on stockpiling of antiviral drugs challenged, *Nature*, 508(7497), pp. 439-440, viewed 22 March 2020, from <https://search-proquest-com.uplib.idm.oclc.org/docview/1523911096?OpenUrlRefId=info:xri/sid:wcdiscov ery&accountid=14717> .

- [41] Nguyen, H.T., Fry, A.M. and Gubareva, L.V., 2012, Neuraminidase inhibitor resistance in Influenza viruses and laboratory testing methods, *Antiviral Therapy*, 17(1), p.159-173.
- [42] Cady, S.D., Mishanina, T.V. and Hong, M., 2009, Structure of amantadine-bound M2 transmembrane peptide of Influenza A in lipid bilayers from magic-angle-spinning solid-state NMR: the role of Ser31 in amantadine binding, *Journal of Molecular Biology* 385(4), pp.1127-1141.
- [43] Chan-Tack, K.M., Murray, J.S. and Birnkrant, D.B., 200, Use of ribavirin to treat Influenza, *New England Journal of Medicine* 361(17), pp.1713-1714.
- [44] Gilbert, B.E. and Knight, V.E.R.N.O.N., 1986, Biochemistry and clinical applications of ribavirin, *Antimicrobial Agents and Chemotherapy* 30(2), p.201.
- [45] Van Vlierberghe, H., Delanghe, J.R., De Vos, M. and Leroux-Roel, G., 2001, Factors influencing ribavirin-induced hemolysis, *Journal of Hepatology* 34(6), pp.911-916.
- [46] Crotty, S., Maag, D., Arnold, J.J., Zhong, W., Lau, J.Y., Hong, Z., Andino, R. and Cameron, C.E., 2000, The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen, *Nature Medicine* 6(12), pp.1375-1379.
- [47] Barry, W., Cockburn, F., Cornall, R., Price, J.F., Sutherland, G. and Vardag, A., 1986, Ribavirin aerosol for acute bronchiolitis, *Archives of Disease in Childhood* 61(6), pp.593-597.
- [48] Sidwell, R.W., Robins, R.K. and Hillyard, I.W., 1979, Ribavirin: an antiviral agent, *Pharmacology & Therapeutics* 6(1), pp.123-146.
- [49] Graci, J.D. and Cameron, C.E., 2006, Mechanisms of action of ribavirin against distinct viruses, *Reviews in Medical Virology* 16(1), pp.37-48.
- [50] Boltz, D.A., Aldridge, J.R., Webster, R.G. and Govorkova, E.A., 2010, Drugs in development for Influenza, *Drugs* 70(11), pp.1349-1362.
- [51] Smee, D.F., Hurst, B.L., Wong, M.H., Bailey, K.W. and Morrey, J.D., 2009, Effects of double combinations of amantadine, oseltamivir, and ribavirin on Influenza A (H5N1) virus infections in cell culture and in mice, *Antimicrobial Agents and Chemotherapy* 53(5), pp.2120-2128.

- [52] Ilyushina, N.A., Hay, A., Yilmaz, N., Boon, A.C., Webster, R.G. and Govorkova, E.A., 2008, Oseltamivir-ribavirin combination therapy for highly pathogenic H5N1 Influenza virus infection in mice, *Antimicrobial Agents and Chemotherapy* 52(11), pp.3889-3897.
- [53] Nguyen, J.T., Hoopes, J.D., Le, M.H., Smee, D.F., Patick, A.K., Faix, D.J., Blair, P.J., De Jong, M.D., Prichard, M.N. and Went, G.T., 2010, Triple combination of amantadine, ribavirin, and oseltamivir is highly active and synergistic against drug resistant Influenza virus strains in vitro, *Public Library of Science One* 5(2), p.e9332.
- [54] Katakya, A. and Handique, P.J., 2010, A brief overview on *Andrographis paniculata* (Burm. f) Nees., a high valued medicinal plant: Boon over synthetic drugs, *Asian Journal of Applied Science and Technology* 6, pp.113-118.
- [55] Rates, S.M.K., 2001, Plants as source of drugs, *Toxicon* 39(5), pp.603-613.
- [56] Mehrbod, P., Abdalla, M.A., Njoya, E.M., Ahmed, A.S., Fotouhi, F., Farahmand, B., Gado, D.A., Tabatabaian, M., Fasanmi, O.G., Eloff, J.N. and McGaw, L.J., 2018, South African medicinal plant extracts active against Influenza A virus, *BMC Complementary and Alternative Medicine* 18(1), p.112, viewed 29 April 2020, from <https://link.springer.com/article/10.1186/s12906-018-2184-y> .
- [57] Vimalanathan, S. and Hudson, J., 2014, Anti-influenza virus activity of essential oils and vapours, *American Journal of Essential Oils and Natural Products* 2(1), pp.47-53.
- [58] Light, M.E., McGaw, L.J., Rabe, T., Sparg, S.G., Taylor, M.B., Erasmus, D.G., Jäger, A.K., Van Staden, J. and Eloff, J.N., 2002, Investigation of the biological activities of *Siphonochilus aethiopicus* and the effect of seasonal senescence, *South African Journal of Botany* 68(1), pp.55-61.
- [59] York, T., De Wet, H. and Van Vuuren, S.F., 2011, Plants used for treating respiratory infections in rural Maputaland, KwaZulu-Natal, South Africa, *Journal of Ethnopharmacology* 135(3), pp.696-710.
- [60] Rezanka, T., Siristova, L. and Sigler, K., 2009, Antiviral Sesqui-, Di- and Sesterterpenes, *Anti-Infective Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Infective Agents)* 8(3), pp.169-192.

- [61] Sohail, M.N., Rasul, F., Karim, A., Kanwal, U. and Attitalla, I.H., 2011, Plant as a source of natural antiviral agents, *Asian Journal of Animal and Veterinary Advances* 6, pp.1125-1152.
- [62] Zhang, L., Cheng, Y.X., Liu, A.L., Wang, H.D., Wang, Y.L. and Du, G.H., 2010, Antioxidant, anti-inflammatory and anti-influenza properties of components from *Chaenomeles speciose*, *Molecules* 15(11), pp.8507-8517.
- [63] Fennell, C.W., Light, M.E., Sparg, S.G., Stafford, G.I. and Van Staden, J., 2004, Assessing African medicinal plants for efficacy and safety: agricultural and storage practices, *Journal of Ethnopharmacology* 95(2-3), pp.113-121.
- [64] Hartzell, J.F., 2011, Response of the Endangered Medicinal Plant: *Siphonochilus Aethiopicus* (Schweif) BL Burt. to Agronomic Practices, PhD Thesis, Pietermaritzburg, University of KwaZulu-Natal.
- [65] Van Wyk, B.E., 2011, The potential of South African plants in the development of new medicinal products, *South African Journal of Botany* 77(4), pp.812-829.
- [66] Menzel, T.R., Weldegergis, B.T., David, A., Boland, W., Gols, R., van Loon, J.J. and Dicke, M., 2014, Synergism in the effect of prior jasmonic acid application on herbivore-induced volatile emission by Lima bean plants: transcription of a monoterpene synthase gene and volatile emission, *Journal of Experimental Botany* 65(17), pp.4821-4831.
- [67] Ramakrishnan, M.A., 2016, Determination of 50% endpoint titer using a simple formula, *World Journal of Virology* 5(2), p.85.
- [68] Piñero, J.C. and Dorn, S., 2007, Synergism between aromatic compounds and green leaf volatiles derived from the host plant underlies female attraction in the oriental fruit moth, *Entomologia Experimentalis et applicata* 125(2), pp.185-194.

DEET **(2)** has been the most widely used synthetic insect repellent on the market. With each new generation, insects build a resistance towards synthetic compounds and are manufacturers are required to increase the concentration of these compounds.^[6] This increase in DEET concentration has caused the absorption of the compound through the human skin, resulting in many health issues.^[6] These health issues have caused concern and has increased the demand for more natural insect repellents.^[2,9,10]

5.1.2 Natural insect repellents

There are about 344 species of plants already discovered with insect repellent properties, but it is possible that many more are still undiscovered^[11,12] In some cultures, people have been using plant-based insect repellents long before synthetic compounds were introduced.^[10,11,13] They are considered to be safe, trustworthy and better for the environment, but are in some countries the only affordable and available solution.^[13-15] Most plant-based insect repellents are used in the form of essential oils since they are easily isolated and environmentally friendly.^[3,15] Some of these oils include citronella, lavender, peppermint, eucalyptus, orange and lemon essential oils and are very popular worldwide.^[12,14,16] Figure 5.2 shows four commercialized insect repellents sold in South Africa containing essential oils with repellent properties:

- Wild Basil & Lemongrass Mosquito Repellent **(3)**: basil, thyme, camphor, eucalyptus and lavender.
- Earthsap Bugs Away Roll-on **(4)**: lemongrass, citronella, eucalyptus, mint and camphor.
- The Apothecary Insect Repellent Spray **(5)**: citronella, lemongrass, lavender, cinnamon and eucalyptus.
- Natural Orange House Hold Insect repellent **(6)**: orange, lemongrass oil and eucalyptus.



Figure 5.2 Commercialized insect repellents containing different essential oils; Wild Basil & Lemongrass mosquito repellent **(3)**, Earthsap Bugs Away roll-on **(4)**, The Apothecary insect repellent spray **(5)** and Natural Orange House Hold insect repellent **(6)**.

Essential oils have also been researched for their insect repellent properties with the aim of identifying their active compounds for commercialization.^[13,15,16] These compounds are known as the secondary metabolites that protects the plant against its natural enemies.^[17-19] The activity of these metabolites are dependent on their concentration in the plant or extract.^[20-22] Many of these compounds have been identified and used for commercialization but the demand for more has increased over the years.^[3,13,23,24]

5.1.3 Noot-a-Bug

In this chapter a natural insect repellent sample was provided from Applied Protein Biotechnologies (Pty) Ltd. (APBio). The company owns and have trademarked the Noot-a-Bug product, which contains a proprietary essential oil. The essential oil was prepared through the reaction of natural sweet orange oil blended with a naturally sourced enzyme cocktail.

The purpose of the analysis of Noot-a-Bug was to confirm the conversion of valencene, a precursor to nootkatone in the sweet orange oil with the help of the naturally sourced enzymes. Nootkatone is a well-known insect repellent, but its synthesis is very expensive and there is a need for more economical methods for the production of the compound.^[25] Once the conversion is confirmed, the essential oil containing nootkatone could be used as a natural insect repellent in households, restaurants and food markets by means of diffusion of the essential oil.

5.1.3.1 Nootkatone as an insect repellent compound

Nootkatone (**7**) is a popular natural aromatic compound from citrus fruit and grapefruit peels and is used for its insect repellent properties.^[26-29] It has been the targeted compound by many synthetic biological companies for large-scale production.^[23,24,30,31] This has proven problematic because of low yields and the danger its isolation poses to the environment.^[31,32]

The synthetic production of nootkatone by the oxidation of valencene has also proven troublesome.^[23,24,31,33] Up to recent times there has been no easier, sustainable, natural conversion of valencene to nootkatone. One possibility of achieving this conversion is by using an essential oil known to contain valencene.

Sweet orange oil was selected since it's well-known for its insect repellent properties and contains valencene, and many other repellent compounds.^[16,34] Valencene was used for the conversion to nootkatone, resulting in the production of Noot-a-Bug.

5.1.3.2 Sweet orange oil

Sweet orange oil has an aromatic, sweet smell and is used in fragrances.^[21,35] The essential oil contains about 100 compounds whereas 85 – 99% are volatiles and 1 – 15% non-volatiles.^[21,35,36] It is well-known for containing many compounds with repellent properties that acts better synergistically for the plant's protection.^[20,21,34] The oils contain different classes of compounds with repellent properties. The most popular compounds

are monoterpenes, such as α -pinene (**8**), β -myrcene (**9**), cymene (**10**), linalool (**11**), carvone (**12**) and limonene (**13**) and the sesquiterpene valencene (**14**).^[29,37,38]

Limonene (**13**) is the main constituent of the essential oil of sweet orange oil and other citrus fruit, and is therefore the major compound.^[26,27,39,40] It is one of the most economical insect repelling compounds in nature and works best synergistically with other repellent compounds.^[40,41]

Valencene (**14**) is a very popular, inexpensive, commercially available and easily accessible repellent compound.^[27] It is used in the natural conversion to nootkatone by the allylic hydroxylation of valencene.^[24,42]

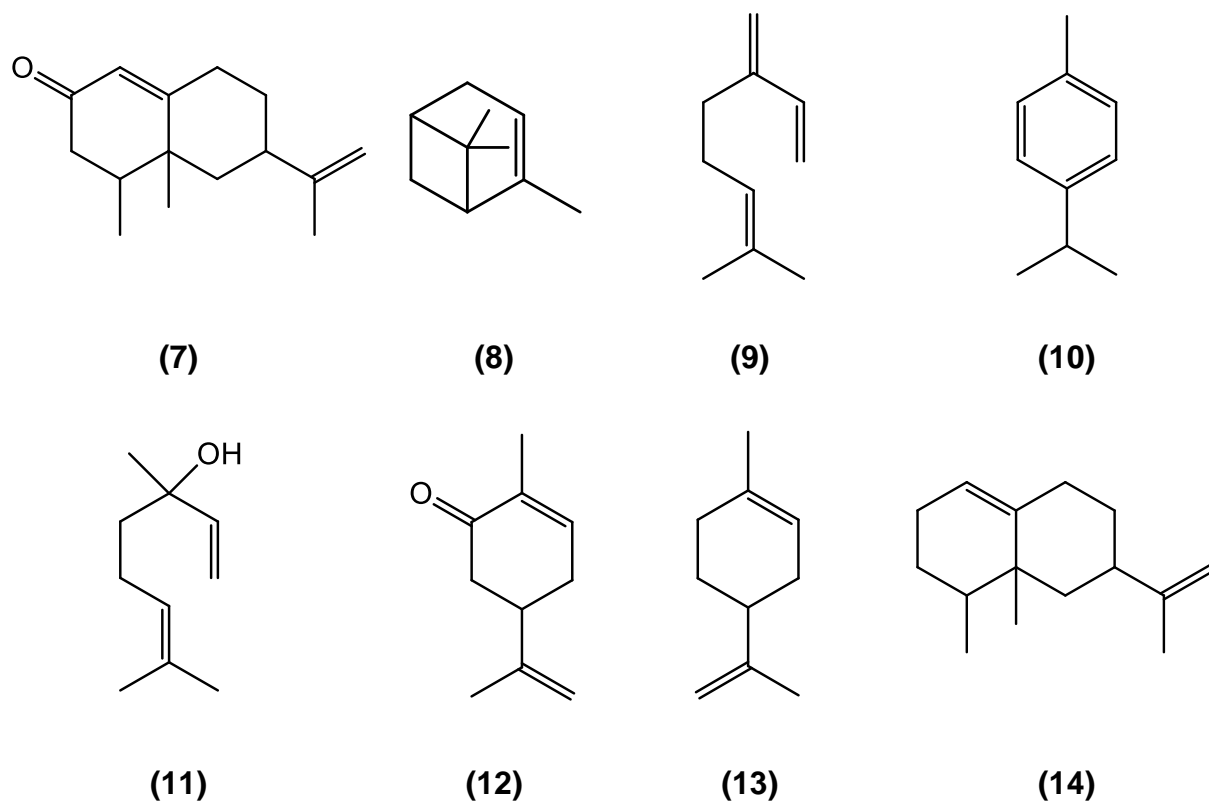


Figure 5.3 Active compounds with insect repellent properties.^[27,38,40,43]

There is an increase in demand for more natural products, as synthetic compounds are slowly being replaced by natural compounds.^[3] The use of sweet orange oil for the production of nootkatone is no different. The analysis of Noot-a-Bug to confirm the conversion was done through gas chromatography-mass spectroscopy (GC-MS) and for

the commercial diffuser formulation, through headspace-solid phase microextraction (HS-SPME).

The use of natural insect repellents however results in the loss of effective repellency due to the short-lived volatile compounds.^[2,3] It is thus necessary to investigate how long the active compound, nootkatone, is still present in the Noot-a-Bug sample upon release as a vapour using HS-SPME. This will assist in detecting the presence of the compounds in the vapours after being diffused into the open air for a known period of time.^[44]

5.1.4 GC-MS and HS-SPME analysis

GC-MS is the method of combining gas-liquid chromatography and mass spectrometry to qualitatively and quantitatively determine phytochemical substances within a sample.^[45,46] It is used for drug detection, environmental analysis, explosives investigation, and identifying unknown samples.^[46,47]

GC-MS analysis can be used to identify the presence of a specific compound or determine the chemical composition of an essential oil or extract.^[48,49] Compounds are charged with a high beam of energy that separates the ions into charged fragments. These fragments have a certain mass/charge ratio which is detected and provides the relative abundance of the compounds.^[50,51] Compounds are then identified by comparing retention times and mass spectra of compounds to known compounds in a data library.^[45,48,49] This method was used to determine the chemical profiles of the sweet orange oils and Noot-a-Bug to confirm the conversion of nootkatone.

HS-SPME has many applications including environmental, food, fragrances, forensics, pharmaceuticals and natural products.^[52-54] It is a preparative technique used to qualitatively and quantitatively identify compounds of a solid or liquid sample.^[53] The vapour of the sample is directly exposed to a solid coated fibre, the compounds adsorb onto it and then gets inserted into the GC for analysis and identification.^[54-56] It is solvent free and an effective method of analysing essential oils to identify volatile compounds that are more concentrated.^[57-59]

Essential oils are mostly used as natural insect repellents and are used in many households. These essential oils are used in diffusers and vaporised into the open air. Many consumers use candles as well, but previous research has confirmed that diffusers are more effective than candles as insect repellents.^[60] Air sampling has become very popular and can be used to determine the compounds released from the vapours of essential oils.

To our knowledge HS-SPME has not yet been used to confirm the presence of compounds with repellent properties in essential oils that are being released into the open air as a vapour from a diffuser or vaporiser and as this investigated.

5.2 Problem statement and justification

Nootkatone is a compound with repellent properties of low yield, high cost and its synthesis can be dangerous to the environment. Nootkatone can easily be oxygenated from valencene by using sweet orange oil and a naturally sourced enzyme cocktail. This makes for an easier, natural and cost-effective method of producing nootkatone. The chapter consists of two parts: 1) Analysis of the sweet orange oil and Noot-a-Bug through GC-MS to confirm the oxygenation of valencene to nootkatone and 2) investigate the longevity of nootkatone in Noot-a-Bug in its vaporised form, after being diffused, through HS-SPME-GC-MS. The continuous diffusion of Noot-a-Bug over 24 hours was investigated to confirm nootkatone and other repellent compounds were still being released after this time.

5.3 Goal and objectives

- To investigate the profiles of sweet orange oil and Noot-a-Bug using GC-MS;
- To use the GC-MS data to confirm the conversion of valencene to nootkatone in the samples;
- To identify other repellent compounds in both samples using the NIST library;
- To diffuse Noot-a-Bug into a controlled room over 24 h and use HS-SPME-GC-MS to analyse the vapours diffused at fixed time intervals;

- To identify whether active compounds are still being diffused after 24 h by analysing the GC-MS data; and
- Confirmation of the presence of other repellent compounds during and after the 24 hours of diffusion.

5.4 Methodology

5.4.1 Standards

Valencene (C₁₅H₂₄; 204.36 g/mol) and nootkatone (C₁₅H₂₂O; 218.34 g/mol) both had a purity >98% and were purchased from Sigma Aldrich. The compounds were used as standards to confirm their presence in the oils.

5.4.2 Noot-a-Bug

The Noot-a-Bug samples provided by Applied Protein Biotechnology (Pty.) Ltd (www.apbio.co.za). They own and have trademarked the Noot-a-Bug sample. It was prepared by blending neutral sweet orange oil with a naturally sourced enzyme cocktail.

5.4.3 GC-MS analysis for identification of targeted compounds

Standards, valencene and nootkatone, sweet orange oil and Noot-a-Bug were directly injected into the GC-MS to determine the presence of the targeted compounds. The same sweet orange oil used to manufacture the Noot-a-Bug, was used for GC-MS analysis to identify presence of valencene and nootkatone. The standards and sweet orange oil were prepared by dissolving 1.00 mg of sample in 1.00 mL hexane (1.00 mg/mL) and Noot-a-Bug by dissolving 1.00 mg in 1.00 mL methanol, and injecting 1.00 µL into the inlet of the GC at 250°C.

Analyte separation was done using a LECO Pegasus 4D GC–TOF–MS including an Agilent 7890 GC (LECO Africa (Pty) Ltd., Kempton Park, South Africa) on an apolar Rxi-5Sil MS 30 m×0.25 mm ID×0.25 µm df (Restek, Bellefonte, PA, USA) capillary column. The carrier gas, helium, was of ultra-high purity (UHP) grade (Afrox, Gauteng, South

Africa) and was set at a flow rate of 1.00 mL/min in the constant flow mode. The GC inlet was operated in the split mode (50:1 split ratio). The GC oven temperature program was held for 3 min at 40°C to 5 min at 300°C at 10°C/min, with a run time of 35.9 min. The MS transfer line temperature was set at 280°C and the ion source temperature was set at 230°C. The electron energy was 70 eV in the electron impact ionization mode (EI+), the data acquisition rate was 10 spectra/s, the mass acquisition range was 40–500 Daltons, and the detector voltage was set at 1750 V. A NIST 14 Mass Spectral Library version 2.2 was used to identify and confirm compounds detected based on their molecular ions and percentage similarities (%).

After confirmation of nootkatone in the Noot-a-Bug sample it was used to determine the longevity of the compound in the sample after being diffused over a period of 24 hours.

5.4.4 Diffusing of Noot-a-Bug into the environment

An ALUMI-China, Aroma Nebulizer Rechargeable Battery Inside Diffuser was used for continued diffusion of the insect repellent into the environment. The experiment was conducted in a controlled 3.00 m³ fume hood and the diffuser's airflow was set at its lowest area coverage (10 m³). The repellent was diffused for 24 h at which air samples were taken at different times (0 h, 1 h, 2 h, 4 h, 6 h and 24 h).

5.4.5 HS-SPME-GC-MS for identification of targeted compounds in the diffused vapour

The longevity and sustainability of the Noot-a-Bug sample was determined by using HS-SPME and inserting it into the GC-MS. Sorptive extraction was done with a SPME device which was fitted with a 2 – 50/30 µm DVB/Carboxen/PDMS StableFlex fiber (Supelco, Sigma-Aldrich (Pty) Ltd. Kempton Park, South Africa). The fiber was exposed to the head space above the exit of the diffuser for 5 min at each time interval, sampling the vapours of the Noot-a-Bug being released in its vapour form. After extraction the compounds were desorbed from the SPME fiber for 5 min in the injection port of a GC–MS at 250°C.

The same method used to analyse the standards and samples above (section 5.4.4), was used for the HS-SPME-GC-MS analysis.

5.5 Results and discussions

5.5.1 GC-MS analysis of insect repellent

Nootkatone is one of the most well-known insect repellent compounds around, but unfortunately has proven troublesome with synthesise.^[26,27,31] The oxygenation from valencene to nootkatone has also presented its own problems and a more sustainable method of production of nootkatone is necessary.^[23,24,31] This was attempted by APBio who blended sweet orange oil with a naturally sourced enzyme cocktail to produce Noot-a-Bug. The aim of this conversion was to produce a natural insect repellent in diffusers for the hospitality industry or household use. To achieve this the conversion to nootkatone first had to be confirmed and was done through GC-MS analysis. The chemical profile of sweet orange oil and Noot-a-Bug was determined to confirm the presence of valencene and nootkatone in both samples.

The first analysis was performed by injecting the standards of the targeted compounds, valencene and nootkatone. The retention times and MS data from the NIST library of the standards were also used to identify the compounds in the oil samples.

Figure 5.4.(1) shows the chromatogram of the standard valencene with an intense peak at 134.50 s, which is the solvent peak of hexane. There is also another intense peak at 1015.80 s, a m/z of 204 and molecular formula $C_{15}H_{24}$.^[26,61] The peak had a 94% match to the NIST library to valencene and was used for further analysis to confirm valencene in the oils.

Figure 5.4.(2) shows the chromatogram of the standard nootkatone with an intense peak at 132.30 s, which is the solvent peak of hexane. A less intense peak at 1258.60 s was matched as nootkatone based on the NIST library (94%). It showed a m/z of 218 and molecular formula $C_{15}O_{22}O$.^[62]

The retention times and NIST library matches of both standards were used to confirm the presence of two compounds in the sweet orange oil, Noot-a-Bug and the vapours diffused (section 5.5.2).

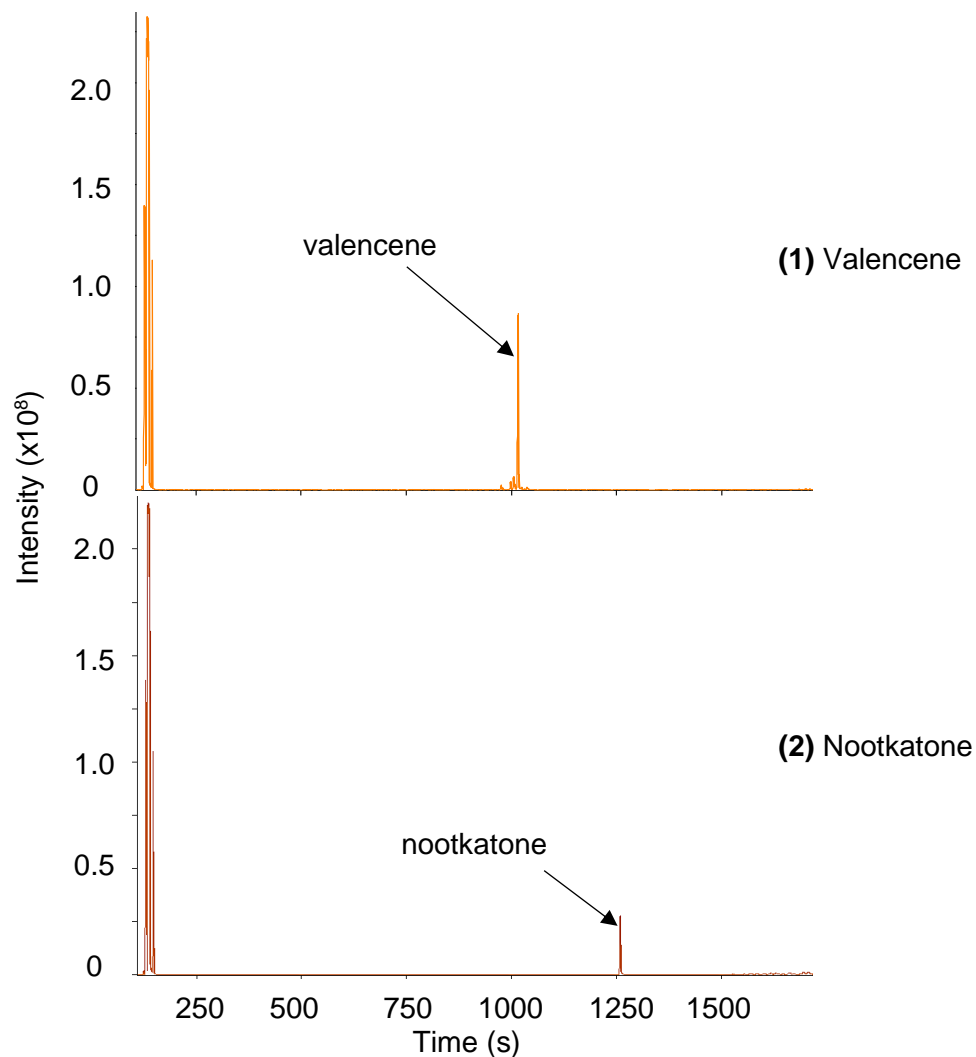


Figure 5.4 Chromatograms from GC-MS analysis of **(1)** valencene; 1015.80 s and **(2)** nootkatone; 1258.60 s.

Figure 5.5.(1) shows the chromatogram of the sweet orange oil. The two intense peaks at 126.90 and 129.40 s were the solvent peaks of hexane. The intensity of the peak caused the splitting of the peak, therefore the two peaks were seen for hexane. Two other distinct peaks were observed at 531.50 and 1016.10 s. The peak at 1016.10 s was confirmed to be valencene when compared to the standard (1015.80 s) (Figure 5.4.(1)).

The NIST library also matched the compound to valencene which was present in Noot-a-Bug.

The NIST library matched the peak at 531.50 s to limonene (**13**) (91% match) with a m/z of 136 and molecular formula of $C_{10}H_{16}$. Based on the intensity of the peak, this indicated it to be the major compound and was also confirmed by literature.^[26,27,39,40]

No nootkatone was found in the sweet orange oil, but other compounds with repellent properties were identified by the NIST library in the sweet orange oil: α -pinene (**8**), β -myrcene (**9**), cymene (**10**), linalool (**11**) and carvone (**12**). However, these compounds were not confirmed by the retention times of their standards.

Figure 5.5.(2) shows the chromatogram of the Noot-a-Bug sample indicating a very distinct peak at 518.00 s which was confirmed by the NIST library to be limonene (93% match), the major compound of sweet orange oil. The NIST library also confirmed the presence of valencene, at 1015.00 s, with a less intense peak than limonene. This was also confirmed by the standard at 1015.80 s.

The NIST library confirmed the presence of nootkatone (93% match) at 1259.50 s, which was a minor peak. This was also confirmed by the standard at 1258.60 s (Figure 5.4.(2)). This suggested that nootkatone was present in the in the Noot-a-Bug sample, confirming the successful conversion of valencene to nootkatone.

Other compounds with repellency properties identified by the NIST library to be present in the Noot-a-Bug sample were: α -pinene (**8**), β -myrcene (**9**), cymene (**10**), linalool (**11**) and carvone (**12**). Most of these compounds were only present in small amounts in the oil based on the intensities of the peaks, however could contribute to the repellent properties of the oil.^[63]

The area percentage (area %) of the total chromatogram of each of the peaks corresponding to the compounds with repellent properties was also investigated and shown in Table 5.1. The table shows the percentage area for the compounds of the total chromatographic area for sweet orange oil and Noot-a-Bug.

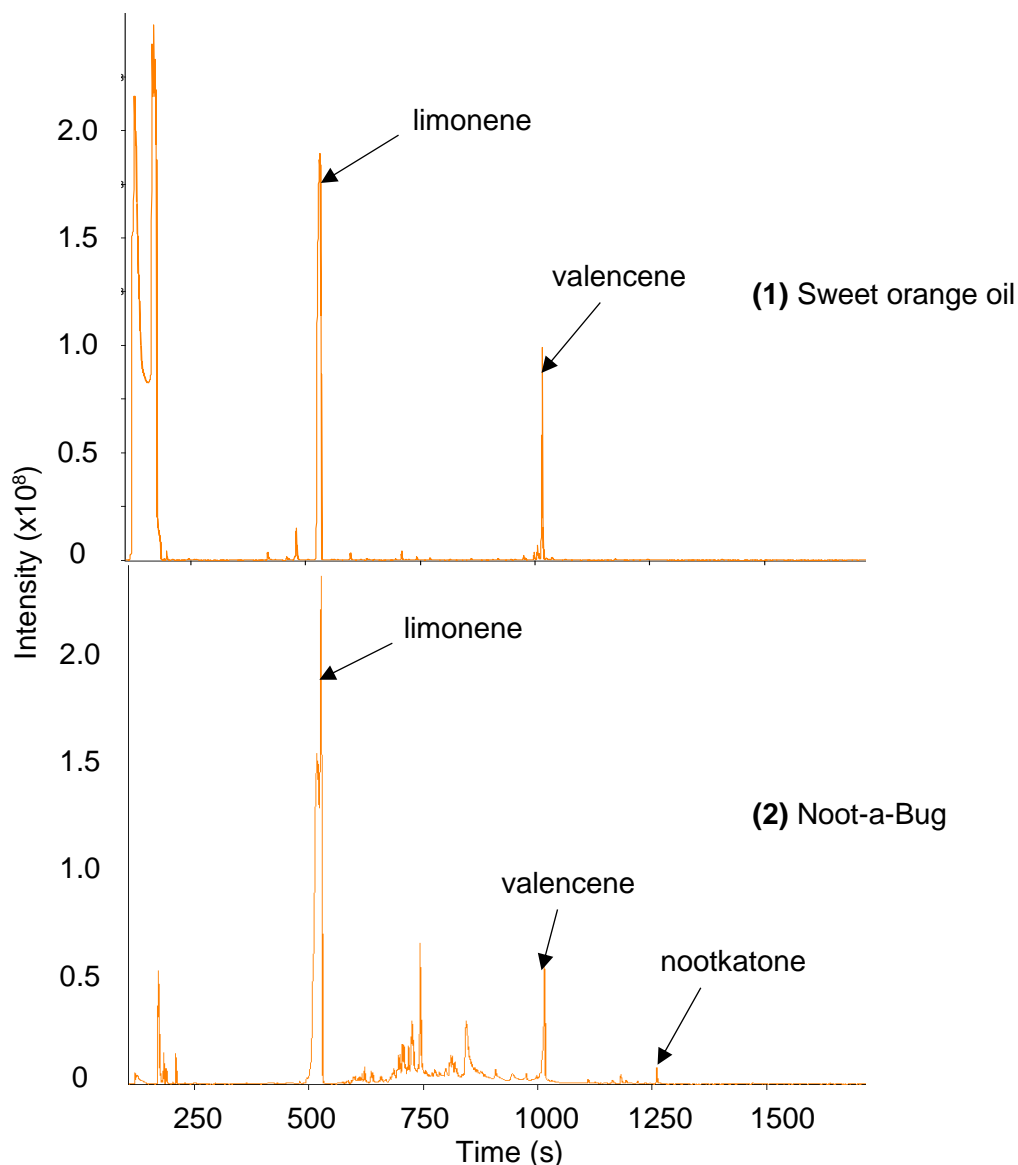


Figure 5.5 Chromatograms from GC-MS analysis of **(1)** Sweet orange oil and **(2)** Noot-a-Bug.

The area percentage of the total chromatogram for valencene in the sweet orange oil was 1.07% with nootkatone not being detected. This confirmed that there was an adequate amount of valencene available for the oxygenation. The Noot-a-Bug sample showed the area percentage of the total chromatogram for nootkatone to be 0.18% which confirmed the successful oxygenation of valencene to nootkatone. Even though the conversion was

successful there was still valencene present (1.35%) in the Noot-a-Bug sample indicating that the conversion does not go to completion (Figure 5.5.(2)) and hence further optimization of the enzyme conversion should be investigated.

Table 5.1 The area percentage of the total chromatogram of valencene, nootkatone and other compounds with known repellency properties.

Area percentage of the total chromatogram of each repellent compound						
Sample	Valencene	Nootkatone	Limonene	β -myrcene	Linalool	Carvone
Sweet orange oil	1.07	Not detected	6.25	0.21	0.04	0.02
Noot-a-Bug	1.35	0.18	14.2	0.02	0.01	0.01

Limonene (**13**) showed a significantly high area percentage of the total chromatogram in both the sweet orange oil (6.25%) and Noot-a-Bug (14.2%) which confirmed it to be the major compound in both samples.^[21] Smaller amounts of other compounds with repellency properties are also shown in Table 5.1. The large differences in the area percent of the compounds between the sweet orange oil and the Noot-a-Bug was attributed to the formulation and the addition of various other ingredients such as enzymes, co-factors and media that were added to Noot-a-Bug.

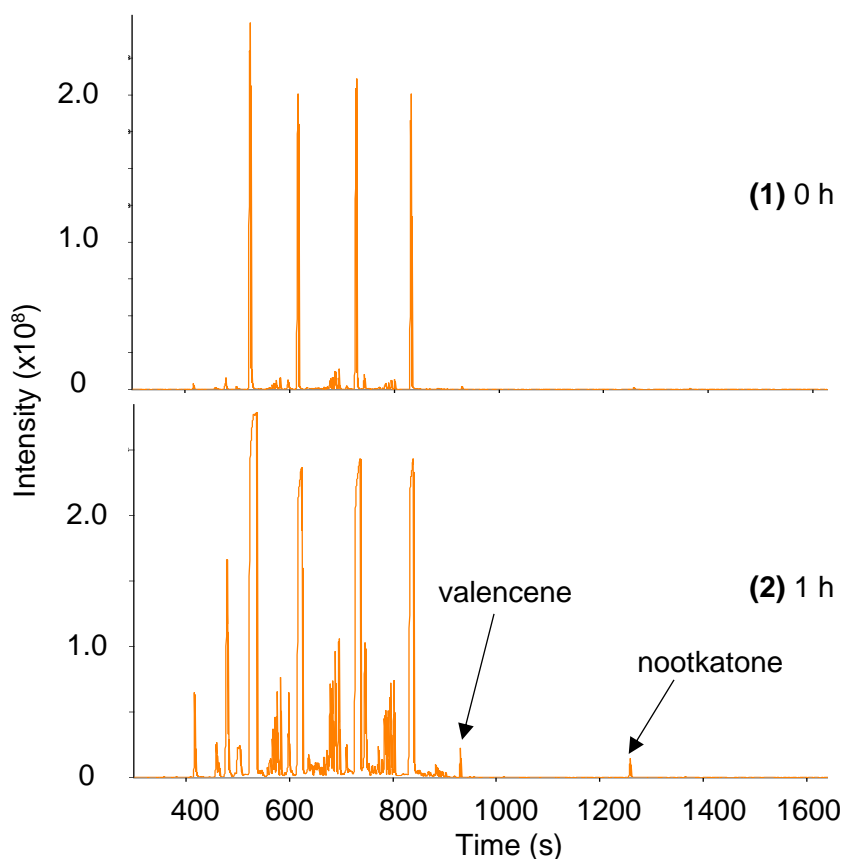
5.5.2 HS-SPME-GC-MS analysis of Noot-a-Bug released as a vapour

The successful conversion of nootkatone from valencene in Noot-a-Bug was confirmed and further investigated to determine the longevity of nootkatone and other repellent compounds in the sample. One of the major problems with natural insect repellents is the lack of longevity of the active compound(s).^[6]

The presence of nootkatone and other repellent compounds in the insect repellent was determined by HS-SPME-GC-MS over a period of 24 h of continuous diffusion. The insect repellent was diffused in a fume hood and a fibre was exposed to the continuous release

of vapours at the outlet of the diffuser. This was injected into the GC-MS where nootkatone, valencene and other repellent compounds were determined.

Figure 5.6 shows the chromatograms of the vaporised Noot-a-Bug sample released from the diffuser at different time intervals and shows that nootkatone is present in all the chromatograms at different times. At the start of the experiment (0 h) nootkatone showed a low intensity peak (Figure 5.6.(1)) and was continuously present as the experiment progressed including the 24-h time point. This indicated that the compound is present in the vapours as the oil is vaporised using the diffuser suggesting that the repellency properties will be effective over a 24-h period.



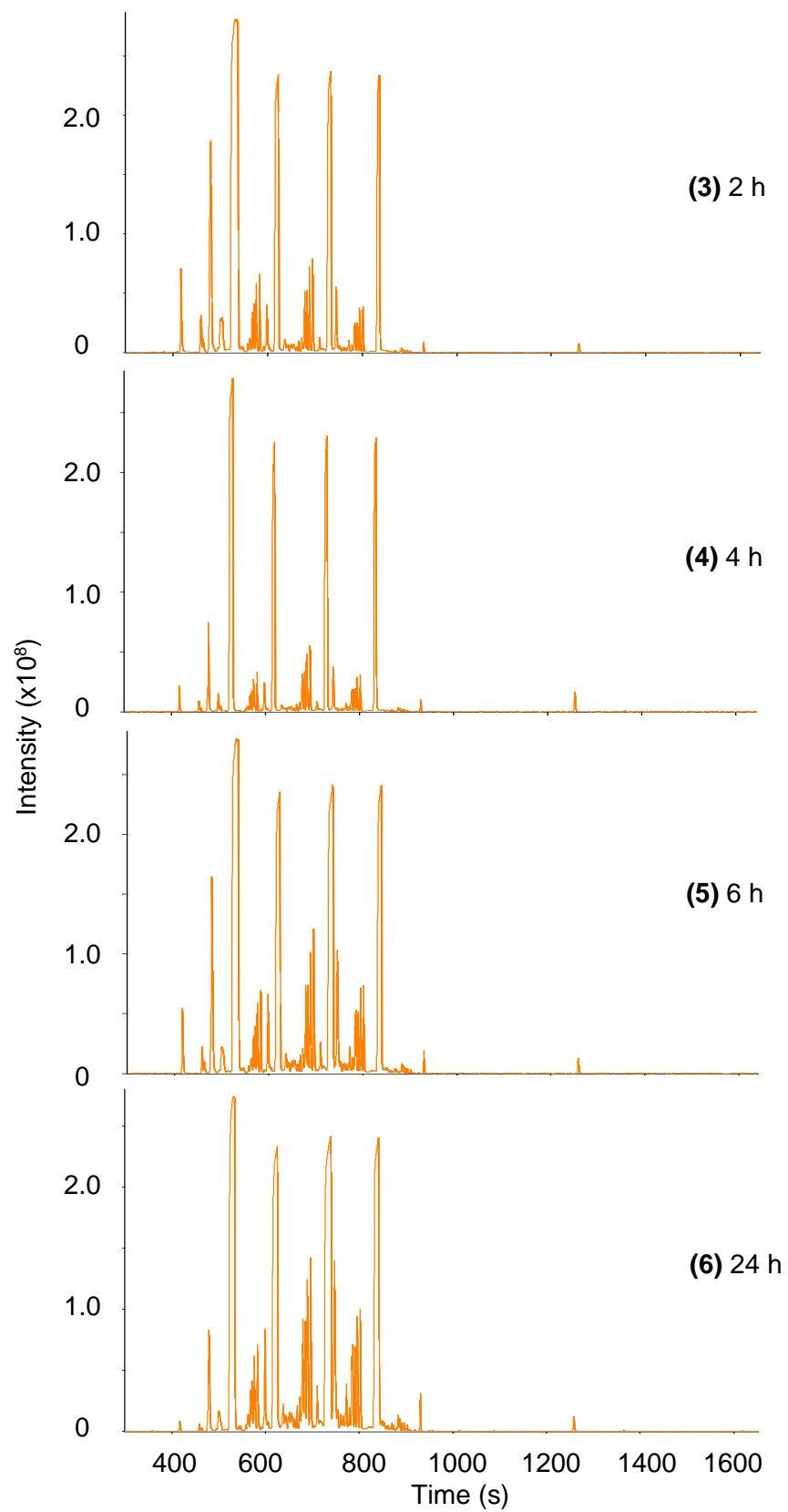


Figure 5.6 Chromatograms from GC-MS of Noot-a-Bug at **(1)** 0 h, **(2)** 1 h, **(3)** 2 h, **(4)** 4 h, **(5)** 6 h and **(6)** 24 h.

Table 5.2 shows the area percentage of the total chromatogram of nootkatone, valencene and other repellent compounds released from the diffusion of Noot-a-Bug at different time intervals and shows that at all time intervals, nootkatone was consistently being released.

At the start of the experiment there was no valencene detected by the GC-MS (Figure 5.6.(1)), but was detected as the experiment progressed. Even though valencene had a more intense peak and area percentage of the total chromatogram than nootkatone in Noot-a-Bug (Figure 5.5.(2)), this ratio changed when the sample was vaporised and released into the air, in all likelihood due to the difference in the volatility of the two compounds.

Other repellent compounds were also released and identified through the NIST library. Table 5.2 shows that based on the area percentage of the total chromatogram of limonene, it was still the major compound in the vaporised Noot-a-Bug. Other minor repellent compounds like α -pinene (**8**), β -myrcene (**9**), cymene (**10**), linalool (**11**) and carvone (**12**) were also still being released at trace amounts after the continuous diffusion of 24 h. Only cymene was not detected after 4 h of diffusion. The release of these repellent compounds will improve the repellency of Noot-a-Bug since the compounds could work synergistically to repel insects.

The analysis of the results obtained from the HS-SPME-GC-MS confirmed that there is a continuous release of nootkatone over a 24-h period and the sample should be used for further repellency testing on life specimens.

Table 5.2 The retention time and area percentage of the total chromatogram of repellent compounds in Noot-a-Bug diffused over a 24-h period.

% Area of the total chromatogram at different time intervals (hours)						
Name	0 h	1 h	2 h	4 h	6 h	24 h
α -Pinene	0.14	0.62	0.81	0.31	0.43	0.05
β -myrcene	0.34	2.42	3.24	1.22	1.85	0.68
Cymene	0.01	0.06	0.08	-	-	-
Limonene	27.9	6.37	4.91	29.8	4.09	8.03
Linalool	0.28	0.61	0.46	0.40	0.53	0.56
Carvone	0.39	1.23	0.67	0.59	1.04	1.30
Valencene	-	0.01	0.002	0.01	0.004	0.01
Nootkatone	0.05	0.13	0.07	0.25	0.10	0.07

5.6 Conclusion

The demand for natural insect repellents have increased over the years since synthetic compounds hold many side effects to the human body and the environment. This demand has led to the search for naturally occurring repellent compounds for which certain essential oils are very popular for this use. A very popular insect repellent compound, nootkatone, is used as an insect repellent and can be synthesized or converted from valencene, a cheaper precursor. The main problem with nootkatone availability is that its synthetic and natural production are very expensive.

APBio a privately owned company investigated the oxygenation of valencene present in sweet orange oil into nootkatone using an enzyme cocktail and trademarked the final ingredient as Noot-a-Bug. Noot-a-Bug with nootkatone, being one of the compounds in the product, could then be used as an insect repellent. The success of the conversion was investigated through the use of analysis using GC-MS of the Noot-a-Bug sample. The results of this study confirmed the presence of the valencene in the sweet orange oil

which was used for the conversion. This provided the evidence that the pre-cursor compound was present in sufficient levels in the oil for any successful enzyme conversion. The analysis of the Noot-a-Bug sample indicated the presence of nootkatone thereby confirming the success of the enzymatic conversion. However, although this was not a quantitative study, the area percentage of the nootkatone of the total area of the chromatograms indicated this was not a major compounds and large amounts of the valencene remained unconverted and that further optimization of the enzymatic conversion would be recommended. Other known compounds with reported repellency properties were also identified in small levels in Noot-a-Bug which could support a synergistic effect of the various compounds towards repelling insects.

The longevity of the nootkatone during diffusion (release) was analysed using HS-SPME-GC-MS. The sample was vaporised from a diffuser over a period of 24 h and vapours absorbed onto a SPME fiber at different time intervals (0 h, 1 h, 2 h, 4 h, 6 h and 24 h), and introduced into the GC-MS.

The GC-MS data and comparison of the mass spectral data with the NIST library confirmed that nootkatone and other insect repellent compounds were being diffused after 24 h of diffusion.

5.7 References

- [1] Tavares, M., da Silva, M.R.M., de Siqueira, L.B.D.O., Rodrigues, R.A.S., Bodjolle-d'Almeida, L., Dos Santos, E.P. and Ricci-Júnior, E., 2018, Trends in insect repellent formulations: A review, *International Journal of Pharmaceutics* 539(1-2), pp.190-209.
- [2] Maharaj, R., Maharaj, V., Newmarch, M., Crouch, N.R., Bhagwandin, N., Folb, P.I., Pillay, P. and Gayaram, R., 2010, Evaluation of selected South African ethnomedicinal plants as mosquito repellents against the *Anopheles arabiensis* mosquito in a rodent model, *Malaria Journal* 9(1), p.301.
- [3] Nerio, L.S., Olivero-Verbel, J. and Stashenko, E., 2010, Repellent activity of essential oils: a review, *Bioresource Technology* 101(1), pp.372-378.

- [4] Licciardi, S., Hervé, J.P., Darriet, F., Hougard, J.M. and Corbel, V., 2006, Lethal and behavioural effects of three synthetic repellents (DEET, IR3535 and KBR 3023) on *Aedes aegypti* mosquitoes in laboratory assays, *Medical and Veterinary Entomology* 20(3), pp.288-293.
- [5] Sharp, B.L., 1983, *Anopheles merus* (Donitz) its biting cycle in relation to environmental parameters, *Journal of the Entomological Society of Southern Africa* 46(2), pp.367-374.
- [6] Blum, M., Roitberg, M. and Burlington Bio-Medical and Scientific Corp, 1999, *Natural insect repellent formula and method of making same*, U.S. Patent 5,885,600.
- [7] Ditzen, M., Pellegrino, M. and Vosshall, L.B., 2008, Insect odorant receptors are molecular targets of the insect repellent DEET, *Science* 319(5871), pp.1838-1842.
- [8] National Center for Biotechnology Information, 2020, PubChem Compound Summary for CID 3036, *Clofenotane*, viewed 12 August 2020, from <https://pubchem.ncbi.nlm.nih.gov/compound/Clofenotane>.
- [9] Fradin, M. and Day, J., 2002, Comparative Efficacy of Insect Repellents against Mosquito Bites, *New England Journal of Medicine* 347(1), pp.13-18.
- [10] Schultz, G., Peterson, C. and Coats, J., 2006, Natural insect repellents: activity against mosquitoes and cockroaches, *Natural Products for Pest Management* 927(13), pp. 168-181.
- [11] Gillij, Y.G., Gleiser, R.M. and Zygadlo, J.A., 2008, Mosquito repellent activity of essential oils of aromatic plants growing in Argentina, *Bioresource Technology* 99(7), pp.2507-2515.
- [12] Jaenson, T.G., Pålsson, K. and Borg-Karlson, A.K., 2006, Evaluation of extracts and oils of mosquito (Diptera: Culicidae) repellent plants from Sweden and Guinea-Bissau, *Journal of Medical Entomology* 43(1), pp.113-119.
- [13] Maia, M.F. and Moore, S.J., 2011, Plant-based insect repellents: a review of their efficacy, development and testing, *Malaria Journal* 10(S1), p.S11.
- [14] Paluch, G., Bartholomay, L. and Coats, J., 2010, Mosquito repellents: a review of chemical structure diversity and olfaction, *Pest Management Science* 66(9), pp.925-935.

- [15] Batish, D.R., Singh, H.P., Kohli, R.K. and Kaur, S., 2008, Eucalyptus essential oil as a natural pesticide, *Forest Ecology and Management* 256(12), pp.2166-2174.
- [16] Adusei-Mensah, F., Inkum, E., Mawuli Agbale, C. and Eric, A., 2014, Comparative Evaluation of the Insecticidal and Insect Repellent Properties of the Volatile Oils of *Citrus Aurantifolia* (Lime), *Citrus sinensis* (Sweet Orange) and *Citrus limon* (Lemon) on *Camponotus nearcticus* (Carpenter Ants), *International Journal of Novel Research in Interdisciplinary Studies* 1(2), pp.19-25.
- [17] Gololo, S.S., Shai, L.J., Agyei, N.M. and Mogale, M.A., 2016, Effect of seasonal changes on the quantity of phytochemicals in the leaves of three medicinal plants from Limpopo province, South Africa, *Journal of Pharmacognosy and Phytotherapy* 8(9), pp.168-172.
- [18] Croteau, R., Kutchan, T.M. and Lewis, N.G., 2000, Chapter 24: Natural Products (Secondary Metabolites), Buchanan, B.B., Grisse, W. and Jones, R.L., *Biochemistry and Molecular Biology of Plants*, 2nd ed., American Society of Plant Biologists, pp.1250-1319.
- [19] Figueiredo, A.C., Barroso, J.G., Pedro, L.G. and Scheffer, J.J., 2008, Factors affecting secondary metabolite production in plants: volatile components and essential oils, *Flavour and Fragrance Journal* 23(4), pp.213-226.
- [20] Effiom, O.E., Avoaja, D.A. and Ohaeri, C.C., 2012, Mosquito repellent activity of phytochemical extracts from peels of citrus fruit species, *Global Journal of Science Frontier Research* 12(1), pp.1-8.
- [21] Qiao, Y., Xie, B., Zhang, Y., Zhang, Y., Fan, G., Yao, X. and Pan, S., 2008, Characterization of aroma active compounds in fruit juice and peel oil of Jincheng sweet orange fruit (*Citrus sinensis* (L.) Osbeck) by GC-MS and GC-O, *Molecules* 13(6), pp.1333-1344.
- [22] Sritabutra, D., Soonwera, M., Waltanachanobon, S. and Pongjai, S., 2011, Evaluation of herbal essential oil as repellents against *Aedes aegypti* (L.) and *Anopheles dirus* Peyton & Harrion, *Asian Pacific Journal of Tropical Biomedicine* 1(1), pp.S124-S128.
- [23] Fraatz, M.A., Berger, R.G. and Zorn, H., 2009, Nootkatone—a biotechnological challenge, *Applied Microbiology and Biotechnology* 83(1), pp.35-41.

- [24] Fraatz, M.A., Riemer, S.J., Stöber, R., Kaspera, R., Nimtz, M., Berger, R.G. and Zorn, H., 2009, A novel oxygenase from *Pleurotus sapidus* transforms valencene to nootkatone, *Journal of Molecular Catalysis B: Enzymatic* 61(3-4), pp.202-207.
- [25] Sauer, A.M., Crowe, W.E., Laine, R.A. and Henderson, G., Louisiana State University and Mechanical College, 2006, *Efficient and economic asymmetric synthesis of nootkatone, tetrahydronootkatone, their precursors and derivatives*, U.S. Patent 7,112,700.
- [26] Elston, A., Lin, J. and Rouseff, R., 2005, Determination of the role of valencene in orange oil as a direct contributor to aroma quality, *Flavour and Fragrance Journal* 20(4), pp.381-386.
- [27] Furusawa, M., Hashimoto, T., Noma, Y. and Asakawa, Y., 2005, Highly efficient production of nootkatone, the grapefruit aroma from valencene, by biotransformation, *Chemical and Pharmaceutical Bulletin* 53(11), pp.1513-1514.
- [28] Hong, B., Lebeuf, R., Delbaere, S., Alsters, P. and Nardello-Rataj, V., 2016, One-pot synthesis of (+)-nootkatone via dark singlet oxygenation of valencene: The triple role of the amphiphilic molybdate catalyst, *Catalysts* 6(12), p.184.
- [29] Sowden, R.J., Yasmin, S., Rees, N.H., Bell, S.G. and Wong, L.L., 2005, Biotransformation of the sesquiterpene (+)-valencene by cytochrome P450 cam and P450 BM-3, *Organic & Biomolecular Chemistry* 3(1), pp.57-64.
- [30] Bomgardner, M. 2016, *Nootkatone tested as a mosquito repellent*, Chemical & Engineering News, viewed on 25 August 2020, from <https://cen.acs.org/articles/94/i14/Nootkatone-tested-mosquito-repellent.html>
- [31] Wriessnegger, T., Augustin, P., Engleder, M., Leitner, E., Müller, M., Kaluzna, I., Schürmann, M., Mink, D., Zellnig, G., Schwab, H. and Pichler, H., 2014, Production of the sesquiterpenoid (+)-nootkatone by metabolic engineering of *Pichia pastoris*, *Metabolic Engineering* 24, pp.18-29.
- [32] Wilson III, C.W. and Shaw, P.E., 1978, Synthesis of nootkatone from valencene, *Journal of Agricultural and Food Chemistry* 26(6), pp.1430-1432.
- [33] Hunter, G.L.K. and Brogden Jr, W.B., 1965, Conversion of valencene to nootkatone, *Journal of Food Science* 30(5), pp.876-878.

- [34] Cholke, P.B., Bhor, A.K., Shete, A.M. and Sonawane, R.K., 2017, Extraction and GC-MS analysis of orange (*Citrus sinensis*) peel oil, *Research Journal of Agriculture and Biological Sciences* 2(5), pp.41-51.
- [35] Liu, K., Chen, Q., Liu, Y., Zhou, X. and Wang, X., 2012, Isolation and biological activities of decanal, linalool, valencene, and octanal from sweet orange oil, *Journal of Food Science* 77(11), pp.C1156-C1161.
- [36] Acar, Ü., Kesbiç, O.S., Yılmaz, S., Gültepe, N. and Türker, A., 2015, Evaluation of the effects of essential oil extracted from sweet orange peel (*Citrus sinensis*) on growth rate of tilapia (*Oreochromis mossambicus*) and possible disease resistance against *Streptococcus inia*, *Aquaculture* 437, pp.282-286.
- [37] Ahmad, M.M., Iqbal, Z., Anjum, F.M. and Sultan, J.I., 2006, Genetic variability to essential oil composition in four citrus fruit species, *Pakistan Journal of Botany* 38(2), p.319.
- [38] Behr, A. and Johnen, L., 2009, Myrcene as a natural base chemical in sustainable chemistry: a critical review, *ChemSusChem: Chemistry & Sustainability Energy & Materials* 2(12), pp.1072-1095.
- [39] Tao, N.G., Liu, Y.J. and Zhang, M.L., 2009, Chemical composition and antimicrobial activities of essential oil from the peel of bingtang sweet orange (*Citrus sinensis* Osbeck), *International Journal of Food Science & Technology* 44(7), pp.1281-1285.
- [40] Thomas, A.F. and Bessiere, Y., 1989, Limonene, *Natural Product Reports* 6(3), pp.291-309.
- [41] Hosni, K., Zahed, N., Chrif, R., Abid, I., Medfei, W., Kallel, M., Brahim, N.B. and Sebei, H., 2010, Composition of peel essential oils from four selected *Tunisian Citrus* species: Evidence for the genotypic influence, *Food Chemistry* 123(4), pp.1098-1104.
- [42] Cankar, K., Jongedijk, E., Klompmaker, M., Majdic, T., Mumm, R., Bouwmeester, H., Bosch, D. and Beekwilder, J., 2015, (+)-Valencene production in *Nicotiana benthamiana* is increased by down-regulation of competing pathways, *Biotechnology Journal* 10(1), pp.180-189.

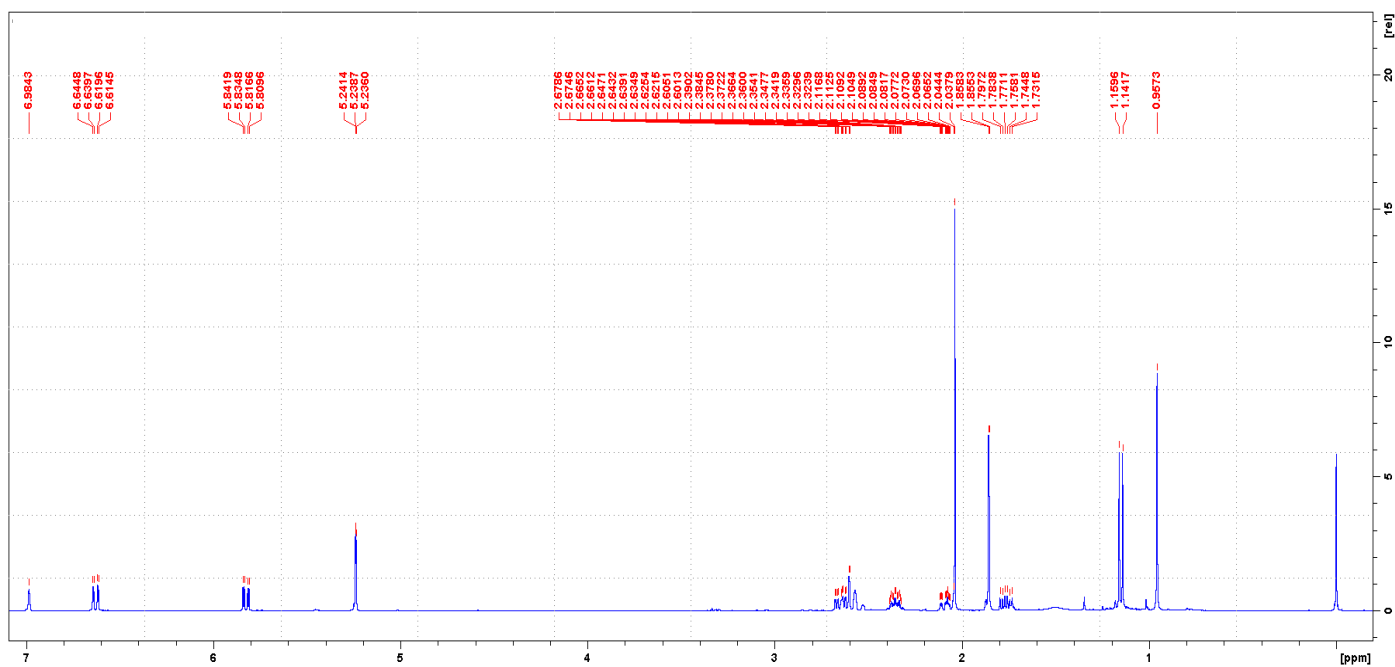
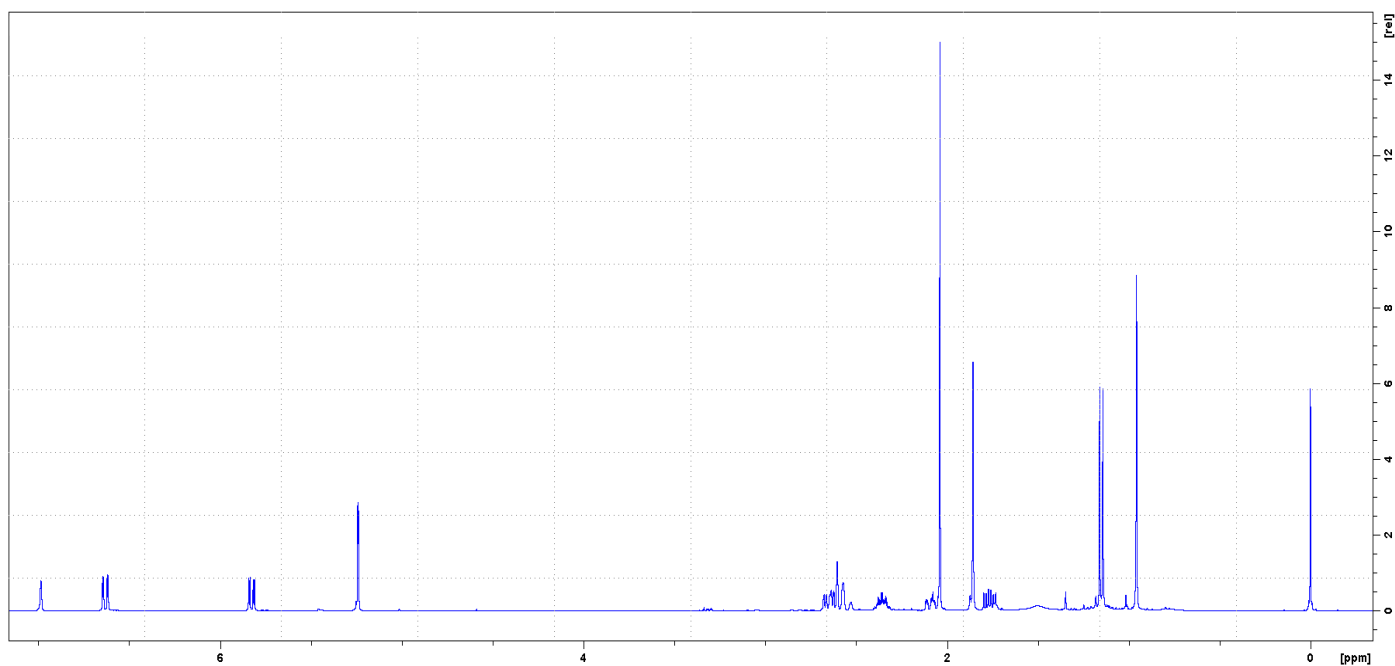
- [43] Ravizza, R., Gariboldi, M.B., Molteni, R. and Monti, E., 2008, Linalool, a plant-derived monoterpene alcohol, reverses doxorubicin resistance in human breast adenocarcinoma cells, *Oncology Reports* 20(3), pp.625-630.
- [44] Pietrogrande, M.C. and Basaglia, G., 2007, GC-MS analytical methods for the determination of personal-care products in water matrices, *Trends in Analytical Chemistry* 26(11), pp.1086-1094.
- [45] Daferera, D.J., Ziogas, B.N. and Polissiou, M.G., 2000, GC-MS analysis of essential oils from some Greek aromatic plants and their fungitoxicity on *Penicillium digitatum*, *Journal of Agricultural and Food Chemistry* 48(6), pp.2576-2581.
- [46] Dhivya, R. and Manimegalai, K., 2013, Preliminary phytochemical screening and GC-MS profiling of ethanolic flower extract of *Calotropis gigantea* Linn.(Apocynaceae), *Journal of Pharmacognosy and Phytochemistry* 2(3), pp.28-32.
- [47] Cone, E.J., Presley, L., Lehrer, M., Seiter, W., Smith, M., Kardos, K.W., Fritch, D., Salamone, S. and Niedbala, R.S., 2002, Oral fluid testing for drugs of abuse: positive prevalence rates by Intercept™ immunoassay screening and GC-MS-MS confirmation and suggested cutoff concentrations, *Journal of Analytical Toxicology* 26(8), pp.541-546.
- [48] Gherman, C., Culea, M. and Cozar, O., 2000, Comparative analysis of some active principles of herb plants by GC/MS, *Talanta* 53(1), pp.253-262.
- [49] Wang, F.C.Y., Qian, K. and Green, L.A., 2005, GC×MS of diesel: a two-dimensional separation approach, *Analytical Chemistry* 77(9), pp.2777-2785.
- [50] EL-SCIENCE, 2019, *The Gas Chromatograph (GC)*, viewed 11 August 2019, from <https://elscience.co.uk/our-lab/gcms-work/>.
- [51] InnovaTech, 2014, *How Stuff Works: GC/MS Analysis*, viewed 11 August 2019, from <https://www.innovatechlabs.com/newsroom/642/stuff-works-gcms-analysis/>.
- [52] Lamas, J.P., Sanchez-Prado, L., Lores, M., Garcia-Jares, C. and Llompарт, M., 2010, Sorbent trapping solid-phase microextraction of fragrance allergens in indoor air, *Journal of Chromatography A* 1217(33), pp.5307-5316.

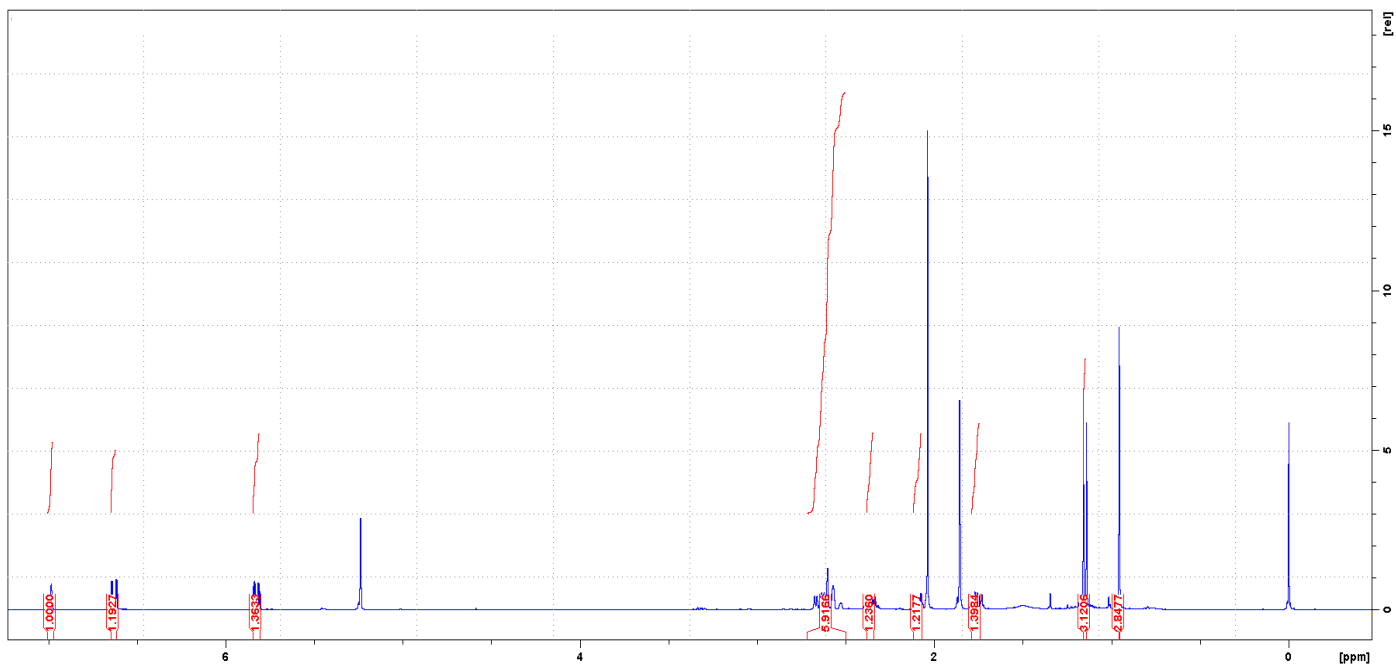
- [53] Snow, N.H. and Slack, G.C., 2002, Head-space analysis in modern gas chromatography, *Trends in Analytical Chemistry* 21(9-10), pp.608-617.
- [54] Zhang, Z. and Pawliszyn, J., 1993, Headspace solid-phase microextraction, *Analytical Chemistry* 65(14), pp.1843-1852.
- [55] Field, J.A., Nickerson, G., James, D.D. and Heider, C., 1996, Determination of essential oils in hops by headspace solid-phase microextraction, *Journal of Agricultural and Food Chemistry* 44(7), pp.1768-1772.
- [56] Oliveira, A.P., Silva, L.R., de Pinho, P.G., Gil-Izquierdo, A., Valentão, P., Silva, B.M., Pereira, J.A. and Andrade, P.B., 2010, Volatile profiling of *Ficus carica* varieties by HS-SPME and GC-IT-MS, *Food Chemistry* 123(2), pp.548-557.
- [57] Nekoei, M. and Mohammadhosseini, M., 2017, Chemical composition of the essential oils and volatiles of *Salvia leriifolia* by three different extraction methods prior to gas chromatographic-mass spectrometric determination: Comparison of HD with SFME and HS-SPME, *Journal of Essential Oil Bearing Plants* 20(2), pp.410-425.
- [58] Meshkatalasadat, M.H., Salahvarzi, S.A.B.A.H., Aminiradpoor, R. and Abdollahi, A., 2012, Identification of essential oil constituents of caraway (*Carum carvi*) using ultrasonic assist with headspace solid phase microextraction (UA-HS-SPME), *Digest Journal of Nanomaterials and Biostructures* 7(2), pp.637-640.
- [59] Md Othman, S.N.A., Hassan, M.A., Nahar, L., Basar, N., Jamil, S. and Sarker, S.D., 2016, Essential oils from the Malaysian Citrus (Rutaceae) medicinal plants, *Medicines* 3(2), p.13.
- [60] Müller, G.C., Junnila, A., Butler, J., Kravchenko, V.D., Revay, E.E., Weiss, R.W. and Schlein, Y., 2009, Efficacy of the botanical repellents geraniol, linalool, and citronella against mosquitoes, *Journal of Vector Ecology* 34(1), pp.2-8.
- [61] Swamy, M.K. and Sinniah, U.R., 2015, A comprehensive review on the phytochemical constituents and pharmacological activities of *Pogostemon cablin* Benth.: an aromatic medicinal plant of industrial importance, *Molecules* 20(5), pp.8521-8547.

- [62] National Center for Biotechnology Information, 2020, PubChem Compound Summary for CID 1268142, *Nootkatone*, viewed 13 August 2020, from <https://pubchem.ncbi.nlm.nih.gov/compound/Nootkatone>.
- [63] Behle, R.W., Flor-Weiler, L.B., Bharadwaj, A. and Stafford III, K.C., 2011, A formulation to encapsulate nootkatone for tick control, *Journal of Medical Entomology* 48(6), pp.1120-1127.

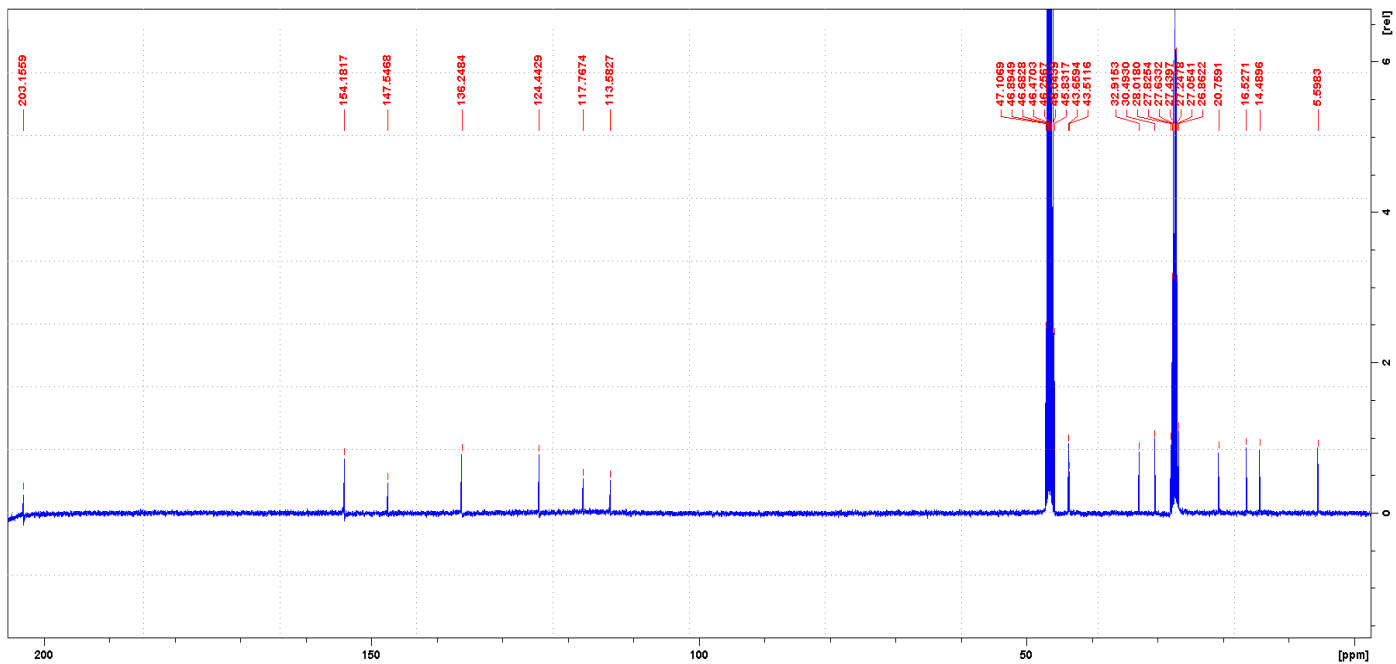
Supplementary Data

Supplementary data 1: ^1H NMR spectrum of siphonochilone

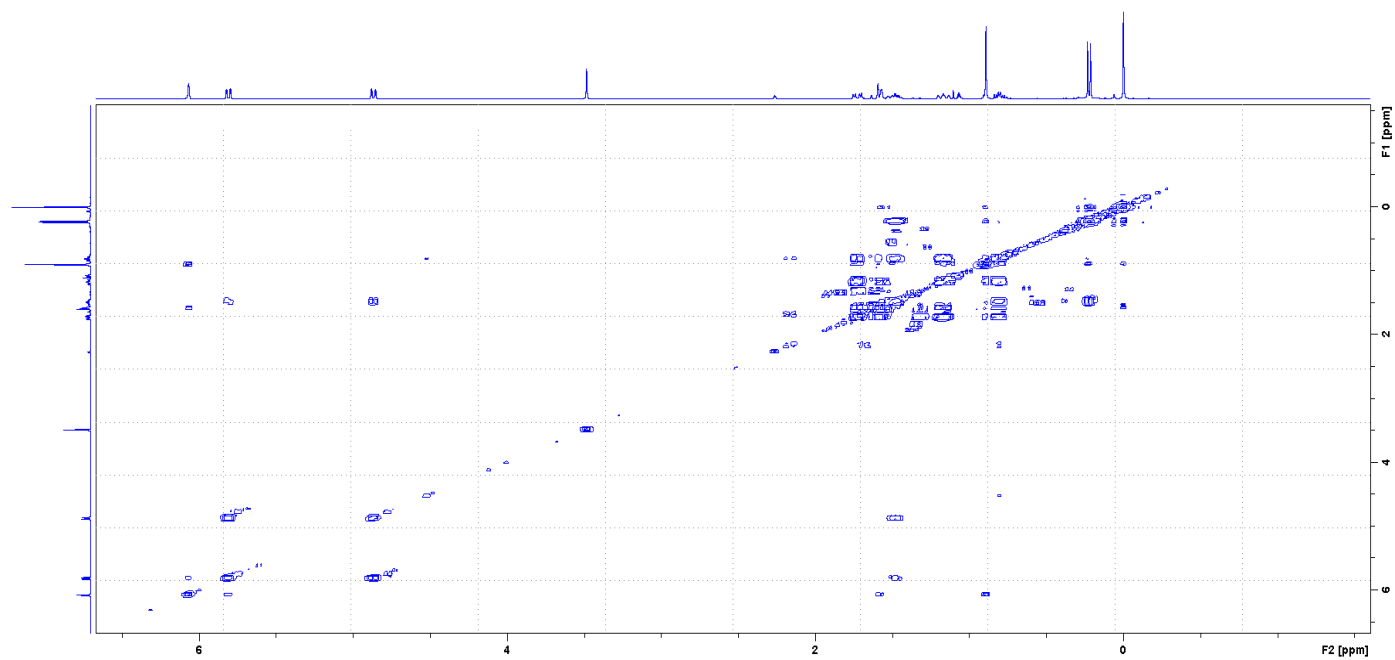




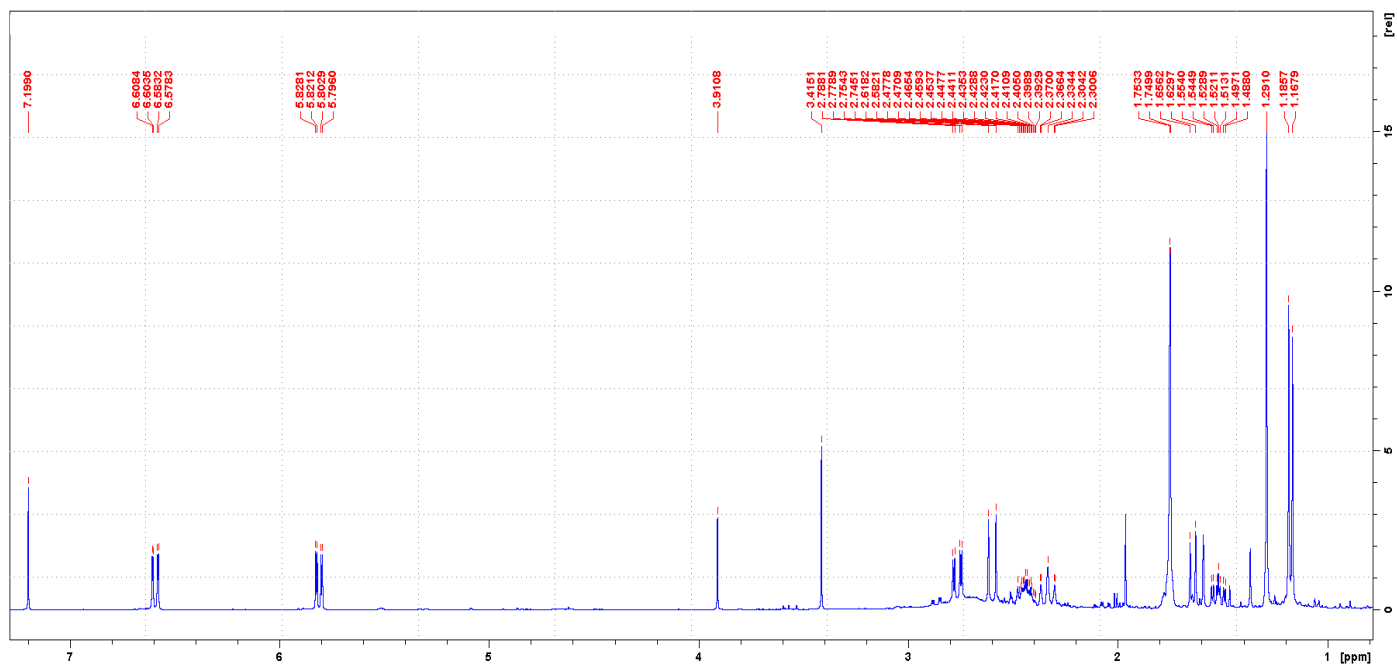
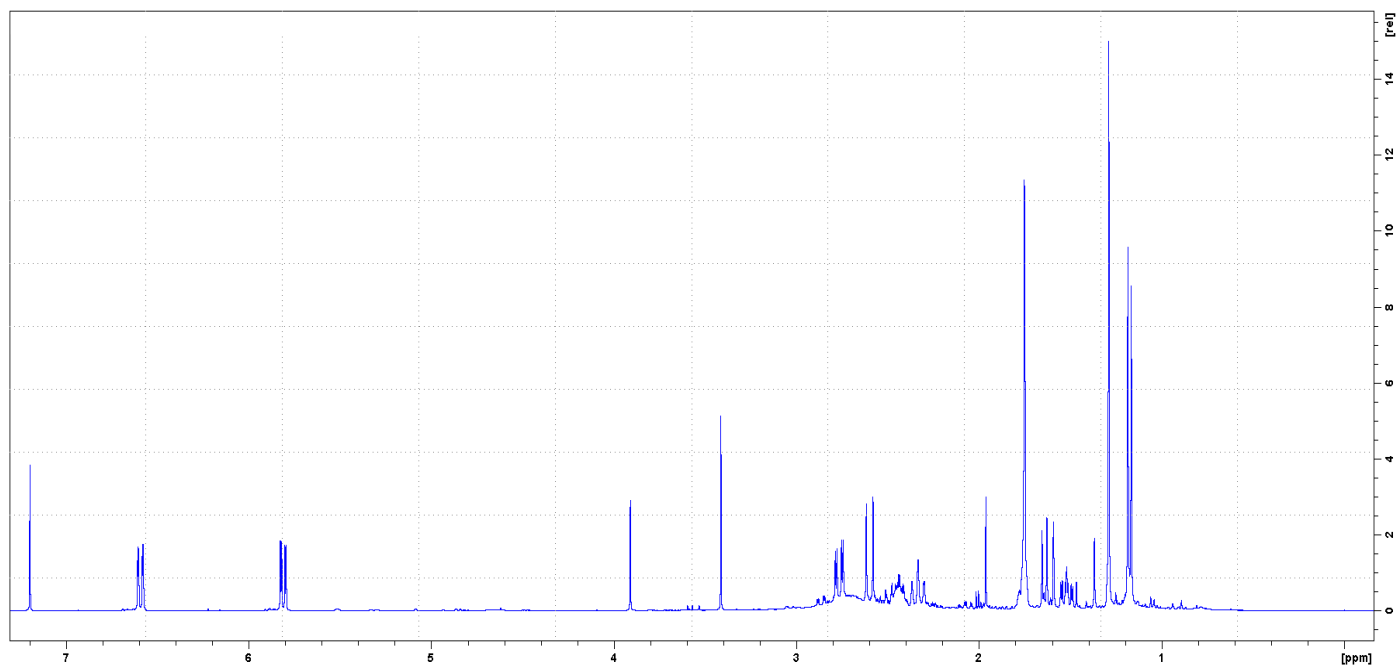
Supplementary data 2: ¹³C NMR spectrum of siphonochilone

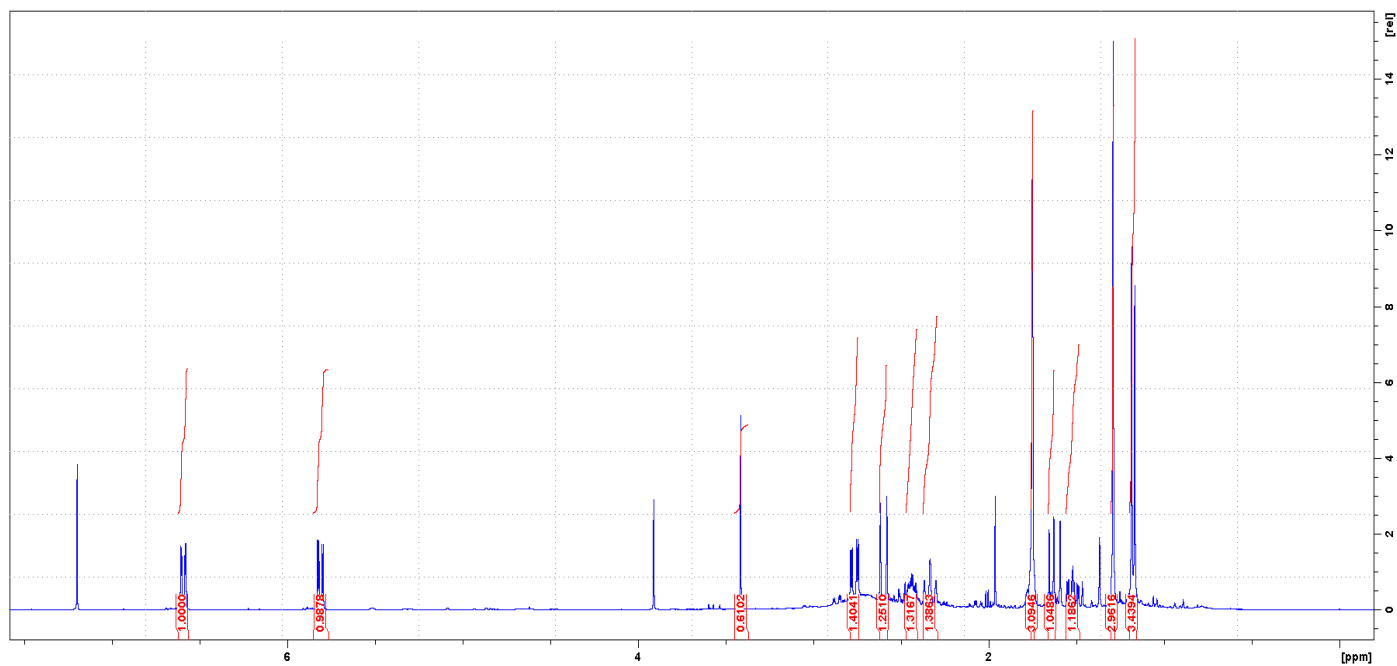


Supplementary data 3: ^1H - ^1H COSY data of siphonochilone

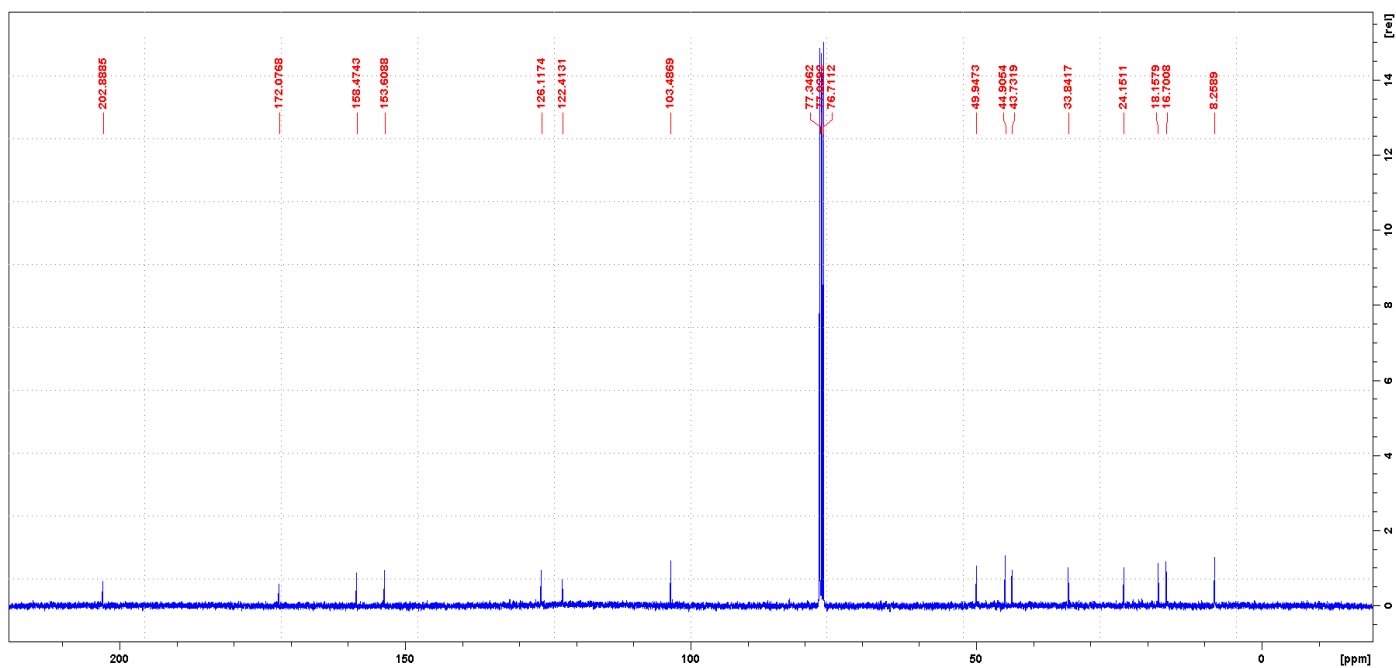


Supplementary data 4: ¹H NMR spectrum of the hydroxylated lactone

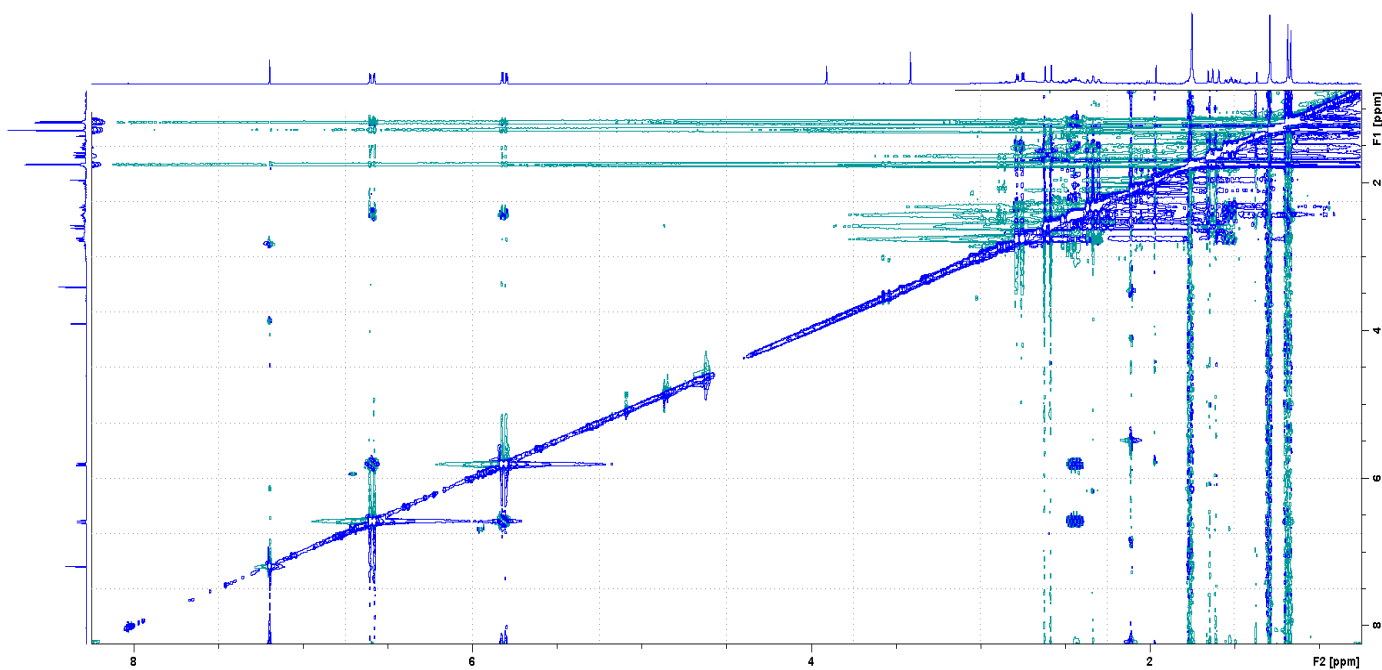
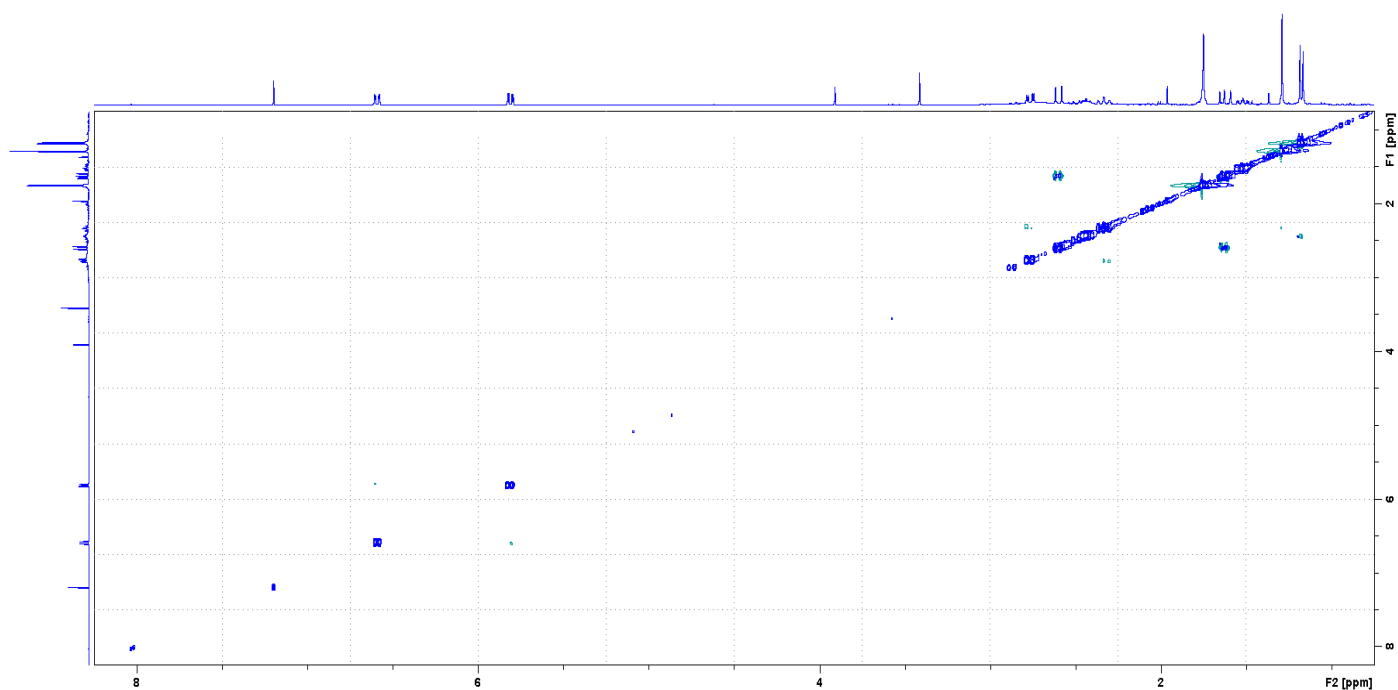




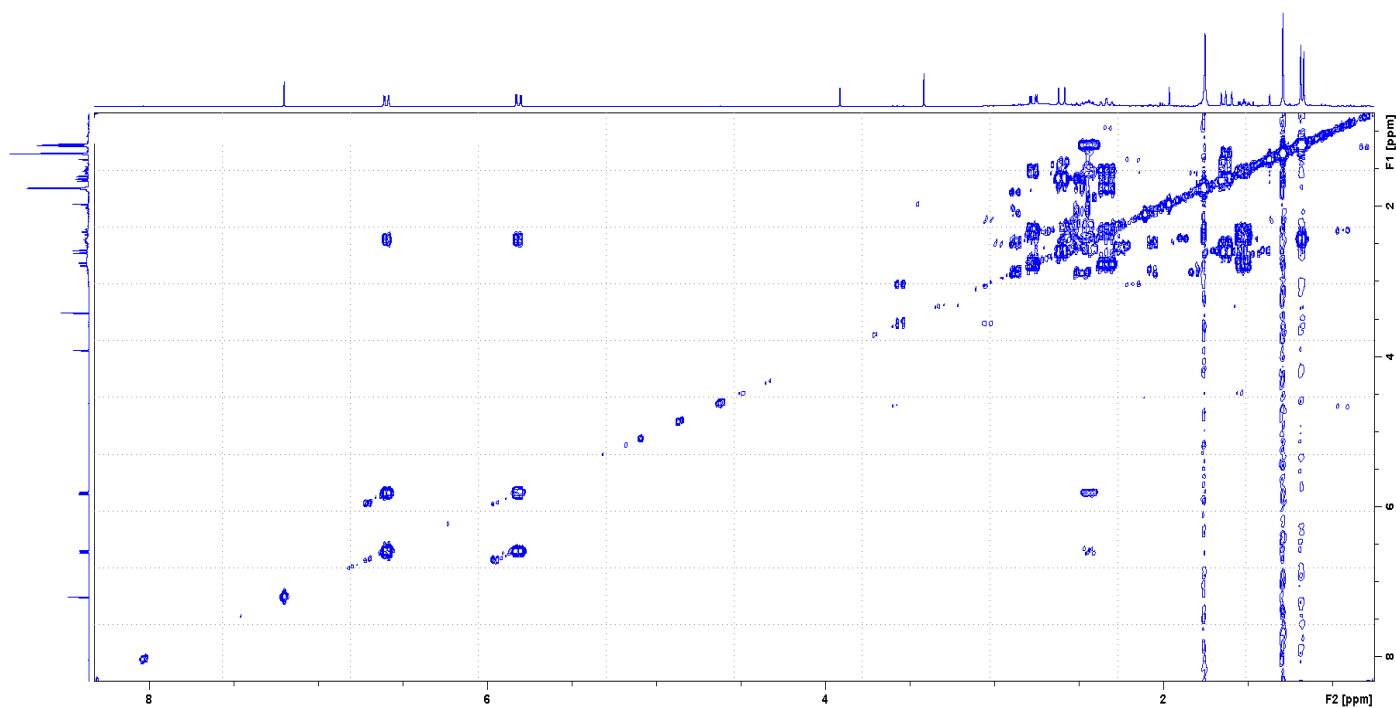
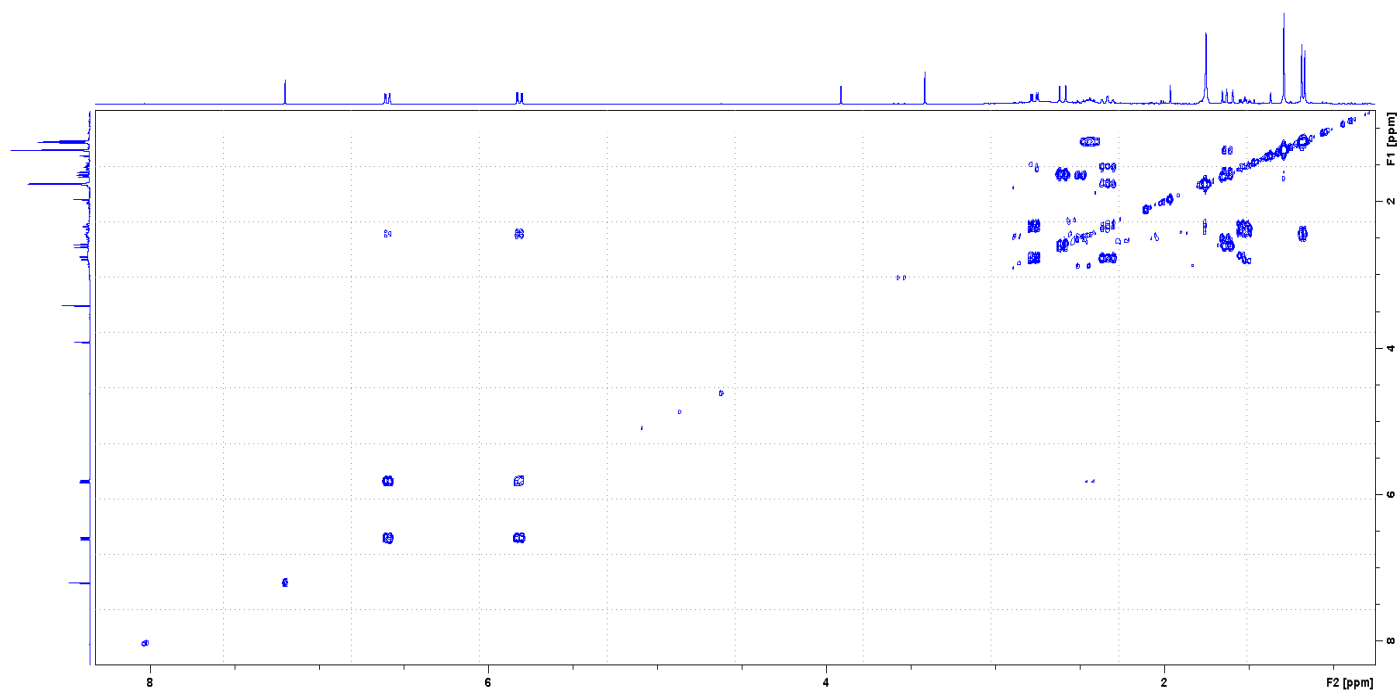
Supplementary data 5: ¹³C NMR spectrum of the hydroxylated lactone



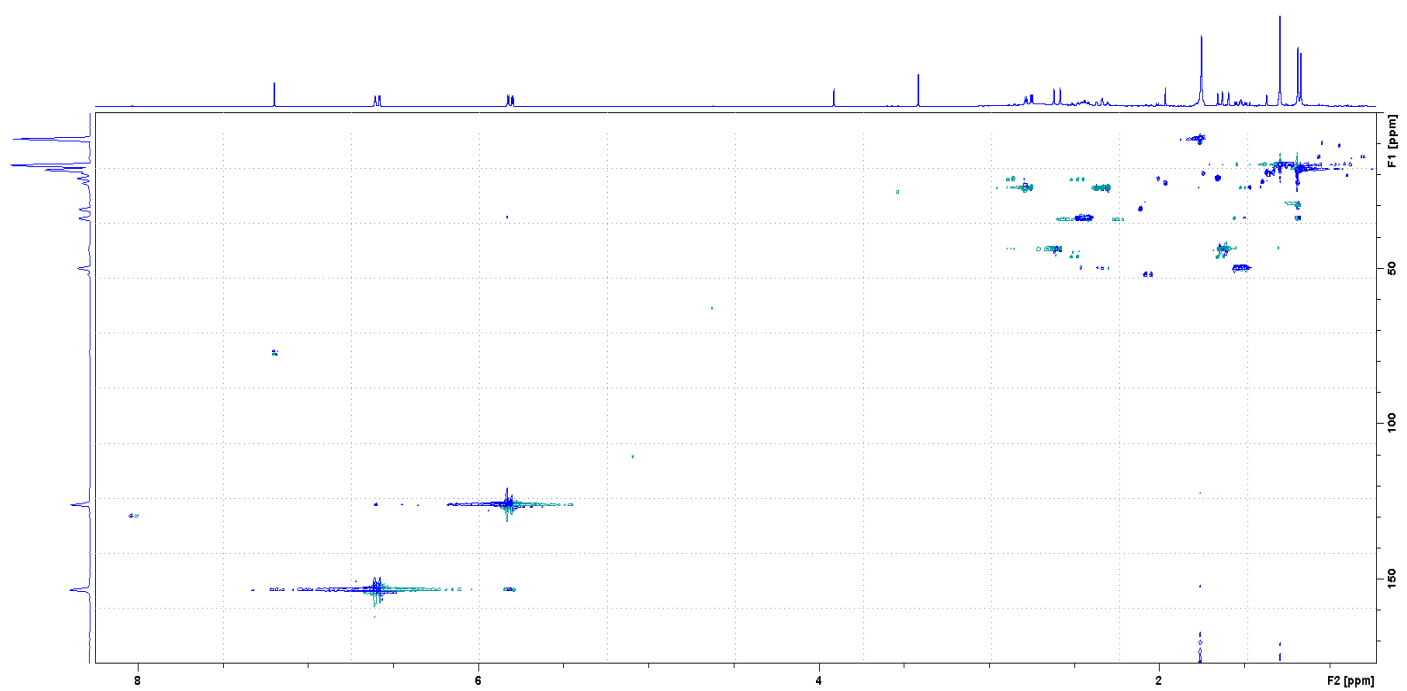
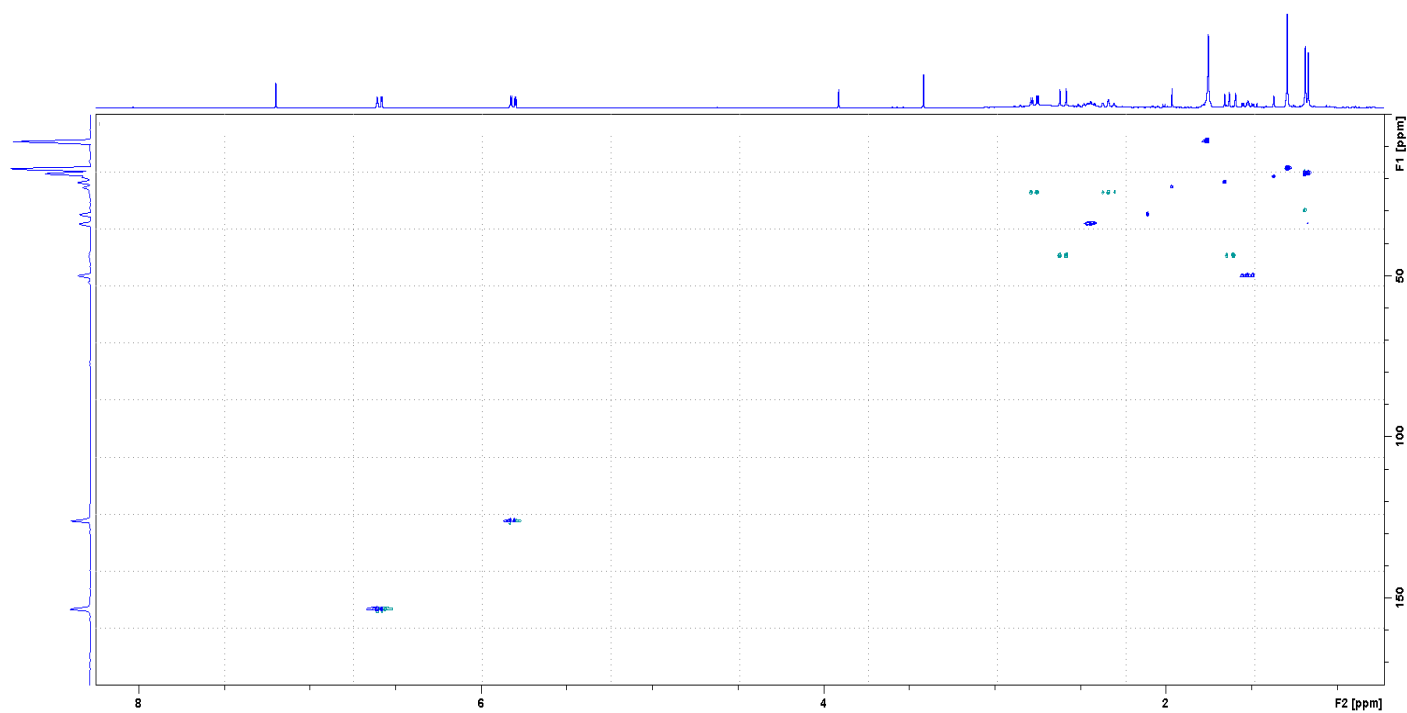
Supplementary data 6: ^1H - ^1H NOESY data of the hydroxylated lactone



Supplementary data 7: ^1H - ^1H COSY data of the hydroxylated lactone



Supplementary data 8: HSQC data of the hydroxylated lactone



Supplementary data 9: HMBC data of the hydroxylated lactone

