Isolation and characterization of bio-active compounds from *Lippia javanica*

Dissertation submitted in the fulfillment of the requirements for the degree

Master of Technology

in

Chemistry

in the

Faculty of Science

at the

University of Johannesburg

by

Thobile Prudence Dlamini

Supervisor: **Mrs. D. K. Olivier**

Co-supervisor: **Dr. R. W. M. Krause**

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DECLARATION

I declare that this thesis attached for submission for the research qualification

MASTERS DEGREE IN CHEMISTRY

to the University of Johannesburg, Department of Chemical Technology is, besides the assistance of the project supervisors, my own work and has not previously been submitted by me to another institution to obtain any research qualification.

_____ on this _____ day of _____

(Candidate)

_____ on this _____ day of _____

(Supervisor)

_____ on this _____ day of _____

(Co-supervisor)

DEDICATION

I dedicate this work to my husband S. N. Dlamini, my mother in-law, my mum and dad and my natural products yet to come. May the God almighty shower you with all the blessings that you deserve.

ACKNOWLEDGEMENTS

My sincere appreciation to all those who in one way or another through their assistance, ideas and advices made it possible that this dissertation could finally exist, and in particular the following:

- **D. K. Olivier, R.W.M. Krause,** for their supervision, advices and their invaluable assistance.

- **Sabelo D. Mhlanga** and **Susan Travis** for helping with IR, **Richard Mampa** for performing NMR experiments and **Tommy van der Merwe** for performing FAB-MS all at the University of Witwatersrand.

- **K. L. Salipira, S. D. Mhlanga** and **E. N. Nxumalo** for their invaluable assistance in so many things.

- My husband **Nkosinathi S. Dlamini** for being so patient, encouraging and supportive, his keen interest in the project, **love** and all the sacrifices he made to enable me to pursue my degree. He surely has been everything to me, thank you!

- The National Research Foundation **(NRF)** and the University of Johannesburg Postgraduate Bursary for financial support.

- Finally, yet most important, all my sincere gratitude to the God all mighty!- His grace, support, the strength and ability,* the patience and the opportunity–if it wasn't for him…….

TO GOD BE THE GLORY, GREAT THINGS HE HAS DONE…..WORTHY GOD!!!!!!!!!

* "Nginemandla ekwenta konkhe ngaye Kristu longicinisako" Kubasefiliphi 4:13

ABSTRACT

Lippia javanica is an erect, small woody shrub that grows up to two metres in height, popularly known as “fever tea” or “koorsbossie”. It is distributed throughout southern Africa covering almost the entire country of Swaziland and large parts of South Africa. Its uses range from that of caffeine free tea with fever and pain-relieving activities to treatment of microbial infections such as coughs, colds and other bronchial ailments as well as the basic symptoms of HIV and AIDS. Topical uses also include disinfection and treatment of skin disorders such as dermatitis and dry skin, and even for the treatment of lice and scabies. Furthermore, it is used in combination with *Artemisia afra* as a remedy against malaria and as a prophylactic against dysentery and diarrhoea.

In view of the traditional medicinal importance of *L. javanica*, it was surprising that not much is known on the polar fraction of this plant. This work was done to explore the presence of the polar compounds of *L. javanica* which may contribute to the plants’ medicinal properties.

The chemical screening was conducted using both the non-polar and polar extracts of the aerial parts of *L. javanica* collected from various localities in South Africa and Swaziland. The screening involved extraction of the aerial parts of the plant in solvents of different polarities after which TLC showed the presence of essential oils, phenolic glycosides, amino acids, diterpenoids, triterpenoids and other phenolic compounds which may include flavonoids. The chemical variation was further investigated by means of chromatographic techniques such as HPLC and GC/MS.

The essential oils of *L. javanica* demonstrated a dramatic variation both in quality and in quantity within and between natural plant populations. The variation was found to be random and it was not correlated to the geographical distribution of this plant. The polar extracts also showed variation in the chemical compounds,

with clear differences observed between Swaziland and South African populations. Even among the amino acids a considerable variation was observed on a geographical level. Although only the aerial parts of the plants were used in each case, the observed variation could be due to the different developmental stages of the plants and the different harvesting times.

We have also established that there are different compounds present in *L. rehmannii* (toxic *Lippia* species) and *L. javanica* which are similar to each other in appearance and they grow in the same regions and similar environments. The results demonstrated the importance of performing chemical variation studies before using these plants and for quality control purposes for marketed products.

The extracts of *L. javanica* were subjected to the isolation of compounds and structural elucidation. Two phenylethanoid glycosides, verbascoside β-[(3,4-dihydroxyphenyl)-ethyl]-(3′-*O*-α-L-rhamnopyranosyl)-(4′-*O*-caffeoyl)-β-D-glucopyranoside and isoverbascoside β-[(3,4-dihydroxyphenyl)-ethyl]-(3′-*O*-α-L-rhamnopyranosyl)-(6′-*O*-caffeoyl)-β-D-glucopyranoside, previously unknown in *L. javanica* were isolated. The structures were established by extensive spectroscopic investigation (IR, UV, FAB-MS, 1D- and 2D-NMR). The discovery of these compounds gave the most important new insight in this study and they are known to possess antioxidant activities[1]. This activity is still to be investigated in *L. javanica*.

[1] Delazar A., Gibbons S., Kumarasamy Y., Nahar L., Shoeb M., Sarker S. *Biochem. Syst. and Ecol.* 2005, **33**, 87-90.

TABLE OF CONTENTS

3.0 MATERIALS AND METHODS

LIST OF ABBREVIATIONS

Deleted: ¶

AIDS	: Acquired immune deficiency syndrome
BuOH	: Butanol
C_2H_3N	: Acetonitrile
$C_6H_5CH_3$	: Toluene
CD4	: Target cells
HCO_2H	: Formic acid
DNA	: Deoxyribonucleic acid
DPPH	: 1,1-diphenyl-1-picrylhydrazyl
EtOAc	: Ethyl acetate
F_{254}	: Fluorescence at 254nm
FAB-MS	: Fast atom bombardment-mass spectrometry
gp. 120	: HIV glycoprotein 120
HIV	: Human Immunodeficiency virus
HPLC	: High performance liquid chromatography
IHN-L	: Indigenous healing network list
IR	: Infra-red
LC/MS	: Liquid chromatography-mass spectrometry
LSDI	: Lubombo spatial development initiative
m/z	: Mass to charge ratio
MeOD	: deuterated –methanol
NMR	: Nuclear magnetic resonance
PDA	: Photodiode array
R	: Variable group
RNA	: Ribonucleic acid
RT	: Reverse transcriptase enzyme
SA	: South Africa
SD	: Standard
SPE	: Solid phase extraction
spp.	: Species

SWD	: Swaziland
TLC	: Thin layer chromatography
UV-vis	: Ultra-violet visible
v/v	: volume by volume
WHO	: World health organization

LIST OF FIGURES

MATERIALS AND METHODS

RESULTS AND DISCUSSIONS

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Background

1.1. Medicinal plants

Plants have been a basic source of medicine for many years throughout the world. Before the introduction of modern medicine, African countries were only exposed to traditional medicines. Many of these traditional medicines are still in use today. In southern Africa, large volumes of medicinal plants or their extracts are sold in the informal and commercial sectors of the economy.[1]

The use of plants in medicine is expanding worldwide and is gaining popularity.[2] For example, in the United Kingdom, approximately £65 million was spent by consumers on herbal medicinal products in 2000, which represents a 50% increase since 1995. Plants are not only used for primary health care of the poor in emerging countries, but also in countries where modern medicine is common in the national health care systems.[3]

Traditional healing takes a holistic approach to disease; it assumes that the mind and the spirit are inseparable from the body, and that spiritual and mental abnormalities influence physical conditions. A traditional healer for instance will try to understand why the patient became ill from both a physical and spiritual point of view, and treatment prescribed will address the possible spiritual and mental causes in addition to physical causes. The healer will also prescribe other

[1] Gurib-Fakim A. *Mol. Aspects Med*. 2006, **27**, 1-93.

[2] An introduction to Medicines from Plants http://www.rainforesteducation.com/medicines/PlantMedicines/rfmedicines.htm accessed 2005.11.28, 1-6.

[3] Van Wyk B -E., Wink M. 2004. *Medicinal Plants of the World*. Briza Publications, Pretoria.12

therapies to relieve the signs and symptoms of the condition, while a medical doctor may diagnose a disease in terms of a physical symptom, and treat it by simply prescribing the necessary medication.[3]

About 75 - 80% of the world's population is estimated by the World's Health Organization (WHO) to consult traditional medical practitioners for plant medicine to meet their health problems, in addition to medical doctors.[3,4] Many of these people use plant medicines because of their low costs; as they cannot afford the costs of pharmaceutical drugs.[5]

Since traditional medicine is able to accommodate both the poor and the rich, and also because of its effectiveness in treating illnesses, studies are trying to bridge the gap between modern and traditional medicine. The drive for this bridge is spreading throughout the world. As an example, in the United States, Canada, India and Germany authorities are trying to lobby for the absorption of local medicinal herbs into modern medicine. The Indigenous Healing Network List (IHN-L) in Australia is also promoting that the Australian people should be educated on the practice of indigenous methods of healing, and a centre for scientific research has been established for such a purpose in Ghana.[6] This trend has also been observed in Southern Africa, where the South African Parliament has already adopted a bill to regulate South Africa's traditional healers while discussions on the same subject are underway in the parliament of Swaziland.[6]

Medicinal plants provide a basis for approximately 50% of all drugs in medical use in the world. For example, the anti-malarial drug Artemisinin, isolated from *Artemisia annua*, is used for the treatment of multi-drug-resistant malaria.[1]

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[4] Cunningham A. B. *African medicinal plants, setting priorities at the interface between conservation and primary healthcare, people and plants working paper.* 1993, 1-39.

[5] Tadeg H., Mohammed E., Asres K., Gebre-Mariam T. *J. Ethnopharmacol.* 2005 1-8.

[6] True Love Magazine, March 2005, **313**, 165 -170.

The active ingredients of plants are chemical compounds that act directly or indirectly to prevent or treat diseases and maintain health.[3] African medicinal plants contain a great variety of these compounds, many of which have not been investigated and could be sources of new prescription drugs.[1,7] These compounds are used in preparations that in many instances have been shown to be clinically effective in treating illnesses.[8]

1.2. Problem statement

There have been numerous studies on the wide range of chemical extracts used in traditional healing from the *Lippia* species as will become evident in **Chapter 2**. Most of the previous studies on *Lippia javanica* (a species used medicinally) have focused almost exclusively on the volatile oils with only a few reporting on the amino acids, sugars, flavonoids, alkaloids, saponins and iridoids contained in the species. Consequently, this study will focus intensely on the non-volatile compounds of *L. javanica.* Since the plant is more often administered in the form of teas, the compounds most likely to be extracted for traditional medicinal use would be the less volatile and more polar fractions, such as the ones that were studied in this project. Also very few studies report on the regional variation of the compounds in *L. javanica* and almost all of these studies were done in one region (country), this has been another important part of this study.

Thus, the objectives of the study were the following:

- To do a chemical variation study on plants collected from different localities in South Africa and Swaziland using TLC, GC-MS and HPLC as screening methods.
- To extract the polar compounds from *Lippia javanica.*

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[7] Medicinal Plants (Science Tracer Bullet-science reference services) Library of congress.http://www.loc.gov/rr/scitech/tracer-bullets/medicplantstb.html accessed 2005/11/22, 1-18.

[8] Heinrich M., Barnes J., Gibbons S., Williamsons E. M., 2004. *Fundamentals of pharmacognosy and phytotherapy*, Elsevier Science Limited, Churchill Livingstone. 5

- To isolate these compounds using column chromatography
- To utilize IR, UV, FAB-MS and NMR spectroscopic techniques to determine the molecular structures of the isolated compounds.
- To do biological testing of the crude extract and isolated compounds to determine the potential medicinal effects.

CHAPTER 2

LITERATURE REVIEW

Introduction

In this chapter the literature survey on the botany, ethnopharmacology, distribution and the chemical composition of the *Lippia* species will be presented. The main focus was on *Lippia javanica*, which is widely used in traditional medicine for the treatment of many illnesses such as coughs, colds and fevers. The plant is more often administered in the form of teas to serve these purposes. In view of the medicinal effects from the teas, it was important to investigate the chemistry and the presence of the polar compounds found in this plant.

2.1. *Lippia*: Botanical overview

The genus *Lippia* (Houst.) of the Verbenaceae family has approximately 200 species of herbs, small trees and shrubs. Amongst the Verbenaceae genera, *Lippia* is differentiated by the two sepals, which are generally, 2-4 toothed. These plants possess three or four petals, four stamens and an ovary, which has two chambers with one ovule per chamber. The flowers are small, short and dense on long stalks that grow on adjacent sides (**Figure 2.1**).[1] The *Lippia* species possess a strong smell when their leaves are crushed. They are distributed all over the Tropical and Southern African countries.[2,3]

[1] Compton R. H. *S. Afr. J. Bot.* 1976, **11**, 484-485.

[2] Pascual M.E., Slowing K., Carretero E., Sanchez D., Villar A. *J. Ethnopharmacol.* 2001, **76**, 201-214.

[3] Viljoen A. M., Subramoney S., Van Vuuren S. F., Başer K.H.C., Demirci B., *J. Ethnopharmacol.* 2005, **96**, 271-277.

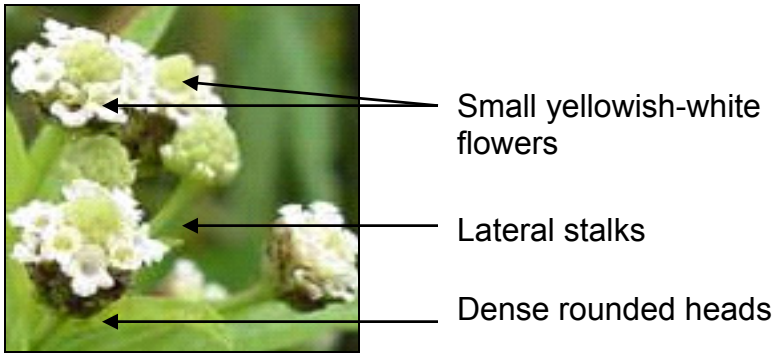


Figure 2.1: Illustration of *Lippia javanica* flowers

2.1.1. Medicinal uses of *Lippia*

Lippia species are commonly used both by traditional healers and lay people for the treatment of several ailments as summarized in **Table 2.1**. The leaves and stems are used in the form of tea infusions to serve these purposes.[2]

Table 2.1: The medicinal uses of the *Lippia* species

Species	Medicinal uses
L. javanica	See section 2.2.2.3
L. scaberrima	See section 2.3.1
L. multiflora	Coughs, colds, dysentery, diarrhea,[3] malaria,[4] diabetes,[5]
L. alba	Coughs, colds,[2] stomach ache,[3] mouth disinfectants, menstrual disorders syphilis[3]
L. graveolens	Dysentery, diarrhoea, diabetes,[3] mouth disinfectants [3]
L. chevalieri	Influenza, asthma, bronchitis,[2] dysentery, malaria, diabetes ,
L. oaxacana	Dysentery, diarrhoea[3]
L. dulcis	Influenza, asthma, bronchitis,[2] indigestion, stomach ache,[3]
L. origanoides	Indigestion, stomach ache,[3]
L. turbinate	Indigestion, stomach ache[3]
L. reptans	Indigestion, stomach ache[3]
L. micromera	Influenza, asthma, bronchitis,[2] indigestion, stomach ache,[3]
L. triphylla	Indigestion, stomach ache[3]
L. citriodora	Indigestion, stomach ache,[3]
L. nodiflora	Malaria, menstrual disorders, gonorrhea[3]
L. geminate	Menstrual disorders, gonorrhea[3]
L. sidoides	Activity against human leukemia cell lines

[4] Hernandez N.E., Tereschuk M.L., Abdala L. R. *J. Ethnopharmacol*. 2000, **73**, 317-322.

[5] Manenzhe N.J., Portgieter N., Van Ree T. *Phytochem*. 2004, **65**, 2333-2336.

2.1.2. Pharmacology of *Lippia*

The medicinal uses of these *Lippia* species are due to some pharmacological properties of the chemical compounds. Studies on the pharmacological activities of the *Lippia* species have mainly focused on the antimicrobial, larvicidal, antifungal and antiseptic properties of the essential oils.[2]

In a recent antiviral activity study of six medicinal plants from the Brazilian Atlantic Tropical Forest, it was only the ethyl acetate extracts of *L. alba* that showed antiviral activity against polio.[6] On the other hand a screening study for antimicrobial activity against Gram-positive (*Staphylococcus, Streptococcus, Cryptococcus*) and Gram-negative (*Escherichia coli, Pseudomonas, Klebsiella*) microorganisms and parasites such as *Plasmodium* has been observed with *L. turbinata*. The flavonoid extract of this species showed major activity against these bacteria,[7] while the hexane extract of *L. multiflora* had antimicrobial activity on *Candida albicans* resulting from carvacrol (**Figure 2.2**) isolated from this extract.[8] These results confirm the traditional uses of *L. multiflora* in the treatment of ailments such as coughs and colds, which are due to microbes.

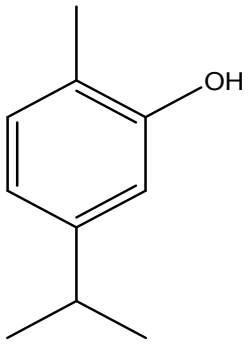


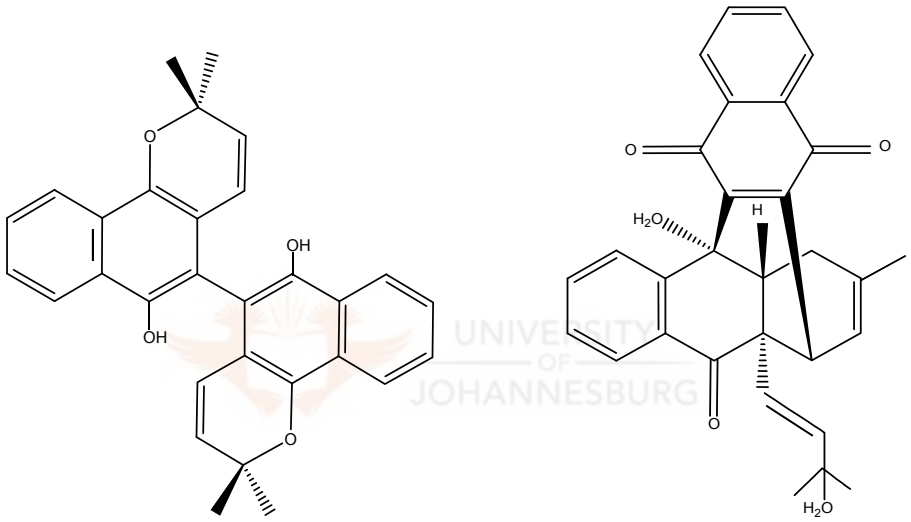
Figure 2.2: Carvacrol, the compound responsible for antimicrobial properties in *L. multiflora*

[6] Andrighetti-Frohnera C.R., Sinceroa T. C. M., Da Silvaa A. C., Savia L. A., Gaidoa C. M., Bettegaa J. M. R., Mancinib M., De Almeidab M. T. R., Barbosab R. A., Fariasb M. R., Barardia C.R.M., Simoesa C. M. O. *Fitoter.* 2005, **76**, 374-378.

[7] Rastrelli L., Caceres A., Morales C., De Simone F., Aguino R. *Phytochem*. 1998, **49(6)**, 1829-1832.

[8] Kunle O., Okogun J., Egamana, Emojevwe E., Shok M. *Phytomed*. 2003, **10**, 59-61.

The volatile oils of *L. chevalieri* also exhibited strong antibacterial and antifungal activities.[9] Recently, the essential oils of *L. origanoides* were tested against the fungi *Cryptococcus albicans*, *C. parapsilosis, C. guilliermondii, C. neoformans,* as well as the bacteria *Staphylococcus aureus,* and *S. mutans*. These oils inhibited all the listed microorganisms.[10] Tectol and lippsidoquinone (**Figure 2.3**) are phenolic compounds that have been isolated from the ethanol extracts of *L. sidoides*, and exhibited significant activity against human leukemia cell lines.[11] These results are in agreement with the ethnopharmacological uses of this plant species.



Tectol **Lippsidoquinone**

Figure 2.3: The phenolic compounds isolated from *L. sidoides* that possess activity against human leukemia cells.

2.1.3. Chemical composition

A wide range of natural products from the *Lippia* plants is the reason for these diverse pharmacological activities. The compounds discussed in this section have been isolated from the *Lippia* species.

[9] Mevy J. P., Bessiere J. M., Dherbomez M., Millogo J. *J. Food Chem*. 2006 (in print).

[10] Alviano D.S., Alviano C. S., Leita S. G. *J. Food Chem*. 2006 (in print).

[11] Costa S. M., Lemos T. L. G., Pessoa O.L., Pessoa C., Montenegro R. C., Raimundo B. *J. Nat. Prod.* 2001, **64**, 792-795.

2.1.3.1. Phenolic compounds

Phenolic compounds are the most widely distributed compounds in the plant kingdom. They are phenylalanine derived compounds which have at least one hydroxyl-substituted aromatic ring system. They exhibit a wide range of biological activities such as anti-inflammatory and endocrine disrupting properties. Phenolic compounds include the flavonoids, phenyl glycosides, coumarins, tannins and lignans.[12]

- **Flavonoids**

Flavonoids are a large group of polyphenolic compounds that possess a basic structural skeleton called 2-phenylchromane (**Figure 2.4**). They vary in having the phenyl ring either in the C-2 or C-3 position. Flavonoids are widely distributed in the plant kingdom with several biological properties, such as anti-inflammatory, antioxidant and antimicrobial activities.[13] Some members also possess recently discovered anti-HIV, anti-fungal, immunostimulant and anti-diarrhea activities.[14]

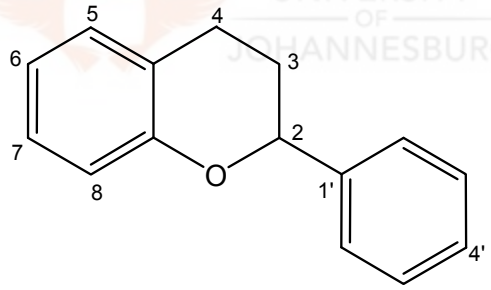


Figure 2.4: Structural skeleton of flavonoids

Very few studies give detailed reports with regards to the flavonoid compounds of *Lippia.* The majority of flavonoids reported are flavone-type aglycones. For instance, flavone mono- and disulphates were obtained from *L. canescens*.[3]

[12] Van Wyk B-E., Wink M. 2004. *Medicinal Plants of the World*. 1st Edition. Briza Publications, Pretoria. 382-392.

[13] Hoffmann D.1996. *The Complete Illustrated Holistic Herbal*. Element Books Inc., Australia. 35.

[14] Gurib-Fakim A. *Mol. Aspects. Med.* 2006, **27**, 1-93.

Fifteen flavones, of which twelve flavone sulphates and three flavone aglycones have been isolated and identified from the aerial parts of *L. nodiflora* and *L. canescens*. The flavone sulphates are mono- and disulphates such as jaceosidin, nepetin, hispidulin, 6-hydroxyluteon and a sodium salt called nodifloretin. Populations of this species found in Saudi Arabia and Malaysia have some flavone trisulphates.[15,16] Phytochemical analysis of *L. triphylla* contained the 7-glucuronylglucosides of apigenin and of luteolin (**Figure 2.5**).[15]

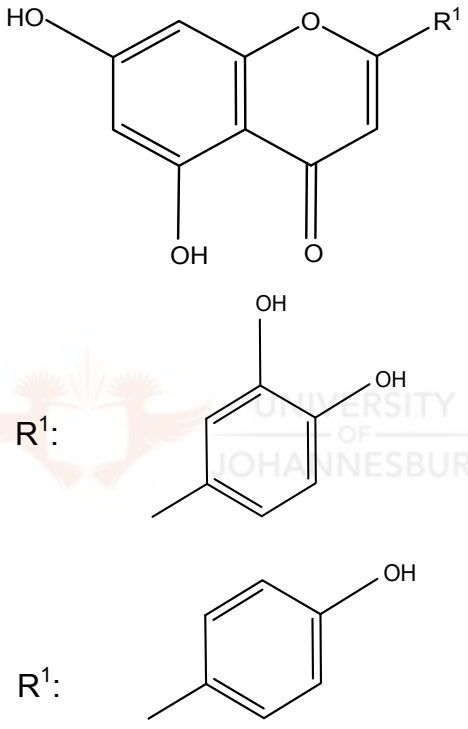


Figure 2.5: The 7-glucuronylglucosides flavonoids from *L. triphylla*

A flavonoid 6,7-dimethoxy-5,4'-dihydroxyflavone (**Figure 2.6**) was found in *L. sidoides*,[2] while tectol and lippsidoquinone (**Figure 2.3**) were isolated from the ethanol extract of *L. sidoides*.[11]

[15] Tomas-Barberan F. A., Harborne J. B., Self R. *Phytochem.*1987, **26(8)**, 2281-2284.

[16] Harbone J. B. 1994. *The Flavonoid Advances in Research Since 1986*. Chapman and Hall. 344.

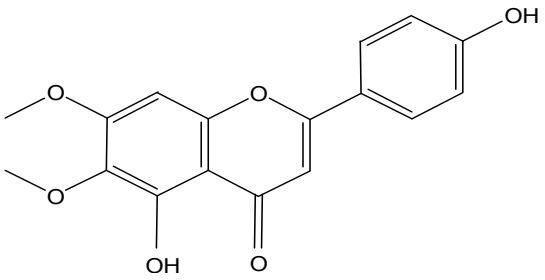


Figure 2.6: Chemical structure of 6,7-dimethoxy-5,4'-dihydroxyflavone the flavonoid compound isolated from *L. sidoides*.

- **Phenolic glycosides**

These are phenolic compounds that either have a 3 carbon side chain (phenylpropanoids) or a 2-carbon side chain (phenylethanoids) attached to an aromatic ring such as caffeic acid (**Figure 2.7**).[13]

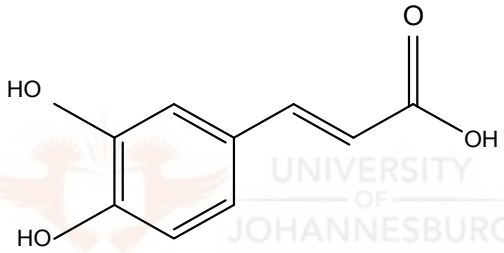


Figure 2.7: The structure of caffeic acid

The phenylethanoid glycosides are often found in the order Lamiales to which the Verbenaceae family belongs. These compounds possess antioxidant properties as they have an ability to scavenge free radicals.[17,18] Some of these compounds have been reported in the *Lippia* species: isonuomioside has been isolated from *L. multiflora* and recently isolated from *L. alba* while decaffeoylverbascoside (**Figure 2.8**) has only been isolated from *L. alba*.[19] Verbascoside has been isolated from *L. citriodora, L. dulcis* and *L. multiflora*. In the latter species isoverbascoside (**Figure 2.9**)[2] was also found.

[17] Ersoz T., Tasdemir D., Calis I. *Turk. J. Chem.* 2002, **26**, 465-471.

[18] Akdemir Z. S., Tatli I. I. *Turk J. Chem.* 2004, 28, 621-628.

[19] Barbosa F. G., Lima M. A. S., Braz-Filho R., Silveira E. R. *Biochem. Syst. Ecol.* 2006, 1-3

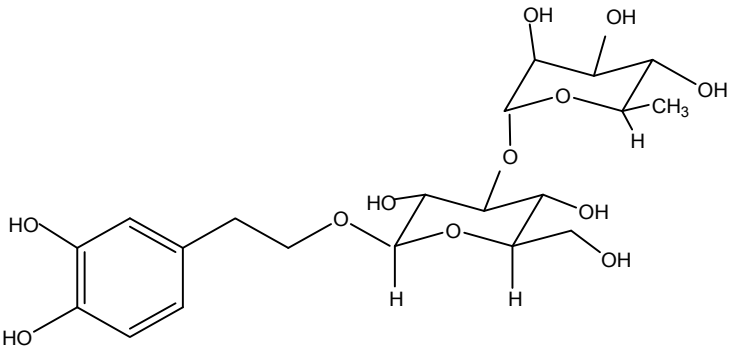
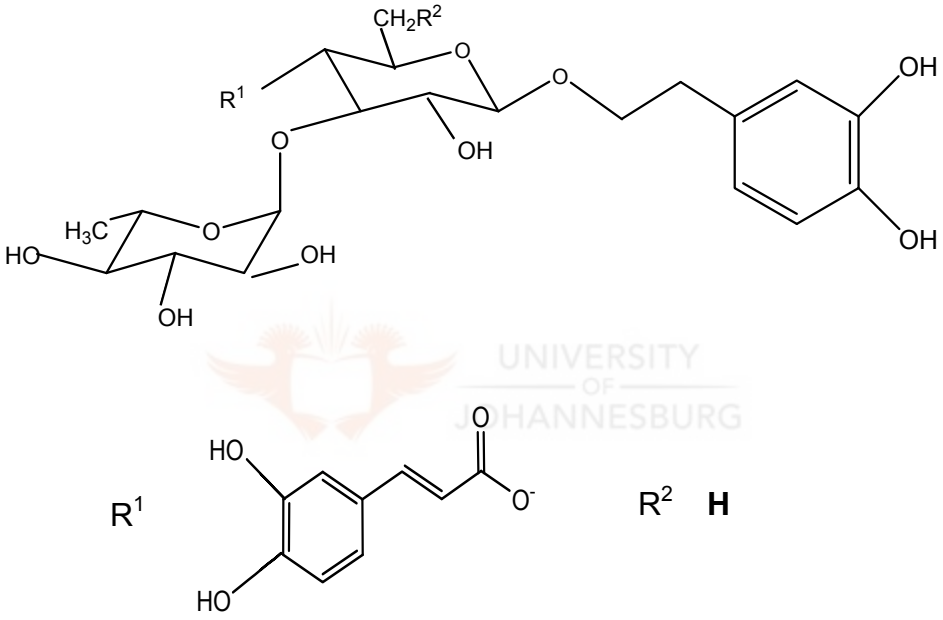
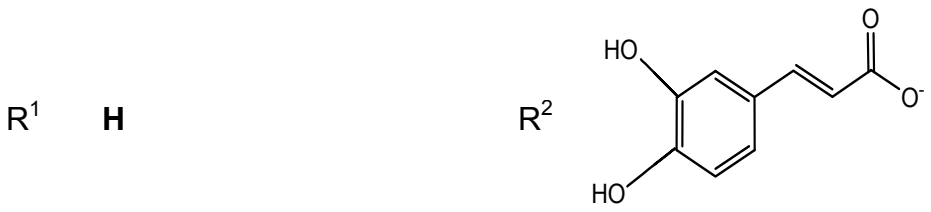


Figure 2.8: Decaffeoylverbascoside, isolated from *L. alba*



Verbascoside



Isoverbascoside

Figure 2.9: Verbascoside isolated from *L. citriodora, L. dulcis* and isoverbascoside from *L. multiflora.*

2.1.3.2. Terpenoids

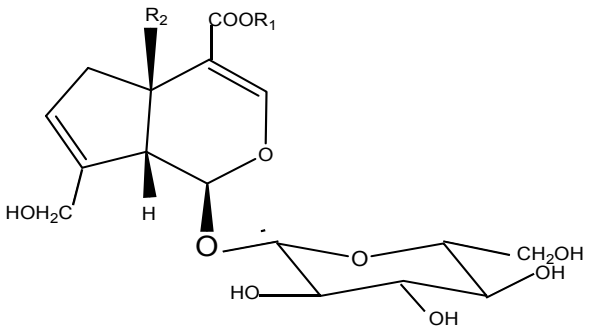
Terpenoids are a class of compounds that are formed in nature by five carbon units called isoprene units that are linked together in a regular pattern. As the chain of the isoprene units build up, the resulting terpenoids are classified by the size, such as the monoterpenoids which are formed by two isoprene units thus have 10 carbon atoms, the sesquiterpenoids are formed by 3 isoprene units and have 15 carbon atoms, the diterpenoids with 4 isoprene units therefore have 20 carbon atoms and the triterpenoids made from 6 isoprene units thus have 30 carbon atoms.[13]

- **Iridoides**

These are monoterpenoid compounds, which form a large group of plant constituents that are often found as glucosides.[20] They have a characteristic cyclopentapyranoid structure known as an iridane skeleton. Iridoides are common constituents of the Verbenaceae and Lamiaceae families.[12,21] Like the flavonoids, they also possess anti-inflammatory properties together with analgesic and antirheumatic properties. They also have a bitter taste and thus contribute to the bitter tonic effect, which is the induction of secretion of bile and digestive juices to improve the function of the digestive system.[12] The iridoides theviridoside and theveside-Na have been found from *L. turbinata* while the iridoid geniposide was isolated from *L. alba* (**Figure 2.10**).[2]

[20] Connolly J. D., Hill R. A.1991. *Dictionary of Terpenoids*. Vol.**1**. Chapman and Hall. xvii.

[21] Heinrich M., Barnes J., Gibbons S., Williamson E. M. 2004. *Fundamentals of Pharmacognosy and Phytotherapy*. Elsevier Science Limited. Churchill Livingstone. 80-81.



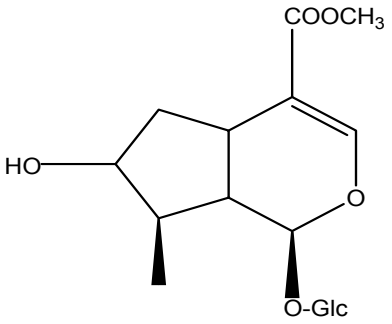
Theviridoside	R_1 **= CH_3**	R_2 **=OH**
Theveside-Na	R_1 **= Na**	R_2 **=OH**
Geniposide	R_1 **= CH_3**	R_2 **= H**

Figure 2.10: Iridoid compounds isolated from *L. turbinata* and *L. alba*

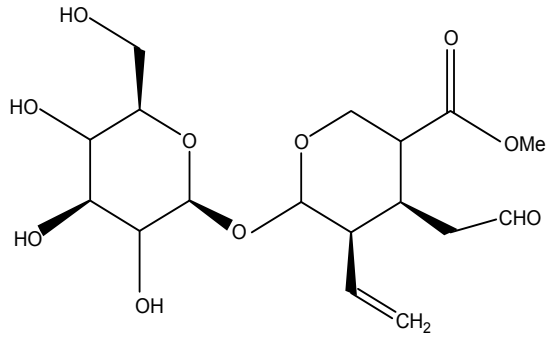
Ten iridoid and secoiridoid glucosides as well as their ester derivatives have been isolated from the leaves of *L. graveolens*. Major constituents were the caryoptosidic acid with an ester at the C-6 position of a glucose molecule and lippioside, which had caffeoyl residues. Minor constituents were loganin, secologanin, loganic acid (**Figure 2.11**), caryoptoside, secoxyloganin, dimethylsecologanoside, secoxyloganin and 8-epi-loganic acid.[7]

The iridoid contents of the genera belonging to the Verbenaceae family from Brazil, examined through a cladistic analysis (a system of biological arrangement that will group organisms on the basis of their shared features in order to decide their common ancestors), yielded (i) C4-carboxy-iridoids such as theviridoside**,** theveside, geniposide (**Figure 2.10**) mussaenoside, ipolamiide and lamiide together with (ii) C4-decarboxylated iridoids such as aucubin, mellitoside, agnuside, harpagide and reptoside as shown in **Figure 2.12**.[22]

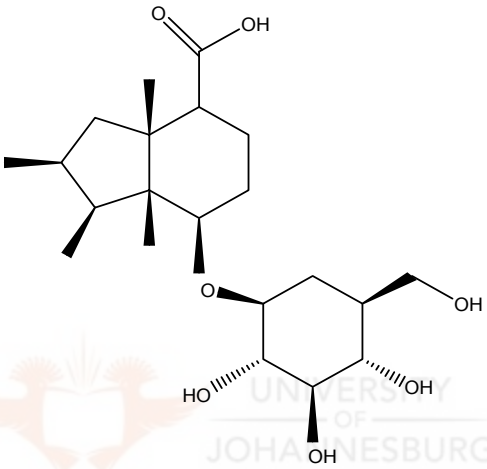
[22] Von Poser G. L., Toffoli M. E., Sobral M., Henriques A. T. *Pl. Syst. Evol*. 1997, **205**, 265-287.



Loganin



Secologanin

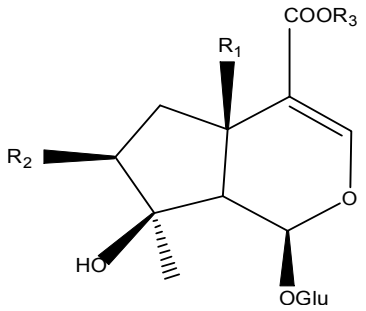


Loganic acid

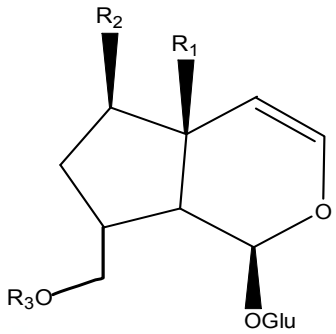
Figure 2.11: Iridoid compounds isolated from *L. graveolens*

A diterpenoid compound, 1(6)-Lippifolien-5-one has previously been isolated from *L. intergrifoli,* which is a species of which no literature could be found.[23]

[23] Dictionary of Natural Products. http://-www.chemnetbase.com. Accessed 2006.11.17



Mussaenoside	R_1 **= H**	R_2 **= H**	R_3 **= CH**
Ipolamiide	R_1 **= OH**	R_2 **= H**	R_3 **= CH_3**



Aucubin	R_1 **= H**	R_2 **= OH**	R_3 **= H**
Melittoside	R_1 **= OGlc**	R_2 **= OH**	R_3 **= H**
Harpagide	R_1 **= OH**	R_2 **= OH**	R_3 **= H**

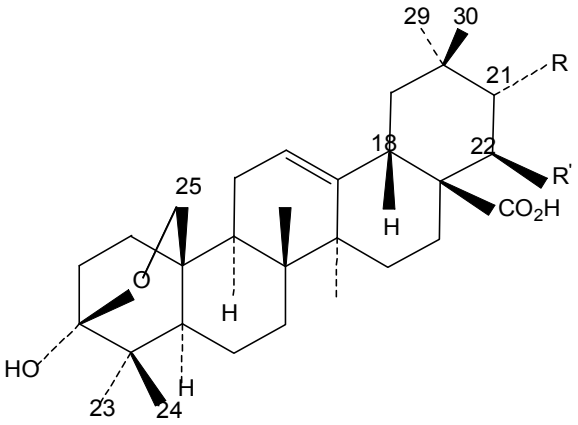
Figure 2.12: The C4-carboxy and C4-decarboxylated iridoids of the Brazilian Verbenaceae family.

- **Triterpenoids**

Triterpenoids have six isoprene units and often occur in plants with one or more sugar compounds attached, making them soluble in water.[24] Most of the triterpenoid compounds are infamous for their toxic properties and they are medicinally used for their antimicrobial, anti-inflammatory and their amarum (bitter) activities.[12] Four novel 3,25-epoxy acids (**Figure 2.13**) have been isolated from the MeOH/CH_2Cl_2 extract of *L. turbinata* together with some known triterpenoids such as camaric acid, lantanolic acid, lantanilic acid and rehmannic

[24] Van Wyk B-E., Van Heerden F., Van Oudtshoorn B. 2002. *Poisonous Plants of South Africa*. 1st Edition. Briza Publications. 140.

acid (known as lantadene A).[25] Icterogenin and rehmannic acid (**Figure 2.14**) are toxic triterpenoids that have been isolated from *L. rehmannii*.[24]



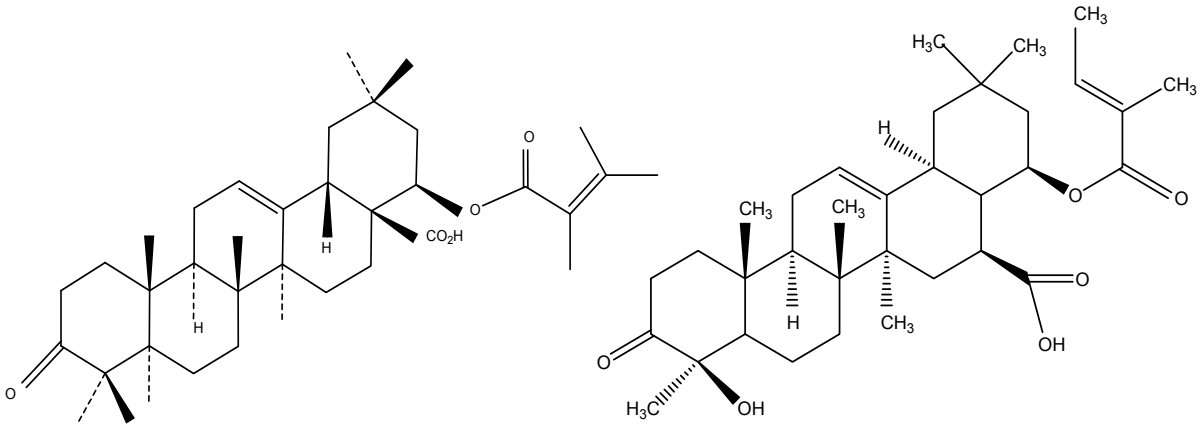
R = **OH** R' = **3-methylbut-2-ene-1-oyloxy**

R = **OH** R' = **tigloyloxy**

R = **H** R' = **2-methylbutan-1-oyloxy**

R = **OH** R' = **angeloyloxy**

Figure 2.13: The 3,25-epoxy acid triterpenoid compounds isolated from *L. turbinata*



Rehmannic acid **Icterogenin**

Figure 2.14: Toxic triterpenoid compounds isolated from *L. rehmannii*

[25] Wachter G. A., Valcic S., Franzblau S. G., Suarez E., Timmermann B. N. *J. Nat. Prod.* 2001, **64**, 37-41.

2.2. Botanical and Ethno-botanical description of *Lippia javanica*

2.2.1. *Lippia javanica*

Umsutane (SiSwati), Fever Tea (English), Koorsbossie (Afrikaans), Mosukudu (Tswana)

2.2.2.1. Plant description

Lippia javanica (Burm.f.) (**Figure 2.15**) is popularly known as "fever tea", "koorsbossie" or mosukudu. It is an erect, small woody shrub that grows up to two metres in height and is multi-stemmed.[26]

Figure 2.15: *Lippia javanica,* the woody multi-stemmed shrub

It bears dense cream white flowers, which are born in round, short flower stalks. Its fruits are found just below the flowers and are very small and dry. The small, hairy leaves are simple, opposite and found in circles of 3. These leaves are rough with a sharp apex where the base tapers and runs down the petiole (**Figure 2.16**). The margins of the leaves are saw-like and have very noticeable

[26] Van Wyk B-E., Van Oudtshoorn B., Gericke N. 2000. *Medicinal Plants of South Africa*. 2nd Edition. Briza Publications. Pretoria. 168.

veins,[27] with a strong lemon-mint smell when they are crushed. *L. javanica* is said to be one of the most aromatic among South Africa's indigenous shrubs.[28]

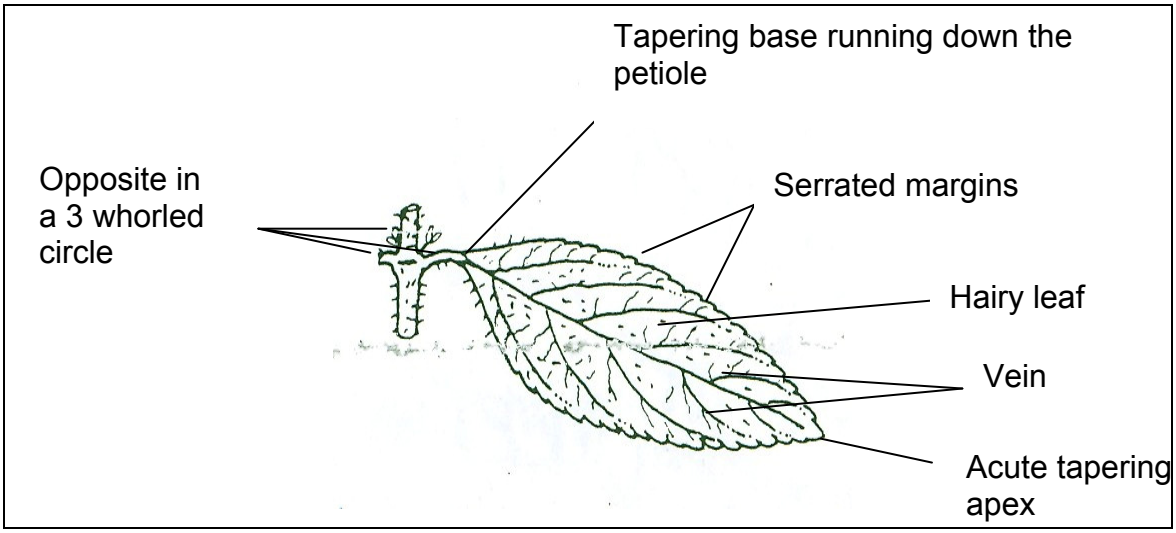


Figure 2.16: Leaf morphology of *L. javanica* [27]

2.2.2.2. Distribution and propagation

This plant species is distributed throughout large parts of South Africa, growing from the Eastern Cape extending to the tropical African countries,[26] which include Swaziland, Mozambique, Malawi, Tanzania, Zambia, Botswana and Kenya. The distribution in South Africa is shown in **Figure 2.17**.

[27] Palgrave M. C., Drummond R. B., Moll E. J. 2003. *Trees of Southern Africa*. 3rd Edition, Struik, Cape Town. 976.

[28] Watt J. M., Breyer-Brandwijk M. G.1962. *The Medicinal and poisonous plants of Southern and Eastern Africa*. 2nd Edition. E and S Livingstone LTD, 1051.

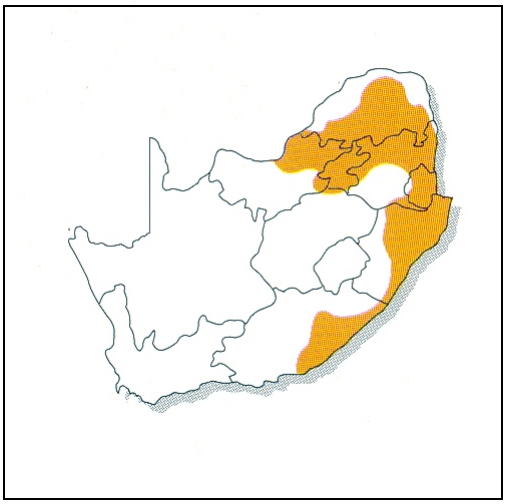


Figure 2.17: Distribution of *L. javanica* in Southern Africa [26]

The plant can be propagated from seeds but will also grow from cuttings. It grows comparatively fast in sunny areas and grows well in most soil types. It invades disturbed areas like woodlands grasslands and bushvelds and can survive in difficult circumstances with little maintenance.[27]

2.2.2.3 Uses, cultural aspects and medicinal properties

L. javanica is a well-known medicinal plant to many African tribes and herbalists. It is used as a caffeine-free tea (**Figure 2.18**) substitute in Botswana,[5,27] while in the Mpumalanga and Limpompo Provinces of South Africa the tea is used for its fever and pain-relieving activities. In Malawi and Zimbabwe it is used as a nerve tonic or generally used as a health tonic.[5,29] The Xhosa use this plant as a disinfectant against anthrax infected meat.[27] People who have been in contact with a corpse use it ritually in a cleansing ceremony. Others believe that using it would protect them against dogs, crocodiles and lightning while the Masai include this plant in making red ointment to decorate their bodies.[3,5]

[29] Victor J., Siebert S., Hoare D., Van Wyk B. 2005. http:www.fao.org/ag/AGP/AGPC/doc/Show/SAfrica/saessay/htm accessed 2005.11.03.

www.exporters.bw/teaboxes-1.jpg

Figure 2.18: Tea made from the leaves of *Lippia javanica,* mostly known as mosukudu in Tswana

It can also be used in preparation for embodiment formulations prepared as lotions or as lotion bars for soothing and treating skin disorders like itching, psoriasis, dermatitis, rough skin and cracking.[30] Skin disorders like heat rashes, scratches, stings and bites can be treated with cooled tea applied as a lotion.[3,27] Some of these lotions are used for the treatment of stretch marks in pregnant women (**Figure 2.19**).

It is widely used in traditional herbal preparations both by lay people and traditional healers in treating microbial infections which include coughs, colds and generally for bronchial troubles including shortness of breath and chest.[3,26] Smoke from burning leaves is inhaled for respiratory conditions like asthma, chronic coughs and pleurisy. The Lobedu people stuff the nose with crumpled leaves to stop nasal hemorrhage and colds.[27] In South Africa it is also extensively used for the treatment of various chest ailments, stomach problems and headaches.[5]

[30] Sprinstead P. R. 2005. United States Patent Application. UTMAN Law offices LTD, 1-8.

www.aromatherapyapa.com

Figure 2.19: Skin lotions made from *Lippia*, which are used, as insect repellents, colic relief and for treating stretch marks in pregnant women.

A recent study on HIV and AIDS patients at Ngwelezane hospital in Northern KwaZulu Natal in South Africa reports that fresh leaves of *L. javanica* are used by the patients to make tea infusions that fight against the basic symptoms of AIDS.[31] Furthermore, *L. javanica* is used synergistically with *Artemisia afra* as an effective remedy against malaria, measles and as a prophylactic against lung infections, dysentery and diarrhea.[3,27]

The therapeutic actions of *L. javanica* include analgesic, anti-inflammatory, antipyretic, antispasmodic, antiaggregant, antihistaminic, antiseptic, icterogenic, insectifugic, antimalarial and phototoxic activities.[3,32] Leaf infusions of *L. javanica* are known to be responsible for the anthelmintic property of the plant while its

[31] Can Traditional Medicine help AIDS? http://www.i-sis.org.uk/CTMHAids.php accessed 2005.11.03.

[32] Durrheim D. N., Govere J. M., Braack L., Gericke A., Spear R. *Exploiting Nature's Bounty for Malaria Control.* http://www.tropmed.org/rreh/vol1__12htmarea accessed 2005.11.03.

essential oils possess antimicrobial activities which explains its use for treatment of coughs, colds and respiratory disorders.[3]

In recent studies, the main compounds contained in the leaf essential oils of *L. javanica* are limonene, linalool, myrcene, *p*-cymene, caryophellene, myrcenone, dihydro-tagetone, *cis*–tagetone, (*E*)- and (*Z)*- targetenone.[3] An *in vitro* study of the essential oils of this species from the Limpompo Province in South Africa showed a low activity against *Escherichia coli* and *Staphylococcus aureus* but these oils showed high activity against *Plasmodium falciparum* in micromolar concentrations of 8 μg/ml. In contrast, *L. javanica* from Zimbabwe possess the highest activity against the mentioned bacteria. This plant also showed activity against *Salmonella gallnarum, Candida albicans, Pseudomonas aeruginosa* and *Klebsiella pneumoniae.*[5] A collective sample of *L. javanica* from Fairland in South Africa tested against *Cryptococcus neoformans* and *K. pneumoniae* showed positive results which supported the use of this plant in the treatment of respiratory infections such as fevers and bronchitis.[3]

A current research study reports the insect repelling properties of the alcoholic extracts of *L. javanica* plants, which provided an appreciable protection of 77% against *Anopheles arabiensis* at a four-hour post application rate.[32] It was also recently discovered that the acetone and methanol extracts of *L. javanica* are active against the bacteria *S. aureus, Bacillus subtilis, Proteus mirabilis* and *Serratia marcescens.*[32] The chemical compounds and microbial activities of this plant were observed to vary with their geographical location. Thus, *L. javanica* plants collected at different times and from different regions might have different activities on microorganisms.[33] A previous study on the polar extracts of *L. javanica* detected terpenoids, flavonoids,[34] and a number of amino acids including alanine, asparagine and arginine as the major amino acids.[35]

[33] Samie A., Obi C. L., Bessong P. O., Namrita L. *Afr. J. of Biot.* 2005. **4(12)**, 1443-1451.

[34] Bell E.A. *Biochem. J.* 1962, **83**, 225 in [35] Neidlein R., Stahle R. *Deut. Apoth. Ztg.* 1974. **40**, 1588-1592.

[35] Neidlein R., Stahle R. *Deut. Apoth. Ztg.*1974. **40**, 1588-1592.

In view of the traditional medicinal importance of *L. javanica*, it was surprising that not much is known on the polar fractions of this plant. The research reported in this dissertation was done to explore the presence of the polar compounds of *L. javanica*, which may contribute to these medicinal properties.

2.3. Other South African *Lippia* species

2.3.1. *Lippia scaberrima*

Lippia scaberrima **(Figure 2.20)** known as beukesbossie[24] or *musukujane*[36] is one of the *Lippia* species found in Southern Africa and it is similar to *L. javanica* in its physical features. It is a deciduous perennial shrub,[37] which differs from *L. javanica* and the other species by its large conspicuous bracts below its cream white flowers, which are borne in long rounded heads.[24,36] The leaves of *L. scaberrima* are long and narrow and have a smell that resembles lavender.[37] Like *L. javanica*, the leaves are opposite in circles of 3 and bears white, small flowers.[36]

L. scaberrima is also used in traditional medicine as a tea in the treatment of coughs, colds, bronchial troubles, fever[24,36] and also as a caffeine free tea like *L. javanica*.[37]

[36] Van Wyk B-E., Gericke N. 2000. *People's plants and useful plants of Southern Africa.* 1st Edition. Briza Publications. 128.

[37] http://www.exporters.bw/catalogue/herbal/thusano_lefatsheng/index.html accessed 2006.08.17.

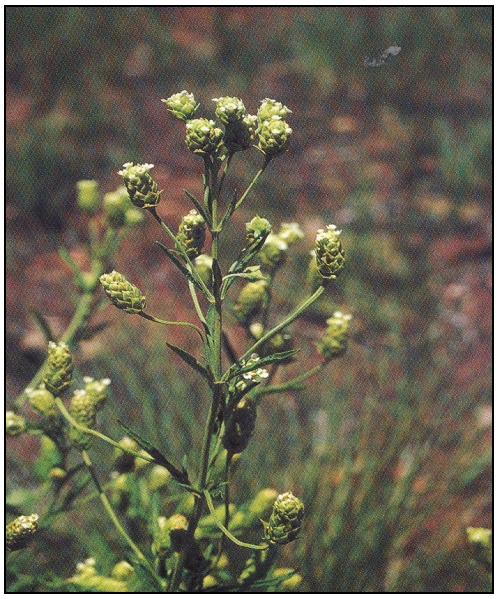


Figure 2.20: The aerial parts of *L. scaberrima*[31]

L. scaberrima occurs most abundantly in the Northern Cape Province and in the North West Province along the Suikerbosrand and Witwatersrand regions,[24] (**Figure 2.21**) growing very well in the desert veld of Botswana.[37].

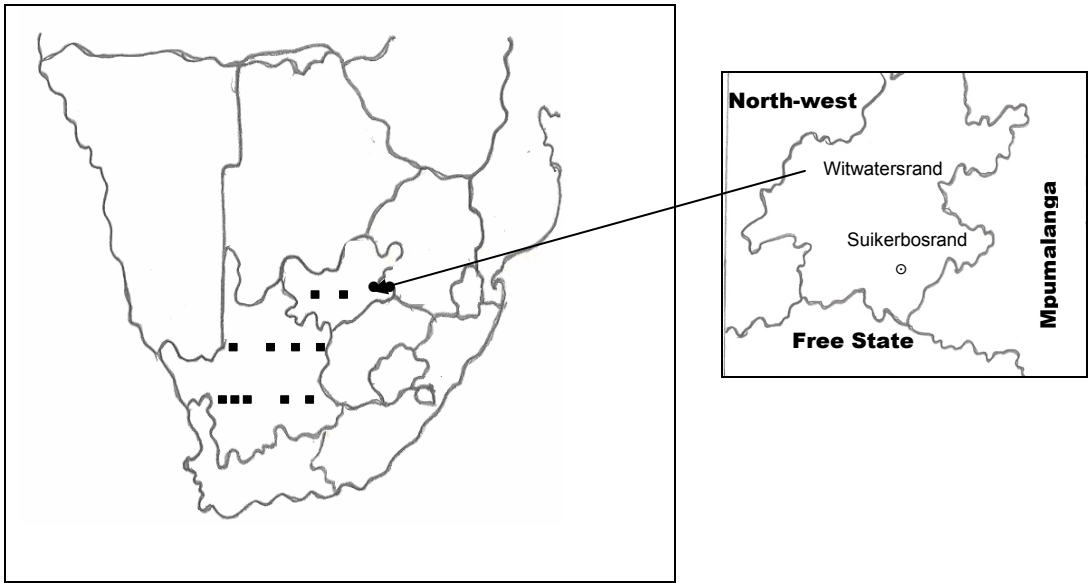


Figure 2.21: Distribution of *L. scaberrima* in South Africa

2.3.2. *Lippia rehmannii*

L. rehmannii, well known as laventelbossie, is an aromatic plant like *L. javanica* and *L. scaberrima* but shorter with a height of 0.5 m as compared with the latter which grows to a height of 2 m high. It has erect branches that grow from the ground level (**Figure 2.22**). The leaves are prominently veined, rough on the touch with saw-like margins and are opposite in pairs of two. Like both *L. javanica* and *L. scaberrima* it has small cream white flowers but which are borne in short conical spikes along the axils of the leaves and yet unlike the other species, the flower bracts are longer than the flowers.[24]

Figure 2.22: The woody shrubby nature and the flower heads of *L. rehmannii*

While *L. javanica* and *L. scaberrima* are used as traditional medicines, *L. rehmannii* is a toxic plant containing triterpenoids such rehmannic acid and icterogenin (**Figure 2.14**). These triterpenoids are toxins, which causes liver damage and jaundice. *L. rehmannii* plants are distributed in the Northern parts of South Africa mainly in the Magaliesberg region of Pretoria as shown in the map in **Figure 2.23**.[24,38]

[38] Van Wyk B-E., Malan S. 1998. *Field Guide to the Wild Flowers of the Highveld*. Struik Publishers (Pty) Ltd. 182.

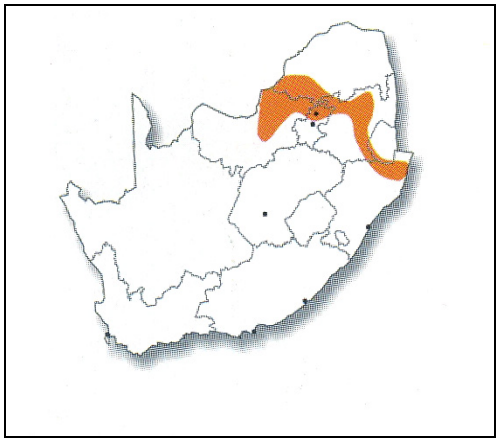


Figure 2.23: Map of Southern Africa showing the distribution of *L. rehmannii* [24]

It is very likely that this plant could be mistaken for *L. javanica* because of the close relationship that they have in their physical features and the fact that they are both found in the same environment. The experimental methods elaborating on the phytochemical differences between *L. javanica* and *L. rehmannii* follow in **Chapter 3** and results are presented in **Chapter 4**.

2.3.3. *Lippia wilmsii*

Another South African *Lippia* species is *L. wilmsii.* Apart from the fact that it hybridizes readily with *L. javanica* and it is thus very unlikely to obtain a pure *L. wilmsii* species, not many localities for this species are known. Also, the scope of the study did not include the species. For these reasons, no focus fell on *L. wilmsii* in this dissertation.

CHAPTER 3

MATERIALS AND METHODS

Introduction

A geographical chemical variation study of the aerial parts of *Lippia javanica* found in Swaziland and South Africa was done. This was achieved by extracting plants from a total of 20 locations (11 from Swaziland and 9 from South Africa) and subjecting them to various chromatographic techniques such as: TLC, CC, HPLC and GC/MS. Two polar compounds from the methanol extracts have been isolated and characterized using IR spectrometry, 1D and 2D NMR spectrometric techniques FAB/MS. A further comparison was made between the chemical compounds found in *L. javanica* and *L. rehmannii* (which is known as a toxic *Lippia* species). Due to the late rains it was not possible to get *L. scaberrima* to compare its chemical compounds with those of the other species (*L. javanica* and *L. rehmannii*).

3.1 Chemical variation

3.1 .1. Collection and extraction

The aerial parts of *L. javanica* were collected from different sites in Swaziland and South Africa and those of *L. rehmannii* were collected from Hartbeespoort, Protea-Ridge and Sterkfontein in the north east and north west of the Gauteng Province in South Africa. The plant samples from Swaziland were collected from the four regions (Hhohho, Shiselweni, Manzini and Lubombo) of the country. Voucher specimens were deposited in the herbarium at the University of Johannesburg, Department of Botany and Plant Biotechnology, (Kingsway campus).

The plant material was dried in open air and crushed into fine powder using a Waring blender. Three different extractions were made using 2 cm^3 boiling H_2O, 1 cm^3 ether/hexane (1:1) and 3 cm^3 MeOH, respectively with 0.3 g dried plant

material in each case. Similarly, boiling H_2O and MeOH extractions were done with the same amount of plant material, respectively for *L. rehmannii* (no ether/hexane extract was used in this case since the non-polar compounds were not the focus of the project). For each of the extracts, the materials were mixed by shaking the test tubes in which they were prepared for a minute and leaving the tubes for a period of 48 hours at room temperature. Both the *L. javanica* and *L. rehmannii* extracts were filtered using pasteur pipettes and cotton wool to separate the crude liquid extract from the plant residue. The MeOH and ether/hexane extracts were evaporated to dryness in the fume hood over a period of 48 hours while the H_2O extracts were freeze dried also for a period of 48 hours. The yields are given in **Table 4.1**.

The MeOH extracts were used to screen for polar compounds such as flavonoids and triterpenoids as well as medium polarity compounds such as phenyl propanoids. The ether/hexane extracts were used to screen for the compounds of medium polarity as well as the non-polar essential oils which are known for their antimicrobial activities in *L. javanica.*[1] The boiling H_2O extracts were done to mimic the extraction methods prescribed when using *L. javanica* as teas, tinctures or infusions. These extracts would contain the compounds that may be responsible for the pharmacological activities that are attributed to this plant when used in the listed applications. The activities may include treating coughs and skin disorders together with the antidiarrhoeal activity for which carbohydrates mucilages are responsible. Compounds in the boiling water extracts include mono- or disaccharides and amino acids but also triterpenoids, phenyl propanoids and flavonoids.[2]

Since *L. rehmannii* has similar physical features than *L. javanica,* at first glance, the two could easily be mistaken for each other, such that people may use either

[1] Viljoen A. M., Subramoney S., Van Vuuren S. F., Başer K. H. C., Demirci B. *J. Ethnopharmacol.* 2005, **96**, 271-277.

[2] Van Wyk B-E., Wink M. 2004. *Medicinal Plants of the World*. 1st Edition. Briza Publications, Pretoria.394.

thinking that they are using *L. javanica*. The boiling H_2O extracts were also used to compare the chemical compounds contained in the tea infusions of the two species to determine if the toxic components could be extracted in this manner.

3.1.2. Thin layer chromatography (TLC)

Thin layer chromatography (TLC) plates of the ether/hexane, MeOH and boiling H_2O extracts were developed on glass-backed silica gel F_{254} (20 x 20 cm, 0.25 mm) plates from Machery Nagel. A volume of 50 μL of extracts reconstituted in 1ml of the solvent was loaded onto TLC plates at a distance of 1 cm from the bottom of the plate. The plates were developed in a developing tank with the appropriate solvent system for compound separation. To detect the compounds TLC plates were dipped into visualizing reagents, and sometimes baked depending on the visualization technique. These experimental procedures are outlined in **APPENDIX A**. Specific procedures for the development of the ether/hexane, methanol and water extracts are given in the sections that follow.

3.1.2.1. Ether/hexane extracts

The ether/hexane extracts were re-suspended in 1.0 cm^3 of ether/hexane (1:1) and applied on a TLC plate. The plate was developed in an ether/hexane (2:3) solvent system and the compounds were detected by dipping the plate in 1% vanillin/EtOH followed by 5% H_2SO_4/EtOH reagents. The plate was then baked at 100°C for 6 minutes. Such treatment facilitates the detection of monoterpenoid alcohols as blue to blue violet spots, where phenyl propanoid compounds would exhibit colors from blue, blue-violet, red-violet or orange-red.[3] Results are discussed in **Chapter 4**.

3.1.2.2. Methanol extracts

The MeOH extracts were re-suspended in 1.0 cm^3 MeOH and loaded onto two plates, each developed in a different solvent system. One of the plates was developed with $CHCl_3$/MeOH/H_2O/CH_3COOH (6:3:0.8:0.6) followed by dipping in

[3] Wagner H., Bladt S. *Plant Drug Analysis. A thin layer chromatography atlas*. 1996. 2nd Edition Springer-Verlag Berlin Heidelberg, New York. 166.

1% vanillin/EtOH and 5% H_2SO_4/EtOH and consequently baked for 6 minutes for flavonoid screening. The other plate was spotted with *L. rehmannii* extracts together with two representative extracts of *L. javanica.* This plate was developed in EtOH/toluene/formic acid (50:50:20) and visualized using an anisaldehyde/H_2SO_4 reagent for the screening of triterpenoids.

3.1.2.3. Boiling water (tea) extracts

Water extracts were re-suspended in 1.0 cm^3 MeOH/H_2O (1:1). These were spotted on a plate and developed in BuOH/CH_3COOH/H_2O (4:1:2) followed by visualization by means of 2% ninhydrin as a screening for amino acid. A summary of the TLC solvent systems and visualizing reagents for the different extracts is given in **Table 3.1**. Results are discussed in **Chapter 4**.

Table 3.1: TLC solvent systems and spray reagents used for the chemical variation study of *L. javanica* from different localities.

Classes of compounds	Solvent/ visualizing reagents	Water extract	Methanol extract	Ether/hexane extract	Expected colour
AMINO ACIDS	Solvent System	BuOH/ CH_3COOH/H_2O (4: 1: 2)	-	-	Pink to orange
	visualizing reagent	2% ninhydrin	-	-	
FLAVONOIDS	Solvent System	$CHCl_3$/MeOH/H_2O/ CH_3COOH (6: 3: 0.8: 0.6)	$CHCl_3$/MeOH/H_2O/ CH_3COOH (6: 3: 0.8: 0.6)	-	Yellow
	visualizing reagent	1% vanillin/EtOH and 5% H_2SO_4/EtOH 100°C	1% vanillin/EtOH and 5% H_2SO_4/EtOH 100°C	-	
TRITEPENOIDS	Solvent system	-	Ethyl acetate/toluene/ formic acid (50:50: 20)	-	Violet (purplish-blue)
	visualizing reagent	-	Anisaldehyde/H_2SO_4 100°C	-	
NON-POLAR (PHENOLIC COMPOUNDS)	Solvent System	-	$CHCl_3$/ MeOH 60:30	$CHCl_3$/ MeOH 60:30	Purple/ Pink
	visualizing reagent	-	1% vanillin /EtOH and 5% H_2SO_4/EtOH 100°C	1% vanillin/EtOH and 5% H_2SO_4/EtOH	
VERY NON POLAR COMPOUNDS	Solvent System	-	ether/ hexane 2:3	ether/hexane 2:3	
	visualizing reagent	-	1% vanillin /EtOH and 5% H_2SO_4/EtOH 100°C	1%vanillin/EtOH and 5% H_2SO_4/EtOH	Blue-blue violet, red, orange

3.1.3. Gas chromatography-mass spectrometry (GC/MS)

Gas chromatography (GC) was used as a separating technique while mass spectroscopy (MS) was used to identify and quantify the separated compounds. With GC/MS, it is possible to characterize components in a mixture of compounds.

3.1.3.1. Non-polar compounds

The ether/hexane extracts of *L. javanica* from Swaziland and South Africa (discussed in 3.1.1) were re-suspended in 4 cm^3 of 1:1 (ether/hexane) and analyzed by GC/MS using a Varian 3800 capillary GC that is coupled with a Saturn 2000 MS. The analysis conditions are given in **Table 3.2**. The results are discussed in **Chapter 4**.

Table 3.2: Conditions for GC/MS analysis for the non-polar compounds

Column type	**RTX-5MS (5% diphenyl-95% dimethylpolysiloxane, 30 m X 0.25 mm)**
Injection temperature	**60°C for 1 min raised to 300°C at 100°C/min for 5 min decreased to 60°C at 200°C/min and maintained for 33.40 min**
Injection volume	**1.0 µL**
Carrier gas	**Helium**
Column flow	**1.0 cm^3/min**
Oven programme	**50°C for 1 min increased to 210°C at 4°C/min increased again to 300°C at 45°C/min**

3.1.3.2. Amino acids

There are polar compounds, such as the amino acids, flavonoids, sugars and many more, which cannot be directly analyzed by GC/MS. This is because they are not volatile enough or they are strongly attracted to the stationary phase due to these molecules having polar functional groups such as hydroxyl groups. For the amino acids, derivatization methods have been applied to increase the volatility and thermal stability of these compounds. Derivatization also increases

the detector response, improve separation and reduce tailing.[4] Derivatized amino acids can be analyzed by using techniques such as GC/FID, LC/MS, GC/NPD or GC/MS of which the latter was used in this study due to its availability and ease by which results may be interpreted.

The freeze dried aqueous extracts prepared in section 3.1.1 were used for amino acid determination using an amino acid derivatization kit (EZ:faast-free amino acid analysis kit). Essentially the derivatization procedure was as follows: The crude extracts (samples) were re-suspended in MeOH/H_2O (1:1). A volume of 100 μL of extract was combined with 100 μL of an internal standard (0.2 mM norvaline). These samples were then loaded onto a solid phase extraction (SPE) sorbent tip designed to retain the amino acids (**Figure 3.1.**). The SPE sorbent was consequently washed using 200 μL of *n*-propanol to ensure that nearly all compounds such as proteins, lipids and inorganic salts that could interfere with the chromatographic analyses were removed. The extracted amino acids were eluted from the stationary phase in the sorbent tip by means of 200 μL solution of sodium hydroxide and *n*-propanol (3:2), respectively. The amino acids were deprotonated at the same time. A chloroform solution (50 μL) containing the derivitizing agent (alkyl chloroformate) was consequently added to the extracted amino acids. The derivatization reaction is illustrated in **Figure 3.2**.

A vortex mixer was used to thoroughly mix all reagents involved and the mixture was then left for a minute for the reactions to proceed. This was followed by the addition of 100 μL *iso*-octane to extract the derivatized amino acids.

[4] Braithwaite A., Smith F. J. 1996. *Chromatographic Methods*. 5th Edition. Blackie academic and professional, Chapman and Hall. 135.

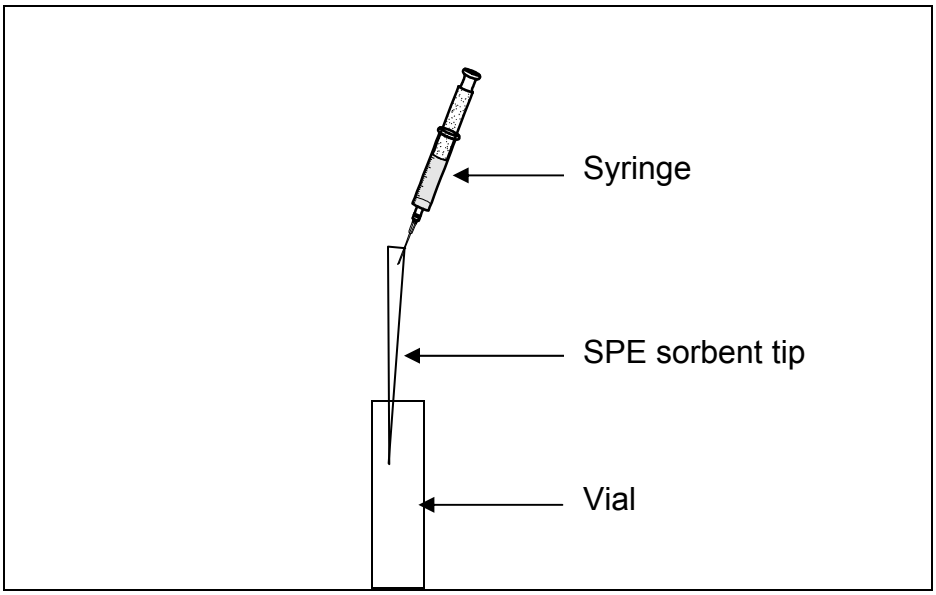


Figure 3.1: Illustration of plant samples loaded onto the sorbent tip for the extraction of amino acids.

The formation of two layers was observed with the organic layer being the bottom layer containing the derivatized amino acids. The organic layer (50 μL) was evaporated under pressurized air and the resulting derivatized amino acid mixture was redissolved in 100 μL of 80% *iso*-octane in chloroform.

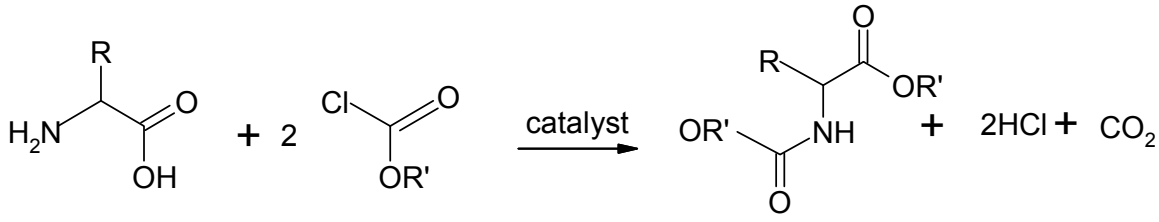


Figure 3.2: Illustration of the EZ: faast derivatization reaction

An EZ: faast-Free Amino Acid analysis method provided with the kit was used for the GC/MS analysis. The derivatized amino acids were then analyzed in the GC/MS using the method outlined in **Table 3.3**.

Table 3.3: EZ: faast -Free (Physiological) Amino acid analysis method by GC/MS

Column type	**Zebron ZB-AAA GC 10 m X 0.25 mm Amino Acid Analysis GC column**
Injector	**Split 1:15 @ 250°C, 1.5 μL (with hot needle)**
Carrier gas	**Helium, constant flow**
Flow rate	**1.1 cm^3/min**
Oven program	**30°C/min from 110° to 320°C (hold 2 min)**
Scan range	**45-450 m/z**
Sampling rate	**3.5 scans/sec**

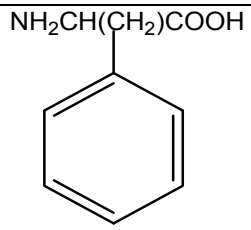
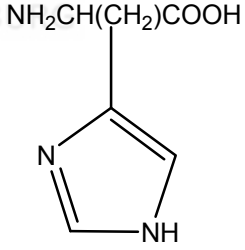
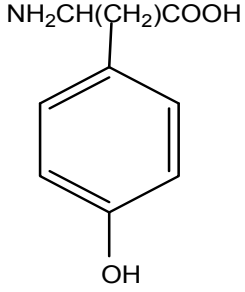
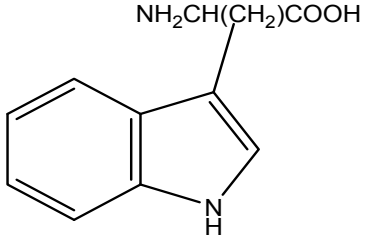
Calibration standards containing mixtures of amino acids used for quantitation purposes were prepared and derivatized using the same method in which the sample mixtures were prepared (the standards provided in the kit contained underivatized amino acids). The final volume for all samples and standards was the same. The structures of amino acids included in the standards are presented in **Table 3.4**. Five calibration levels of different volumes of the standard (SD) solution were prepared as described below. The same volume of the internal standard was added to each SD solution.

- **calibration level I – 50 nmoles/cm^3:** 25 μL SD 1 solution, plus 25 μL of SD 2 solution and 25 μL of SD 3 solution plus 100 μL internal SD.
- **calibration level II – 100 nmoles/cm^3:** 50 μL of each SD solutions 1 to 3 plus 100 μL of the internal SD.
- **calibration level III – 200 nmoles/cm^3:** 100 μL of each of the three SD solutions plus 100 μL of the internal SD.
- **calibration level IV− 300 nmoles/cm^3:** 150 μL of each of the SD solutions plus 100 μL of the internal SD.
- **calibration level V− 350 nmoles/cm^3:** 175 μL each of the SD solutions plus 100 μL of the internal SD.

Even though the volumes of the standard mixtures were different initially, this had no influence on the final concentration of the mixtures injected in to the GC/MS

as all of the standard mixtures were adsorbed onto the sorbent before derivatization.

Table 3.4: The list of amino acids detected in the aerial parts of *L. javanica*

Retention time (min)	**Amino acid**	**Abbreviation**	**Structure**
1.125	α-Aminobutyric acid	ABA	$CH_3CH_2CH(NH_2)COOH$
1.222	Valine	VAL	$NH_2CH(CHCH_3CH_3)COOH$
1.448	Isoleucine	ILE	$CH_3CH_2CH(CH_3)CH(NH_2)COOH$
1.896	Asparagine	ASN	$NH_2CH(CH_2CONH_2)COOH$
2.822	Phenylalanine	PHE	
3.131	α-Aminoadipic acid	AAA	$H_2OCH_2CH_2CH_2CH(NH_2)COOH$
4.157	Lysine	LYS	$NH_2CH(CH_2)_4NH_2COOH$
4.336	Histidine	HIS	
4.619	Tyrosine	TRY	
4.896	Tryptophan	TRP	

Quantification of the data was accomplished by developing a processing method in the GC/MS. This was done by building a compound table of specific mass fragments and comparative signal strengths for each compound to be quantified. When these mass fragments appeared with the correct retention times in the chromatogram of an injected sample, the identity of the targeted compound was obtained. The quantification of these compounds was then achieved by comparing their respective target ion signal intensities with the concentration curves of prepared volume from the different derivatized amino acid standards.

Since norvaline was used as an internal standard, it aided in the correction for variations in the injected volumes and the peak retention times of the amino acids. Calibration curves for each compound in the standard mixtures were obtained by the GC/MS plot of the peak area and concentration of the identified compounds. Compound identification was done by means of spectrum matching using an EZvarian amino acid library.

3.1.4. High-performance liquid chromatography (HPLC)

The MeOH and water extracts were resuspended in 4 cm^3 of MeOH and 1:1 MeOH/H_2O respectively and analyzed by HPLC. Some compounds that were isolated (**see section 3.2**) were also re-suspended in 4 cm^3 of MeOH and analyzed together with the extracts. MeOH was used because of its protective role in preventing phenolic compounds from being oxidized by enzymes such as phenoloxidases that could also be present in the mixture.[5]

The analysis of these crude extracts was performed using a Waters 600E HPLC system (Millipore) equipped with a Photodiode Array detector (PDA). A Luna C-18 column (150 X 4.60 mm and a 5 μ stationary phase) was used. The UV spectra were recorded between 210 nm and 400 nm for the identification of phenolic compounds. A constant flow rate of 1.0 cm^3/min and an injection volume of 10 μL was used. Mobile phase **A** consisted of acetonitrile while mobile phase

[5] Proestos C., Sereli D., Komaitis M. *Food Chem.* 2006, **95**, 44-52.

B consisted of 1% acetic cid. Elution was done in an isocratic mode using **A**/**B** (18:82 v/v).[6]

3.2. Isolation and structural elucidation

3.2.1. Collection and extraction

After the chemical variation study, one plant (**TD 57**) with representative natural product distribution was chosen for the isolation and structural elucidation of the compounds listed below. It was also possible to harvest this particular plant in bulk.

The plant material was collected (about 1 kg), dried and crushed as before, but in this case, 75.0 g of the ground aerial parts was extracted with 750 cm^3 methanol, for 23 hours. A sintered-glass filter with 5 g of celite, rinsed with 30 cm^3 methanol, was used to filter the plant material. The solvent was evaporated in a fume hood over a period of 48 hours and a green residue with a mass of 7.64 g was obtained. The extract was partitioned between hexane and 90% methanol-water to allow for the partial separation of the polar (4.61 g) and non-polar (3.01 g) compounds.

3.2.2. Thin layer chromatography (TLC)

Phytochemical investigation was initiated by running TLC plates of the polar extract. Aluminium backed silica gel 60 F_{254} precoated TLC plates (silica layer of 0.2 mm) were used. The solvent systems used for the chemical variation study by TLC were modified for use with column chromatography (CC) during the isolation of the compounds listed further on in this chapter. Modification was done as it is not advisable to use acids in CC solvent systems due to the fact that compounds in the sample might by be hydrolyzed during the drying process. When tracking the progress of separation in CC, TLC plates of the various fractions were viewed under the UV lamp and then dipped into 1% vanillin/EtOH

[6] Lei L., Yang F., Zhang T., Tu P., Wu L., Ito Y. *J. Chrom. A.* 2001, **912**, 181-185.

followed by 5% H_2SO_4/EtOH. The plates were heated at 100˚C in the oven until maximal visualization of spots was obtained.

3.2.3. Column chromatography (CC)

Several compounds were observed from the TLC of the polar extract developed in the $CHCl_3$/MeOH/H_2O/CH_3COOH (6:3:0.8:0.6) solvent system. Yellow/orange and purple compounds were observed to be the major components with Rf (retention factor) values of 0.54 and 0.71, respectively.

The column chromatography of the mentioned extract was carried out using Merck silica gel 60 F_{254} on columns of different diameters. The polar extract (4.61 g) was dissolved in a small amount of solvent and pre-adsorbed on silica gel. After evaporating the solvent by means of a rotatory evaporator the adsorbed extract was then gently loaded onto the column as shown in **Figure 3.3**.

The mobile phase solvent systems used were varying ratios of $CHCl_3$, MeOH and H_2O. Elution generally started with a system containing a large amount of $CHCl_3$, and ended with a solvent system with little or no chloroform but high amounts of methanol or H_2O. An amount of 4.61 g of the polar fraction was subjected to a column chromatography for a preliminary separation of groups of compounds and four major fractions were collected and were numbered as **A** (0.92 g), **B** (1.62 g), **C** (1.01 g) and **D** (1.04 g).

- **Fraction A**

Fraction **A** (0.92 g) was subjected to silica gel column chromatography as a stationary phase on a column with a diameter of 20 mm. It was eluted using a gradient solvent system starting with $CHCl_3$/MeOH/H_2O (50:30:3). The polarity was gradually increased by adjusting the solvent to $CHCl_3$/MeOH/H_2O (40:30:3). The fraction containing Compound **1** was applied onto a column again in order to purify it using a 10 mm diameter column with $CHCl_3$/MeOH/H_2O (50:30:2) followed by $CHCl_3$/MeOH/H_2O (50:20:2).

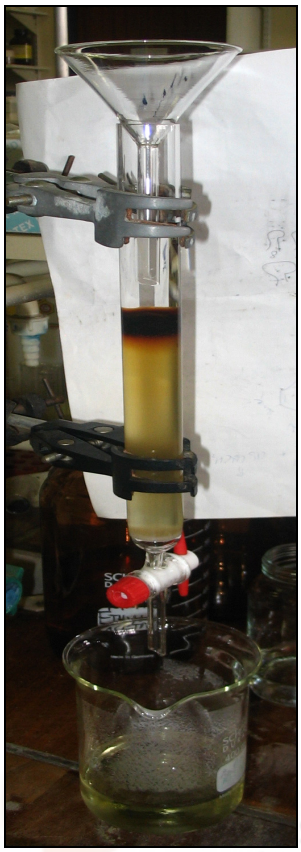


Figure 3.3: Illustration of a column chromatography eluting the polar extract of *L. javanica.*

Compound **1** was still not pure so preparative chromatography was used to purify it. Preparative thin-layer chromatography (TLC) was done on glass-backed silica gel F_{254} (20 x 20 cm, 0.25 mm) plate. The sample was dissolved in (1:1) MeOH/ H_2O and loaded across the plate in a straight line, 2 cm from the bottom of plate using a pipette. The plate was developed twice in the tank using $CHCl_3$/MeOH/H_2O (50:30:2) as a solvent system. Small strips on the sides of the plates were sprayed with 1% vanillin/ EtOH and 5% H_2SO_4/EtOH followed by heating only the sprayed strips on a hot plate for 5 minutes to develop bands of compounds. The compounds were marked and scraped off using a spatula and removed from the silica gel by dissolving this mixture of silica and the compound in warmed acetone and filtered to yield 15 mg of Compound **1**.

- **Fraction B (1.62g)**

An amount of 1.62 g of fraction **B** was subjected to silica gel (170 g) in a 25 mm column eluting with $CHCl_3$/MeOH/H_2O (70:30:0.5) increasing polarity to $CHCl_3$/MeOH/H_2O (70:30:3) to yield an impure Compound **2**. This compound was further purified by column chromatography in a column of a diameter of 20 mm by eluting with $CHCl_3$/MeOH/H_2O (70:30:1) to yield 11 mg of a pure Compound **2**.

- **Fraction C (1.01g)**

Fraction **C** (1.01 g) was applied to a 25 mm column of silica gel (100 g) eluting with a gradient of $CHCl_3$/MeOH (90:10, 85:15 and 80:20) to yield an impure Compound **3**. Further purification of this compound was done using preparative TLC with a solvent system of $CHCl_3$/MeOH (70:30) and yielded two pure Compounds **3** (12 mg) and **4** (7 mg).

- **Fraction D (1.04g)**

Fraction **D** (1.04 g) was subjected to silica gel (100 g) and was purified by gradient elution using $CHCl_3$/MeOH/H_2O (70:30:1, 70:30:3 and 60:40:2). The impure Compound **5** obtained was further purified by means of preparative-TLC. The plate was developed in $CHCl_3$/MeOH/H_2O (60:30:1). The silica band containing Compound **5** was scraped off and removed from the silica by means of warmed acetone followed by filtering to yield 23 mg.

3.2.4. Nuclear magnetic resonance (NMR)

The structures of the isolated compounds were elucidated by comprehensive 1D (^{1}H and ^{13}C) and 2D (COSY, HMBC, HMQC, HSQC) NMR spectroscopy. The 1D experiments were conducted on a Bruker Avance DRX 400, operating at 400.132 MHz for ^{1}H spectra and 100.625 MHz for ^{13}C spectra. The 2D NMR spectra were recorded at 300 MHz for ^{1}H and, 75 MHz for ^{13}C spectra using a Bruker Avance 300 spectrometer. The chemical shifts are reported in parts per million (ppm) using the residual signal *d*-MeOH (δ = 3.30 ppm for ^{1}H and 49.00 ppm for ^{13}C), as the internal reference. Structures are discussed in **Chapter 4**.

3.2.5. Fast atom bombardment-mass spectrometry (FAB/MS)

Since the isolated compounds were not volatile enough to be analyzed with the GC/MS used in section 3.1.3, FAB/MS was used to determine the accurate mass and ion fragments of the compounds to aid in the structural elucidation of the compounds. A sample typically dissolved in a volatile solvent and bombarded with heavy ions such as cesium. When a droplet of the emulsion absorbs the collision energy from the cesium ions it explodes and ionizes.[7]

The analyses were carried out on a Kratos MS902/50, VG 70 SEQ spectrometer at 70 eV and 200 μA in FAB ionization mode with positive polarity at the University of Witwatersrand.

3.2.6. Infrared spectroscopy (IR)

The IR analyses of the isolated compounds were obtained from a Bruker Tensor 27 FTIR spectrometer at the University of the Witwatersrand. IR is useful in the identication of the functional groups present in the compounds. Results obtained from these experimental methods are discussed in the chapter that follows **(Chapter 4).**

[7] McMaster M., McMaster C. 1998.*GC/MS, A Practical User's Guide*. Wiley-VCH. 25.

CHAPTER 4

RESULTS AND DISCUSSION

Introduction

L. javanica is an aromatic woody shrub, which is widely known for its essential oils. These oils account for the plant's use as an insect repellent and during inhalation therapy.[1] However, very little is reported regarding the polar compounds of this species, except for the presence of two iridoides (theveside-Na and theviridoside **Figure 2.10**),[2] amino acids and sugars.[3] The fact that little research has been done on polar compounds of *L. javanica* is unusual since most medicinal preparations of plants are used and taken in the form of teas. The use of water as an extracting solvent would concentrate the polar components. Thus this study was done to investigate the chemical classes of polar components, and to detail the variation of these compounds in plants collected from Swaziland (SWD) and South Africa (SA), together with the isolation and structural elucidation of some of these compounds.

During our investigation it became apparent that the toxic *L. rehmannii* has almost identical physical features as that of *L. javanica*: such as small yellowish white flowers, aromatic leaves and woody, shrubby nature. At first glance the two species could easily be mistaken for each other, such that people may use *L. rehmannii*, which is toxic, thinking that they are using *L. javanica*. In addition to the similarity in appearance, these plants grow in the same regions and similar environments. The MeOH extracts of these plant species were investigated to investigate the presence of the toxic triterpenoids in *L. rehmannii.*

[1] Van Wyk B-E., Wink M. 2004. *Medicinal Plants of the World*. Briza Publications, Pretoria. 384, 415.

[2] Pascual M. E., Slowing K., Carretero E., Sanchez Mata D., Villar A. *J. of Ethnopharmacol*. 2001.**76**, 201-214.

[3] Neidlein R., Stahle R. *Deut. Apoth. Ztg*. 1974, **40**, 1588-1592.

Chemical variation

4.1. Plant collection

Where available, two or three different plants were harvested at each site to allow for comparison within the same (intra-population) growing area. **Figure 4.1** shows these collection sites. The plants were transported in cool aired paper bags or plastic bags out of the sun.

Voucher specimens were deposited in the herbarium at the University of Johannesburg, Department of Botany and Plant Biotechnology (Kingsway campus). Their voucher numbers and the localities are presented in **Table 4.1**.

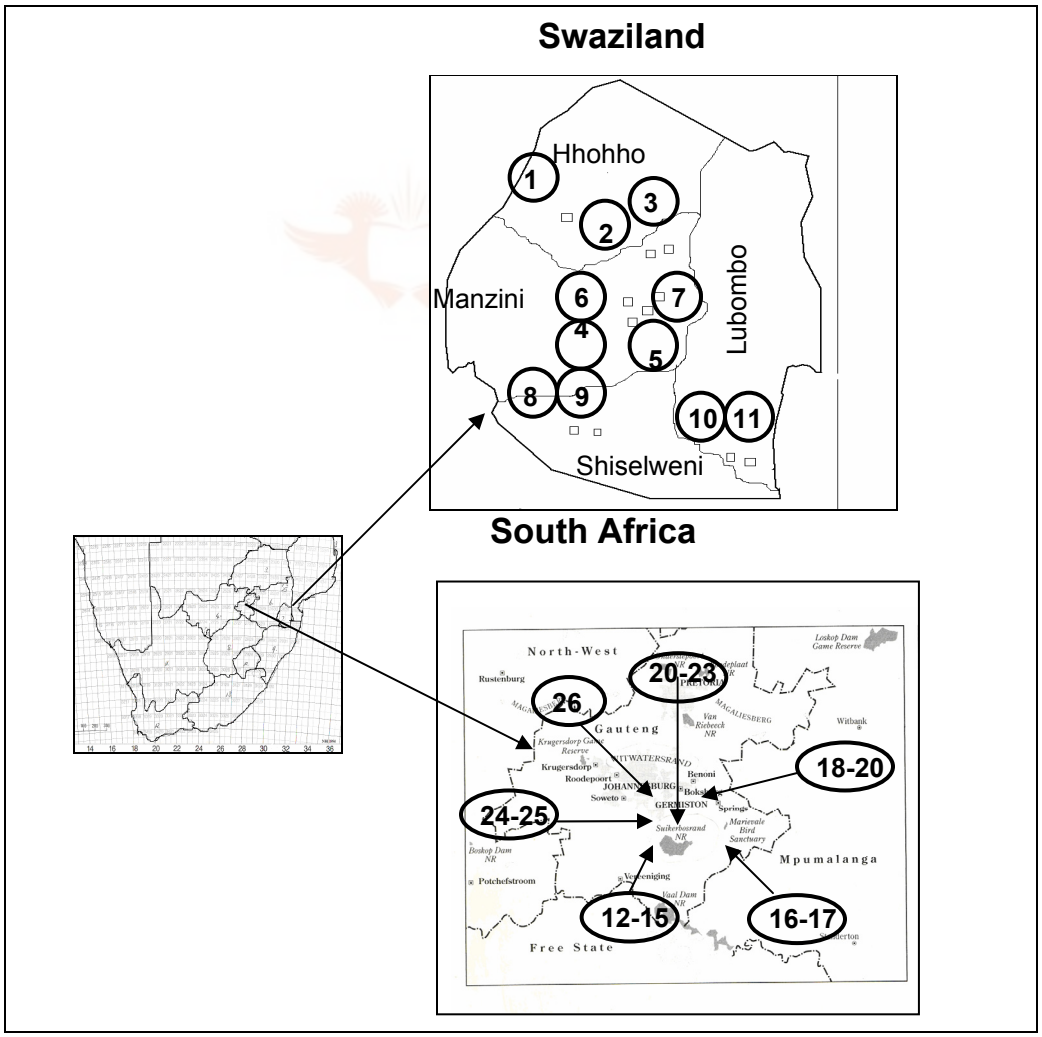


Figure 4.1: The maps of Swaziland and South Africa showing the collection sites of *L. javanica* and *L. rehmannii* plant samples.

The extracts were prepared as described in section 3.1.1. The mass yields (g extract/0.3 g dry material x 100) of the extracted plant samples using ether/hexane, MeOH and water are presented in **Tables 4.1(a)-(c)** (Extract numbers correspond with those in **Figure 4.1** and are used throughout chemical variation section).

Table 4.1(a): Localities of *L. javanica* in SWD and the mass percentage yields of the extracts.

Extract No.	Locality	Voucher No.	Ether/ hexane	MeOH	H_2O
	SWAZILAND				
1	Hhohho region, Mbabane town, Mvutshini area	TD 57	14.2	10.1	8.3
2	Manzini region ,Nkiliji area	TD 58 (a)	22.5	10.7	10.8
3	Manzini region, Nkiliji area	TD 58(b)	12.8	10.7	10.8
4	Manzini region, Matsapha town, Kwaluseni area	TD 59 (a)	17.2	2.8	11.7
5	,,	TD 59 (b)	7.2	7.6	8.3
6	,,	TD 59 (c)	15.0	8.9	10
7	,,	TD 59 (d)	31.2	11.5	16.7
8	Shiselweni region, Nhlangano town Nsongweni area	TD 60	18.7	8.2	11.7
9	Shiselweni region, Nhlangano town, Khiza area	TD 61	17.0	10.8	8.3
10	Lubombo region, Lavumisa town, Mahlabatsini area	TD 62 (a)	17.3	8.8	10.0
11	Lubombo region, Lavumisa town, Mahlabatsini area	TD 62 (b)	16.0	15.8	6.7
		Average	**17.2**	**9.6**	**10.3**

The yields of the ether/hexane extracts were higher than the yields of the MeOH and H_2O extracts and this could probably due to high quantities of chlorophyll contained in these extracts. Amongst the ether/hexane extracts, high yields were observed in the SWD plant samples. For example, extracts **2** and **7** had a mass yield of 22.5% and 31.2%, respectively. An almost insignificant difference in the average mass yield of 18.0% **{Table 4.1(b)}** was observed with the SA plant extracts compared to 17.2% **{Table 4.1(a)}** in the SWD extracts.

Table 4.1(b): Localities of *L. javanica* in SA and the mass yields of the extracts.

Extract No.	Locality	Voucher No.	Ether/ hexane	MeOH	H_2O
	SOUTH AFRICA				
12	Rooderpoort, Walter Sisulu Botanical Gardens, useful plant garden	DO,TD,TN 56 (a)	17.7	12.4	10.0
13	Rooderpoort, Walter Sisulu Botanical Gardens useful plant garden	DO,TD,TN 56 (b)	13.0	10.4	9.2
14	Rooderpoort, Walter Sisulu Botanical Gardens, along path to bushveld	DO,TD,TN 56 (c)	18.8	8.8	10.8
15	Rooderpoort, Walter Sisulu Botanical Gardens, bushveld	DO,TD,TN 56 (d)	18.6	11.4	10.0
16	Melville koppies	DO,TD,TN 57 (a)	16.5	8.8	10.8
17	Melville koppies	DO,TD,TN 57 (b)	20.2	8.2	8.3
18	Buffelspoort	DO 58 (a)	19.0	11.4	10.8
19	Buffelspoort	DO 58 (b)	17.8	9.8	10.0
20	Buffelspoort	DO 58 (c)	20.2	9.3	9.2
		Average	**18.0**	**10.1**	**9.9**

Table 4.1(c): Localities of *L. rehmannii* and the mass yields of the extracts.

Extract No.	Locality	Voucher No.	MeOH	H_2O
21	Hartbeespoort	TD, RK 1	6.7	6.2
22	Hartbeespoort	TD, RK 2	10.0	8.8
23	Hartbeespoort	TD, RK 3	6.8	6.1
24	Protea-Ridge	TD, RK 4	6.7	6.0
25	Protea-Ridge	TD, RK 5	6.7	5.9
26	Sterkfontein	TD, RK 6	7.2	6.4
		Average	**7.4**	**6.6**

The MeOH extracts of SA samples generally showed a slightly higher yield (10.1%) as compared to the SWD extracts (9.6%). This trend was only reversed in the water extracts, where the SWD plants had an average yield of 10.3% compared to the 9.6% for the SA plants. The relatively small numbers of extracts

however make these small variations insignificant. *L. rehmannii,* on the other hand, had much lower yields for both the MeOH extracts (7.4%) and water extracts (6.6%). These are about 25% lower than in the corresponding *L. javanica* extracts. The observed variation cannot be explained by the harvesting times of the plant samples nor can it be explained by the climate of each area of harvest because majority of the plants that were collected on the same day and in the same area do not have similar mass yields. This is observed in exception only in the MeOH and H_2O extracts of the plant samples of *L. javanica* harvested in the Manzini region (plant extract **2** and **3**) and those of *L. rehmannii* from Protea-ridge (plant extracts **24** and **25**).

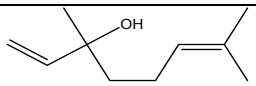
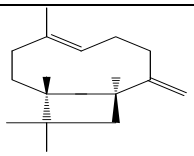
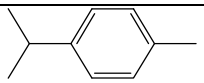
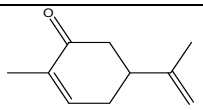
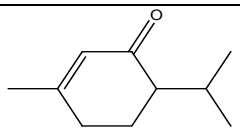
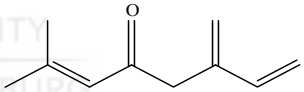
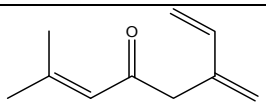
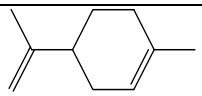
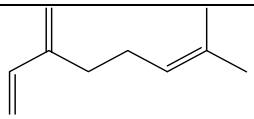
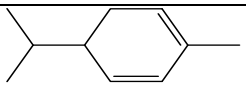
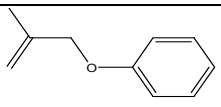
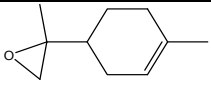
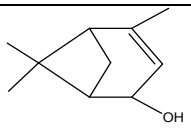
4.2 Essential oils of *L. javanica*

L. javanica is known for its essential oils (volatile compounds), which are widely used for their antimicrobial activities. Previous studies have mostly focused on these oils.[3,4,5] In view of the extensive chemical variation studies of these oils, already reported in literature, this project did not focus on essential oils, but rather on more polar compounds contained in *L. javanica*. However, the non-polar extracts from *L. javanica* were compared with the known essential oils and reported here for completion. **Table 4.2** contains some of the compounds previously identified from the essential oils of *L. javanica* isolated by means of hydro- distillation.

[4] Viljoen A. M., Subramoney S., Van Vuuren S.F., Başer K. H. C., Demirci B. *J. Ethnopharmacol.* 2005, **96**, 271-277.

[5] Omolo M. O., Okinyo D., Ndiege I., Lwande W., Hassanali A. *Phytochem*. 2004, **65**, 2797-2802.

Table 4.2: Chemical structures of the essential oils detected in *L. javanica*

Compound no.	Names of compounds	Structures
1	linalool	
2	caryophyllene	
3	*p*-cymene	
4	carvone	
6	piperitone	
7	ipsdienone	
8	myrcenone	
9	limonene	
10	myrcene	
11	α-phellandrene	
12	*o*-*Iso*-propenylanisole	
13	limonene oxide	
14	*cis*- verbenol	

In this study, solvent extraction was done from the aerial parts of *L. javanica*. The solvent extraction of the aerial parts of *L. javanica* included some of the compounds of the essential oils because of the low polarity of the solvent system, together with other compounds of a medium polarity. The presence of these compounds was investigated using both TLC and GC/MS analysis.

4.2.1 Thin layer chromatography (TLC)

The ether/hexane extracts were re-suspended in 1.0 cm^3 of ether/hexane (1:1) and a volume of 50 μL of the ether/hexane extracts of the plant were applied onto a F_{254} glass-backed silica gel plate **(Figure 4.2)** and developed in a 2:3 ether/hexane solvent system. The compounds were visualized with vanillin and H_2SO_4 as previously discussed in Chapter 3, and resulted in the identification of blue to purple stained compounds, which were assumed to be the monoterpenoid alcohols (essential oils, **EO**). Since the identity of these compounds could not be clearly determined by TLC, GC/MS analyses were used.

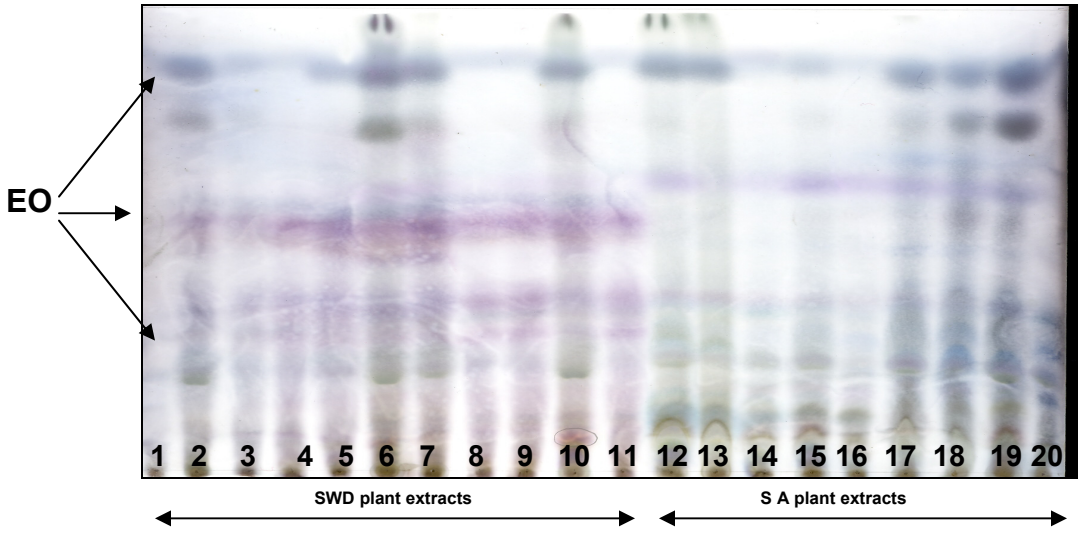


Figure 4.2: TLC results of the ether/hexane extracts of the aerial parts of *L. javanica.*

4.2.2 Gas chromatography-mass spectrometry (GC/MS)

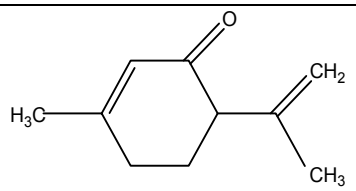
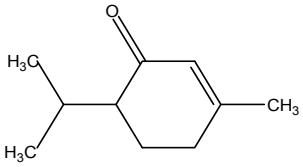
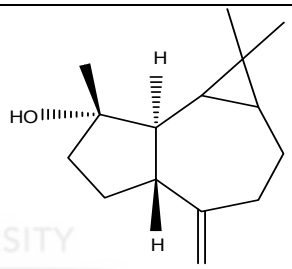
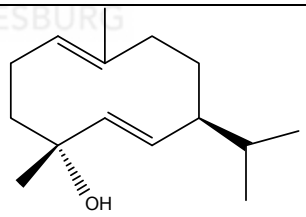
The ether/hexane extracts were re-suspended in 4 cm^3 of 1:1 (ether/hexane) and analyzed by GC/MS using a Varian 3800 capillary GC that is coupled with a Saturn 2000 MS operating in EI mode. A qualitative analysis of the GC/MS was taken because no internal standard was used. Thus the results in this section can only be compared by reference to relative changes in extracts. A total of 16 compounds (**Table 4.3**) were revealed and the identities of 5 were confirmed using a library search of the MS. A literature search showed that the identified compounds, except for 5,8-dimethyoxy-2,4-dimethylquinoline were found as essential oils with low polarity in *L. javanica*[4] and the structures of these compounds are presented in **Table 4.4**.

Table 4.3: Compounds of the non-polar extracts of *L. javanica*

RT	Number	Mass fragments (*M/z*)	Compound
14.05	1	84, 57, 56	-
17.80	2	137, 82, 59, 43, 39	*Iso*-piperitenone
21.51	3	71, 57, 43,41	-
24.70	4	82, 57, 55, 41	-
25.02	5	84, 57, 43, 71, 93, 41	-
28.52	6	217, 202	5,8-dimethyoxy-2,4-dimethylquinoline
30.50	7	71, 57, 39	-
31.21	8	109, 96,71,55	-
31.23	9	109, 96, 82, 57, 56	-
31.54	10	97, 84	-
36.43	11	71, 93, 41	-
37.08	12	96, 82, 69,39	-
37.34	13	94, 84	-
38.37	14	57, 85, 41, 39	Piperitenone
38.41	15	94, 55, 43, 41	Spathulenol
38.72	16	81,43	Germacrene-D-4-ol

Underlined values- major peaks in mass spectra

Table 4.4: Chemical structures in the non-polar extracts of *L. javanica*

Names of compounds	Structures
iso-piperitenone	
piperitenone	
spathulenol	
germacrene-D-4-ol	

4.2.3 Chemical variation

- **Geographical variation**

The 16 identified compounds in the non-polar extracts were compared to see if there were differences between those plants growing in SWD (extracts **1-11**) and those in SA, or even between plants in the same region. The GC chromatograms are presented in **Figures 4.3** and **4.4**. Previous studies on the non-polar compounds of *L. javanica* have each found different compounds to be the major compounds. A study by Viljoen et al (2005)[4] compared the essential oil compounds of *L. javanica* from SWD and SA and found linalool **1** (numbered compounds detected in this study refer to compounds in **Table 4.2**) to be the

major constituent of extracted oils in plants harvested during the same seasons of the year. Compound **2** and **3** were extracted in second and third highest concentrations. Another study (Omolo et al, 2004)[5] on *L. javanica* plants harvested in the Limpompo province found that Compound **4** was the major compound. Compound **12** and *Z*-ocimenone were extracted in the next highest concentrations followed by Compound **1**. While a third study by Neidlein and Stahle (1974) found that Compound **2** was a major compound followed by Compound **1**.[3] In the current study, (GC chromatograms are presented in **Figures 4.3** and **4.4** and a summary of these results is shown in **Table 4.5**) spathulenol (structure in **Table 4.4**) has been found to be the major compound from plants harvested in each location of the two countries, but with varying concentrations. As an example of the observed variation of this compound in our study, *L. javanica* extracts from the Lubombo region (plant extracts **10** and **11**) had lower yields of spathulenol than for instance plant extracts from the Shiselweni region (extracts **8** and **9**) which had very high concentrations of this compound.

One can then assume that spathulenol must be one of the more polar compounds in these extracts like those with the low Rf values on TLC plates (**Figure 4.2**). In one of the previous studies[4] which compared plants from SWD and SA, spathulenol was only found as a minor compound. Only three extracts from SWD out of six that were investigated in that study, had this compound in very low concentrations of 0.08%, 0.07% and 0.25% respectively. In the SA extracts from that study, the compound only had a concentration of 0.55% (confirming that it was minor). In our study, a possible reason for having spathulenol as a major compound while it was minor in the previous study could be due to the extraction method (solvent extraction) that was performed to obtain the non polar compounds.

Variation was also observed in the other compounds of the non-polar extracts of this study: plant extracts from SWD were observed to have high yields of Compounds **7** and **11** (numbered compounds detected in our study refer to

compounds in **Tables 4.3** and **4.5**) as compared to the yields of the same compounds in the SA extracts. Even though *iso*-piperitenone and Compound **1** were present in average yields in the extracts from SA in this study, they were either not present or found in trace amounts in the SWD extracts. This observation is in contrast with the study by Viljoen (2005) where *iso*-piperitenone was present in 5 out of six of the SWD extracts with generally having higher yields (0.12%, 0.86%, 0.96% and 1.27%) as compared to the SA extracts where *iso*-piperitenone was found in only 4 out of 10 extracts with low yields (0.10%, 0.14%, 0.78% and 0.90% respectively).[4] A similar variation in our study was also observed with Compounds **3**, **4**, **5** and 5,8-dimethyoxy-2,4-dimethylquinoline: most of these compounds were not present in the extracts from SWD. Our study did not present any evidence of similarity in terms of their mass yields from the non-polar compounds of *L. javanica* with respect to their geographical locations.

- **Inter-population variation**

Inter-population variation is the difference in compounds that could be observed between plants of different areas within the same locality. A considerable population variation in the non-polar compounds found in the aerial parts of *L. javanica* was observed (**Figures 4.3** and **4.4**, **Table 4.5**). For example, *iso*-piperitenone was present in small amounts in plant extracts **2**, **4** and **5** which were collected in the Manzini region in SWD, whereas this compound only appeared in trace amounts in the other extracts from SWD. This is in agreement with the study by Viljoen et al (2005) where *iso*-piperitenone was found in mass yields of 0.86%, 0.12%, 1.27% and 0.96% respectively in four of the five plants from SWD investigated.[4] This compound was present in lower yields in extracts from Rooderpoort investigated in this study (extracts **12-15**). *Iso*-piperitenone can be assumed to be one of the more polar compounds in these extracts and could be one of those staining purple with vanillin reagent (e.g. **12-20**) with low Rf values on the TLC plate in **Figure 4.2**.

On the other hand, uniformity seems to exist where the presence of germacrene-D-4-ol in the populations of SA is concerned: all the extracts in all the populations

contained this compound in roughly the same low yields. The non-polar compounds of *L. javanica* only show a slight variation in an inter-population level.

- **Intra-population variation**

A higher chemical difference was observed from plant-to-plant (**Figures 4.3** and **4.4**, **Table 4.5**) as compared to the inter-population variation. Within the SWD populations, extracts **2** and **4** had higher yields of one of the major compounds (Compound **11**) than in extracts **3**, **5**, **6** and **7** which were within the same population and growing within a few kilometers of each other. Plant extracts from Buffelspoort displayed an intense variation where each of the extracts showed a different pattern in the compounds contained, especially in the presence of Compound **12**. Extract **20** did not have Compound **12** at all, and in extract **18** it was found in average yields as compared to the high yields in extract **19**. The same observation in the presence of Compound **12** was true for extract **3** (where it was found as a trace compound) and **4** (present in high yields) which were from the same population and growing next to each other. An intense variation within plant populations is observed in the non-polar compounds of *L. javanica.*

Table 4.5: A summary of the GC/MS results of the ether/hexane extracts of *L. javanica*

		← SWAZILAND →											← SOUTH AFRICA →								
RT	**Compound**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
14.05	1	-	+	-	tr	tr	-	-	-	tr	-	-	+	+	+	+	-	-	tr	tr	+
17.80	*Iso*-piperitenone	-	+	tr	+	+	tr	tr	tr	tr	tr	tr	oo	oo	oo	oo	+	+	+	+	oo
21.51	3	tr	-	-	tr	-	-	-	tr	-	-	-	tr	tr	+	+	-	tr	tr	tr	+
24.70	4	+	+	-	-	-	-	-	-	-	-	-	+	-	tr	tr	-	+	tr	-	-
25.02	5	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	tr	tr	tr
28.52	5,8-Dimethyoxy-2,4-dimethylquinoline	-	+	-	-	-	-	-	tr	-	-	-	+	tr	+	tr	-	-	tr	tr	-
30.50	7	oo	•••	oo	•••	•••	oo	•••	oo	oo	oo	oo	•••	oo	+	oo	+	+	oo	oo	oo
31.21	8	+	oo	oo	oo	oo	+	+	+	+	+	+	oo	+	+	oo	+	+	+	+	+
31.23	9	+	+	-	tr	tr	tr	+	+	tr	-	tr	+	-	-	tr	_	tr	tr	tr	+
31.54	10	oo	oo	+	oo	oo	+	+	+	+	-	+	oo	+	oo	+	+	oo	+	+	+
36.43	11	•••	•••	•••	•••	•••	oo	oo	•••	•••	oo	•••	oo	oo	oo	oo	oo	oo	oo	oo	oo
37.08	12	+	+	•••	-	-	oo	•••	tr	tr	•••	+	oo	tr	-	-	oo	_	oo	•••	-
37.34	13	+	+	+	+	tr	tr	+	+	tr	tr	+	+	tr	+	+	+	_	+	+	+
38.37	Piperitenone	+	oo	+	oo	+	+	+	oo	+	+	+	oo	+	+	oo	+	oo	oo	oo	oo
38.41	Spathulenol	•••	•••	•••	•••	•••	•••	•••	•••	•••	•••	•••	•••	•••	oo	•••	oo	•••	•••	•••	oo
38.72	Germacrene-D-4-ol	+	+	+	+	+	+	tr	+	+	+	tr	+	+	+	+	+	+	+	+	+

Key: **tr** trace **-** not detectable **+** clearly detectable **oo** present in average yields

••• present in high yields/major compound

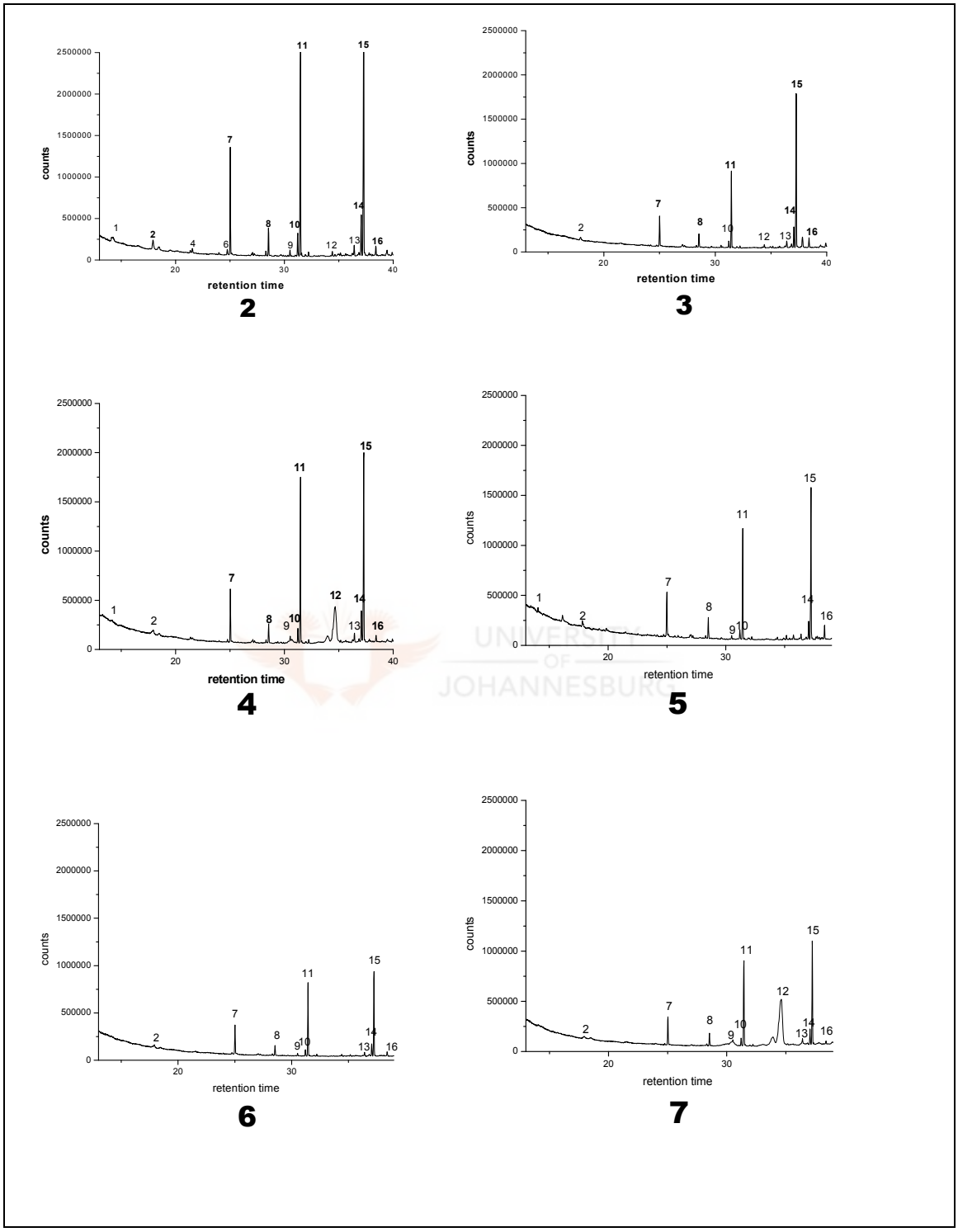


Figure 4.3: GC/MS chromatograms of the aerial parts of the ether/hexane extracts of *L. javanica* from SWD. *Chromatograms were scaled to the same arbitrary "counts" value for comparison.

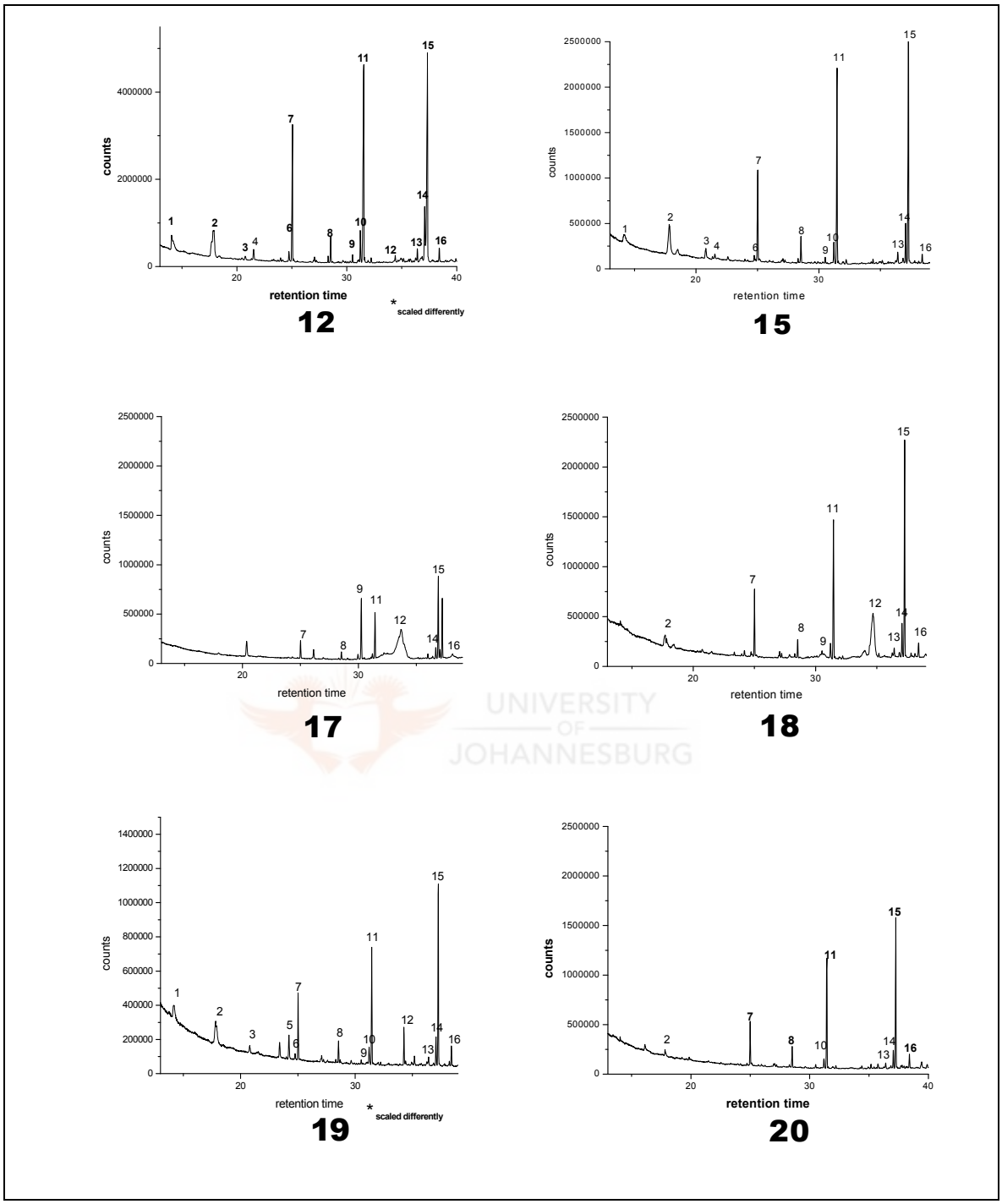


Figure 4.4: GC/MS chromatograms of the aerial parts of the ether/hexane extracts of *L. javanica* from SA. *Two of the chromatograms are not scaled to the Same arbitrary "count" value because the peaks could not fit on a similar scale.

4.3 Amino acids

Amino acid compounds are important for general good health and they are essential in any diet. Some amino acids are known for the treatment of neurological damage and mental retardation in children.[6] A previous study (Neidlein and Stahle, 1974) has been done[3] on the amino acids of *L. javanica.* In an attempt to compare our study on these compounds to the previous study and also to investigate the variation in their quality and quantity we used TLC and GC/MS (after derivatization as discussed in Chapter 3).

4.3.1 Thin layer chromatography (TLC)

The boiling water extracts were re-suspended in 1.0 cm^3 $MeOH/H_2O$ (1:1). These were spotted (50 µL) on a plate and developed in $BuOH/CH_3COOH/H_2O$ (4:1:2) followed by visualization by means of 2% ninhydrin for the detection of amino acids (as discussed in more detail in Chapter 3). The detected amino acids were observed with an orange color for the amine amino acids such as tyrosine and the blue-violet colour which suggested the presence of α-amino acids such as tryptophan.[7] Very little variation appeared to be present as observed from the TLC plate in **Figure 4.5**. Even though the two types of compounds with the highest Rf values exhibiting pink spots were first considered to be amino acids, it was confirmed that pink is not a characteristic colour for amino acids when ninhydrin is used as a visualizing reagent.[8] These compounds were later found to be phenyl ethanoid glycosides as the Rf's compared well with the yellow spots obtained when using a different indicating spray reagent (**section 4.4.1**).

[6] Fried B., Sherma J.1996. *Practical Thin-Layer Chromatography, A multidisciplinary Approach*. CRC Press, New York, Inc. 141.

[7] Shriner R. L., Fuson R. C., Curtin D. Y., Morril T. C.1980. *The Systematic Identification of Organic Compounds*. John Wiley and sons, United States of America. 304-305.

[8] Wagner H., Bladt S., Zgainski E. M. 1984. *Plant Drug Analysis*. Springer-Verlag Berlin Heidelberg, New York. 288.

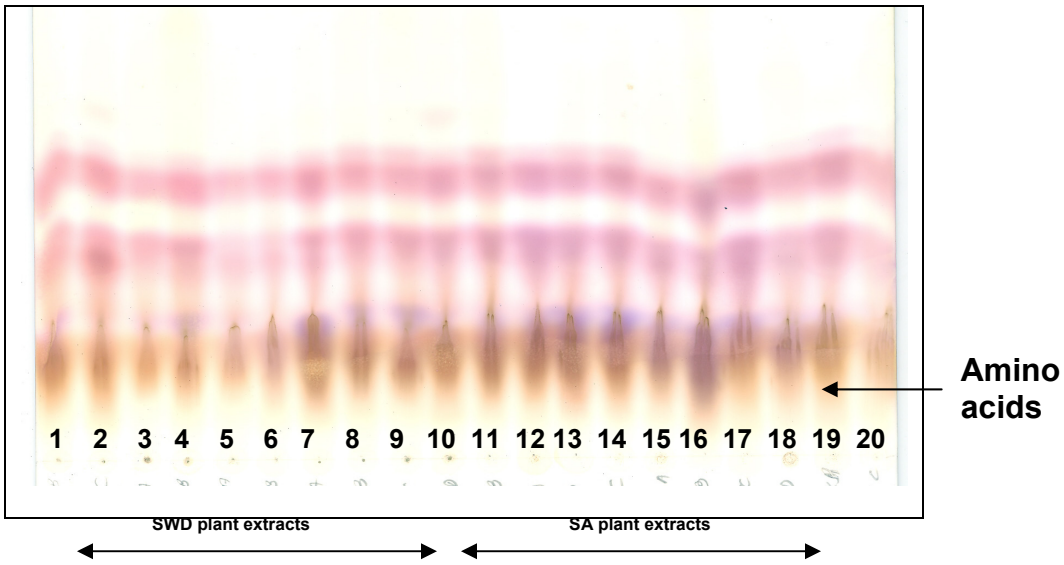


Figure 4.5: TLC plate of the amino acids detected in the aerial parts of *L. javanica* from SWD and SD. Numbers correspond to sample numbers in **Tables 4.1**.

4.3.2 Gas chromatography-mass spectrometer (GC/MS)

While TLC is good for qualitative analyses, GC/MS indicates quantity much more accurately; especially when an internal standard is used (in this case norvaline was used as an internal standard). Boiling water extracts of *L. javanica* were tested for their amino acid content using GC/MS after derivatization as discussed in detail in Chapter 3. A total of 19 amino acids were identified from the 20 plant extracts under investigation. The compounds were identified using a spectral Ezvarian MS library for amino acid derivatives. The percentage recovery of the amino acid content present in the extracts relative to the standard are presented in **Table 4.6** and the typical chromatograms are presented in **Figures 4.6** and **4.7**. The chromatograms were not scaled to the same value (some peaks could not fit on a similar scale for the chromatograms) thus comparison of compounds was only based on the data in Table 4.6. Isoleucine (ILE) was identified as a major compound because of its high concentrations in almost all the *L. javanica* plants from SWD and SA. This is in contrast with the previous study, where alanine (a compound that was not detected in our extracts) was found as the major amino acid.[3] It was actually observed that this study and the previous study

(Neidlein and Stahle, 1974) did not have the same composition of amino acids. For example, glycine, proline, serine, glutamine acid, β-alanine, alanine and glutamine were not present in our case. Amino acids such as α-aminobutyric acid, β-aminoisobutyric acid, 4-hydroxyproline and tryptophan were not present in the previous study. Both methods used derivatization methods before the analysis of these compounds. The previous study used n-trifluoroacetal. In our case new techniques of derivatization using an amino acid derivatization kit (EZ:faast-free amino acid analysis) were used which could have made it possible for us to detect the new amino acids. The origin of the plant samples used for the determination of amino acids in this literature source is not evident and could possibly be commercial. The data obtained from literature was used as a guide and some correlation could be established between the current and previous studies.

4.3.3 Chemical variation

- **Geographical variation**

Considerable variation in the geographical distributions of the plant samples was observed in the total percentage of amino acids in the extracts as summarized in **Table 4.6**. As an example, the amino acid lysine was present in all the extracts of the SA plants except for extracts **18** and **19**. The same was observed in the extracts from SWD where the absence of lysine was observed only in extracts **2**, **8** and **9**. The geographical locations of *L. javanica* plants have no effect in terms of the content of the amino acid compounds. This observation confirms the very slight variation that was observed from the TLC plate in **Figure 4.5**.

Table 4.6: Amino acid content present (% recovery) in water extracts of *Lippia javanica* from SWD and SA

Peak No.	Abbreviation	TD 57	TD 58 (a)	TD 58 (b)	TD 59 (a)	TD 59 (b)	TD 59 (c)	TD 59 (d)	TD 60	TD 61	TD 62 (a)	TD 62 (b)	DO 58 (a)	DO 58 (b)	DO 58 (c)	DO,TD,TN 56 (a)	DO,TD,TN 56 (b)	DO,TD,TN 56(c)	DO,TD,TN 56 (d)	DO,TD,TN 57 (a)	DO,TD,TN 57 (b)
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
A	ABA	1.93	-	3.30	2.58	2.57	0.92	3.43	-	-	-	4.38	0.17	2.03	3.69	2.05	2.46	2.13	-	-	1.59
B	VAL	5.32	6.74	6.37	7.22	7.39	3.94	9.55	6.63	10.18	3.03	12.69	6.45	10.35	11.43	9.12	7.96	9.50	9.28	4.22	4.18
C	B-AiB	0.97	1.41	1.48	1.14	1.04	2.95	1.25	1.19	1.40	1.95	8.71	2.65	1.01	1.97	**17.30**	3.15	2.54	5.13	1.01	2.24
D	ILE	**47.72**	**80.93**	**34.51**	**57.05**	**56.32**	21.88	**69.02**	**73.13**	**52.44**	**45.60**	**39.58**	**70.98**	**61.68**	**32.30**	8.35	9.60	20.72	**60.54**	23.66	**62.99**
E	ASN	15.18	5.02	16.43	5.34	10.38	**24.39**	7.05	9.41	15.89	6.19	3.07	10.44	6.0	20.14	5.91	1.20	**39.14**	5.30	7.46	5.67
F	ASP	0.42	1.37	2.37	1.66	0.44	3.16	0.86	1.04	1.77	5.84	5.54	1.82	1.40	2.22	4.45	7.64	5.28	0.51	**30.83**	1.15
G	4HYP	0.53	0.19	3.0	1.23	0.38	1.29	0.92	0.65	0.92	1.82	2.73	0.13	0.62	0.78	4.55	1.46	6.30	0.69	-	-
H	PHE	4.50	2.43	4.53	1.83	3.20	4.75	3.46	1.70	4.28	4.59	4.55	4.23	6.10	3.86	7.60	10.88	4.52	6.41	4.65	4.15
I	UNK	0.33	0.69	3.55	0.60	0.37	1.75	2.16	0.93	1.49	5.26	2.51	0.39	2.33	0.82	5.20	**18.95**	2.13	6.84	23.96	0.90
J	AAA	1.20	0.37	2.75	0.93	1.04	0.92	0.77	0.87	1.04	-	-	1.56	0.52	0.85	7.19	5.85	0.80	1.87	-	2.09
K	LYS	4.95	-	3.30	7.43	5.47	2.81	0.05	-	-	8.69	6.71	-	1.11	8.44	11.54	9.91	0.30-	-	-	2.62
L	TYR	9.35	-	13.87	7.50	7.35	20.78	0.33	1.87	6.24	8.01	4.33	-	0.10	5.13	6.10	10.45	1.78	1.57	-	8.50
M	TRP	6.98	-	2.94	4.31	3.40	9.62	-	1.72	2.48	8.28	2.62	-	5.55	6.37	8.74	9.23	2.87	0.55	1.06	2.87
	TOTAL	99.38	99.15	98.40	98.82	99.35	99.16	98.85	99.14	98.13	99.26	97.42	98.82	98.69	98.0	98.1	98.74	98.01	98.69	96.85	98.95

Norvaline- Internal standard

ABA-α-Aminobutyric acid, **VAL**-Valine, **β-AiB**- β-Aminoisobutyric acid, **ASN**- Asparagine, **ASP**-Aspartic acid, **4HYP**-4-Hydroxyproline, **ILE**-*Iso*-leucine, **PHE**- Phenylalanine, **UNK**- Unknown amino acid, **AAA**-α-Aminoadipic acid, **LYS**-Lysine, **TRY**-Tyrosine, **TRP**-Tryptophan

- **Inter-population variation**

Variation was still observed to be considerable in the amino acid contents between plant populations of *L. javanica,* as it is evident from **Table 4.6.** For example, plant extract **1** from the Hhohho region of SWD had 1.9% content of α-Aminobutyric acid while this compound was found in contents of 2.6% and 0.9% in extracts **5** and **6** of the Manzini region. These regions are less than 50 km away from each other. This was a similar observation in extract **14** from Rooderport with 3.9% content of phenylalanine. Extract **17** from Melville koppies had a phenylalanine content of 4.5%. These areas are also a few kilometers away from each other.

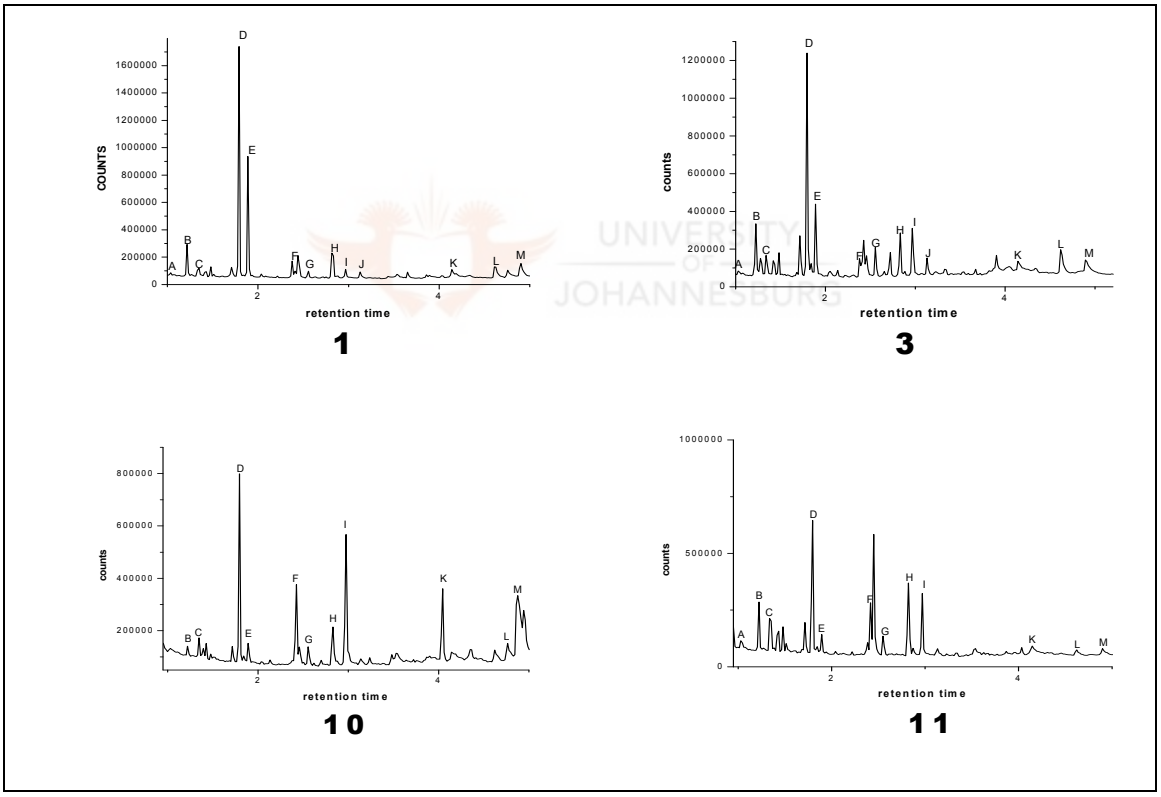


Figure 4.6: Typical chromatographic profiles (GC/MS) of amino acids of the aerial parts of *L. javanica* from SWD depicting the difference of amino acid levels in the extracts. Amino acid numbers correspond with those listed in **Table 4.6** and the plant samples with those in **Table 4.1**

- **Intra-population variation**

An intense population variation in terms of the amino acid contents was observed within the populations of SA and SWD extracts (**Table 4.6**). Variation observed in the population from Manzini region in Nkiliji community was intense in the content of tyrosine. For example, extracts **2** and **3** were harvested in the same site but tyrosine was not present at all in extract **2** while sample **3** had a percentage content of 13.8% tyrosine. The same observation was true for plant extracts **12** and **13** which were both harvested in the useful plants garden in Rooderpoort area, where tryptophan was not present in extract **12** and a percentage content of 5.6% was observed in extract **13.** Chemical variation was observed to be random in the amino acid contents of *L. javanica* from plant to plant; hence in retrospect it is not unusual to find the same random variation between geographical regions. The GC/MS chromatograms of the derivatized and extracted amino acids are presented below, and show this variation clearly.

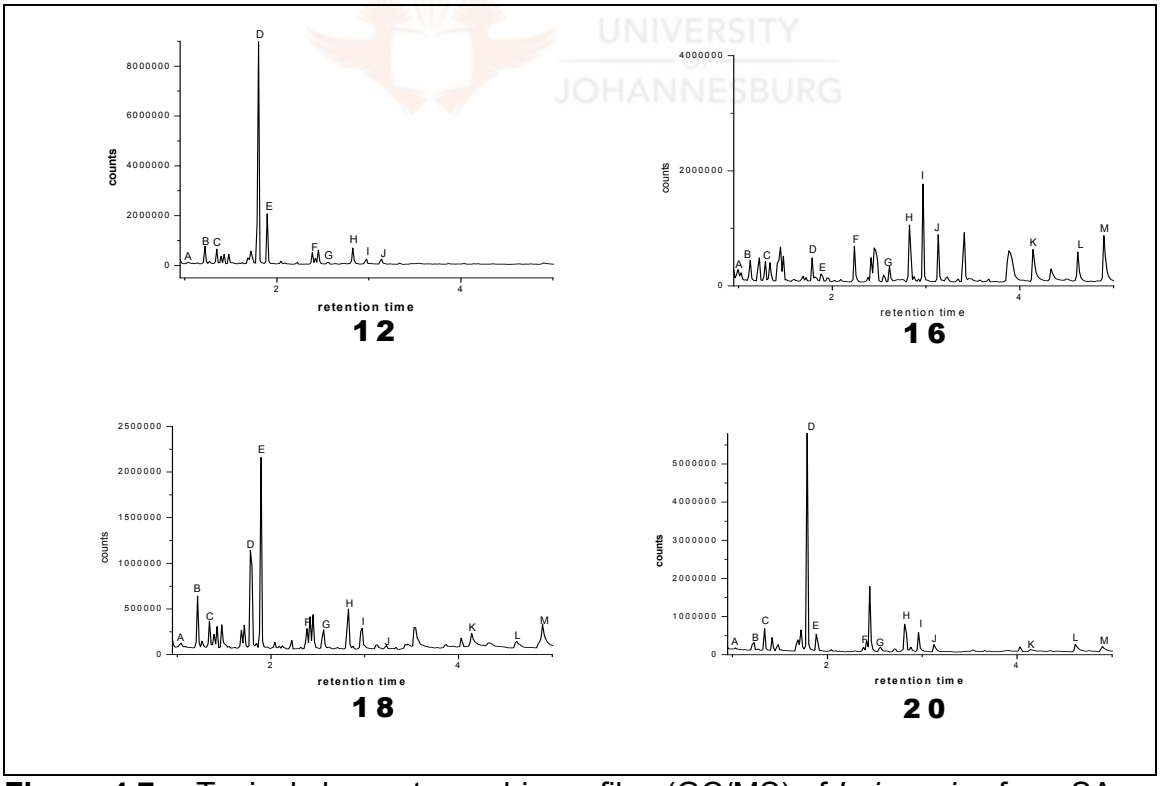
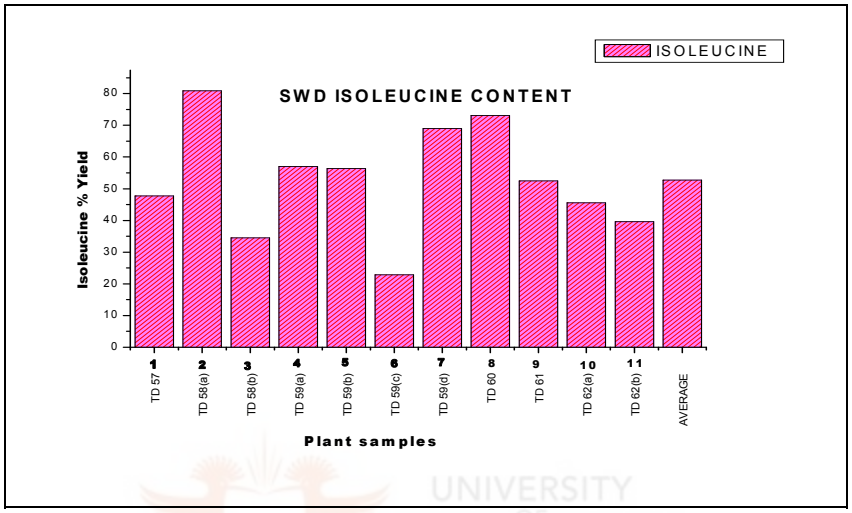


Figure 4.7: Typical chromatographic profiles (GC/MS) of *L. javanica* from SA depicting the difference of amino acid levels in the extracts.

- **Isoleucine content**

There was an evident quantitative variation of the major compound, *iso*-leucine between SWD and SA plants, with the lowest content of 8% and the highest 80%. **Figure 4.8** is a summary of the percentage content of this compound in *L. javanica* plants that was observed in **Table 4.6** (major compound in bold).

A



B

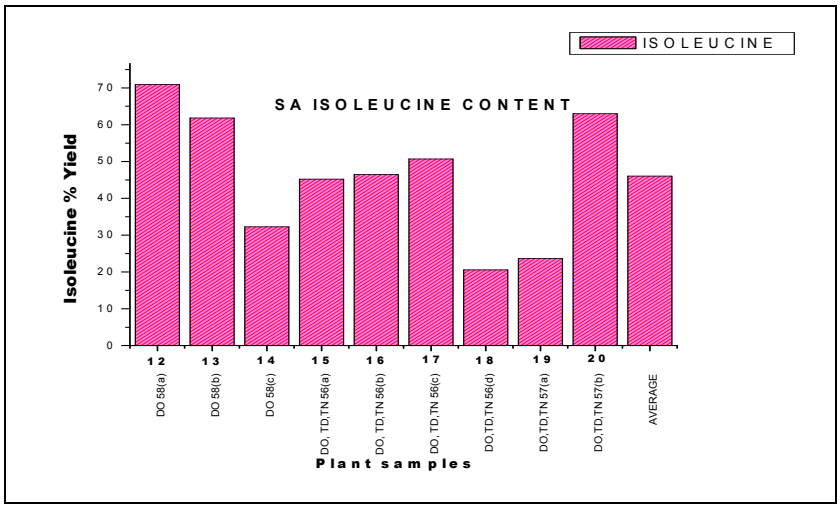


Figure 4.8: Showing the content of the major amino acid compound (ILE) in the SWD (**A**) and SA (**B)** plants.

Intrapopulation variation was evident in most of the plant extracts in the content of *iso*-leucine. For example, extract **6** had an *iso*-leucine content of 21.9% and was growing in the same population with extracts **2**, **3**, **4**, **5** and **7**, but the latter had an *iso*-leucine content of 80.9%, 34.5%, 57.1%, 56.3% and 69.0%, respectively (**Figure 4.8**).

The variation is also evident in extracts **14** and **15** of plants that grew next to each other in Rooderpoort and also from extracts **19** and **20** of the Buffelspoort plants. The observed variation in this major amino acid compound could not be correlated to the geographical distribution of the plants. It would be worthwhile to test if this variation is genetically linked or not by propagating *L. javanica* plants with high *iso*-leucine content to investigate if it would still give high contents of this compound in the daughter plants. This could be very important in growing the *L. javanica* plants commercially, since *iso*-leucine is one of the compounds that are responsible for the treatment of neurological damage and mental retardation in children. It can be observed from Table 4.6 that SWD plants almost always have *iso*-leucine as a major amino acid, where those from SA do not. This is significant for commercialization on these plants.

4.4 Phenolic compounds

Phenolic compounds are the most widely distributed compounds in the plant kingdom. They are phenylalanine derived compounds which have at least one hydroxyl-substituted aromatic ring system. Phenolic compounds include the flavonoids, phenyl glycosides, coumarins, tannins and lignans.[9] The polar nature of phenolic compounds means that they are more likely to be extracted in the aqueous or MeOH extracts. They can be visualized using 1% vanillin/EtOH and 5% H_2SO_4/EtOH reagents and in our case they were investigated using TLC and quantified using HPLC.

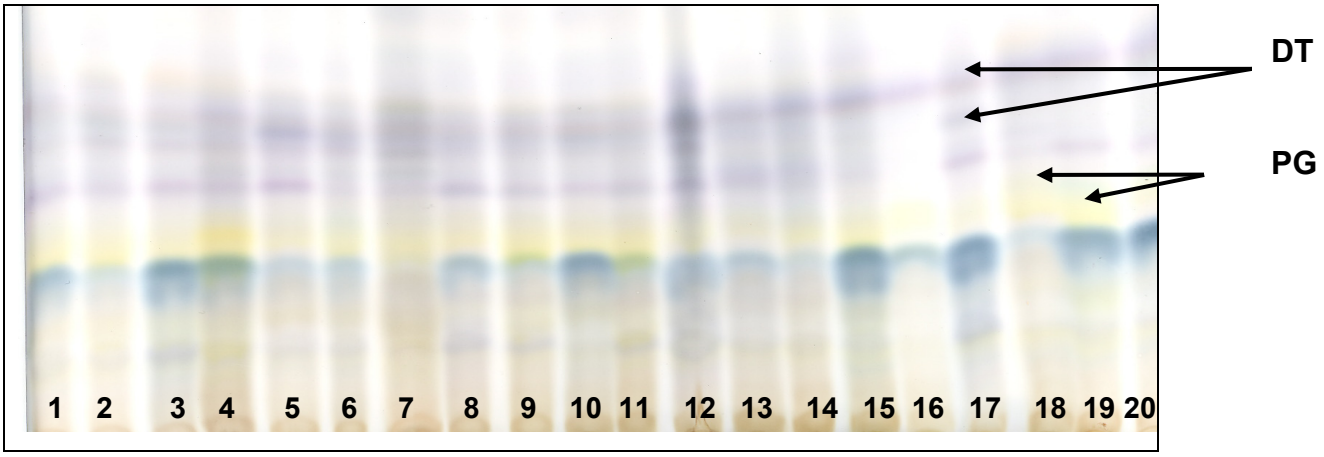
[9] Van Wyk B-E., Wink M. 2004. *Medicinal Plants of the World*. 1st Edition. Briza Publications, Pretoria. 382-392.

4.4.1 Thin layer chromatography (TLC)

Water and MeOH extracts re-suspended in 1.0 cm^3 MeOH/H_2O (1:1) and MeOH respectively were spotted (50 µL) on a TLC plate and developed in $CHCl_3$/MeOH/H_2O/CH_3COOH (6:3:0.8:0.6). This procedure was followed by the visualizing of the compounds with vanillin and H_2SO_4 as discussed in Chapter 3. The survey was aimed at detecting the yellow compounds which were assumed, because of the color and because they were found in the MeOH extracts, to be the flavonoids which are known for a wide range of biological activities such as the anti-inflammatory properties which are known to be present in *L. javanica*.[2] From the TLC plates it was observed that the same compounds were present in both the MeOH and H_2O extracts. Low concentrations of these compounds were observed in the water extracts while they were extracted in higher concentrations in the MeOH extracts. This is evident from the TLC plates in **Figure 4.9**, where the first 11 extracts are the SWD plants and the others are the SA plants.

Because of the apparent concentrations of the yellow-staining spots in the MeOH extracts, it was decided to isolate them using column chromatography. At the same time we decided to include the purple-staining compounds because they were well separated and also showed an apparent high amount in most of the extracts. Four of the yellow and one of the purple compounds were isolated. They were identified using IR, UV and NMR experiments (sections 4.7 to 4.11). Interestingly, after structural elucidation and comparison of data with literature two of the yellow compounds were found to be phenylethanoid glycosides. The structure elucidation of the other two yellow compounds could not be completed due to the small isolated quantity for one and impurities with the other. The purple compound was a diterpenoid. These compounds are labeled as **PG** and **DT** respectively in **Figure 4.9**.

A



B

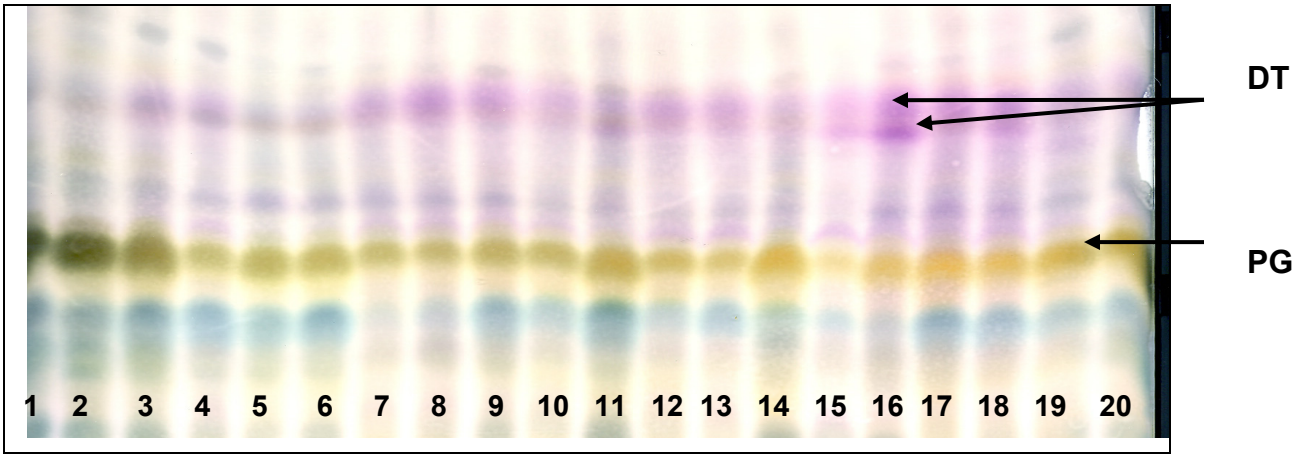


Figure 4.9: TLC plates of the H_2O (**A**) and MeOH (**B**) extracts of the aerial parts of *L. javanica.*

The two isolated phenylethanoid glycosides of which the structures have been solved have been detected in *Lippia* species such as *L. multiflora, L. dulcis* and *L. citriodora*[2] but not in *L. javanica.* These compounds have been found to possess antioxidant properties, suggesting that they have the ability to scavenge free radicals,[10] which may be a very important property to be found in *L. javanica*.

[10] Akdemir Z.S., Tatli I.I., Bedir E., Khan I. A. *Turk. J. Chem*. 2004, **28**, 227-234.

These compounds were quantified using HPLC for chemical variation.

4.4.2 High performance liquid chromatography (HPLC)

The MeOH and H_2O extracts together with the isolated compounds were re-suspended in 4 cm^3 of MeOH and analyzed by HPLC using a Waters 600E HPLC system (Millipore) equipped with a Photodiode Array detector (PDA). HPLC analyses were done for the determination of the presence and the retention times of the phenylethanoid glycosides. Quantification was not possible because some of the chromatograms did not have straight baselines. This was later traced to be a problem with our HPLC pump therefore, only a qualitative analysis of the HPLC was taken. A summary of the HPLC results is presented in **Table 4.7**. A typical HPLC chromatogram in **Figure 4.10** shows the four of the five compounds under investigation in the MeOH extracts, together with other unidentified compounds.

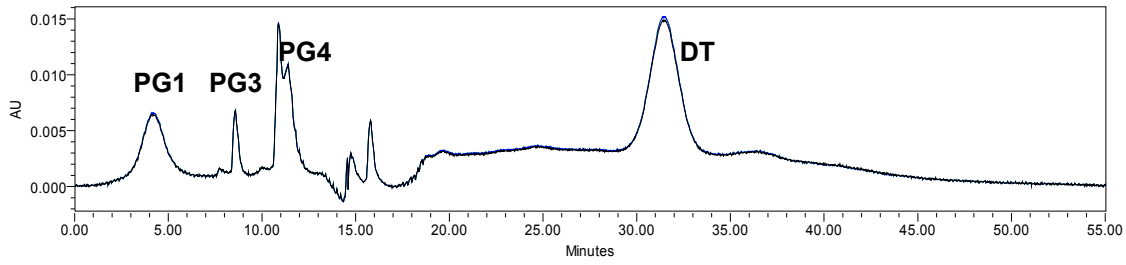


Figure 4.10: Showing a typical methanol extract with some of the isolated compounds

The five compounds were revealed at retention times of 4.04 min (**PG1**), 5.96 min (**PG2**), 9.78 min (**PG3**) and 12.53 min (**PG4**) and 30.64 min for the diterpenoid (**DT**) compound (discussed in **section 4.5**) as shown in **Figure 4.11**. The UV-Vis data of the first three compounds (**PG1-3**) were obtained as expected for phenyl glycoside compounds. PG4 had a different UV, but could not be identified as its structure was not elucidated due the quantity of this compound being too small.

4.4.3 Chemical variation

- **Geographical variation**

An extreme variation in the presence of the **PG** compounds was observed in the two geographical localities in *L. javanica* extracts as presented in **Table 4.7**. The shaded part of the table shows the extracts of the SA plants and the unshaded those of the SWD plants. This big variation was clearly seen in the presence of **PG4** only in plant extract **16** of the SA samples and yet it was found in 6 extracts from SWD. **PG2** was present in 5 extracts from SWD and in 3 from SA. Basically, 73% of the extracts from SWD contained PGs while only 44% of those from SA contained PGs. The presence of the different PG compounds was observed to be random in *L. javanica* plants and cannot be explained by their geographical locations.

- **Inter-population variation**

Considerable variation between populations was observed in the presence of the phenylethanoid glycoside compounds (**Table 4.6**). Two populations from each of the two geographical localities (SWD and SA) had **PG1** and **PG3** and all the populations, except for the population from Buffelspoort possessed **PG2**. **PG4** was found in all populations, except for the Buffelspoort (extracts **18-20**) and Melville koppies (extracts **16** and **17**) populations. Generally none of these compounds were present in the population from Buffelspoort while all the compounds were present in the SWD extracts.

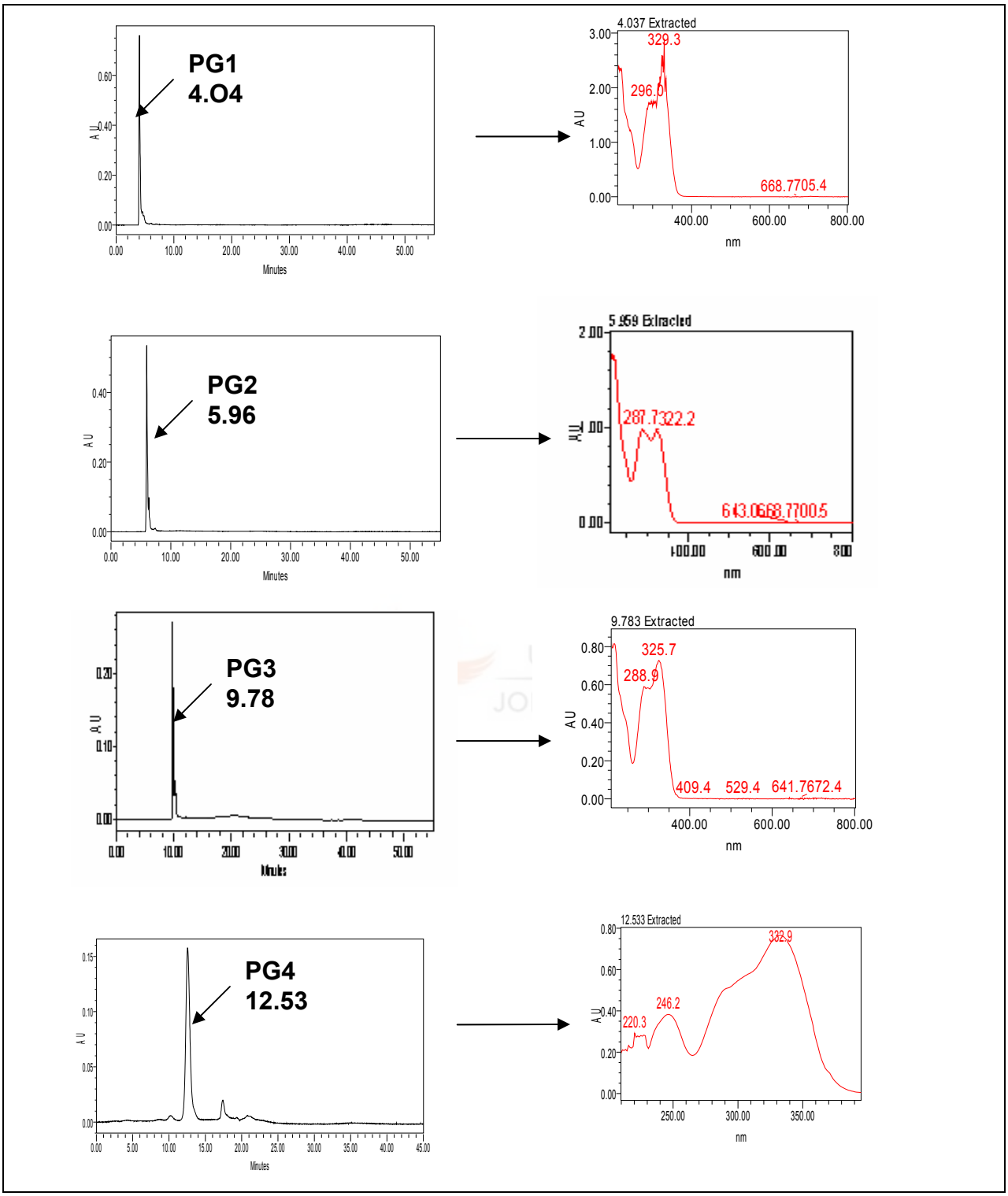


Figure 4.11: The HPLC chromatograms for the **PG** compounds of *L. javanica*, together with the extracted spectra.

- **Intrapopulation variation**

High variation within populations was observed with the PG compounds (**Table 4.6**). Extracts **1** from SWD had all 4 compounds, while extract **20** from the SA extracts had only 3. Some of the plant extracts did not have any of the compounds and majority of them were from the SA extracts. In the SWD extracts it was extract **2** and **3**, both from the Manzini region (Nkiliji community) which did not have any of the PG compounds. This variation shows that each plant has its own pattern in the presence of the phenylethanoid glycosides.

Table 4.7: The HPLC results showing the PG and DT compounds in the MeOH extracts of *L. javanica*.

	PG1	PG2	PG3	PG4	DT5
Sample No.	4.04	5.96	9.78	12.53	30.63
1	√	√	√	√	√
2	-	-	-	-	-
3	-	-	-	-	√
4	√	-	-	-	-
5	-	√	√	√	-
6	-	-	-	√	-
7	-	-	√	√	√
8	-	√	√	√	-
9	-	√	-	-	-
10	√	√	√	-	-
11	-	-	√	-	-
12	-	-	-	-	-
13	-	-	-	-	-
14	-	-	-	-	√
15	-	-	-	-	-
16	-	-	√	√	-
17	-	-	√	-	-
18	√	√	-	-	-
19	-	-	-	-	√
20	√	√	√	-	-

4.5 Diterpenoids

The diterpenoids are a large class of plant products with around 20 carbon atoms. They are commonly found in the Lamiaceae family which is closely related to the Verbenaceae family. These compounds have a number of biological activities such as antimicrobial and antibacterial activities but they are toxic if consumed in large doses.[1] The MeOH extracts were re-suspended in 1.0 cm^3 MeOH. These were spotted on a plate and developed in $CHCl_3$/MeOH/H_2O/CH_3COOH (6:3:0.8:0.6) followed by dipping in 1% vanillin/EtOH and 5% H_2SO_4/EtOH as discussed in **sections 3.1.2.2** and **4.4.1**. The compounds {labeled as **(DT)**} were observed with a purple to pink color after developing TLC plates **(Figure 4.9)**.

From the TLC results the diterpenoids were observed to be present in high concentrations with Rf values (approximately 0.7) higher than those of the phenyl glycosides. Therefore an attempt was made to isolate and identify them as discussed in **section 4.4.1**. The diterpenoids were extracted together with the PGs during the extraction with MeOH and some of them were exhibited as peaks during the PG variation study by means of HPLC as shown in **section 4.4**. These compounds were revealed at a retention time much later than that of the PGs as presented in **Figure 4.12** and the extracted UV spectrum for this compound characteristic of diterpenoids compounds which usually show absorptions at 225, 275 and 405 nm.[11]

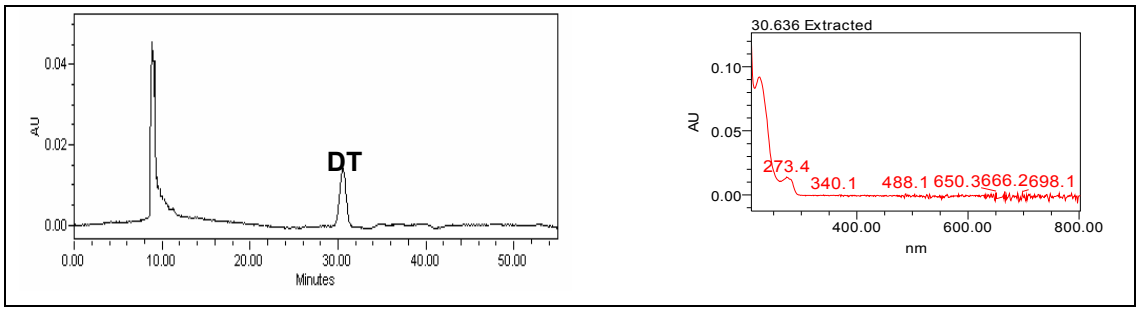


Figure 4.12: Shows the chromatogram for a typical diterpenoid compound together with the extracted UV spectrum.

[11] Mahmoud A., AL-Shiry S., Son B. *Phytochem*. 2005, **66**, 1685-1690

Diterpenoids are not phenolic and will only show a good response factor on the PDA detector if at least one double bond is present in the structure. Even though the TLC shows more than one diterpenoid, the HPLC may not reflect all of them as they may not all contain double bonds. The diterpenoid variation by means of TLC will thus be much more meaningful than that conducted by means of HPLC.

4.5.1 Chemical variation

The diterpenoid compounds were observed to be present in all the extracts that were investigated as shown by TLC results in **Figure 4.9**. Extracts 1-6 from the SWD plants seemed to have much lower concentrations than those from SA as observed from TLC. High quantities and probably more than one diterpenoid are observed in extracts **16**, **17**, **18** from SA together with **8** and **9** from SWD plants.

The diterpenoids with double bonds were present in only five of the twenty plants investigated as observed from the HPLC analyses (**Table 4.7**). Intrapopulation variation in the presence of these compounds was evident in most of the investigated populations. For example, plant extract **14** had high levels of diterpenes while the plants growing next it (extracts **15-17**) did not have it at all **(Table 4.7)**. The variation was observed in almost all the plant populations investigated.

4.6 Triterpenoids

Triterpenoids are tetracyclic alcoholic compounds (containing about 30 carbons) that have a wide distribution in plants.[12] Some medicinally important triterpenoids have been isolated from other *Lippia* species and no data has been found on the isolation of these compounds from *L. javanica.*[2]

All *Lippia* species contain toxic triterpenoids.[13] It is assumed that since *L. javanica* and *L. scaberrima* are used as medicinal plants these triterpenoids

[12] Heinrich M., Barnes J., Gibbons S., Williamson E., 2004. *Fundamental of pharmacognosy and phytotherapy.* Elsevier Science, Spain. 85-88.

are found in small quantities. On account of the toxicity of these compounds, using them in high doses for a long period of time may be harmful according to Van Wyk, et al (2002). *L. rehmannii* is known as a toxic *Lippia* species and is consequently not used as a medicinal plant.[13] In an attempt to differentiate between these two species we used TLC to identify the triterpenoids, which should color purple or violet in the anisaldehyde/H_2SO_4 visualizing reagent and have an Rf range of 0.3-0.55 on using ethyl acetate/toluene/formic acid (50:50:20) as a TLC developing solvent system (as discussed in more detail in Chapter 3).[8]

4.6.1 Thin layer chromatography (TLC)

Boiling water extracts re-suspended in 1.0 cm^3 MeOH/H_2O (1:1) were spotted (50 µL) on a plate and developed as discussed above. The concentration of the triterpenoids was estimated from the TLC analysis of *L. javanica* extracts and compared with those of *L. rehmannii* as shown by the TLC results in **Figure 4.13**.

The purple compounds labeled as **TRP** in extracts **5-6** in the TLC plate were assumed to be the toxic triterpenoids, icterogenin and rehmannic acid (**Figure 2.14**) as mentioned by literature.[13] Since literature reports that triterpenoids are found in *Lippia* species which would also include *L. javanica*, then we could then conclude from the TLC of *L. javanica* extracts **(1-4)** in **Figure 4.13** that these compounds may indeed be present in very low concentrations. This investigation enhances the use of *L. javanica* as a medicinal plant while the origin of toxicity in *L. rehmannii* is not yet established.

[13] Van Wyk B-E., Van Heerden F., Van Oudtshoorn B. 2002. *Poisonous Plants of South Africa*. 1st Edition. Briza Publications, Pretoria, South Africa. 140.

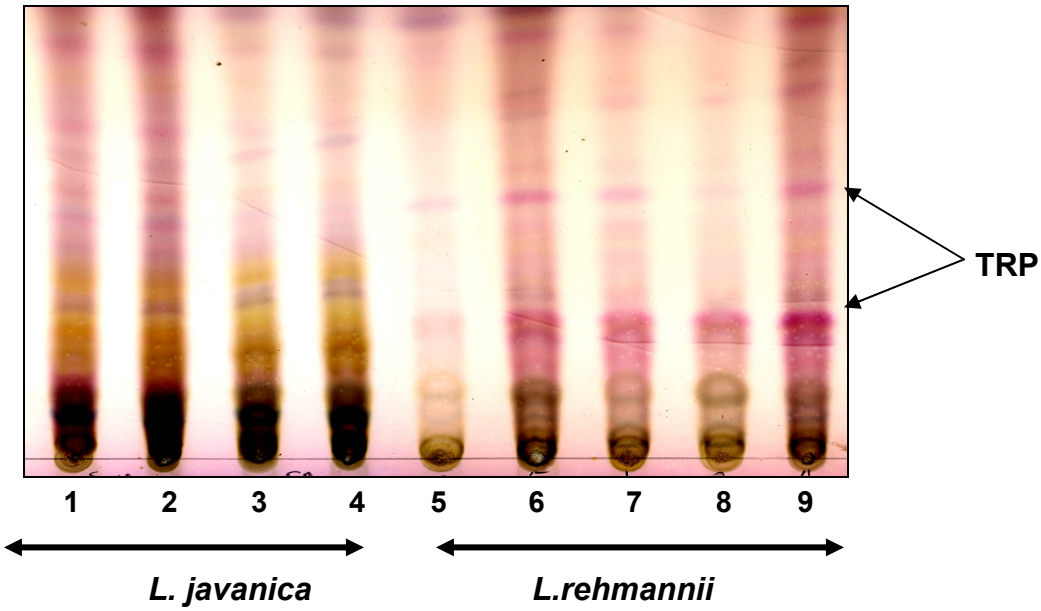


Figure 4.13: TLC results showing the prominent purple triterpenoids **(TRP)** in *L. rehmannii.*

Besides the presence of the purple in *L. rehmannii*, all other chemical compounds were observed to be different from those of *L. javanica.* This variation confirms the importance of chemical variation studies for quality control purposes. This simple color test could be developed for use as a screening for the toxic compounds in commercial use.

Structural elucidation of isolated compounds

Phenylethanoid glycosides were isolated from the aerial parts of *Lippia javanica* by fractionation of the methanolic extract through a silica gel column and preparative TLC (discussed in more detail in Chapter 3). According to 1 and 2D NMR and UV spectra, one phenylethanoid glycoside must be verbascoside numbered as **1** and another phenylethanoid glycoside is isoverbascoside labeled number **2**. The structures of the other two phenylethanoid glycosides could unfortunately not be solved due to their concentrations being too low, but the UV spectra of one of them compares with those of Compounds **1** and **2** and it can thus be concluded that one might be phenylethanoid glycoside, but further work must still be done to resolve the structures.

The phenylethanoid glycosides are characteristic compounds of the order Lamiales to which the Lamiaceae and Verbenaceae families belong.[14,15,16] In the *Lippia* genus this verbascoside has previously been isolated from *L. multiflora, L. citriodora* and *L. dulcis* while *iso*-verbascoside has been reported in *L. multiflora.*[2] This is the first report on the isolation of these compounds from *L. javanica*. This section deals with the structural determination of the verbascoside derivatives and a diterpenoid from *Lippia javanica*.

4.7 Compound 1- Verbascoside

The structure of Compound **1** was determined by comprehensive 1D and 2D NMR spectroscopic analyses including COSY, HSQC and HMBC and was confirmed by comparison of its spectral parameters with those described in literature.[17,18] The FAB-MS, IR and UV spectra also serve as confirmation of the assigned structure.

This compound was isolated as a yellow amorphous solid with a molecular formula $C_{29}H_{36}O_{15}$ (calculated) with a mass of *m/z* $[M + Na]^+$ 647. The IR (KBr) $\acute{u}_{max}$ showed absorption bands at 3384 (O-H), 2855 and 2925 (C-H), 1717 (C=O), 1635 (C=C), as well as 1521 and 1385 (aromatic rings) cm^{-1}(**Figure 4.14**) UV (PDA, MeOH) spectra gave λ_{max} at 289 and 326nm.

[14] Barbosa F.G., Lima M. A. S., Braz-Filho R., Silveira E. R. *Biochem. Syst. and Ecol.*. 2006, 1-3 (in print).

[15] Ronsted N., Bello A. M., Jensen R. S. *Phytochem.* 2003, **64**, 529-533.

[16] Ersoz T., Tasdemir D., Calis I. *Turk. J. Chem.* 2002, **26**, 65-471.

[17] Bedir E., Manyam R., Khan A. *Phytochem.* 2003, **63**, 977-983.

[18] Calis I., Tasdemir D., Sticher O., Nishibe S. *Chem. Pharm. Bull*. 1999, **47(9)** 1305-1307.

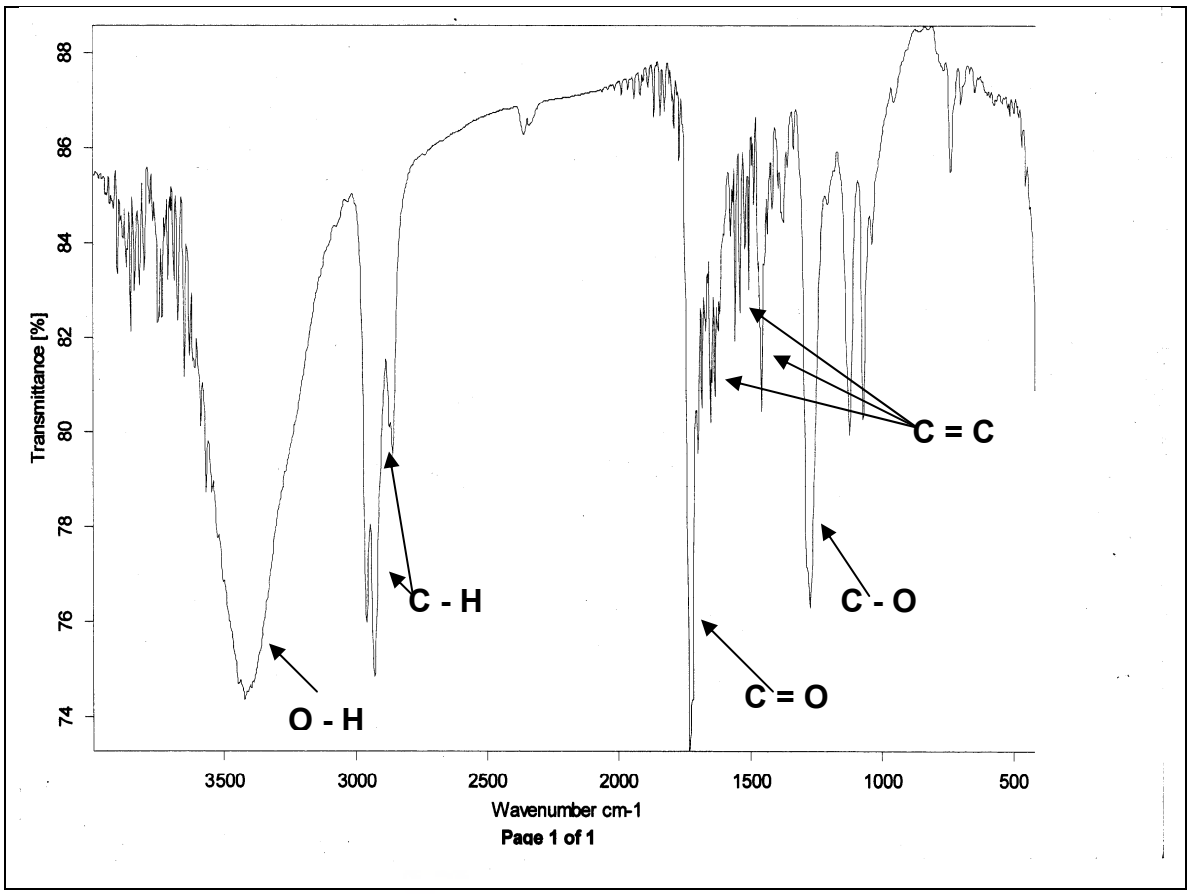


Figure 4.14: IR Spectrum of Compound **1**

The ^{13}C-NMR spectrum showed 29 carbon resonances (**Appendix B**) suggesting 6 carbons more than those suggested by the mass spectrometry. This was due to a sugar molecule that was lost during the fast atom bombardment of the compound during FAB-MS determination.

Two anomeric proton signals were observed at δ_H 4.39 (*d, J* = 7.9 Hz), 5.20 (*d, J* = 1.1 Hz) indicating the presence of two sugar moieties. This was confirmed by their chemical shifts which were consistent with the β-glucopyranose and α-rhamnopyranose data, respectively.[19] The presence of a methyl doublet at δ_H

[19] Ersoz T., Tasdemir D., Calis I. *Turk. J. Chem.* 2002, **26**, 65-471.

1.28 *m* confirmed that one of the sugar residues was a rhamnose. This was further confirmed by the corresponding anomeric carbon resonances at δ_C 104.2 and 103.0. From the 29 carbon resonances, 12 were assigned to these sugar moieties. An acyl group determined to be a caffeoyl moiety, was assumed to be positioned at C-4′ of the glucose unit due to the strong deshielding of the H-4′ signal (δ_H 4.97, *d*, *J* = 9.5 Hz) caused by the carbonyl carbon due to acylation. The HMBC correlation between H-1′′ (δ_H 5.20) of the rhamnose and C-3′ (δ_c 81.7) of the glucose was also observed, indicating an interglycosidic linkage of the C-1′′ *(rha)* $\rightarrow$ C-3′ *(glu).*

The linkage observed from the anomeric proton of glucose H-1′ (δ_H −4.39, *d, J* = 7.9) to C-α (δ_C -72.3) and from H-β (δ_H −2.81 *t, J* = 7.2) to C-1 (δ_C -131.5) of the phenylethyl aglycone confirmed the proposed structure of verbascoside. These determinations were revealed by the HMBC experiment as shown in **Appendix B** and **Figure 4.15**.

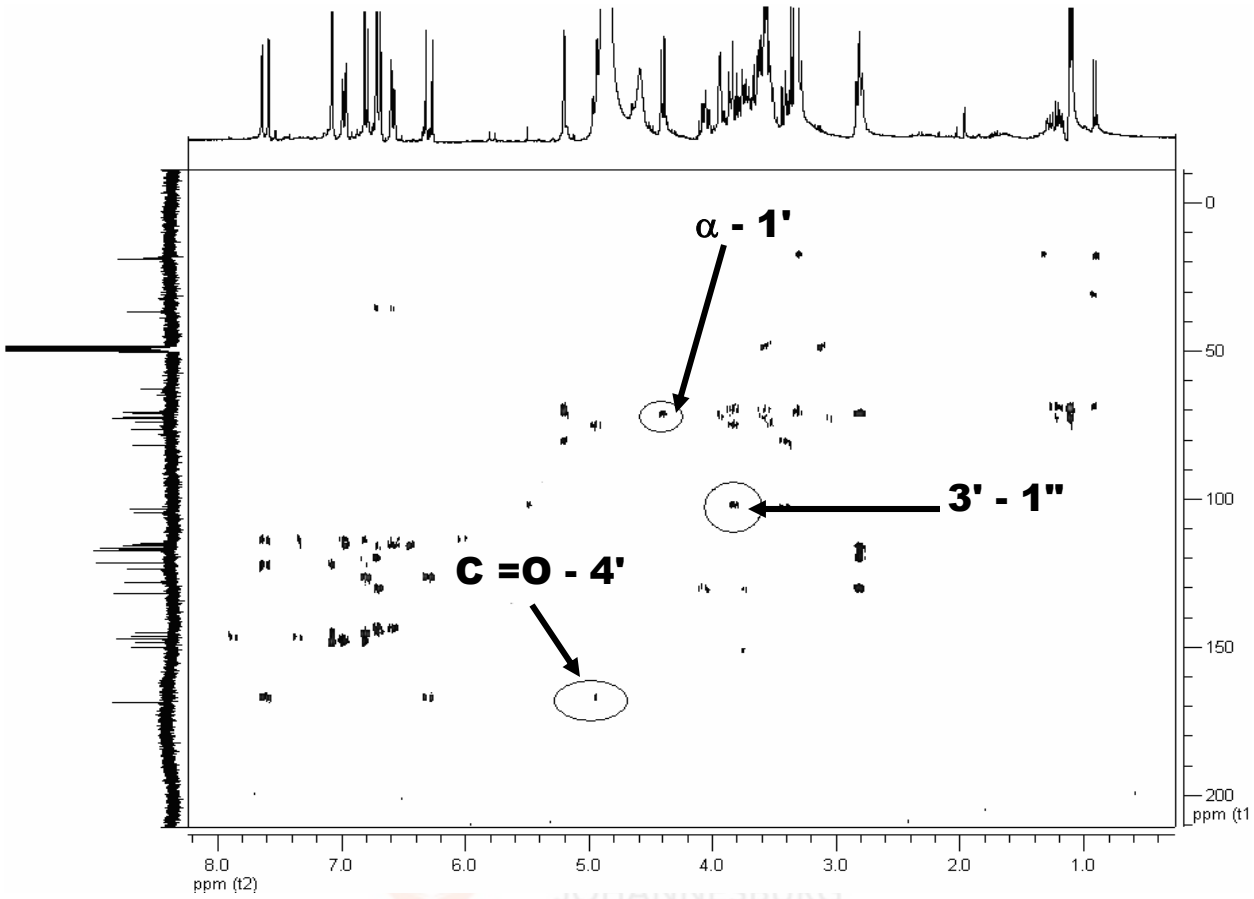


Figure 4.15: The HMBC spectra confirming the interfragmental connectivities for Compound **1**

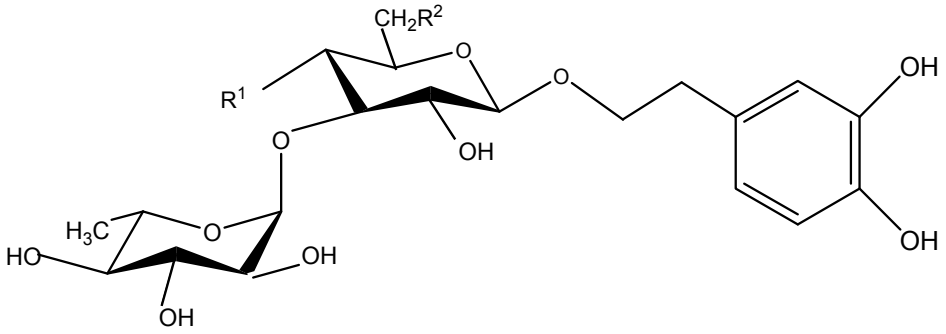
The compound appeared to contain two aromatic rings. These deductions were confirmed by the presence of some aromatic peaks in the aromatic region of the ^{13}C-NMR spectrum. This was further confirmed by the peaks at δ_C-149.8, 146.8, 146.1 and 144.7) which were more deshielded and showed no correlation with protons in the HSQC experiment to be the hydroxyl carbons of the aromatic rings. The presence of a carbonyl carbon was indicated by the signal at δ_C-168.3 which is characteristic of ester carbonyls. Results of all the NMR connectivities are tabulated in **Table 4.8**.

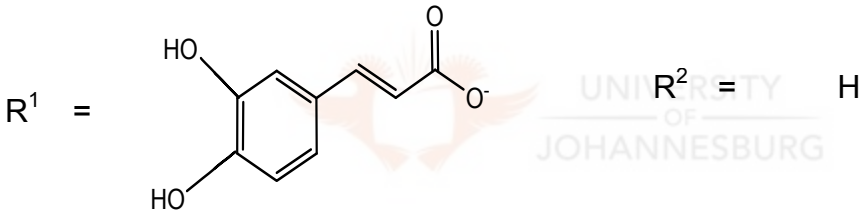
Table 4.8: ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) and 2D NMR {^{1}H (300 MHz) and ^{13}C NMR (75 MHz)} data for verbascoside (CD_3OD)

C Assignments	δ_C(ppm)	δ_H (ppm), *J* (MHz)	HMBC	COSY
Phenylethyl aglycone				
1	131.5	-	5	-
2	116.3	6.69 *d* (2.2)	-	β
3	146.1	-	5	-
4	144.7	-	2, 6	-
5	117.1	6.69 *d* (8.6)	β	6
6	121.3	6.57 *dd* (8.0, 1.7)	4	β, 5
α	72.3	4.05 *m*	**1′**	β
β	36.6	2.81 *t* (7.2)	5, 1	α, 6, 2
Glucose moiety				
1′	104.2	4.39 *d* (7.9)	**α**	2′
2′	76.2	3.41*	-	1′, 3′
3′	81.7	3.84	**1′′**	4′, 2′
4′	70.6	4.97 *m* (9.5)	1′′	3′, 5′
5′	76.0	3.54* *m*	-	-
6′	62.4	3.63*, 3.54*	-	-
Rhamnose moiety				
1′′	103.0	5.20 *d* (1.1)	5′′, 3′, 2′	2′′
2′′	72.3	3.94 *br s*	6′′	1′′, 3′′
3′′	72.0	3.67*	-	-
4′′	73.8	3.28*	4′	5′′
5′′	70.4	3.58*	3’, 6′′	
6′′	18.4	1.28 *m* , 1.10 *d* (6.1)		5′′
Caffeoyl moiety				
1′′′	127.7	-	5′′′, α′	
2′′′	115.2	7.07 *d* (1.8)	6′′′	β′
3′′′	149.8	-		
4′′′	146.8	-		
5′′′	116.5	6.79 *d* (8.2)		6′′′
6′′′	123.2	6.97 *dd* (8.2, 1.6)		5′′′, 2′′′
α′	114.7	6.27 *d* (15.9)	6′′′	β′
β′	148.0	7.54 *d* (15.9)	2′′′, 6′′′	α′
C=O	168.3	-	β′, **4′**, 6′′′, **α**	

Assignments confirmed by HSQC, HMBC and COSY experiments
*Signals unclear due to overlapping

The complete assignment of all proton and carbon resonances was based on the COSY, HSQC, and HMBC experiments **(Appendix B)**. On the basis of the NMR data and literature survey Compound **1** was found to be β-[(3,4-dihydroxyphenyl)-ethyl]-(3′-*O*-α-$_L$-rhamnopyranosyl)-(4′-*O*-caffeoyl)-β-$_D$-glucopyranoside known as verbascoside[20] **Figure 4.16**.





Compound 1 - Verbascoside

Compound 2 - *Iso*-verbascoside

Figure 4.16: Phenylethanoid glycosides isolated from *Lippia javanica*

[20] Ronsted N., Bello A. M., Jensen R. S. *Phytochem.* 2003, **64**, 529-533.

4.8 Compound 2- *Iso*-verbascoside

Compound **2** is an amorphous yellow solid with a mass of FAB-MS [M + Na] *m/z* 647 as determined by the mass spectrum like that of Compound **1**. The IR (KBr) $\acute{u}_{max}$ spectra showed absorption bands at 3405 (O-H), 2927 (C–H), 1698 (C=O), 1630 (C=C), 1523, 1383 (aromatic rings) cm^{-1} (**Appendix B**).UV (PDA, MeOH) spectra gave λ_{max} at 288 and 322 nm.

The ^{13}C-NMR spectrum also showed a number of 29 carbon resonances as with Compound **1** and revealed the same features of a phenylethanoid glycoside discussed previously. The HMBC correlation between H-1′′ (δ_H-5.14) of the rhamnose and C-3′ (δ_c- 81.7) of the glucose was also observed to indicate an interglycosidic linkage of the C-1′′ *(rha)* $\rightarrow$ C-3′ *(glu).* The linkage observed from the anomeric carbon of glucose C-1′ (δ_C −104.1) to H-α (δ_H -3.87, *m*) and from C- 1 (δ_C -131.5) to H-β (δ_H −2.75 *t, J*=7.0) of the phenylethyl aglycone also clearly showed the close resemblance of this compound to Compound **1**. The major difference was concluded to be the attachment site of the caffeoyl and aglycone moieties to the sugar molecules. The assumption that the caffeoyl group was attached to the glucose unit through the C-6′ was confirmed by the HMBC correlation observed between C-5′ (δ_C- 76.0) of the glucose molecule and the carbonyl carbon (δ_C- 168.3) of the caffeoyl moiety. These assumptions still need to be confirmed by further investigation such as LC-ESIMS, HRESIMS and NOESY experiments. This connection is shown in **Figure 4.17**.

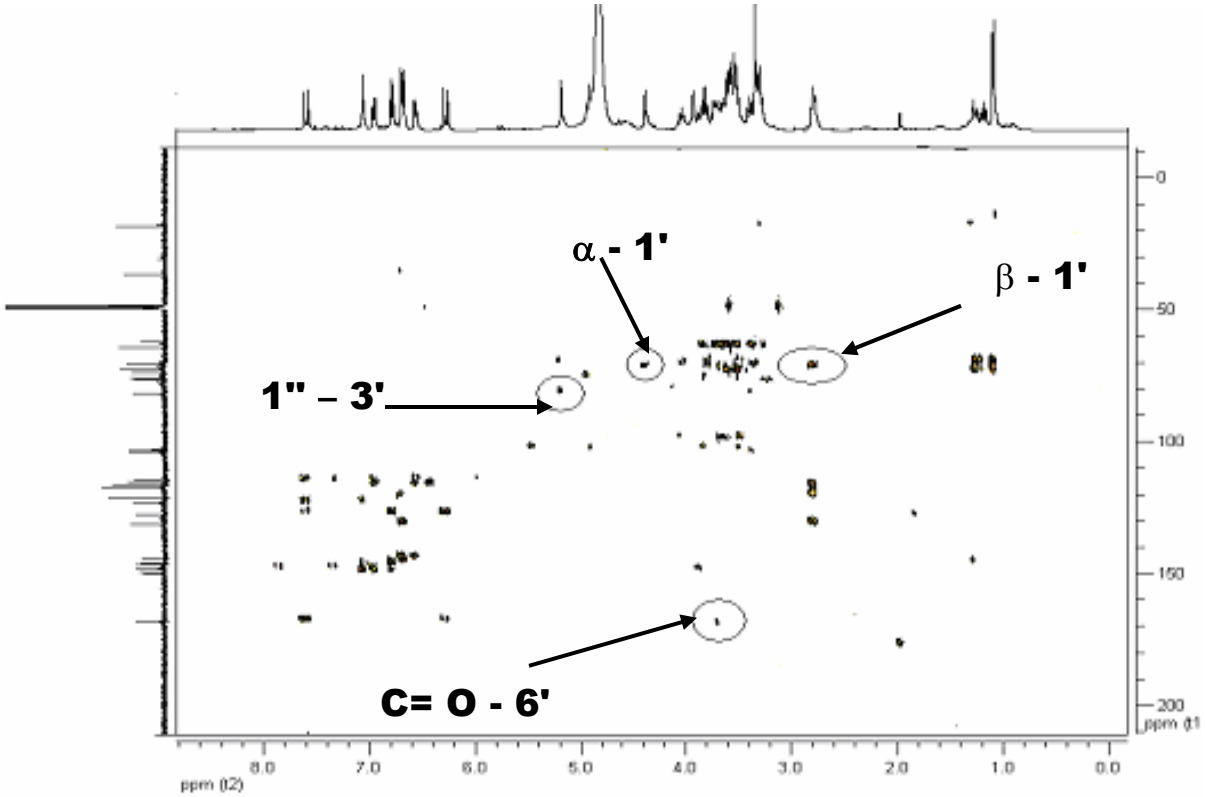


Figure 4.17: The HMBC spectra for Compound **2** showing the interfragmental connectivities and the major difference from Compound **1**

Therefore Compound **2** has the same chemical formula as compound **1**. From the NMR data and literature survey Compound **2** was found to be 2-(3′,4′-dihydroxyphenyl)-ethyl-6-O-caffeoyl-3-O-(-α-$_L$-rhamnopyranosyl)-β-$_D$-glucopyranoside which is commonly known as *iso*-verbascoside (**Figure 4.16**).[21] Results of all connectivities are tabulated in **Table 4.9**.

[21] Kawadi T., Asano R., Makino R., Sakuno T. *J. Wood Sci*. 2002, **48**, 512-515.

Table 4.9: ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) and 2D NMR {^{1}H (300 MHz) and ^{13}C NMR (75 MHz)} data for *iso*-verbascoside (CD_3OD)

C Assignments	δ_C(ppm)	δ_H (ppm), *J* (MHz)	HMBC	COSY
Phenylethyl aglycone				
1	131.5	-	5, β	-
2	116.3	6.62 (2.2)	4	6
3	146.1	-	-	-
4	144.6	-	2, 6	-
5	117.1	6.66 *d* (2.6)		6
6	121.3	6.53 *dd* (8.1, 2.0)	β, 2	2, 5
α	72.3	3.87 *m*	**1**′	β
β	36.5	2.75 *t* (7.0)	5	α, 6, 2
Glucose moiety				
1′	104.1	4.32 *d* (7.9)	5′	2′
2′	76.2	3.38*	2′′, 4′	3′, 1’
3′	81.7	3.84	**1**′′	3′
4′	70.6	4.87 *m* (9.4)	1′′	3′, 2′
5′	76.0	3.50*	3′, **C=O**	4’
6′	62.3	3.59*, 3.54*	2′, 3′	
Sugar moiety				
1′′	103.0	5.14 *d* (1.5)	**3**′, 4′	**3’**, 2′′
2′′	72.2	3.77	2′	1′′
3′′	72.0	3.69*	-	
4′′	73.8	3.26*	2′′	3′′, 5′′
5′′	70.4	3.53*		6′′
6′′	18.4	1.13 *m*, 1.04 *d* (6.2)	3′′	5′′
Acyl moiety				
1′′′	127.6	-	α′, 5′′′	-
2′′′	115.2	7.01 *d* (1.9)	6′′′, β′	6′′′
3′′′	149.7	-	-	-
4′′′	146.8	-	-	-
5′′′	116.5	6.72 *d* (8.2)	1′′′	6′′′
6′′′	123.2	6.92 *dd* (8.3, 2.0)	β′, 2′′′	2′′′, 5′′′
α′	114.7	6.20 *d* (15.9)	6′′′	β′
β′	148.0	7.52 *d* (15.9)	2′′′, 6′′′, C=O	α′
C=O	168.3	-	β′, **5**′	-

Assignments confirmed by HSQC, HMBC and COSY experiments
*Signals unclear due to overlapping.

4.9 Compound 3

Compound **3** is an amorphous yellow solid. The IR (KBr) $\acute{u}_{max}$ showed absorption bands at 3409 (O-H), 2926 (C−C), 1700 (C=O), 1605 (C=C), 1519, 1383 (aromatic rings) cm^{-1}(**Appendix B**). UV (PDA, MeOH)λ_{max} spectra absorbed at 296 and 329.3 nm. The structural elucidation for this compound was not completed because the NMR experiments revealed that the compound had too many impurities which would make it difficult to find the correct structure. This was observed with the ^{13}C-NMR which showed that the compound might have four sugar moieties because of the four peaks observed at the anomeric region (**Figure 4.18**) and yet on FAB/MS it gave a molecular mass (*m/z* 639) as less than that of Compounds **1** and **2** (*m/z* 647). Even though the compound is not clean, based on the NMR and UV spectra it could be noticed that this compound is probably also one of the verbascoside derivatives as shown in **Figure 4.18**.

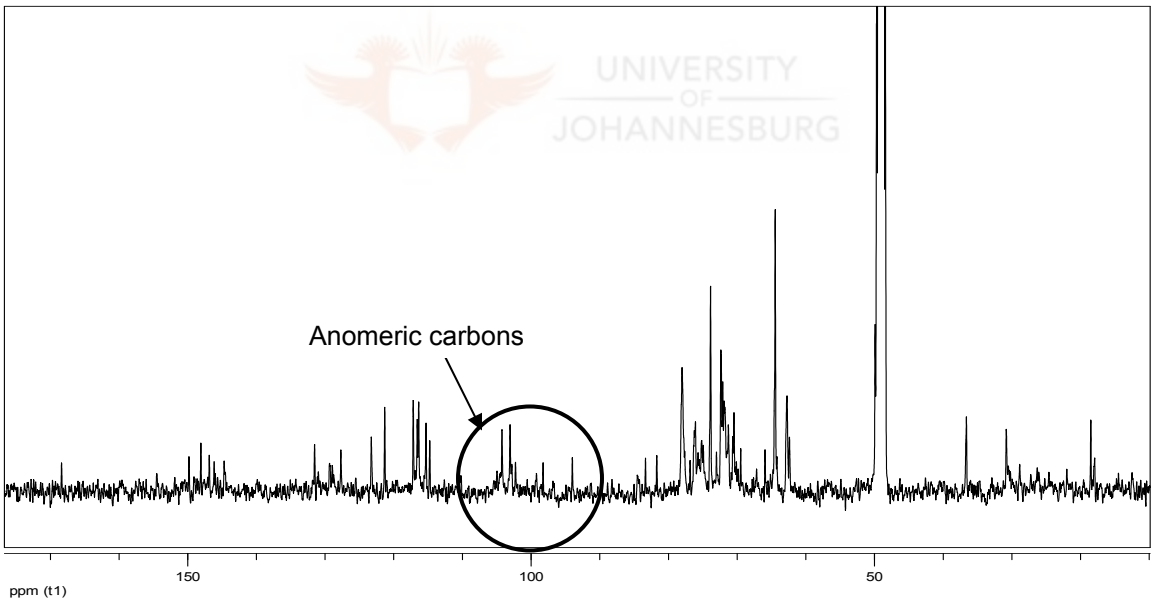


Figure 4.18: Showing the anomeric carbons for Compound **3**

4.10 Compound 4

Compound **4** had a mass of $[M+Na]^+$ 439 as determined by FAB-MS. The IR (KBr) $\acute{u}_{max}$ showed absorption bands at 3409 (O-H), 2926 (C−C), 1699 (C=O), 1605 (C=C), 1519, 1383 (aromatic rings) cm^{-1}. UV (PDA, MeOH) λ_{max} spectra absorbed at 220, 246 and 333 nm suggesting the compounds phenolic nature. The quantity of this compound was very small; therefore NMR could not be used for further characterization. All spectra are presented in **Appendix B**.

4.11 Compound 5

Compound **5** is a diterpenoid compound; this was deduced from the UV spectrum for this compound which is characteristic of diterpenoids compounds which usually show absorptions at 225, 275 and 405 nm.[11] This compound has not been isolated from *L. javanica* before. The IR (KBr) λ_{max} showed absorption bands at 3416 (O-H), 2927, 2892 (C−C), 1699 (C=O), 1263, 1154 (C-O) cm^{-1}.The final structure for this compound still needs to be clarified. The C-H correlations are shown in **Table 4.10**.

Table 4.10: 2D NMR correlations of Compound **5**

C assignment	δ_C (ppm)	DEPT	δ_H (ppm)	COSY	HMBC
1	25.1	CH_3/CH/C	1.99	H-2,3,13	C-3
2	60.6	CH_2	3.43		C-1, 14
3	47.0	CH_3/CH/C	3.40,	H-6, 13,	C-1
			3.29	H-1	-
C=O	174.0		-	-	C-7, 5, 12
5	62.4	CH_3/CH/C	3.57	H-13,12	-
6	29.4	CH_3/CH/C	2.12	H-7	-
			2.26	H-13	-
7	62.7	CH_2	3.97 *dd* (14.9, 2.3)	H-6	C-9
8	23.2	CH_3/CH/C	1.63	H-5, 3, 10,13,11	C-6
9	30.4	CH_3/CH/C	1.27		-
10	44.7	CH_3/CH/C	3.30,	H-10, 11, 13	-
			2.92	H-10, 11, 13	-
11	23.5	CH_3/CH/C	1.88	H-10, 8	-
12	17.4	CH_2	1.44 *d* (7.2)		C-5
13	28.1	CH_3	1.63, 2.10, 2.23	H-2, 8	C-7, 1
14	19.0	-	1.31	-	--

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The aim of this chapter is to give a concise summary of the results and the main outcomes that have risen from this study. From the results obtained the following conclusions can be made:

- The chemical variation study of the non-polar compounds obtained by solvent (ether/hexane) extraction confirmed the results from previous studies on essential oils (non-polar compounds) of *Lippia javanica*: Great variation was observed from plant to plant[1] as compared to the considerable variation, which was observed at population and across population levels. The variation was found to be random and it was not correlated with the geographical distribution of this plant species.[1] Spathulenol was observed as a major compound in varying amounts from plant to plant in SWD and SA samples and the presence of germacrene-D-4-ol was observed in the SA plants.

- A random geographical variation was also observed with the polar extracts containing compounds such as the phenolic compounds and amino acids. This was somewhat unexpected since the plants are essentially used for similar treatments throughout the region in which they were collected. The presence of isoleucine in high concentrations in almost all the plants irrespective of the location was of interest since this compound is known for the treatment of neurological damage and mental retardiation in children. It would be interesting to do a genetic test on *L. javanica* plants by propagating the plants with high isoleucine content

[1] Viljoen A. M., Subramoney S., Van Vuuren S. F., Baser K. H. C., Demirci B. *J. Ethnopharmacol.* 2005, **96**, 271-277.

to see if it would still give high contents of this compound (specifically those found in SWD) in the daughter plants. This could be very important in growing the *L. javanica* plants commercially for treatment of neurological damage and mental retardiation in children.

- A possible contributing factor to the observed chemical variation in both the polar and non-polar compounds of *L. javanica* could be the seasons in which the plants were collected and the developmental stages of the plants which could have been different. The variation in these compounds shows the importance of chemical variation studies especially in commercial sectors (in the production of tonic tea and skin lotions) for quality control purposes.

- There is a high chance that *L. rehmannii* could be mistaken for *L. javanica* because of the close relationship that these species have in their physical features, and the fact that they grow in similar environments. In some cases we found plants from the two species growing next to each other. We have however determined that there are different compounds present in these two species. The results demonstrated the importance of establishing quality control protocols before using these plants commercially. Traditional healers and people who collect the plants need to be taught the differences between the physical features of the plants. The toxicity of *L. rehmanni* should be investigated in a further study.

- The discovery of phenylethanoid glycosides (verbascoside and isoverbascoside) in the aerial parts of *L. javanica* gave important new insight into the uses of the plant. In previous experiments with 1,1-diphenyl-1-picrylhydrazyl (DPPH) these compounds have been found to have antioxidant properties, suggesting that they have the ability to scavenge free radicals. This is a new biological effect to be investigated in *L. javanica.*

- A complete structure elucidation of the isolated compounds was not accomplished since the elemental analysis such as LC-ESIMS (for the determination of the molecular formula); HRESIMS, NOESY (for the determination of the relative stereochemistry of the chiral centers) and their optical rotations were not performed for a confirmation of what was proposed using the 1D and 2D NMR experiments. Further work on these processes is to be done for complete elucidation of the structures.

5.2 Recommendations

From the results obtained in the previous chapter, a number of recommendations for future work on *L. javanica* could be listed;

- Studies on the chemical variation of *Lippia javanica* should focus on a seasonal investigation of both the polar and the non-polar compounds, which could have been a possible distorting factor in the variation of the compounds in this study and also in previous studies.

- The interest of this study also went as far as aiming at comparing *L. javanica* with the other South African *Lippia* species. Unfortunately, due to an overly dry climate in 2006, it was not possible to obtain *L. scaberrima*. Studies on this plant species should also compare the chemical compounds of all these species to make a complete variation study of the South African *Lippia* species.

- The toxicity of the *Lippia* species still needs to be further investigated.

- The biological activities of the extracts and the isolated compounds are an ongoing investigation in our laboratories. The phenylethanoid glycosides of *L. javanica* will in future also be tested for their antioxidant activity, since literature mentions that they are free radical scavengers. More so because there are no literature reports about the antioxidant properties of *Lippia javanica.*

APPENDIX A

TLC Visualizing Reagent Procedures

APPENDIX A

TLC Visualizing Reagent Procedures

A1. Introduction

The technique of compound separation from a plant extract using TLC analyses is completed by the detection of the compounds. Colored compounds can be visually detected, while colorless compounds may be visualized by means of ultra-violet (UV) (254 or 356 nm) radiation or by means of detection reagents (solutions) either by spraying or dipping the plates after development.

In the process of post dipping, the thin layer tank is filled with the detection reagents and the developed dried TLC plates are dipped in the reagent for several seconds and then removed slowly to allow excess reagent to drain back into the tank. The plates are air dried and sometimes heated to visualize the compounds, which then exhibit characteristic colors.

For this study dipping of the plates was preferred over spraying because:

- The dipping procedure is less dangerous (for corrosive reagents)
- It was economical since visualizing reagents can be reused if dedicated reagent dipping containers were used.
- The TLC background was unblemished (which would have not been achieved with spraying) which is important for a true reflection of separated compounds.

The procedures for the preparation of the visualizing reagents are outlined in the section that follows.

A2. Procedure for visualizing reagents preparation

A2.1. Vanillin- sulphuric acid solution[1]

- 1 g vanillin
- 5 cm^3 H_2SO_4 (concentrated)
- 194 cm^3EtOH

The modified preparation of this reagent (Solution II) was used for the detection of the non-polar compounds together with the polar compounds.

- Solution I was prepared with 1% vanillin in ethanol
- Solution II was prepared with 5% sulphuric-acid in ethanol

Each plate was dipped in Solution I, left to dry and dipped in Solution II before heating at 110°C until compounds were revealed.

A2.2. Anisaldehyde-sulphuric acid solution[1]

- 85 cm^3 MeOH
- 5 cm^3 H_2SO_4 (concentrated)
- 0.5 cm^3 anisaldehyde
- 10 cm^3 CH_3COOH (glacial)

For the screening of triterpenoids the visualizing reagent was prepared by dissolving 0.5 cm^3 anisaldehyde in 10 cm^3 of glacial acetic acid, adding 85 cm^3 of methanol and lastly adding 5 cm^3 of concentrated sulphuric acid.[1] TLC plates were dipped into the solution and heated until compounds were revealed at 100°C. This reagent must be prepared fresh before use.

[1] Wagner H., Bladt S. *Plant Drug Analysis. A Thin Layer Chromatography Atlas*. 1996. 2nd edition Springer-Verlag Berlin Heidelberg, New York. 166

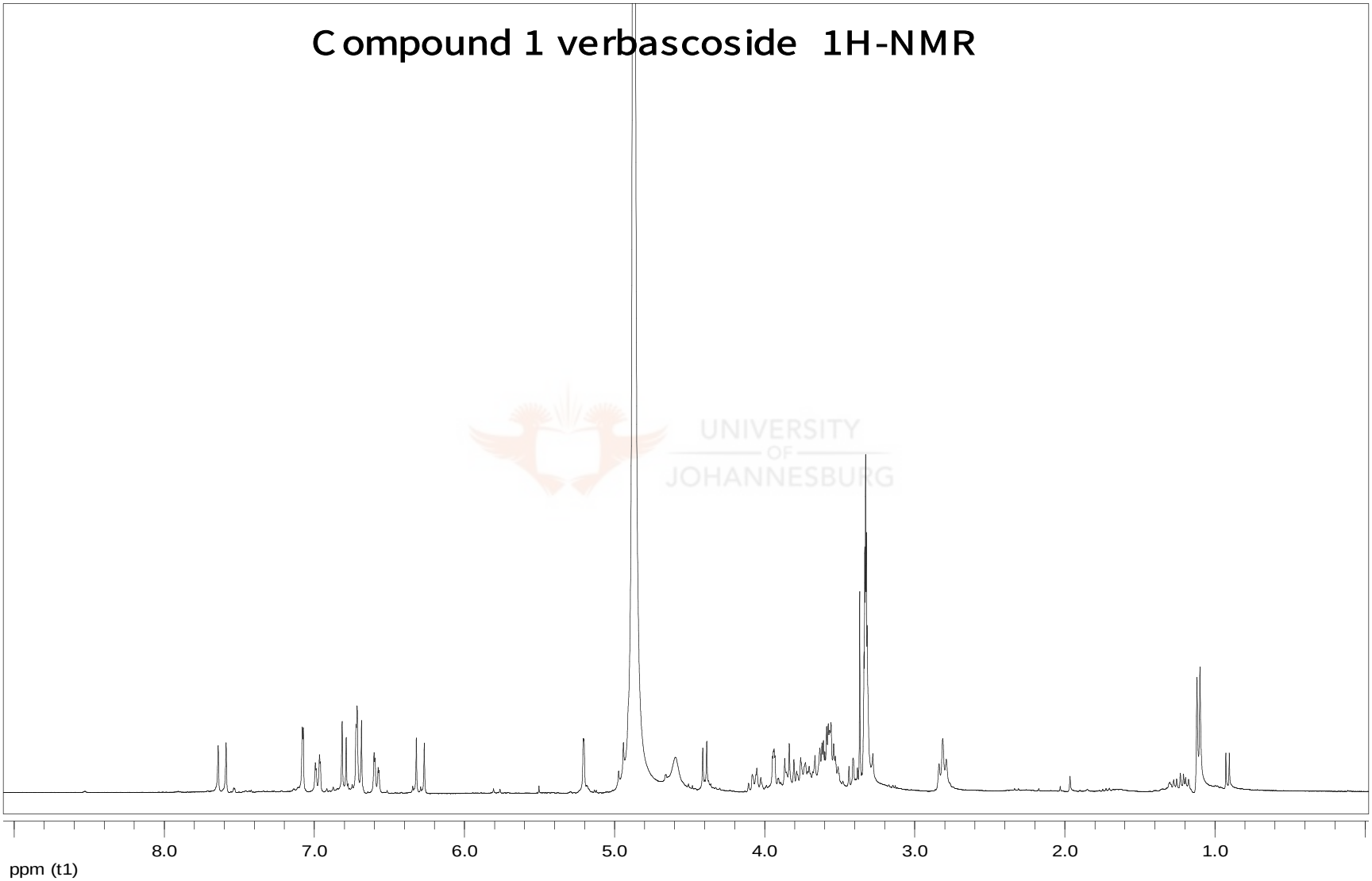
A2.3. Ninhydrin solution[1]

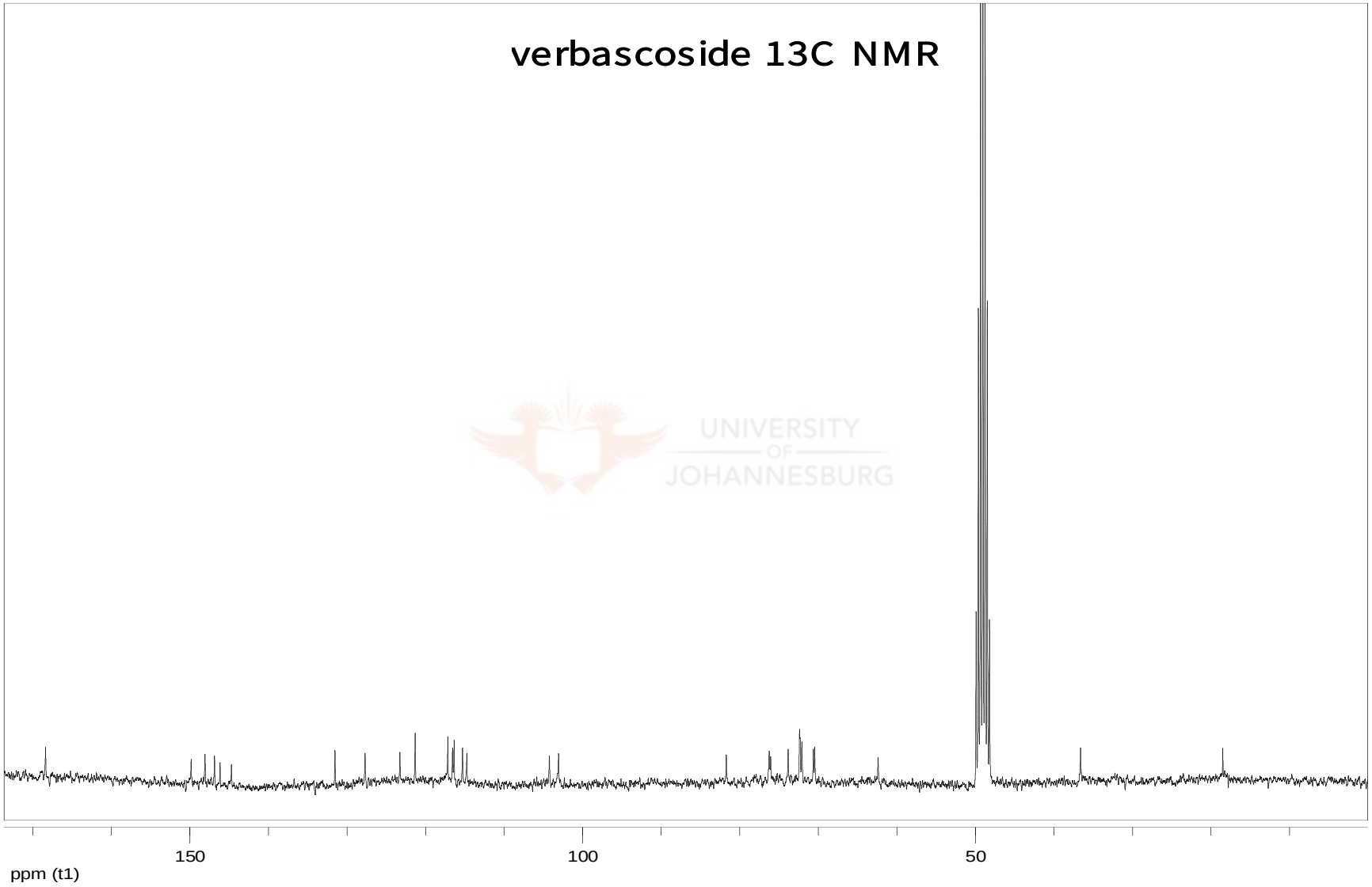
- 30 mg Ninhydrin
- 10 cm^3 *n*-Butanol
- 0.3 cm^3 CH_3COOH (98%)

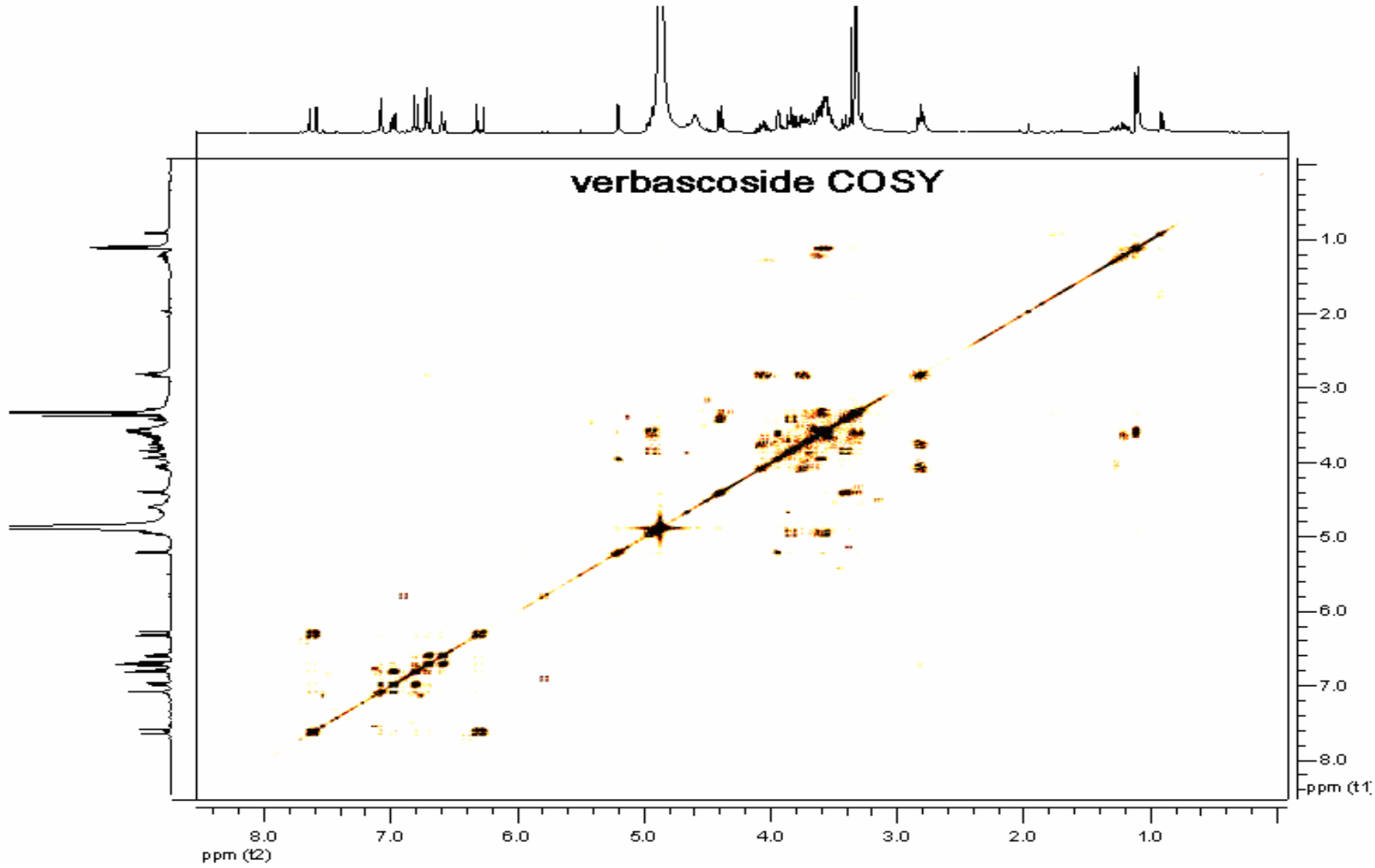
For the detection of amino acids a ninhydrin reagent is prepared by dissolving 30 mg of ninhydrin in10 cm^3 of *n*-butanol and adding 0.3 cm^3 of acetic acid (98%) to the solution. The plate of non-polar compounds was dipped into the solution and heated until compounds were revealed.[1]

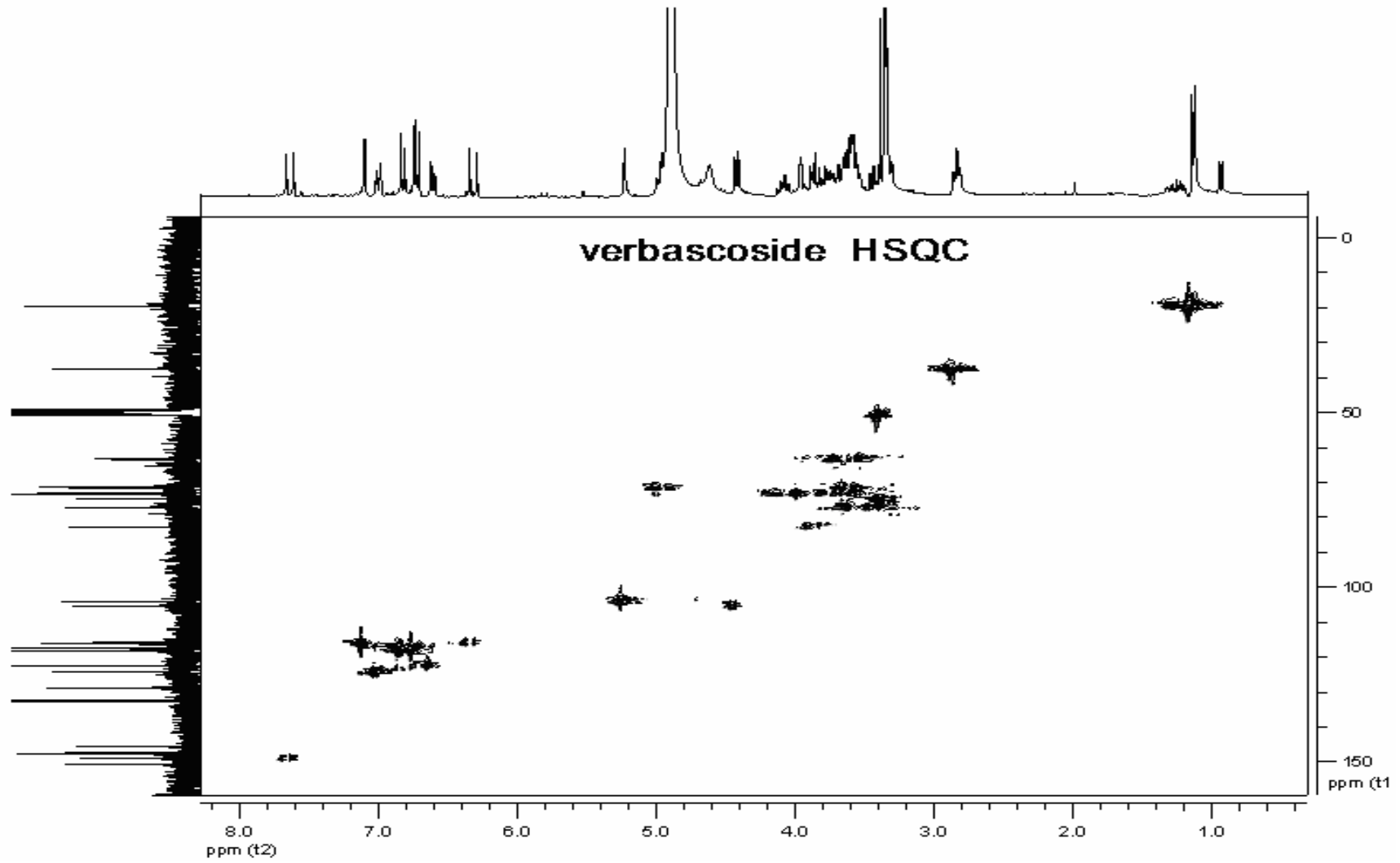
APPENDIX B

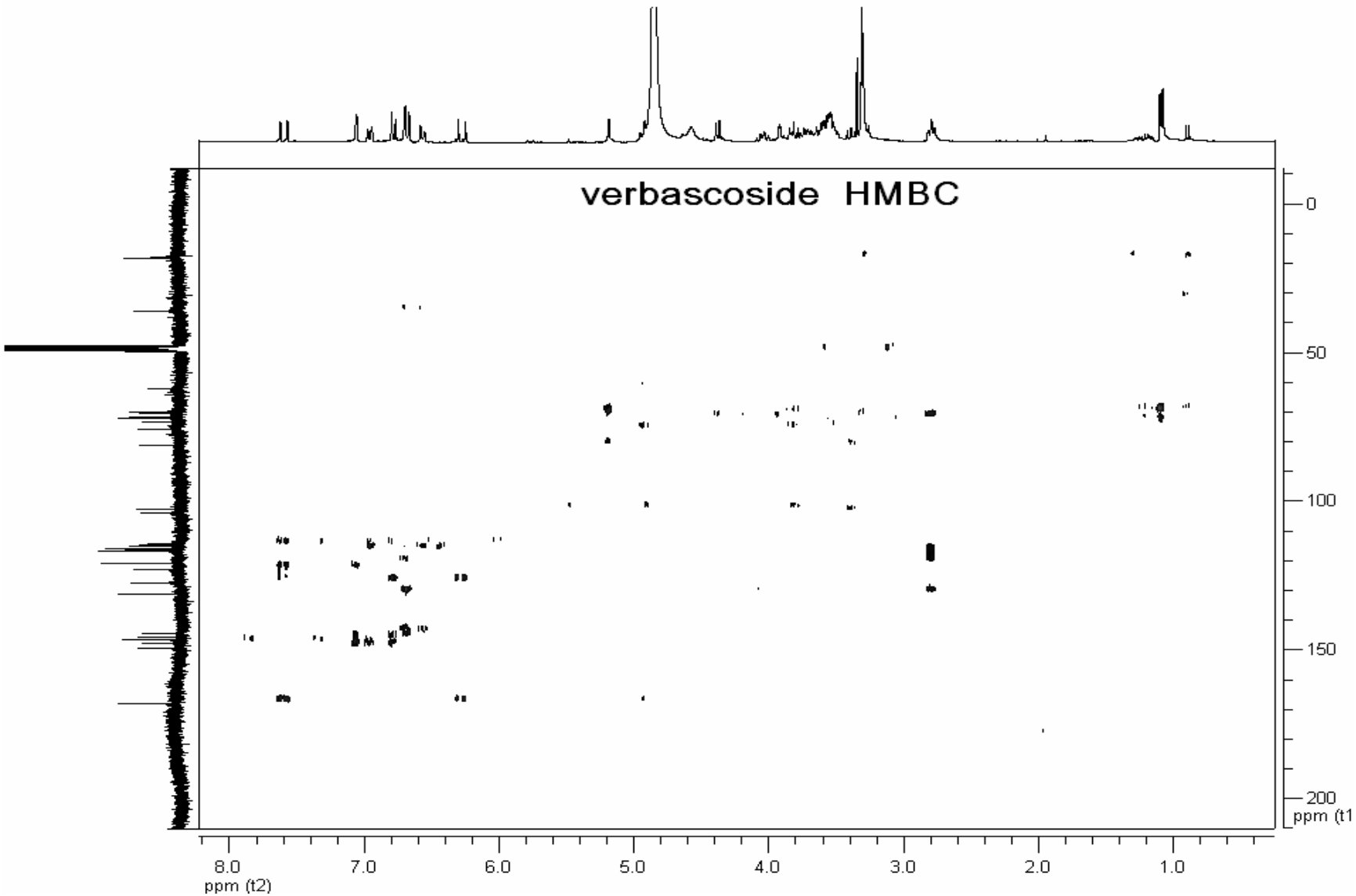
NMR AND IR SPECTRA



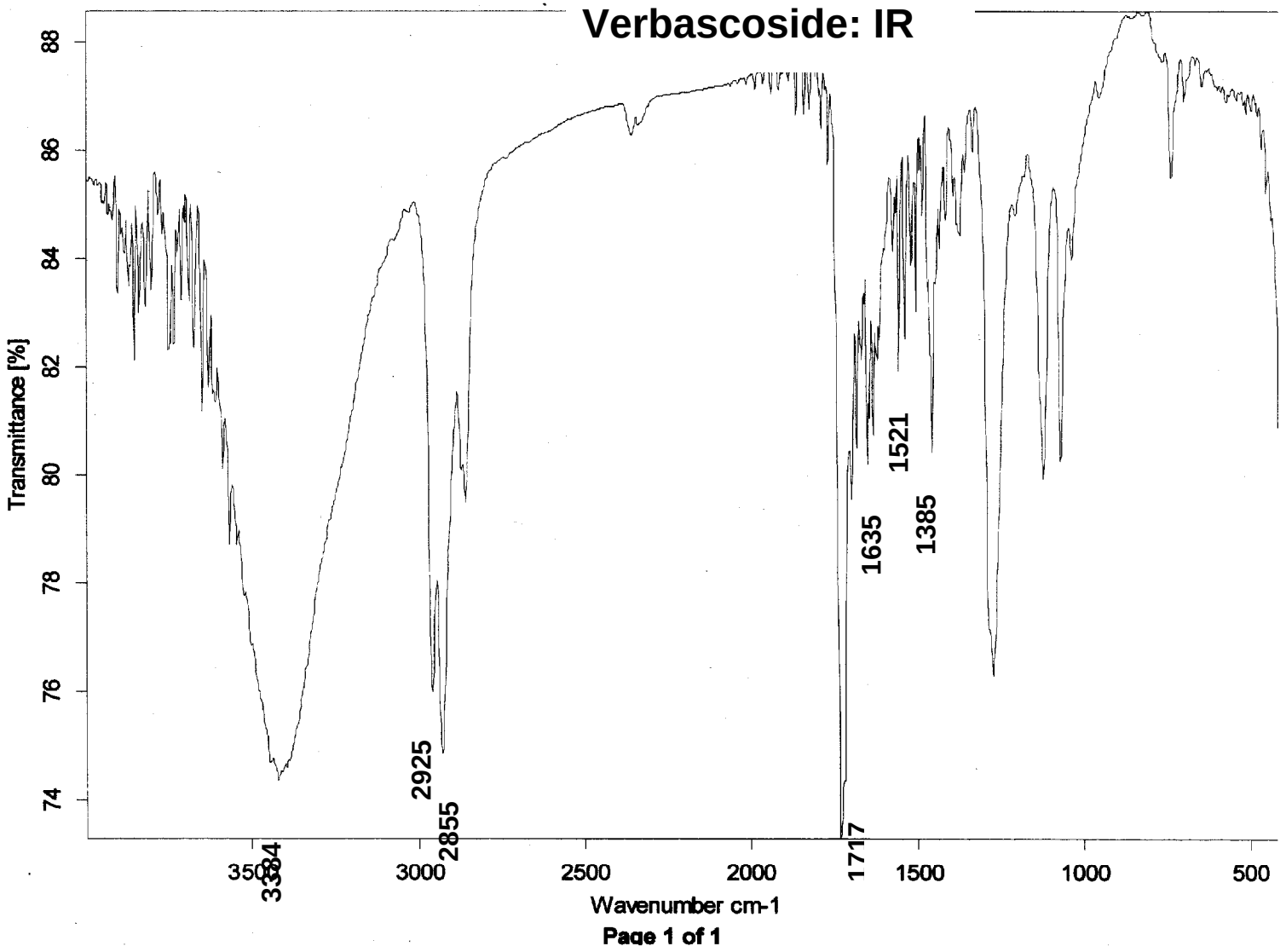

verbascoside 13C NMR
150
100
50
ppm (t1)

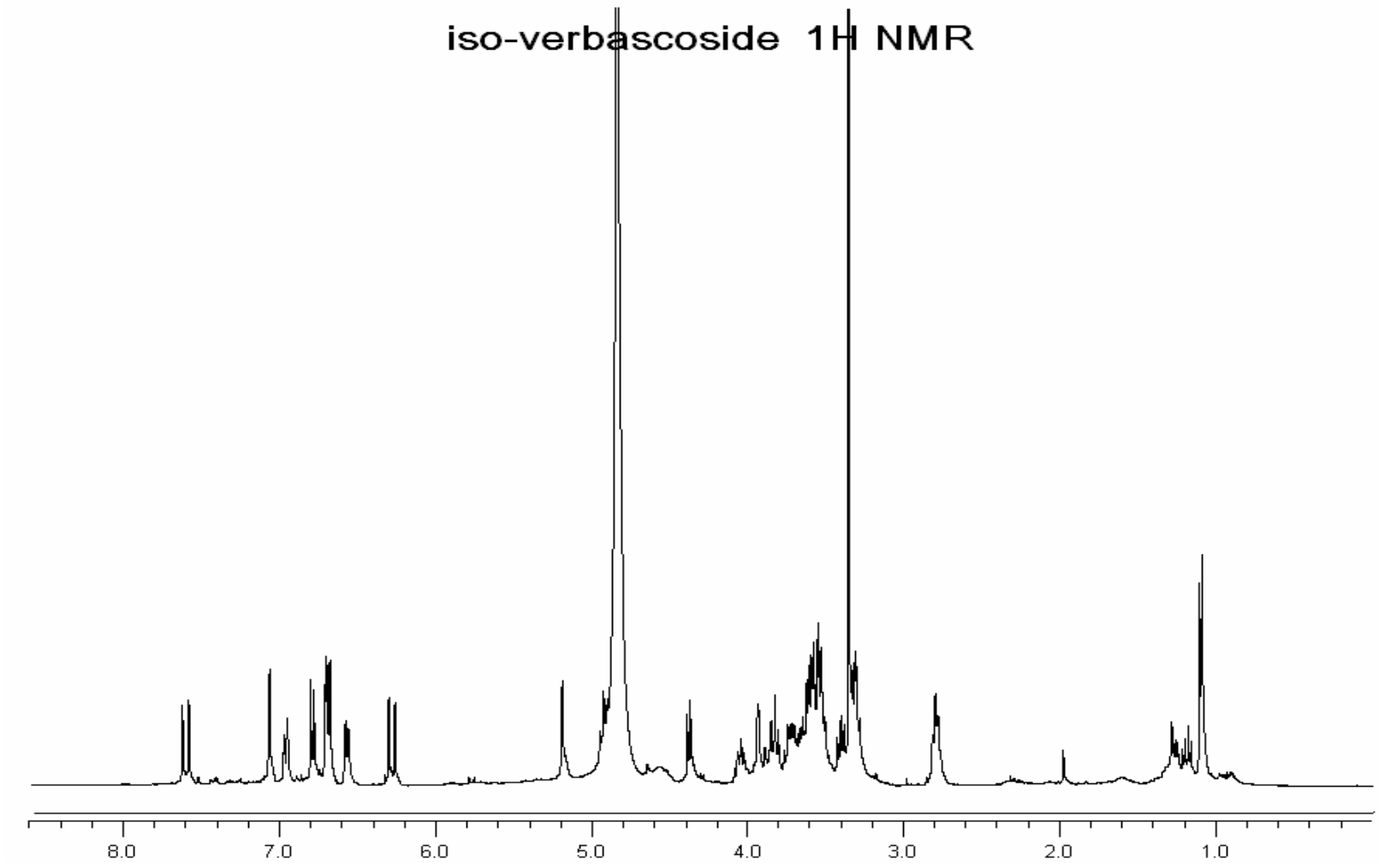

verbascoside COSY
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm (t2)
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
ppm (t1)

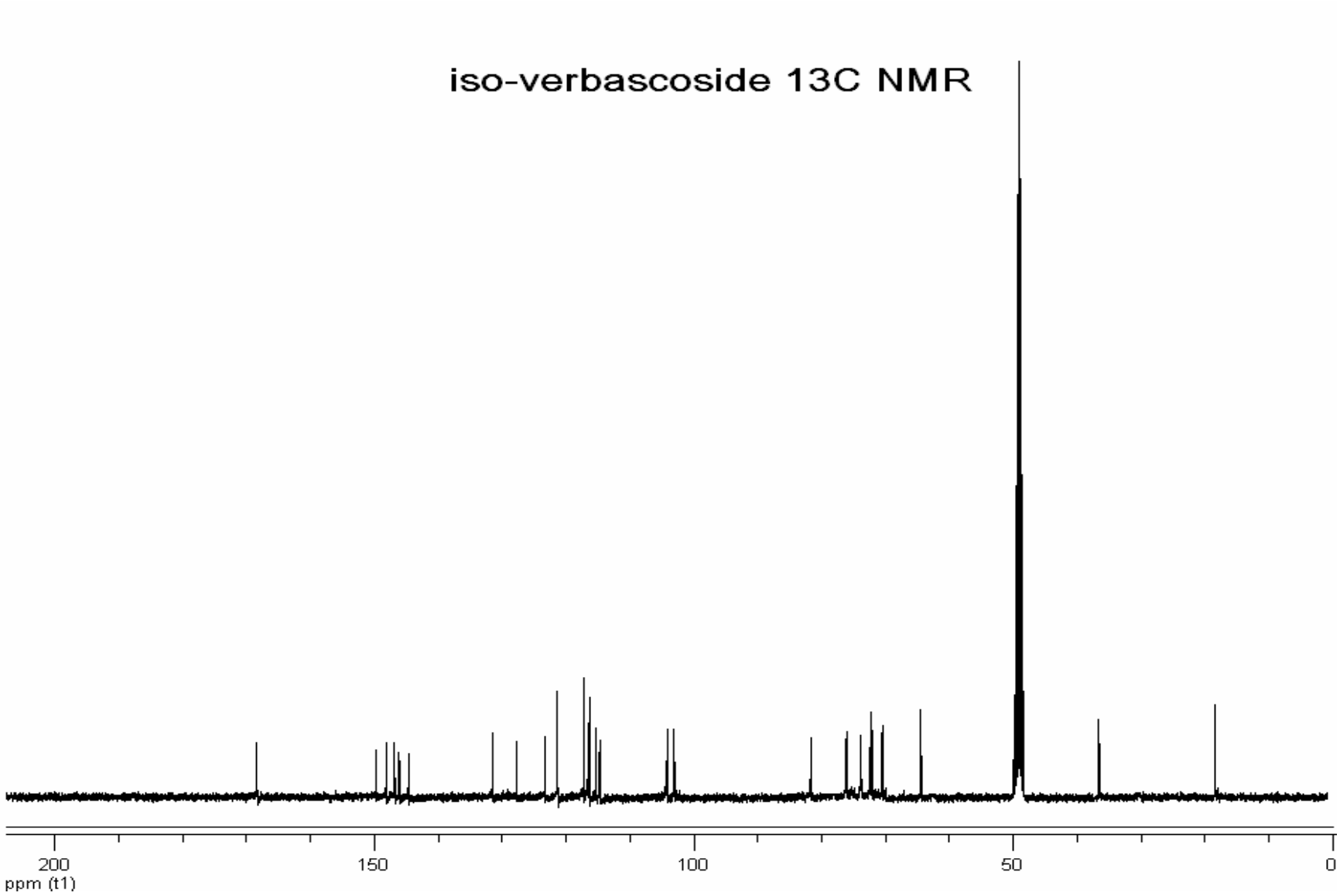

verbascoside HSQC
0
50
100
150
ppm (t1
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm (t2)

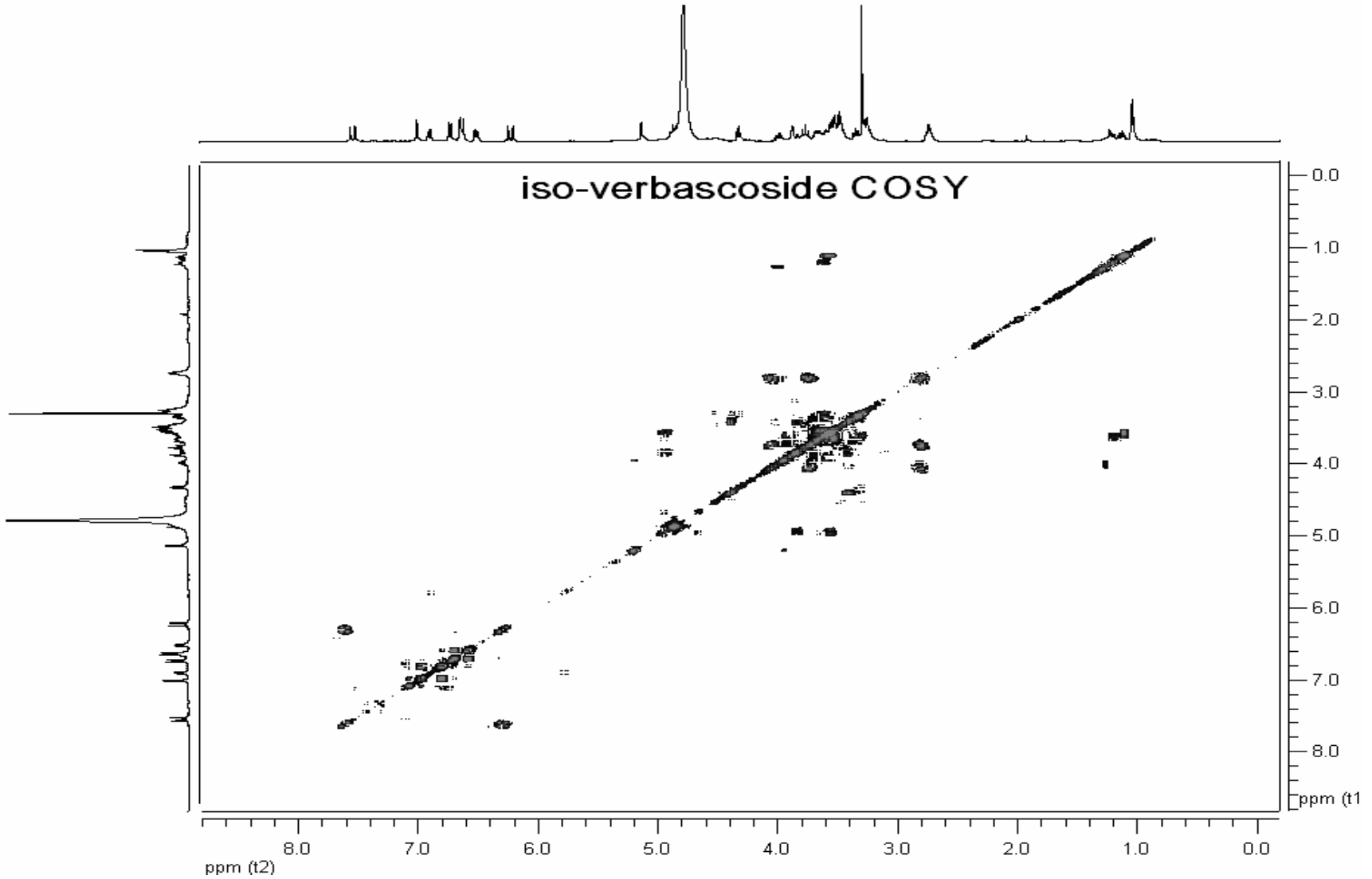

verbascoside HMBC
0
50
100
150
200
ppm (t1
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm (t2)

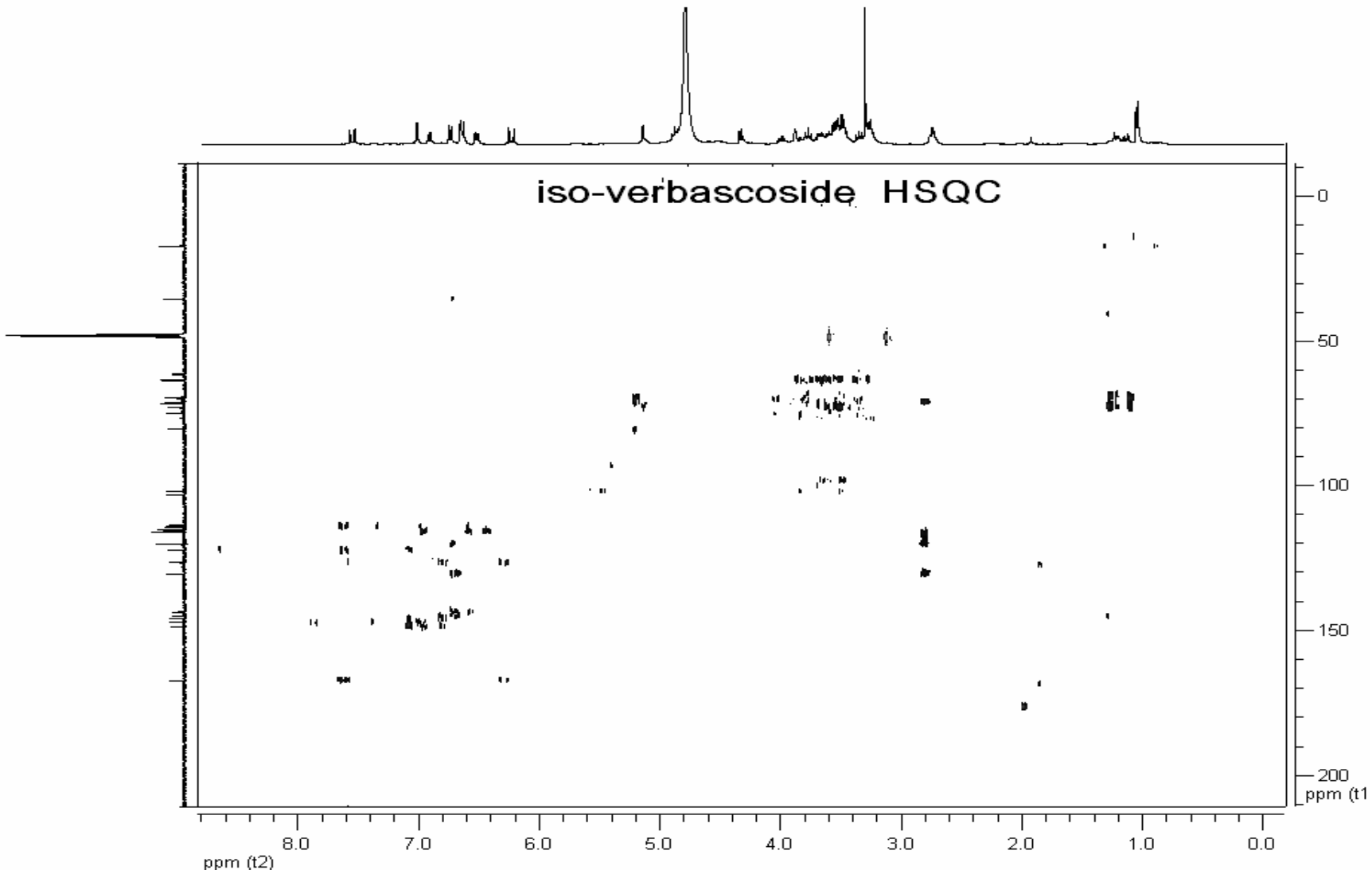
Verbascoside: IR

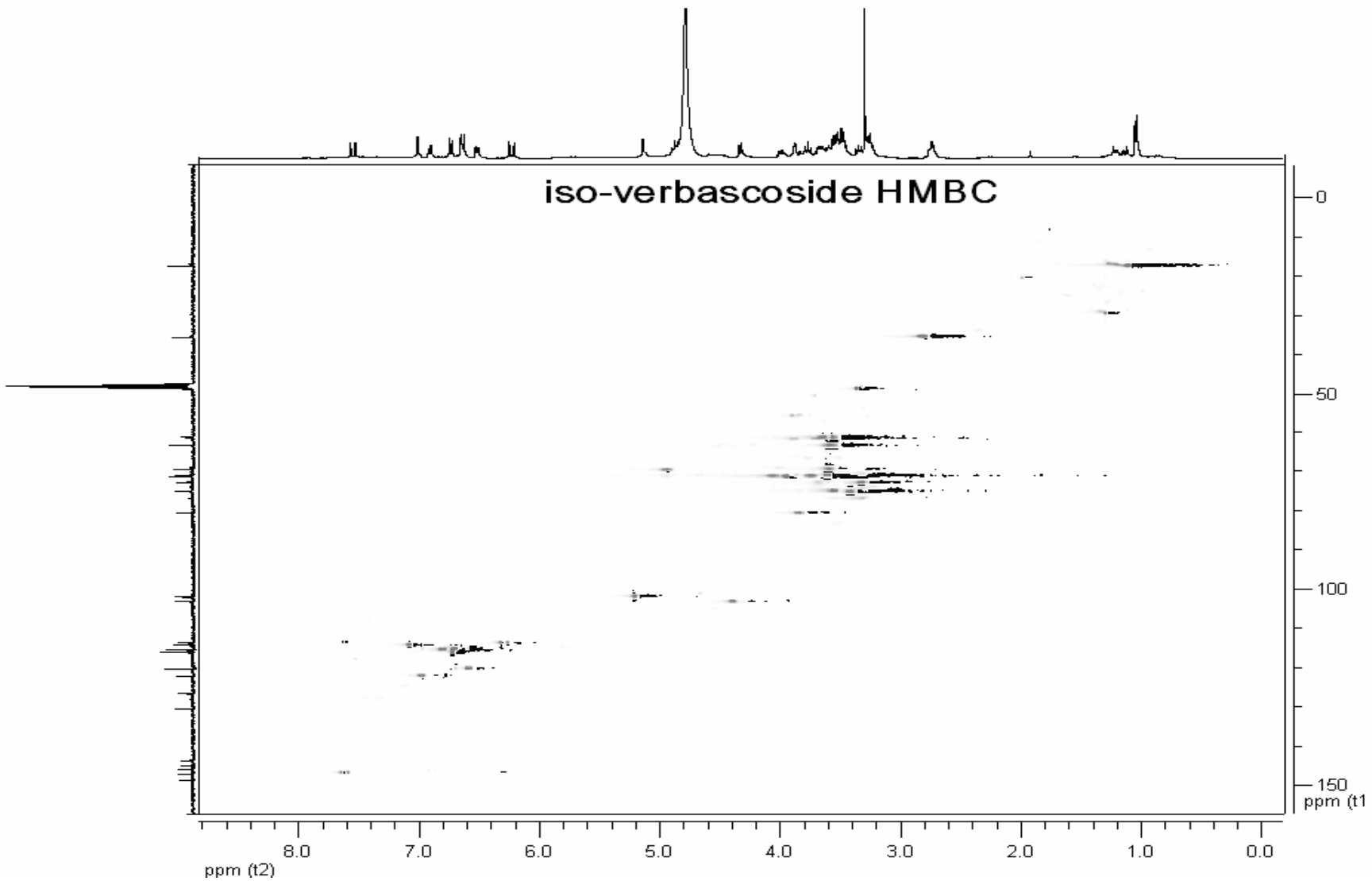


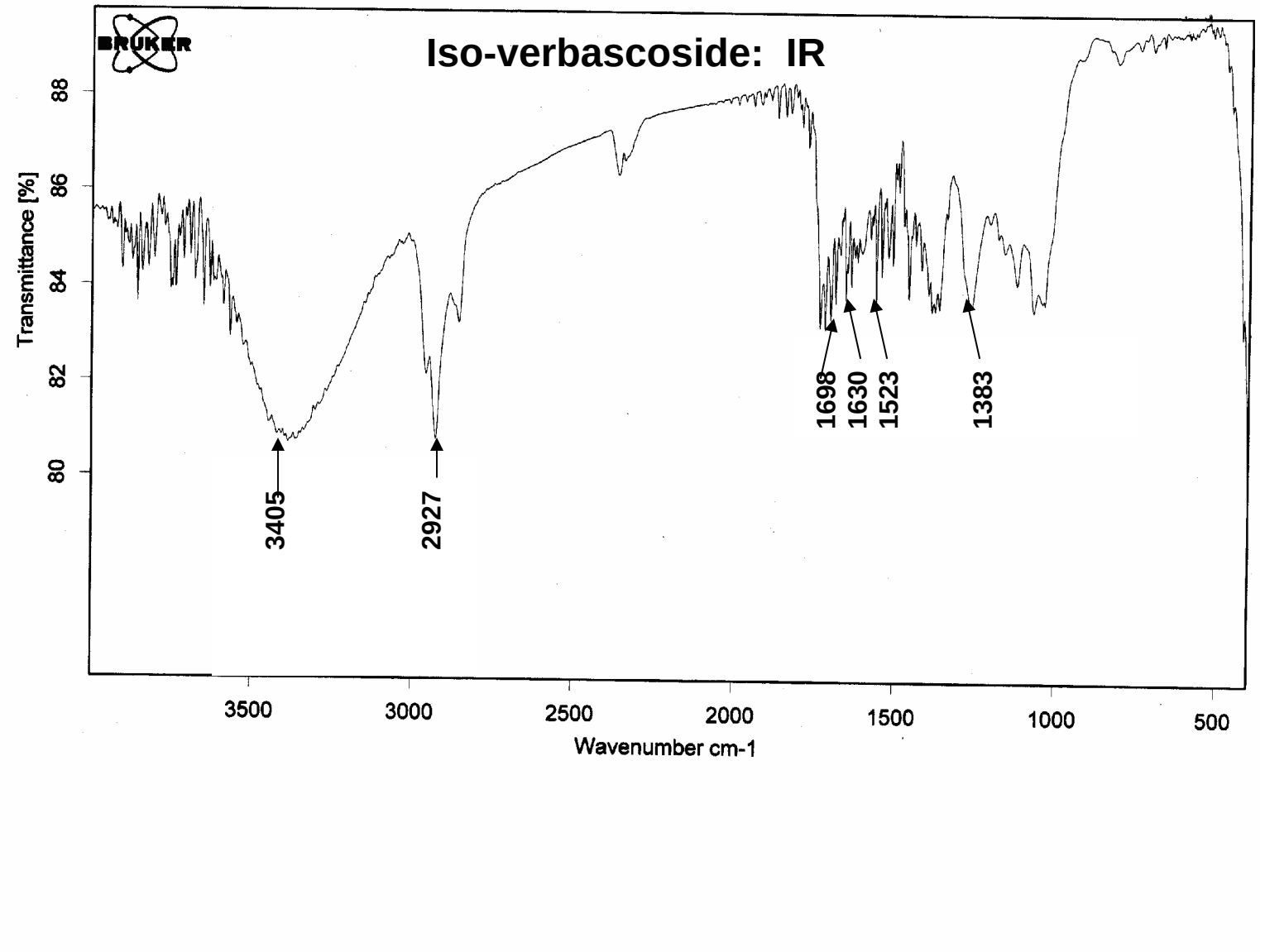

iso-verbascoside 1H NMR
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0

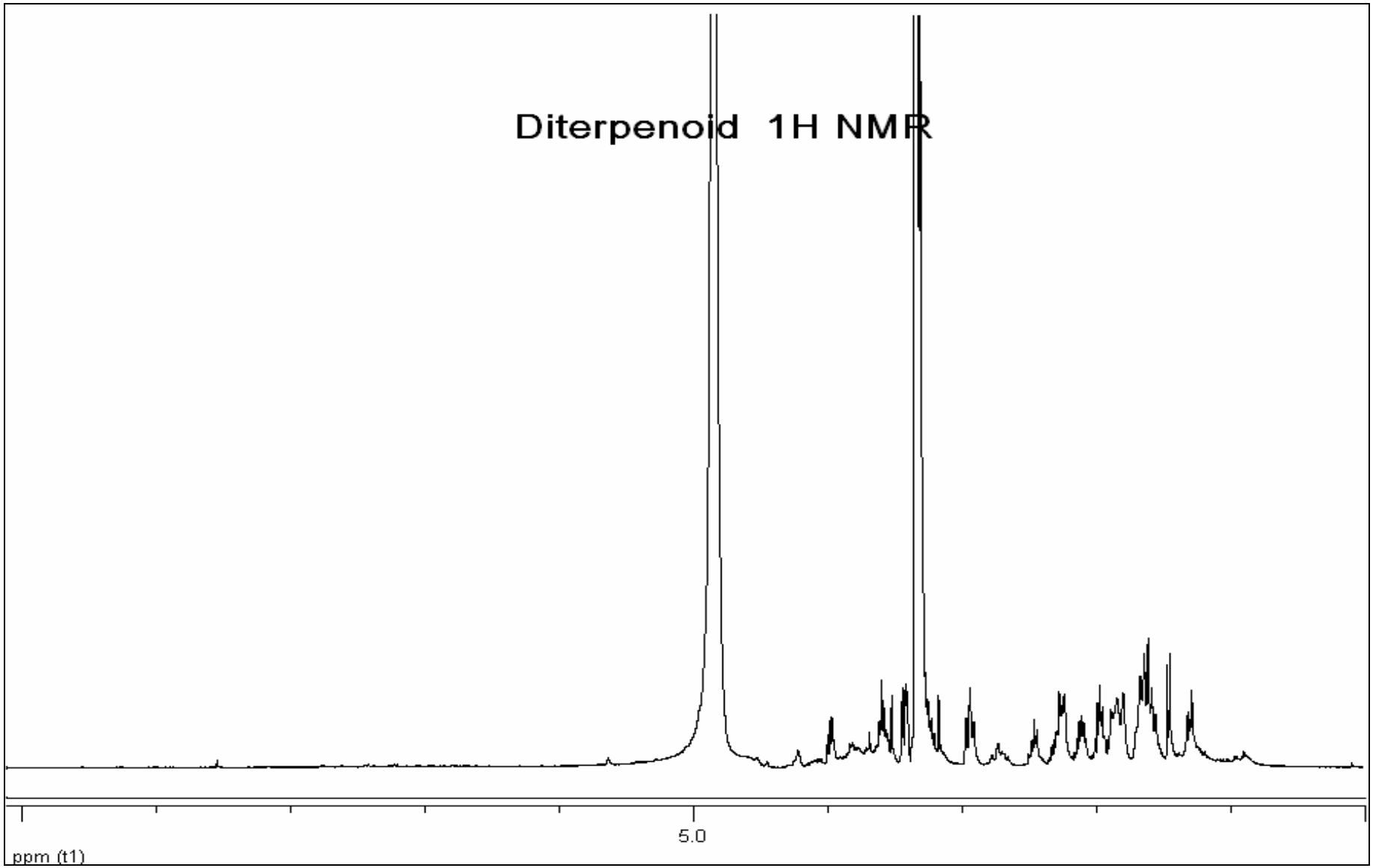

iso-verbascoside 13C NMR
200
150
100
50
0
ppm (t1)

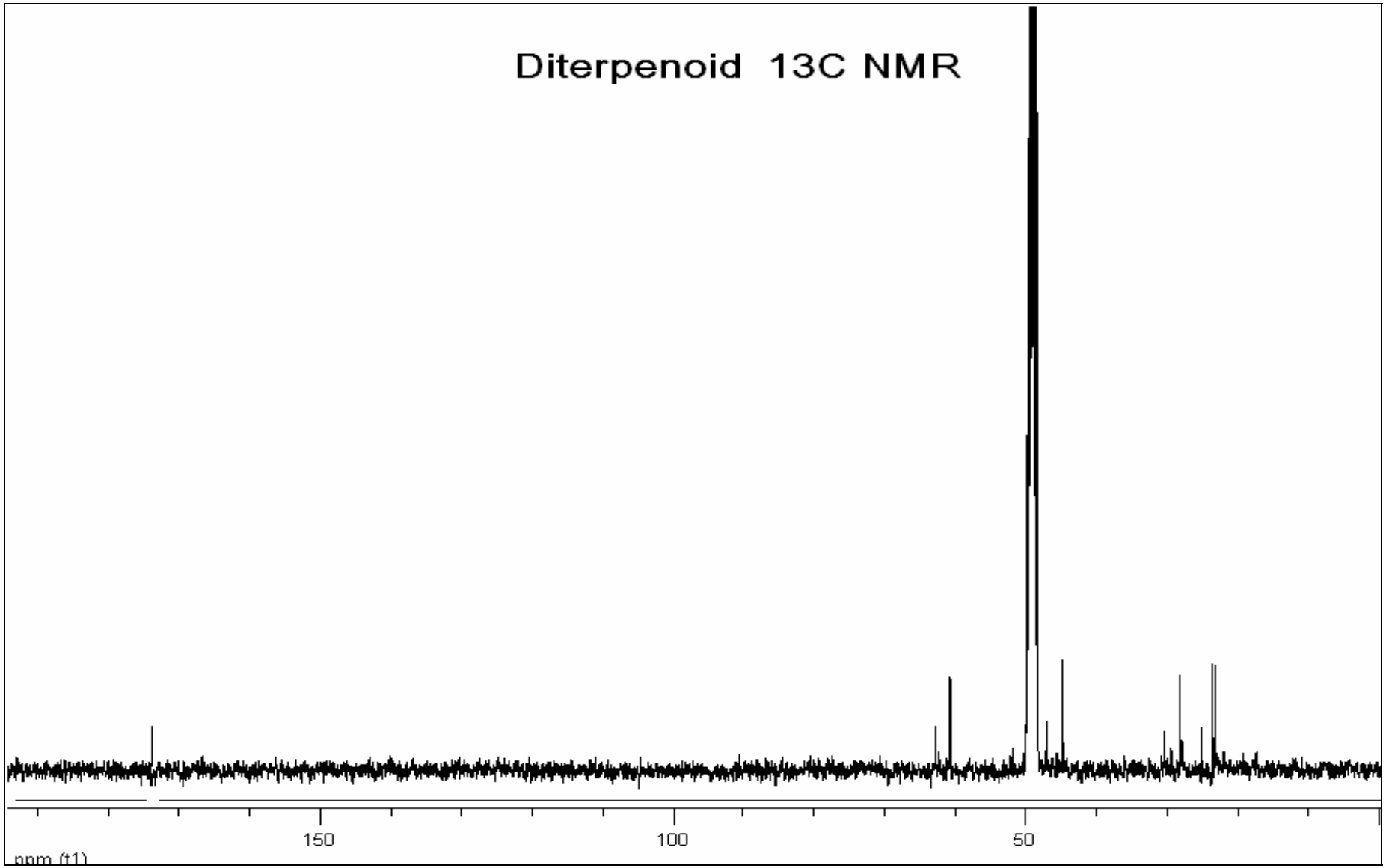

iso-verbascoside COSY
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
ppm (t1
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ppm (t2)


iso-verbascoside HSQC
0
50
100
150
200
ppm (t1
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ppm (t2)

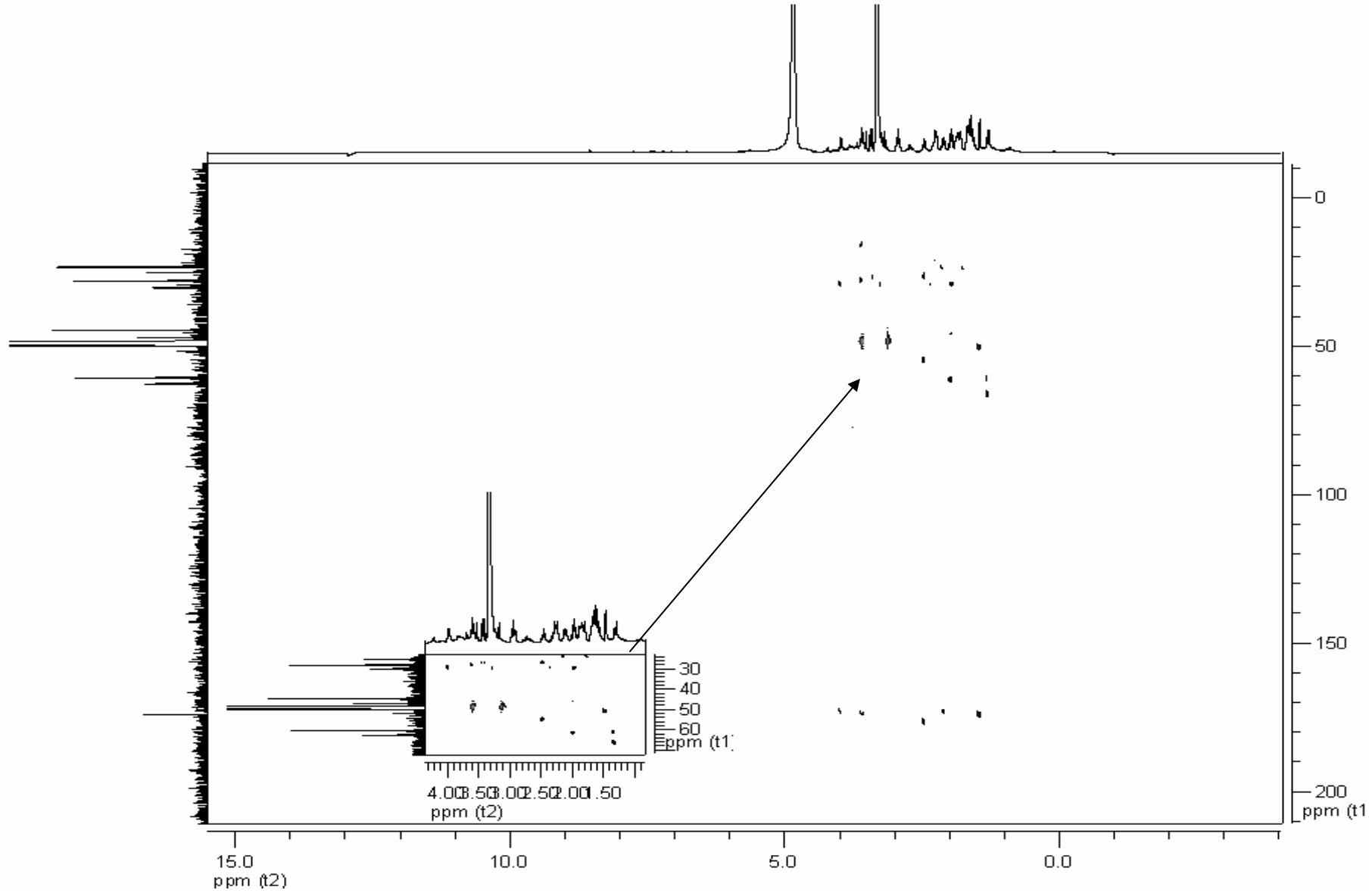

iso-verbascoside HMBC
0
50
100
150
ppm (t1
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ppm (t2)


Iso-verbascoside: IR
BRUKER
Transmittance [%]
88
86
84
82
80
3405
2927
1698
1630
1523
1383
3500
3000
2500
2000
1500
1000
500
Wavenumber cm-1

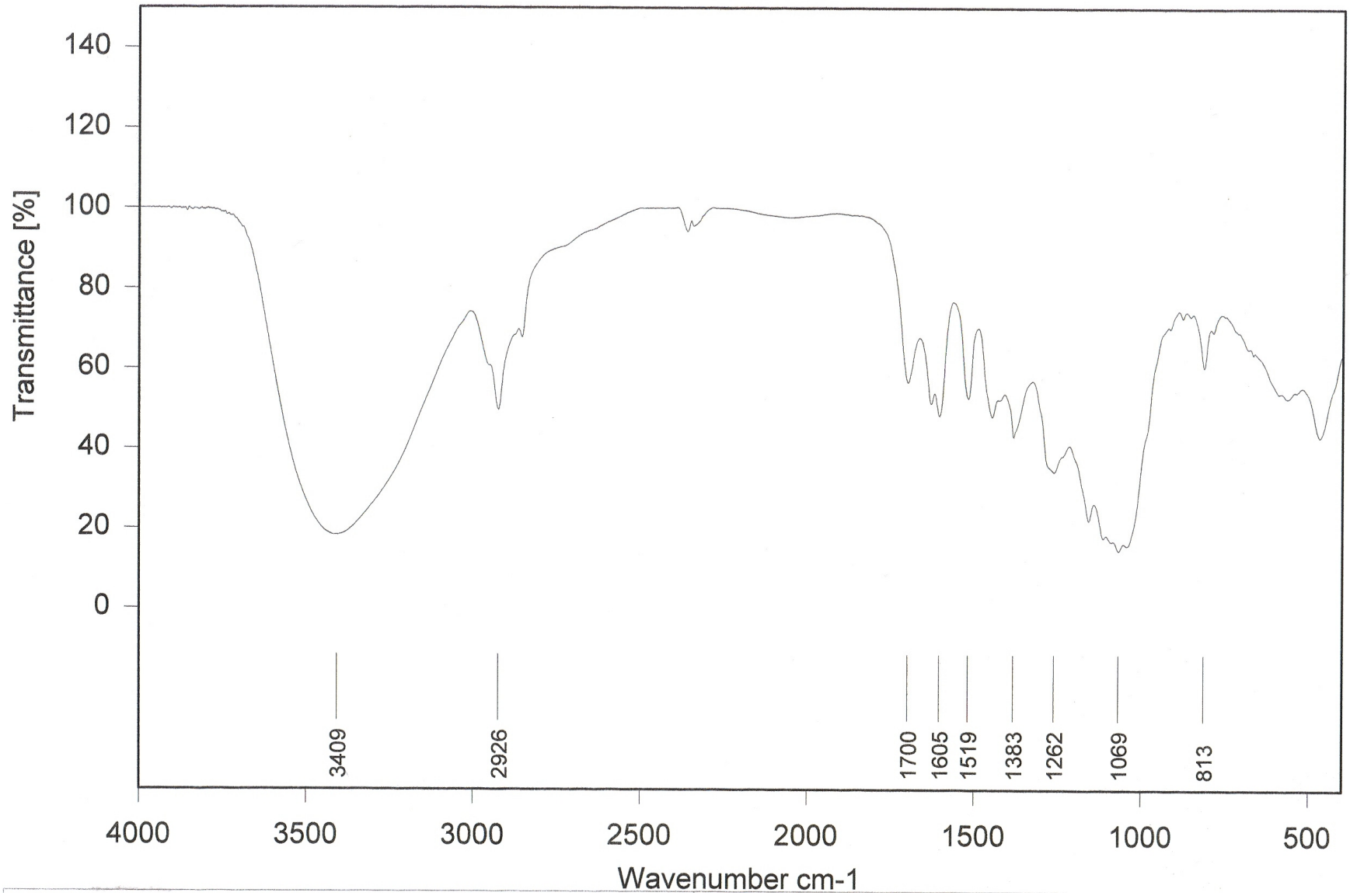

Diterpenoid 1H NMR
5.0
ppm (t1)


Diterpenoid 13C NMR
150
100
50
ppm (t1)

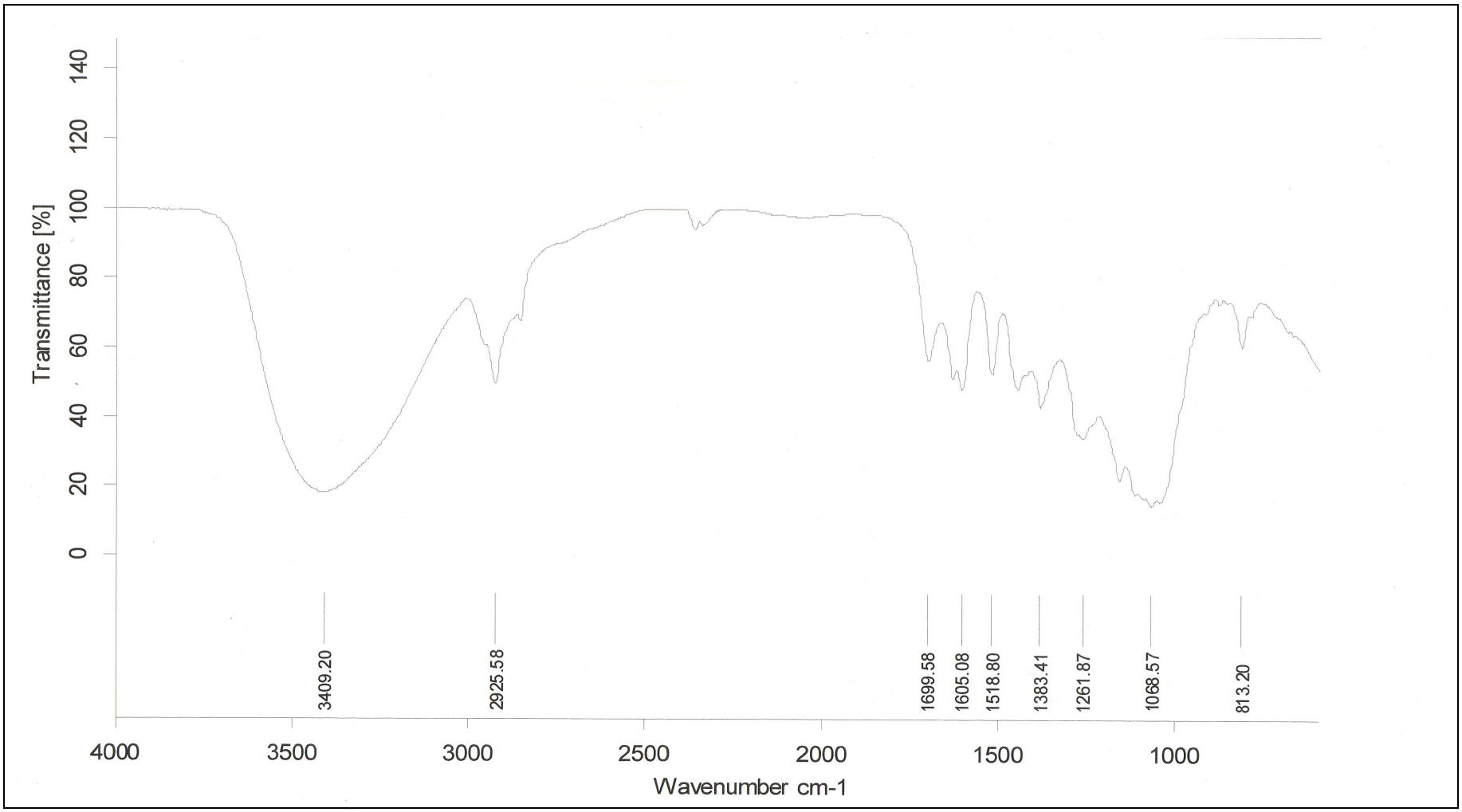
Diterpenoid HMBC

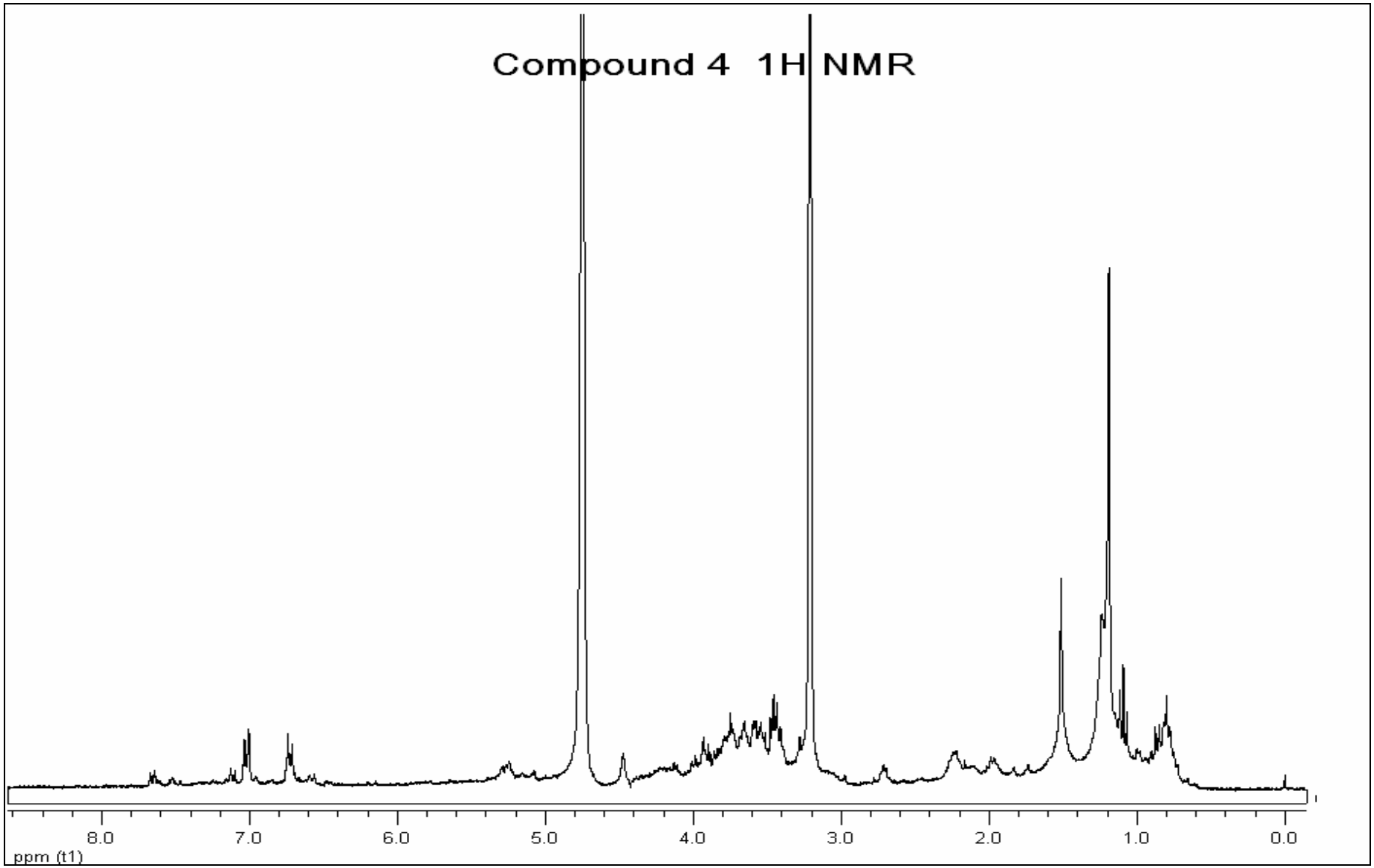


Compound 3 IR

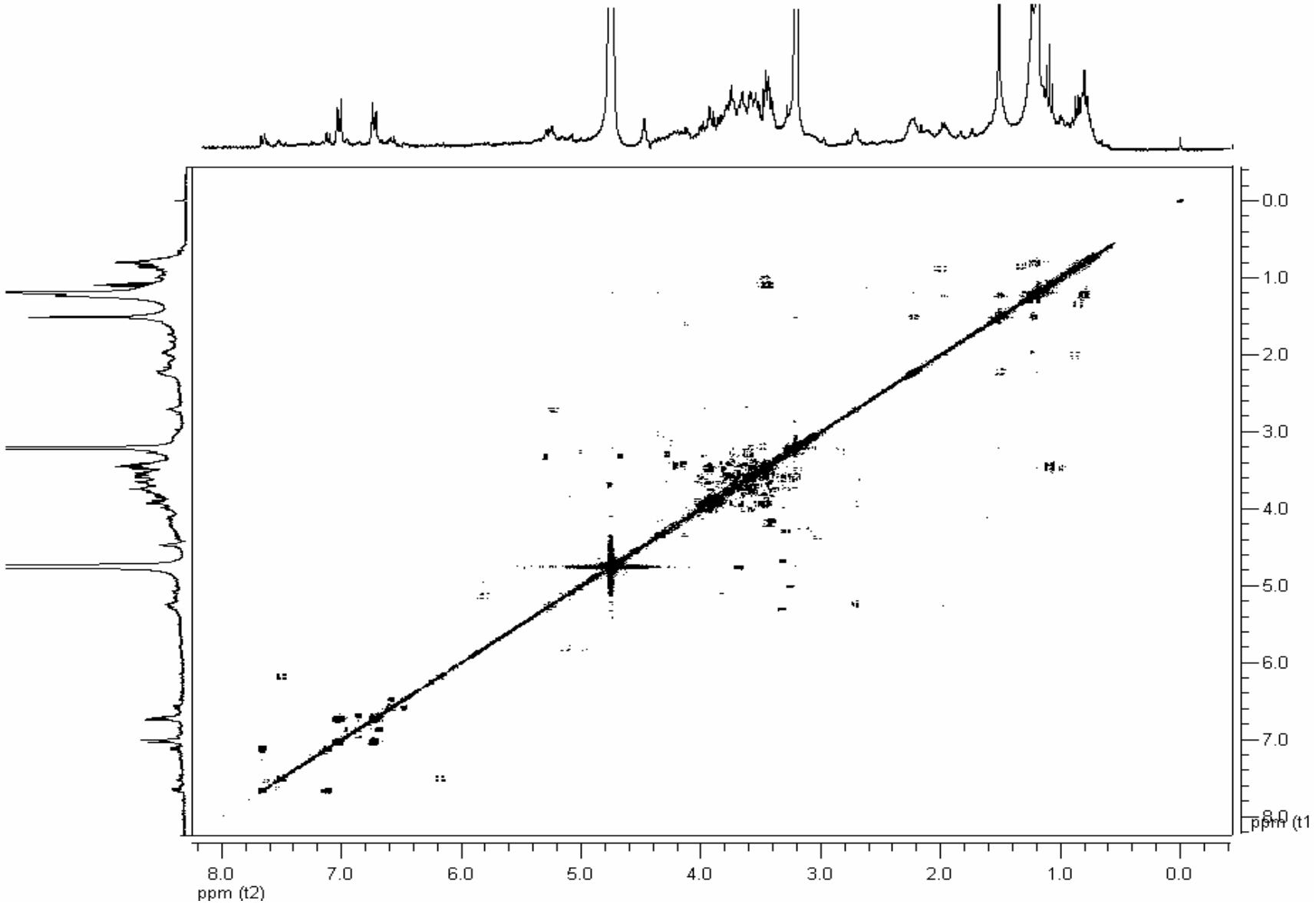


Compound 4 IR

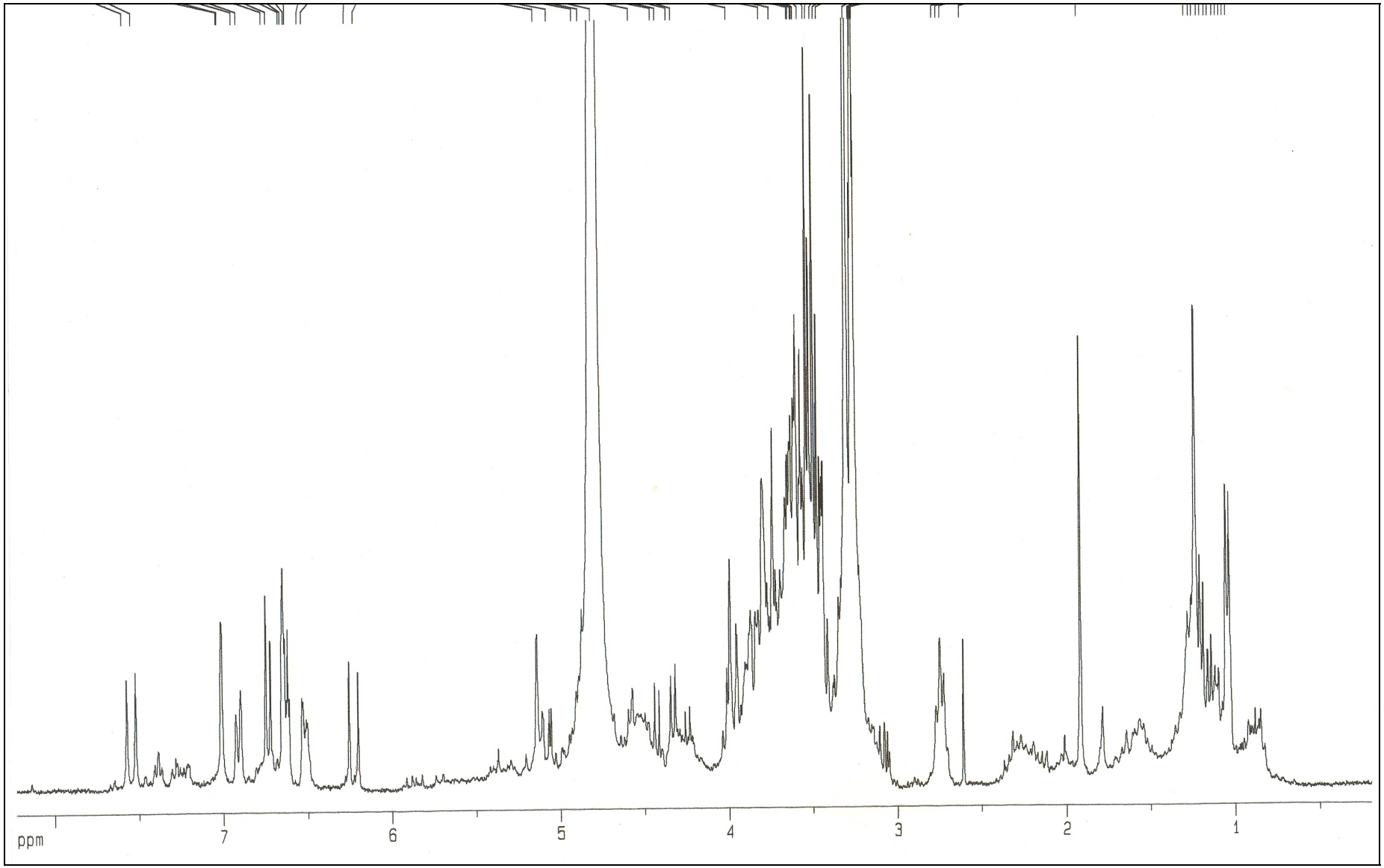



Compound 4 1H NMR
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ppm (t1)

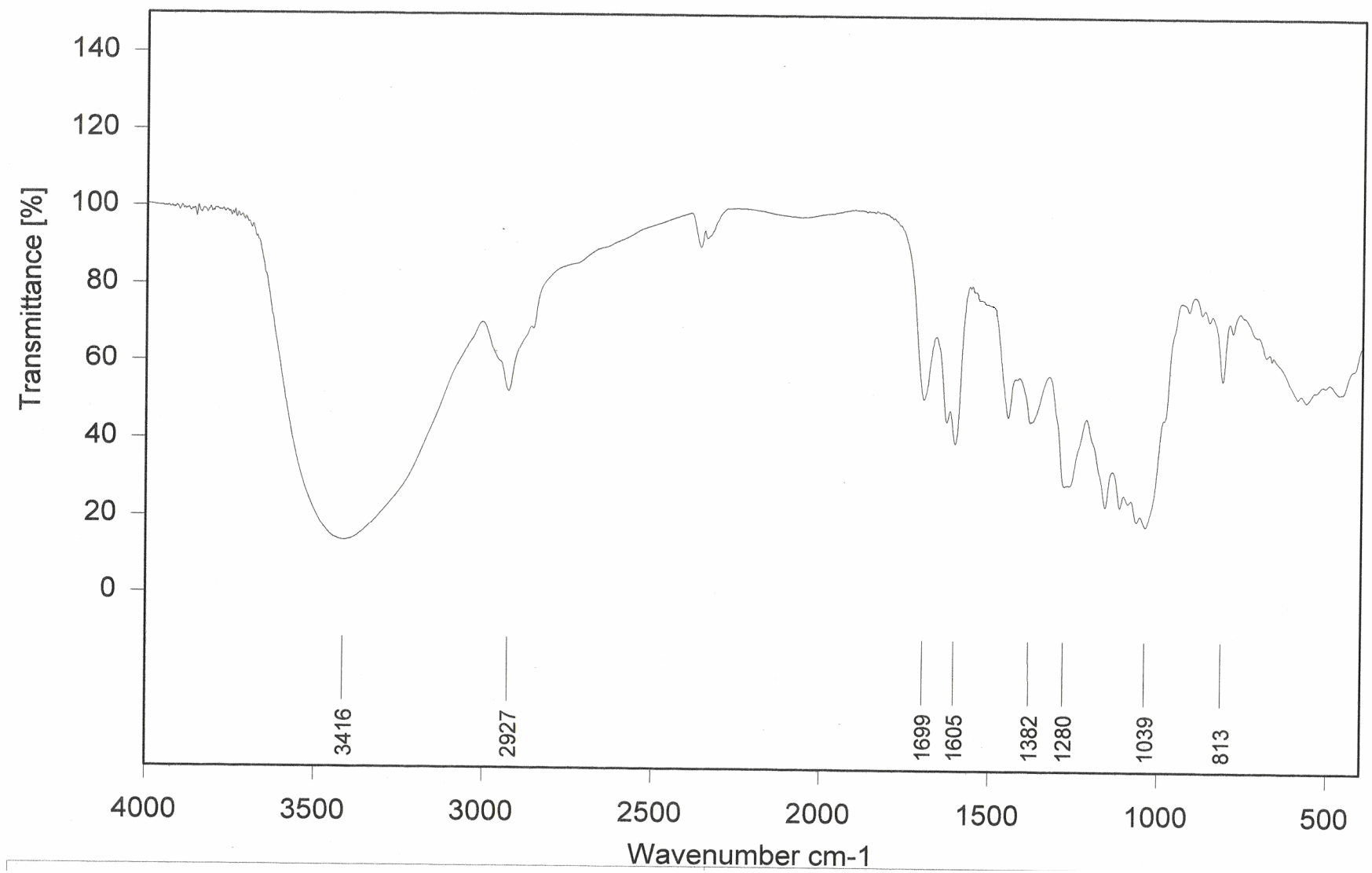
Compound 4 COSY

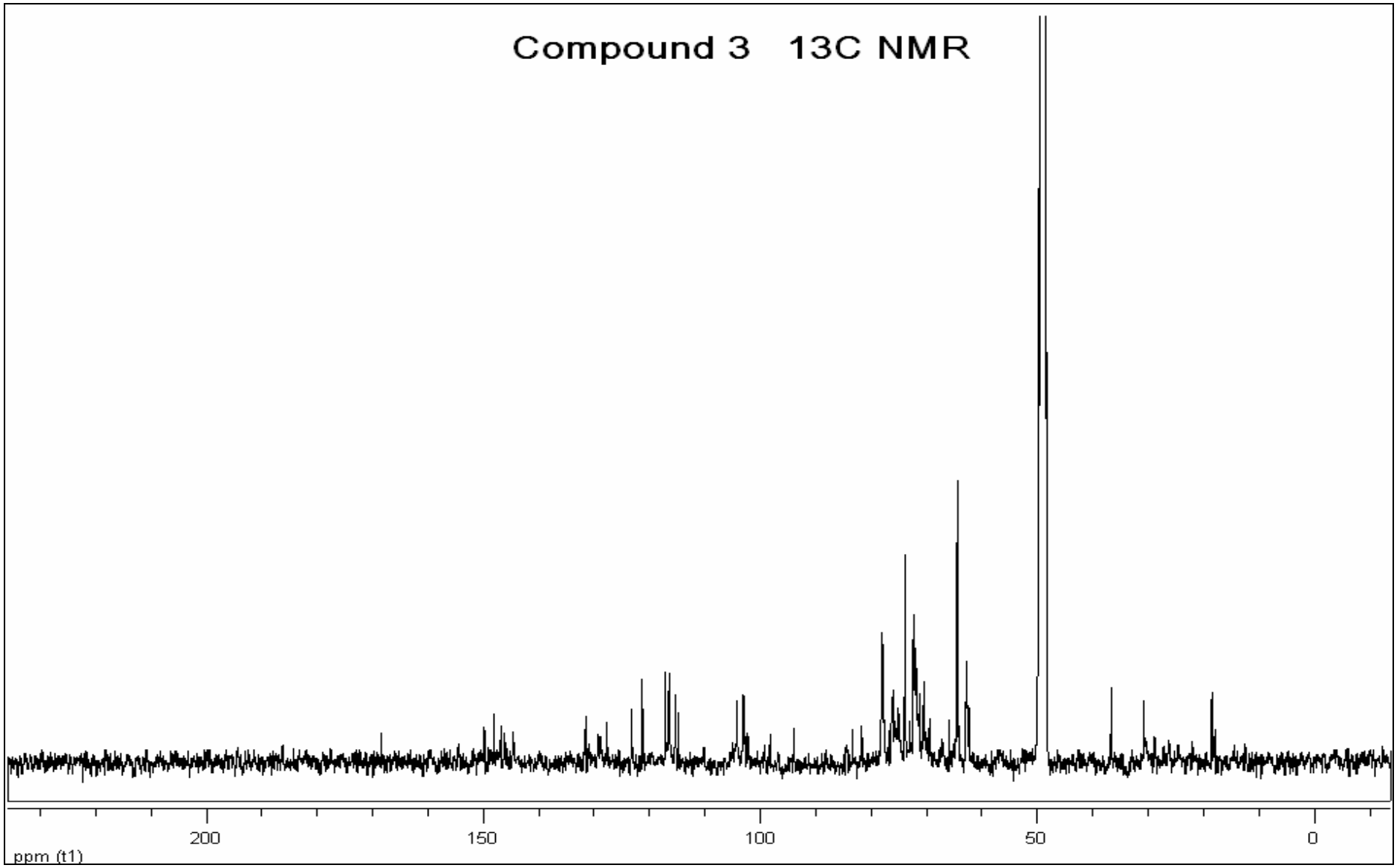


Compound 3: ^{1}H NMR

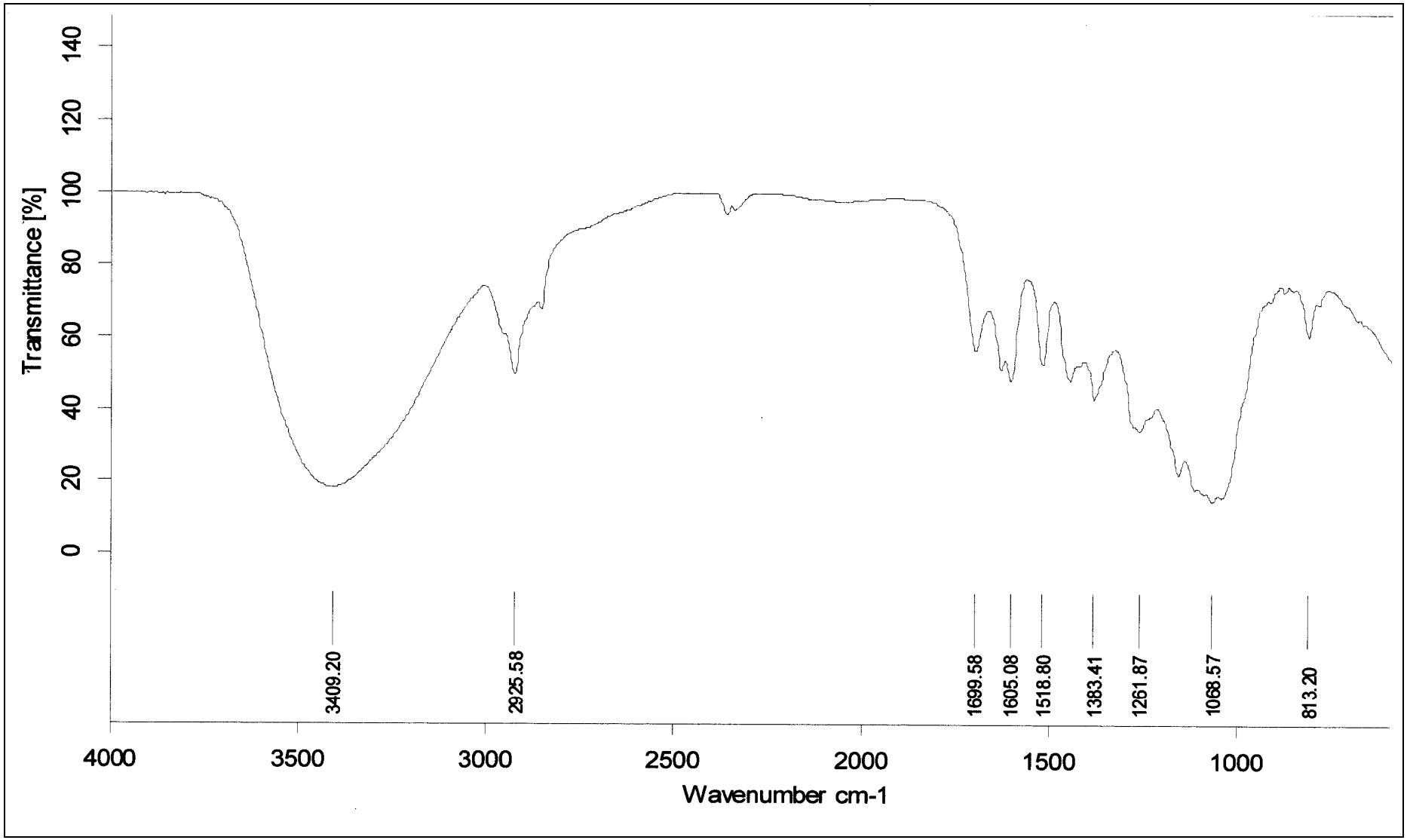


Compound 5: IR

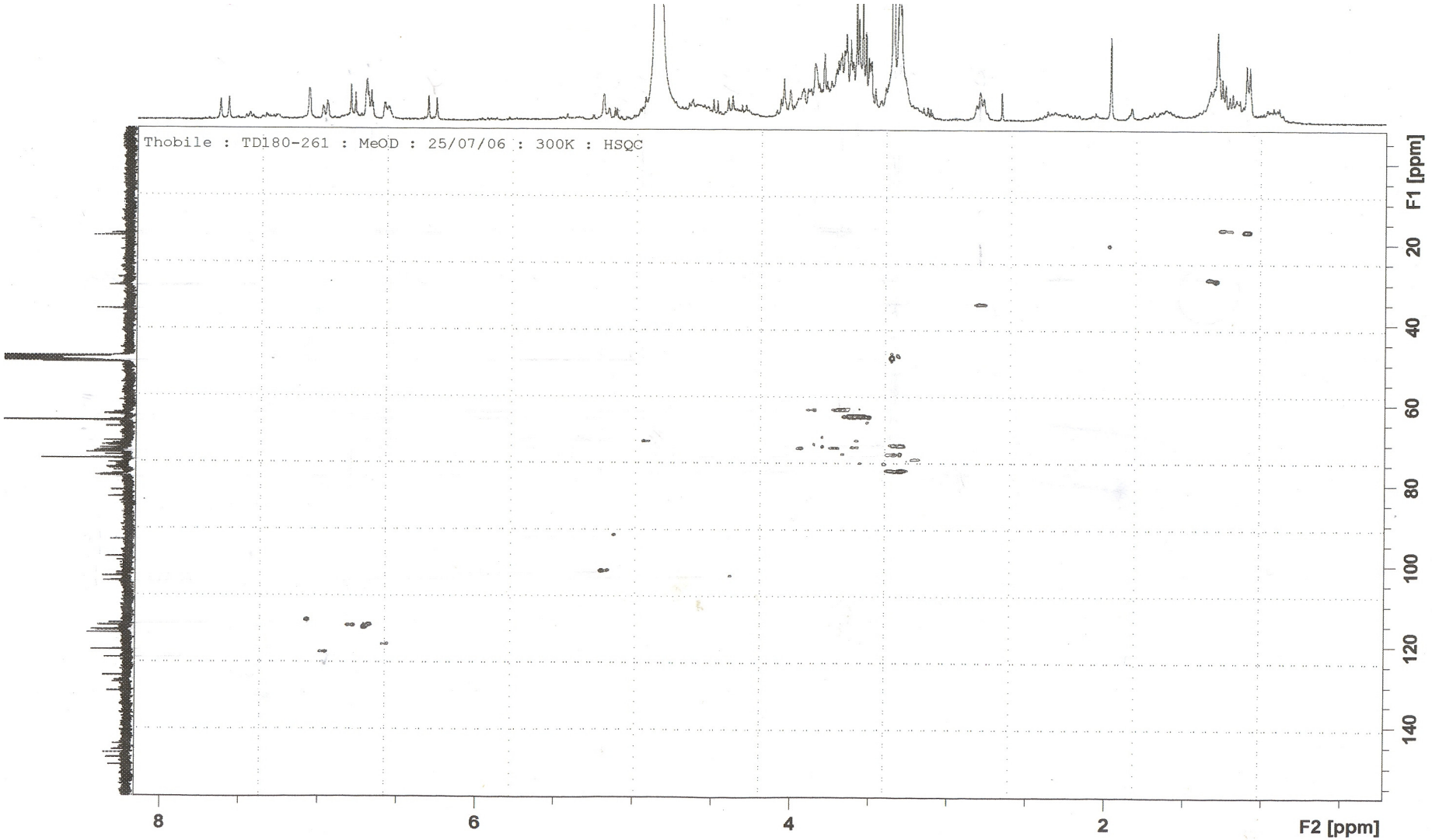



Compound 3 13C NMR
200
150
100
50
0
ppm (t1)

Compound 3: IR



Compound 3: HMBC



Compound 3: HSQC