NITROGEN AND CARBON COSTS OF GROWTH AND ANTIOXIDANT PRODUCTION DURING ACCLIMATION TO ENVIRONMENTAL STRESS IN TWO SPECIES OF *GETHYLLIS*

Christiaan Winston Daniëls

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor Philosophiae: Herbal Science in the Department of the South African Herbal Science & Medicines Institute (SAHSMI), University of the Western Cape.

Supervisors:
Professor Wilfred T. Mabusela
Doctor Alexander J. Valentine
Professor Jeanine L. Marnewick

May 2012

KEYWORDS

Antioxidants

Characterization

Carbon and nitrogen cost

Drought stress

Gethyllis multifolia

Gethyllis villosa

Kukumakranka

Photo-stress

Phytochemical screening

Plant physiology

Succulent Karoo biome

ABSTRACT

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ABSTRACT

Gethyllis multifolia L. Bolus and *G. villosa* Thunb. are winter-growing, summer-blooming, deciduous and bulbous geophytes that grow naturally in the semi-arid succulent Karoo biome of South Africa. Both species grow under full sun conditions and have four distinctive growth phases: a winter (cold and wet) growing phase, leaf senescence phase towards spring, flowering phase during the hot and dry summer months, and fruit and leaf formation phase in autumn. The medicinal uses of this genus (including *G. multifolia* "Kukumakranka" and *G. villosa* "hairy kukumakranka") range from cures for colic, digestive disturbances, teething problems, fatigue, boils, bruises and insect bites, to being used as an aphrodisiac. *Gethyllis multifolia* is threatened in its natural habitat and is listed in the 'Vulnerable' category of the 'Red Data List of Southern African Plants' and the 'IUCN-World Conservation Union List of Plants'. The literature indicate that the habitats of both species are being exposed to drier conditions and is further

threatened by the encroachment of invasive indigenous plant species. It is not known to which extent these factors may pose a threat to the existence of both species.

The first objective of this investigation was to determine the costs of vegetative and reproductive growth during the seasonal life cycle of the plant, using carbon (C) and nitrogen (N) as a physiological currency. The second objective was to elucidate a functional basis to explain the difference in the conservation status of both species in their natural habitat. Both species were subjected to drought and shading as environmental stresses and the plant physiological performance was investigated via photosynthetic gas exchange. The third objective of the study was to evaluate the antioxidant content (total polyphenol, flavonol/flavone and flavanone content) and antioxidant capacity [ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC) and 2,2'-azino-di-3-ethylbenzthiazoline sulphonate (ABTS) radical cation scavenging ability] of natural populations and plant samples that were exposed to photo- and -drought environmental stresses. This study was done to elucidate the antioxidant profile of plant parts of natural populations as well as providing farmers, traditional healers and pharmaceutical companies with cultivation environmental conditions to enhance the antioxidant properties of the species. This investigation also attempted to isolate and characterize, by means of thin-layer chromatography (TLC) and column chromatography (CC), natural compounds from both species to lend support to the purported antioxidant benefit of both species and to further lend support to claims made by traditional healers of the medicinal potential of the

genus. This study, however, did not engage in any *in vivo* studies or human trials to support published literature of the medicinal benefits of the genus.

The photosynthetic adaptation studies indicated that *G. villosa* had a better photosynthetic performance than *G. multifolia* during both drought and low light conditions because of the inability of *G. multifolia* to adapt to a wider range of environmental extremes. The C and N cost of growth and reproduction studies revealed that *G. villosa* had a more efficient resource utilisation strategy for both growth and reproduction. These physiological responses suggest that *G. villosa*, in general, has a more efficient survival strategy and that *G. multifolia* will struggle to adapt to drier environmental conditions, as well as growing in the shade of encroaching invasive plant species. To conclude, this could be a contributing factor as to why *G. multifolia* is threatened in its natural habitat and *G. villosa* not.

The antioxidant content-and -capacity study on natural populations of both species revealed the highest total polyphenol content, FRAP and ORAC values for the flowers and fruits of *G. multifolia* and *G. villosa* compared to other plant parts. These values were found to be in line with and in some cases higher than most commercial fruits and vegetables. The antioxidant activity during drought and photo-stress of the leaves, bulbs and roots was found to be highest in the roots of both species during drought stress. *Gethyllis multifolia*, in general, exhibited higher total polyphenol content than *G. villosa*, with the highest content measured during drought stress in the roots of *G. multifolia*. Phytochemical investigation of

the leaves, bulbs and roots of *G. multifolia* and *G. villosa* revealed the presence of tannins, flavonoids, phenolics, saponins, glycosides as well as essential oils, while alkaloids were absent. The chromatographic profiles of the leaves, bulbs and roots of both species further indicated that the roots of *G. multifolia* contained the highest concentration of natural products, compared to *G. villosa* and other plant parts. Further in-depth studies on the roots of *G. multifolia* led to the isolation and characterization of three known flavonoids, of which one was also isolated as its endogenously acetylated derivative. In contrast to the fact that both species had a high polyphenol content and exhibited high antioxidant activity, the isolated compounds in this study revealed very low antioxidant activity. However, the literature revealed that some of these isolated compounds exhibit antifungal, antibacterial, antiangiogenic and anticarcinogenic properties *in vitro*, which could be ascribed to the medicinal applications of plant parts of certain species belonging to this genus. Furthermore, this study suggests that further chemistry and pharmaceutical research on the genus, *Gethyllis*, in specific the flowers and fruit of these two species, be pursued.

DECLARATION

I declare that "Nitrogen and Carbon Costs of Growth and Antioxidant production during Acclimation to Environmental Stress in two species of *Gethyllis*" is my own work, that it has not been submitted before for any degree or assessment in any other university, and that all the sources I have used or quoted, have been indicated and acknowledged by means of complete references.

C. W. Daniëls May 2012

Signature _____

DEDICATION

I dedicate this doctoral thesis to

my wife,

Naomi Daniëls

and

son,

Lance Daniëls,

without their patience, love, and understanding this achievement could not have been accomplished.

ACKNOWLEDGEMENTS

God Almighty - I acknowledge the Lord for giving me the strength, patience and wisdom to complete this work.

Family - I thank my wife, Naomi, who stood behind me through all the rough times. I love and appreciate you much more than you will ever imagine. My son, Lance, thank you for understanding that the lost time was for a good cause.

Supervisors - I am grateful to my supervisors, Dr. Alex Valentine and Prof. Jeanine Marnewick for their academic guidance, patience and kindness. Thank you to Prof. Wilfred Mabusela for his academic guidance and the interpretation of data for the compound structure elucidation.

Laboratory technicians – I thank Fanie Rautenbach at the Oxidative Stress Research Centre, Cape Peninsula University of Technology and Jelile Babajide at the Chemistry Laboratory, University of the Western Cape, for their technical assistance.

External laboratories - Archeometry Department at the University of Cape Town for the carbon (C) and nitrogen (N) isotope analysis and the nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) laboratories of the Central Analytical Facilities of the University of Stellenbosch, for the phytochemical compound spectroscopy.

Karoo Biodiversity Garden, Worcester - The staff members, Deon Viljoen and Werner Voight, for permission to collect and identification of the plant material.

South African Weather Service - For supplying details on the weather patterns for the Cape Town and Worcester areas.

University Research Fund (URF), Cape Peninsula University of Technology (CPUT) - For payment of my tuition fees, materials and equipment for the research work.

Nursery facilities (Department of Horticultural Sciences, CPUT) - For the use of the greenhouse and shadehouse facilities during environmental stress experiments on the plants.

LIST OF PAPERS PUBLISHED AND SUBMITTED

1. **Daniels, C.W**.; Mabusela, W.M.; Marnewick, J.L.; Valentine, A.J. A comparative drought- and -shade tolerance analysis of two semi-arid desert bulb species of *Gethyllis* (Kukumakranka). Journal: Photosynthetica. **(Accepted for publication - March 2012)**
2. **Daniels, C.W**.; Mabusela, W.M.; Marnewick, J.L.; Valentine, A.J. The carbon costs associated with the growth, reproductive and dormant phases of two semi-arid desert bulb species of *Gethyllis* (Kukumakranka). Journal of Arid Environments. **(Submitted - August 2011)**
3. **Daniels, C.W.**; Rautenbach, F.; Mabusela, W.M.; Valentine, A.J.; Marnewick, J.L. Comparative antioxidant-capacity and -content of leaves, bulbs, roots, flowers and fruit of *Gethyllis multifolia* L. Bolus and *G. villosa* Thunb. species. South African Journal of Botany 2011, 77 (3), 711-717. **(Published - March 2011)**
4. Daniels, C.W.; Rautenbach, F.; Mabusela, W.M.; Valentine, A.J.; Marnewick, J.L. Comparative antioxidant-capacity and -content of *Gethyllis multifolia* L. Bolus and *G. villosa* Thunb. species under drought and photo-stress. Journal of Medicinal Plants Research. **(Submitted - May 2011)**
5. **Daniels, C.W**.; Babajide, J.O; Valentine, A.J.; Marnewick, J.L., Mabusela, W.M. Phytochemical studies of *Gethyllis multifolia* L. Bolus (Kukumakranka) and *G. villosa* Thunb. African Journal of Traditional, Complimentary and Alternative Medicines. **(Submitted - October 2011)**

* It must be noted that the layout and references for the different research papers were formatted according to the requirements of the different journals. The references for the Introduction, Literature review and Final conclusion sections were formatted according to the University of the Western Cape doctoral thesis layout.

CONTENTS

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

PLANT PHYSIOLOGY

C	Carbon
C:N	Carbon and nitrogen isotopic ratio
$\delta^{13}C$	^{13}C Carbon isotope
dap	Days after planting
D_r	Dark respiration rate
DW	Dry weight
E	Transpiration rate
En	Endangered category
G_s	Stomatal conductance
KDNBG	Karoo Desert National Biodiversity Garden
LCP	Light compensation point
N	Nitrogen
$\delta^{15}N$	^{15}N Nitrogen isotope
PH1	Phase 1 - leaf emergence
PH2	Phase 2 - full leaf emergence
PH3	Phase 3 - leaf senescence
PH4	Phase 4 – reproductive stage
P_{max}	Photosynthetic rate
PPFD	Photosynthetic photon flux density
PWUE	Photosynthetic water-use efficiency
RGR	Relative growth rate
SANBI	South African National Biodiversity Institute

SCAR	Specific carbon absorption rate
SCUR	Specific carbon utilisation rate
SLM	Specific leaf mass
SNAR	Specific nitrogen absorption rate
SNUR	Specific nitrogen utilisation rate
V	Vulnerable category

ANTIOXIDANT RESEARCH

AAE	Ascorbic acid equivalent
AAPH	2,2'-azobis (2-amidino-propane) dihydrochloride
ABTS	2,2'-azino-di-3-ethylbenzthiazoline sulphonate
ALA	Alpha lipoic acid
BHA	Butylated hydroxyl anisole
BHT	Butylated hydroxytoluene
CVD	Cardiovascular disease
CoQ10	Coenzyme Q10
DNA	Deoxyribonucleic acid
DNPH	2,4-dinitrophenylhydrazine
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
Gpx	Glutathione peroxidase
HAT	Hydrogen atom transfer

HIV/AIDS	Human immunodeficiency virus/ Acquired immune deficiency syndrome
LDL	Low-density lipoproteins
NE	Naringenin equivalent
nm	Nanometers
ORAC	Oxygen radical absorbance capacity
PE	Phycoerythrin
PPFD	Photosynthetic photon flux density
QE	Quercetin equivalent
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SET	Single electron transfer
SOD	Superoxide dismutase
TAS	Total antioxidant status
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TPTZ	2,4,6-tripyridyl-*s*-triazine
Trolox	6-Hydrox-2,5,7,8-tetramethylchroman-2-carboxylic acid
β-PE	β-phycoerythrin

PHYTOCHEMICAL SCREENING AND CHARACTERIZATION

^{1}H -^{13}C COSY	Heteronuclear Shift - Correlated Spectroscopy
^{1}H -^{1}H COSY	Homonuclear Shift - Correlated Spectroscopy
Alka	Alkaloids

CC	Column chromatography
DCM	Dichloromethane
Esse	Essential oils
Flav	Flavonoids
Glyc	Glycosides
GMM	*Gethyllis multifolia* methanol extract
GT1	*Gethyllis multifolia* compound 1
GT2	*Gethyllis multifolia* compound 2
GT3	*Gethyllis multifolia* compound 3
GVM	*Gethyllis villosa* methanol extract
Hex	Hexane
IR	Infra-red
LC_{50}	Lethal concentration of a chemical that kills 50% of organisms over a specified time period
MP	Melting point
MS	Mass spectroscopy
NMR	Nuclear magnetic resonance
Phen	Phenolics
Sapo	Saponins
Tann	Tannins
TLC	Thin layer chromatography
UV	Ultra-violet
WHO	World Health Organisation

CHAPTER 1: INTRODUCTION

CHAPTER 1
INTRODUCTION

1.1 Background

Gethyllis multifolia L. Bolus and *G. villosa* Thunb. (Family: Amaryllidaceae) are winter-growing, summer-blooming, deciduous, and bulbous geophytes indigenous to South Africa (Du Plessis and Delpierre, 1973). The genus *Gethyllis* consists of 37 currently accepted species and subspecies (Müller-Doblies, 1986). The genus *Gethyllis* is more commonly known as "Kukumakranka" in South Africa and is one of the most extraordinary and poorly researched of all southern African amaryllids (Liltved, 1992). The medicinal uses of this genus range from cures for colic, digestive disturbances, teething problems, fatigue, boils, bruises and insect bites, to being used as an aphrodisiac (Du Plessis and Delpierre, 1973). Apart from its medicinal properties, many members of this genus (including *G. multifolia*) have a highly fragrant, tasty, edible fruit which was used in the past to perfume rooms, cupboards and linen (Lighton, 1992). *Gethyllis* grows under full sun conditions and their natural habitats range from sandy soils to mountainous rocky terrains. The genus have four distinctive growth phases: a winter (cold and wet) growing phase, leaf senescence towards spring, flowering during the hot and dry summer months (when no leaves are present), and fruit and leaf formation in autumn, which is the start of a new growing season (Du Plessis and Duncan, 1989).

Furthermore, *G. multifolia* is threatened in its natural habitat and is classified as 'Vulnerable' according to the Red Data List of Southern African Plants (Hilton-

Taylor, 1996). *G. villosa* grows in the same region but is not threatened at all. The term 'Vulnerable' (V) is defined as taxa believed likely to move into the 'Endangered' category in the near future if the factors causing the decline, continue operating (Hilton-Taylor, 1996). The literature reveals no reports on any plant physiology processes such as photosynthetic activity, respiration, transpiration, stomatal conductance, water-use efficiency, nutrient uptake and nutrient utilisation of these two species to elucidate the difference in their conservation status.

A preliminary study by the author on the antioxidant-capacity and -content of *G. multifolia* and *G. villosa* revealed high antioxidant activity in the leaves and roots of both species (unpublished data). It has also been proposed, through photographic studies of the fruit of *G. multifolia* and *G. villosa*, that the dark red colour in the fruit of *G. multifolia* may be due to the presence of colour pigments i.e. carotenoids or anthocyanins that are known to have antioxidant activity (Daniels, 2007). Anti-inflammatory, anti-bacterial and anti-mutagenic studies were conducted on the roots and bulbs of *G. multifolia* and *G. villosa* (Elgorashi and Van Staden, 2004; Babajide, 2010) and revealed some anti-inflammatory and anti-bacterial activity in both species. A study of head-space volatiles on the fragrance of the fruits of *G. afra* and *G. ciliaris* (Kamatou et al., 2008) was conducted, but this investigation did not include any pharmaceutical studies of the fruit. The fruits and flowers are regarded as the most useful medicinal plant parts, but no literature could be found on any scientific pharmaceutical studies on the fruits and flowers of the genus *Gethyllis*.

In South Africa there is a reluctance amongst traditional healers to use medicinal plants from cultivated sources (Dold and Cocks, 2002) and this mindset results in the plundering of our floral heritage. As mentioned earlier, *G. multifolia* species bear a tasty, fragrant, edible fruit with medicinal potential and is threatened in its natural habitat (Lighton, 1992). This fact emphasizes the need for future cultivation of this species by pharmaceutical companies, traditional healers and farmers. Furthermore, the application of environmental stresses during growth to increase antioxidant capacity, content and possible medicinal potency could prove to traditional healers that cultivated plants can be equally "potent" as plants from natural habitats. This study, however, did not attempt to prove that there is a link between the antioxidant properties of plant parts and the medicinal uses of the genus.

In this investigation, comparative plant nutritional and gas exchange physiology experiments were conducted to investigate whether botanical differences or physiological responses could give an indication as to why there is a difference in the conservation status of *G. multifolia* and *G. villosa*. Secondly, antioxidant studies were conducted to determine the antioxidant-capacity and -content in the various plant parts of natural populations of both species. In addition, both species were subjected to environmental stresses, to ascertain which environmental stress will increase both antioxidant-capacity and -content in the different plant parts. Thirdly, phytochemical screening of both species was performed and natural compounds isolated and characterized in an attempt to

provide a chemistry background to claims made by traditional healers and the Khoi-San people of the medicinal values of the "Kukumakranka", in specific *G. multifolia* and *G. villosa*.

1.2 Problem Statement and Research Motivation

Gethyllis multifolia L. Bolus is threatened in its natural habitat and is classified as 'Vulnerable' according to the Red Data List of Southern African Plants (Hilton-Taylor, 1996). On the other hand, *G. villosa* Thunb. occurs more frequently in the same areas and elsewhere throughout South Africa and is not threatened in any way. No literature could be found on any comparative plant physiology and nutrient uptake and utilisation experiments on *G. multifolia* and *G. villosa* to elucidate the difference in the conservation status of these two species. Environmental impact studies (Daniels, 2007) have been conducted to investigate reasons behind the difference in the conservation status of these two species but a complete botanical/plant physiological study still needs to be conducted to confirm whether plant physiological processes and responses are contributing factors to *G. multifolia*'s 'Vulnerable' status on the Red Data List of Southern African Plants.

A horticultural study by Daniels (2007) revealed that when *G. multifolia* plants were compared to *G. villosa* in their responses to severe drought, *G. villosa* appeared to have better survival strategies. When no moisture was present, *G. multifolia* plants were slower in initiating new leaves at the onset of the new growing season, compared to *G. villosa*. It was also observed, during dry spells in

the natural habitats of both species, that *G. multifolia* plants produced fewer flowers, no flowers at all, flowers with no style or stigma or flowers with fewer stamens than normal, when compared to *G. villosa*. Another interesting phenomenon was that *G. multifolia* plants also aborted flower buds during dry spells, which was not observed in *G. villosa* (Daniels, 2007). In its natural habitat, it was further observed that many *G. multifolia* plants were growing in the shade of *Galenia africana*, an indigenous invasive shrub that grows to a height of 1.5 m. It is, however, not known to what extent the exposure to a lower irradiance poses a threat to the existence of the species.

The above-mentioned statements are based on mere observations and have not been scientifically proven through botanical and plant physiological studies. A complete plant physiological study on the behaviour of *Gethyllis* species during different environmental conditions and growth phases could further our knowledge of our indigenous floral heritage and also facilitate the protection thereof. Current literature revealed limited research on the antioxidant-capacity and -content of the various plant parts (leaves, bulbs, roots, flowers and fruit) of *G. multifolia* and *G. villosa*. Babajide (2009) reported higher polyphenolic content and antioxidant activity in whole plants of *G. multifolia* than in *G. villosa*. Literature searches also revealed no information on the effect of environmental stresses such as drought and different light intensities on the antioxidant production in these two *Gethyllis* species. A preliminary study by the author on the antioxidant content of the leaves, bulbs and roots of *G. multifolia* and *G. villosa* revealed high levels of antioxidants in the leaves and roots of both species

(unpublished data). As mentioned earlier, photographic images revealed a dark red colour in the fruit of *G. multifolia*, which may be due to the presence of colour pigments i.e. carotenoids or anthocyanins that are known to have antioxidant activity (Daniels, 2007). Further antioxidant studies by Babajide (2009) indicate the presence of anthocyanins in water and methanol extracts of whole plants of *G. multifolia*, while none was detected in similar extracts of *G. villosa*.

The genus *Gethyllis* is considered to have medicinal properties and is being used for the following ailments: colic, digestive disturbances, teething troubles, fatigue and an application on boils, bruises and insect bites (Du Plessis and Delpierre, 1973). Further studies by Elgorashi and Van Staden (2004) and Babajide et al. (2010) revealed anti-inflammatory and anti-bacterial activity in both *G. multifolia* and *G. villosa* plant parts. Although previous studies (Elgorashi et al., 2007 and Kamatou et al., 2008) revealed isolation and characterization of natural compounds from *G. afra* and *G. ciliaris* plants parts, no studies have reported on the isolation of natural compounds from G. *multifolia* and *G. villosa* in the quest to create a chemistry background to claims made by traditional healers of the medicinal properties of members of this genus.

In addition to the mentioned medicinal properties of the genus *Gethyllis*, some of the species have a highly fragrant, tasty edible fruit which have been used in the past to perfume linen, rooms and cupboards and is also a sought after snack by children when playing in the veld (Lighton, 1992). *Gethyllis multifolia* has a highly fragrant, tasty and edible fruit and *G. villosa* not, and should environmental

stresses indicate an increase in antioxidant-capacity and -content, the fruit of this species could be of great benefit to the food and beverage industry. Results from this study could benefit farmers, traditional healers and pharmaceutical companies, by providing them with the correct growing conditions to increase phytochemical (i.e. antioxidants) production in the fruit as well as to prevent extinction of the species.

1.3 Objectives of the study

The following are the research objectives outlined:

- Comparative plant physiological experiments of *G. multifolia* and *G. villosa* such as photosynthesis, respiration, transpiration, stomatal conductance, and water-use efficiency to test their performance under drought and shade stress, compare survival strategies and to investigate why there is a difference in the conservation status of both species.
- Comparative carbon and nitrogen acquisition and utilisation experiments of both species during the growth, reproductive and dormant phases to elucidate the difference in the conservation status of the two species.
- To determine the major polyphenolic classes, antioxidant-capacity and -content that are present in the leaves, bulbs, roots, flowers and fruit of natural populations of both species.
- To subject both species to certain environmental stresses, i.e. drought and photo-stresses and ascertain the modulation of the antioxidant content and antioxidant activity as a result thereof.

- To perform phytochemical screening, isolation and characterization on both species to determine the presence of natural compounds in the various plant parts.

1.4 The thesis hypothesis

Botanical/plant physiological experiments could indicate that *G. villosa* has better survival strategies than *G. multifolia* based on the conservation status of both species and horticultural observations during a previous study. Furthermore, certain plant parts of both species may have high antioxidant content and exhibit high antioxidant activity based on a pilot study of the two species, previous literature and the yellow and dark red colours of the fruit of both species. The isolated compounds from both species could possibly create a chemistry background to the medicinal applications of the genus *Gethyllis*.

1.4 Delineation of the study

- The study of the growth cycles of both species was conducted over a period of two years. Weather data for the Worcester and Cape Town areas for the years 2006 and 2007 were supplied by the South African Weather Service (Cape Town office).
- The plant species in this study were limited to *Gethyllis multifolia* and *G. villosa* and both species were collected with the permission of the Karoo Desert National Biodiversity Garden in Worcester (KDNBG), Western Cape in the Worcester area. The exact location of both species is not

disclosed because of the 'Vulnerable' conservation status of *G. multifolia* and warnings by the KDNBG.

- The environmental stresses were limited to 80% shade and drought at 30% field capacity. The environmental stresses and plant physiology experiments were conducted at the nursery of the Department of Horticultural Sciences, Cape Peninsula University of Technology (Cape Town campus), Cape Town, Western Cape.
- Nutrient cost of growth over the different growth and reproductive phases of both species was limited to carbon (C) and nitrogen (N). The isotope ratio analysis for C and N was conducted at the Archeometry Department of the University of Cape Town, Observatory, Western Cape.
- Antioxidant content analysis was limited to polyphenols, flavonols/ flavones and flavanones and the antioxidant capacity to ORAC, FRAP and TEAC assays. The antioxidant profile of both species was determined at the Oxidative Stress Research Centre, Cape Peninsula University of Technology (Bellville campus), Bellville, Western Cape.
- Phytochemical screening and isolation of natural compound methods were limited to thin layer chromatography (TLC) and column chromatography (CC) and were conducted at the Chemistry Department of the University of the Western Cape, Bellville, Western Cape.
- Nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) measurements were limited to COSY, HSQC, HMBC and NOESY and the analysis conducted at The Central Analytical Facility of the University of Stellenbosch, Stellenbosch, Western Cape.

1.6 Brief overview of the chapters

This thesis consists of eight chapters.

- Chapter one presents a general introduction, motivation, thesis statement, research aims and objectives and delineation of the study.
- Chapter two presents and overview of the literature of all the research focus areas pertaining to this study in more detail.
- Chapter three introduces the first of the five research papers. This chapter deals with a series of comparative plant physiological experiments to determine why *G. multifolia* is threatened in its natural habitat and *G. villosa* not, whilst both species are found growing in the same areas. By means of an infra-red gas analyser and the exposure of the two species to artificial drought and shade stress conditions, comparative photosynthetic activity, transpiration, respiration, stomatal conductance and water-use efficiency, were measured during the growth phase.
- Chapter four is a follow-up study on Chapter three and revolves around the carbon and nitrogen content, acquisition and utilisation of both species. The growth and reproduction of *Gethyllis* species takes place over four phases: 1) leaf initiation and fruiting phase, 2) leaf senescence phase, 3) flowering phase, and 4) dormant phase. Firstly the nitrogen and carbon content were measured in the leaves, bulbs and roots during all four phases. Secondly the carbon and nitrogen acquisition and utilisation rates were measured only during the growth and reproductive phases.

- Chapter five firstly reports on the major classes of polyphenolic constituents present in both species. Secondly, the plants of both species from natural populations were excised into leaves, bulbs, roots, flowers and fruit, dried and milled into a fine powder. All the plant parts were then analysed for total polyphenol, flavonol/flavone and flavanone content. Antioxidant activity was measured using the following assays: **1)** Ferric reducing antioxidant power (FRAP), **2)** 2,2'-(1,2-hydrazinediylidene)bis[3-ethyl, 2,3-dihydro]-6-benzothiazolesulfonic acid (ABTS/TEAC) and **3)** Oxygen radical absorbance capacity (ORAC).
- Chapter six is a follow-up study on chapter five whereby the antioxidant-capacity and -content were measured during drought and photo-stress of both species. This experiment was designed to ascertain which plant part of which species produced higher levels of antioxidants and under which environmental stress conditions. The preparation and experimental procedures and chemicals used were similar for both experiments.
- Chapter seven of the thesis reports on phytochemical screening, isolation and characterization of natural compounds found in both species. Plants were authenticated, excised into leaves, bulbs and roots, dried and ground to a fine powder for this set of experiments. Phytochemical screening and isolation of pure natural compounds were performed using different solvents and various separation techniques such as preparative thin layer chromatography (TLC) and column chromatography (CC). Characterization of natural compounds, identification and structure

elucidation were performed using nuclear magnetic resonance (NMR) and mass spectroscopy (MS) techniques.

- Chapter eight reflects on all previous experimental chapters and is a general discussion and conclusion of all the experimental work done in the study and includes recommendations to growers, traditional healers, pharmaceutical companies and future researchers for similar future research projects.

REFERENCES

Babajide, J.O. (2009). Chemical and biological investigation into some selected African indigenous medicinal plants. Doctoral thesis, University of the Western Cape, Bellville, South Africa: pp. 218-225.

Babajide, J.O., Mabusela, W.T., Green, I.R., Ameer, F., Weitz, F., Iwuoha, E.I. (2010). Phytochemical screening and biological activity studies of five South African indigenous medicinal plants. *Journal of Medicinal Plants Research*, 4: 1924-1932.

Daniels, C.W. (2007). *A comparative study of the propagation and cultivation of Gethyllis multifolia and G. villosa.* Masters thesis, Cape Peninsula University of Technology, Bellville, South Africa: pp. 42-57.

Dold, A.P., Cocks, M.L. (2002). The trade in medicinal plants in the eastern Cape Province, South Africa. *South African Journal of Science*, 98: 589-596.

Du Plessis, N., Delpierre, G. (1973). Blommeprag uit eie bodem: Koekemakranka. *Landbouweekblad*, 21 Aug: 37-39.

Du Plessis, N., Duncan, G. (1989). *Bulbous plants of southern Africa.* Tafelberg Publishers, Cape Town, South Africa: pp. 104-109.

Elgorashi, E.E., Coombes, P.H., Mulholland, D.A., Van Staden, J. (2007). Isoeugenitol, a cyclo-oxygenase-1 inhibitor from *Gethyllis cilliaris*. *South African Journal of Botany*, 73: 156-158.

Elgorashi, E.E., Van Staden, J. (2004). Pharmacological screening of six amaryllidaceae species. *Journal of Ethnopharmacology*, 90: 27-32.

Hilton-Taylor, C. (1996). *Red data list of southern African plants.* National Biodiversity Institute, Cape Town, South Africa: pp. 57.

Kamatou, G.P.P., Viljoen, A.M., Őzek, T., Başer, K.H.C., (2008). Head-space volatiles of *Gethyllis afra* and *G. ciliaris* fruits ("kukumakranka"). *South African Journal of Botany*, 74: 768-770.

Lighton, C. (1992). The kukumakranka. *Veld & Flora*, 78: 100-103.

Liltved, W.R. (1992). The kukumakranka, past and present. *Veld & Flora*, 78: 104-106.

Müller-Doblies, D. (1986). Enumeration. *Willdenowia,* 15: 465-471.

CHAPTER 2: LITERATURE REVIEW

CHAPTER 2

LITERATURE REVIEW

This chapter is an elaborated follow up on chapter one. This review covers related research that has been done on the genus *Gethyllis*, specifically the two species *Gethyllis multifolia* and *G. villosa* and why there is a need for further research. The scope of this review can be summarised as follows:

- A general introduction to the genus *Gethyllis.*
- Reasons behind the threatened status of this genus.
- Morphological descriptions of *G. multifolia* and *G. villosa*.
- Botanical and plant physiological studies on *G. multifolia* and *G. villosa*.
- Polyphenolic antioxidant-capacity and -content of related species.
- Medicinal value and application of this genus.
- Scientific pharmaceutical studies on this genus and other Amaryllidaceae species.

2.1 General introduction to the genus *Gethyllis*

2.1.1 History

Horticulturists and botanists became aware of the *Gethyllis* geophyte when it was first introduced to Europe in 1780. For two centuries this plant was known in Europe as the ‘Cape Crocus’ but this name today still remains unused in our Cape Floral Kingdom (Lighton, 1992). Because of the long-necked bulb structure, the Swedish taxonomist, Linnaeus formulated the name *Gethyllis* from

the Greek word *gethullis*, which means a leek or small onion. It was also mentioned by Liltved (1992) that the genus *Gethyllis*, more commonly known as 'Kukumakranka' (English); 'Koekemakranka' (Khoi, Afrikaans), is one of the most extraordinary and poorly researched of all southern African amaryllids. The meaning of the word 'Kukumakranka' is described by farmers as "goed vir my krank maag", meaning cure for an upset stomach (Van der Walt, 2003). Vosa (1986) mentioned that the edible, pulpy, aromatic berry is also called 'Bramakranka' by the Hottentots in Namaqualand.

2.1.2 Taxonomy

The genus *Gethyllis* (family: Amaryllidaceae) consists of 37 currently accepted species and subspecies (Muller-Doblies, 1986). According to Snijman (2004), the genus *Gethyllis* consist of 32 recognized species, 30 of which are found in the summer-arid areas of southern Africa. Manning et al. (2002) reported that the most recent taxonomic literature of *Gethyllis* is an outline by Muller-Doblies (1986), and indicated that keys and complete descriptions of the species are still needed. Further taxonomic studies (Horstmann, 1999) divided *Gethyllis* into several groups, depending on the leaf shape, hairiness and the absence or presence of a cataphyll. The first group consisted of those species with cataphylls visible above the ground (e.g. *G. verticillata* and *G. ciliaris*); the second group consisted of species with prostate leaves arranged in a rosette form (e.g. *G. barkerae* and *G. lata*); the third group with no visible cataphyll (e.g. *G. villosa* and *G. multifolia*) while the final group were those with non-hairy leaves (e.g. *G. afra* and *G. campanulata*).

Interpretation of the species concept of *G. multifolia* follows its original description and classification by Bolus (1929). This interpretation of the species was last used in its 'Red Data Assessment' (Hilton-Taylor, 1996). In the latest 'Red List of Southern African Plants' (SANBI, 2009), *G. multifolia* has provisionally been subsumed under *G. campanulata*, but *G. multifolia* has not formerly been placed into synonomy with *G. campanulata*.

2.1.3 Morphological description of *G. multifolia* and *G. villosa*

Gethyllis multifolia is a bulbous geophyte up to 120 mm in height, with slightly twisted, lightly hairy leaves that are dry at flowering. This species has up to 30 leaves, which can either be straight or spiral. The leaves are uniformly green and may have a reddish colouration towards the base. Fine hairs cover both leaf surfaces. Straight leaves are up to 200 mm in length and about 1-1.5 mm in diameter (Horstmann, 1999). A further study by Daniels (2007) revealed that the leaves vary in length from 85-240 mm and are completely spiral in young plants compared to straight with a slight twirl apically in older plants (Figure 2.2). In mature plants, leaves are completely straight. Leaves tend to spiral up more intensely under extreme dry conditions. Furthermore, leaf spirals are not flat on the ground but have an upright tendency (Figure 2.2). Younger plants have grey coloured foliage, older plants grey-green foliage and mature plants green foliage. The bulb in general is very small and varies from 30-40 mm in diameter with fleshy roots, which varies between 2-4 mm in diameter (Daniels, 2007).

The flowers are large and coloured white to cream with 12 anthers (six pairs) and the flowering period is from November to January (Goldblatt and Manning, 2000). The stamens of open flowers are arranged in six pairs of two, flat against the petals. The stamens are 10-12 mm in length. Flowers are mostly white or light pink in colour and the tips of the tepals are curved downwards (Daniels, 2007) (Figure 2.1). Flowers measure 60-80 mm in diameter with the flower petiole 3 mm in diameter and 20-30 mm in length. The fragrance of flowers varies from not scented to lightly scented. All *Gethyllis* species have an inferior subterranean ovary which develops over a period of three months until it is pushed into view (Manning et al., 2002) (Figure 2.3).

Berries are produced from mid-March to mid-April at the onset of the new growing season (Van Reenen, 1975). Seeds are ripe when the soft berry pushes above ground and topples over (Du Plessis and Duncan, 1989) (Figure 2.3). The berry is highly aromatic and has a passion fruit (more towards pineapple) fragrance. The colour of the berry varies from maroon on the wide end to creamy white towards the narrow end (bottom). The berry is club-shaped and varies in size from 45-80 mm in length and 8-13 mm in diameter on the wide end. Seeds are fleshy and suspended in an even more aromatic and sticky pulp. Seeds are round to slightly oval in shape with the diameter varying from 2-3 mm and vary from 13 to 85 per berry (Daniels, 2007).

Figure 2.1: Flower of *G. multifolia.*

Figure 2.2: Spirally twisted leaves of *G. multifolia.*

Figure 2.3: The dark red berry of *G. multifolia* protruding the soil during autumn.

Gethyllis villosa plants are 30-150 mm in height, with 3-10 green leaves that are 40-120 mm long, between 1.5 and 5 mm wide, flat and loosely spiralled towards the apex, and covered with soft, white, T-shaped hairs (Horstmann, 1999). According to Daniels (2007) the growth habit of the plant varies from spiralled leaves flat on the ground to spiralled leaves above ground which are dry at flowering (Figure 2.5). The diameter of bulbs varies from 18-24 mm.

The flowers are white or pink with tepals 20-40 mm long and consist of six anthers with the style longer than the stamens. The style is curved sideways and has a broad 3-lobed stigma (Manning et al., 2002; Snijman, 2004). Daniels (2007) stated that the flowers of *G. villosa* are star-shaped, mildly scented, white or pink in colour and measure 40-90 mm in diameter (Figure 2.4). The length of the petiole varies from 35-80 mm and the diameter from 2.5-3.5 mm. The flower consists of six distinct tepals, which vary from 26-40 mm in length. The style is more or less 15 mm in length and is flexed to the side of the flower, away

from the stamens, probably to avoid self-pollination. The seed numbers vary from 34 to 115 per berry. *G. villosa*'s berries have no fragrance and are white to cream to yellow in colour (Figure 2.6). The size and shape of the berry and seeds are similar to those of *G. multifolia* (Daniels, 2007).

Figure 2.4: Flowers of *G. villosa* with the stigma and style flexed to one side.

Figure 2.5: Spirally twisted leaves of *G. villosa.*

Figure 2.6: The yellow berry of *G. villosa* protruding the soil during autumn.

2.1.4 Life cycle

According to Van Reenen (1975), *Gethyllis* species are winter growers. Both leaves and fruit reach the soil surface more or less at the same time and this happens in autumn in South Africa. Elvin (2000) reported that when the rainy season (winter) comes to an end and the temperatures rise towards spring, the foliage dies back to the cataphyll and the plants begin to go dormant. Du Plessis and Duncan (1989) documented that the plants reach their flowering phase when the climate is hot and dry (summer) and that the leaves die down before the flowers appear. Flower formation starts in August when flower development takes place at the base of the bulb. The flowering period ranges from October to March (Van Reenen, 1975). Once pollinated, the third and final phase, fruiting, begins (Elvin, 2000). Du Plessis and Duncan (1989) mentioned that after cross-

pollination (probably all species are self-sterile), the ovary begins to swell. The club-shaped berry is gradually formed and pushed above ground during autumn. According to Elvin (2000), it can take more than two months to produce the club shaped, aromatic fruit that ripens just in time to release the seeds for the rainy season. As the fruits ripen, they fall over and, if not eaten or removed, the seeds will germinate to form a dense group of seedlings. Van Reenen (1975) reported that seeds of *Gethyllis* germinate without a preceding resting period and in exceptional cases germination takes place within the berry.

2.1.5 Predators

According to Liltved (1992), tortoises are attracted by the ripening berry and play an important role in the dispersal of the seeds. Snijman (2004) also reported that tortoises, birds and rodents eat the berry's fleshy pulp and act as agents for seed dispersal, but that this remains unconfirmed. Van der Walt (2003) mentioned that humans, tortoises, porcupines, birds, rodents and sheep are common predators of the berry (Figures 2.7 and 2.8).

Figure 2.7: *G. multifolia* leaves grazed by goats in its natural habitat.

Figure 2.8: A rodent hole dug around a *G. villosa* plant in its natural habitat.

2.1.6 Survival strategies

The hair on the leaves of some species serves to trap moisture, prevents excessive transpiration and helps protect the bulb organ from the heat of the sun

(Liltved, 1992; Elvin, 2000). The coiled and curled leaves of geophytes serve the purpose of maximizing photosynthesis by providing leaf surfaces perpendicular to the sun at every hour of the day. Geophytes survive long periods of environmental stress such as summer drought or winter cold by dying back to underground storage organs only to re-sprout the following growing season as in the case with *Gethyllis* species (Figures 2.9 and 2.10). The same underground storage organs also give geophytes a strong tolerance to other stresses such as fire and grazing (Esler and Rundel, 1998). According to Du Plessis and Duncan (1989), the fleshy roots of some gethyllids are extremely long and are attached to an enlarged, fleshy basal plate (Figure 2.11). Bulbs that are eaten by moles or rats regenerate new plantlets from these fleshy eaten basal plates.

Van Reenen (1975) suggested that the reasons why *Gethyllis* plants are found growing in clumps are because they propagate themselves vegetatively through division (Figure 2.11). Several authors (Du Plessis and Duncan, 1989; Manning et al., 2002) reported that young plants frequently produce flowers without a style and also with a reduced number of stamens. It was suggested that this is probably a protective mechanism, to prevent the immature plants from being fertilised and use up all its resources to produce berries. The leafing and fruiting stages occur at the same time and all the nutrients and moisture required by the large developing berry and leaves, have to be drawn from the stored resources in the bulb. It was further suggested by Du Plessis and Duncan (1989) that the sheathing neck left over from the dried leaves, helps to protect the delicate flower from the hot sand (Figure 2.12). It was also mentioned that, because flowers only last for a few

days, it is essential that as many plants as possible flower at the same time to ensure cross-pollination.

Figure 2.9: Dried leaves of *G. multifolia* plants after the growth phase.

Figure 2.10: Initiation of new leaves of *G. multifolia* plants after the dormant phase.

Figure 2.11: Fleshy roots of *G. multifolia* plants attached to the basal plates.

Figure 2.12: The sheathing neck left over from the dried leaves that helps to protect the delicate flower of *G. namaquensis* from the hot sand (Photo taken by Dennis Tsang – Pacific bulb society, 2004).

2.1.7 Distribution and habitat

According to Snijman (2004), the largest number of species occurs in the Succulent Karoo Biome, followed by the Fynbos Biome. Habitats range from coastal forelands to South Africa's high-lying, inland plateau. In most instances the plants prefer open sites, free of competition from shrubs and grasses. The majority of species prefer semi-arid habitats but a few Cape species (*G. afra* and *G. kaapensis)* are found in seasonally moist sites amongst lowland fynbos vegetation. Only *G. transkarooica* and *G. longistyla* are found in the summer-rainfall region's Nama-Karoo Biome.

Gethyllis multifolia occurs naturally on stony clay flats in the Bokkeveld Escarpment as well as the Worcester and Montagu areas (Goldblatt and Manning,

2000). Horstmann (1999) reported the occurrence of this species in the Worcester and De Doorns districts. *Gethyllis villosa* occurs naturally in the Worcester area, Cape Peninsula, western Karoo to Mossel Bay, Namaqualand and the Bokkeveld Mountains (Goldblatt and Manning, 2000; Pacific Bulb Society, 2004). This species is also found over a wide area from Kamieskroon in the north to Bredasdorp in the south (Horstmann, 1999).

2.1.8 Propagation and cultivation

Even though it is mentioned that certain *Gethyllis* species can regenerate new bulblets from the basal plate remains after mole or rat predation and that it is possible for *G. ciliaris* to be propagated by basal bulb cuttings, the genus *Gethyllis* is difficult to propagate asexually (Du Plessis and Duncan, 1989; Elvin, 2000). Neither *G. multifolia* nor *G. villosa* have ever been propagated by means of *in vitro* propagation and only a single report on the *in vitro* propagation of *G. linearis* L Bol. could be found (Drewes and Van Staden, 1994). Even though previous studies indicated no *in vitro* culture studies on *G. multifolia* and *G. villosa*, Daniels (2007) reported slow responses but successful *in vitro* culture results for both species. Literature studies revealed that *Gethyllis* species, however, are easily propagated from seed (Du Plessis and Delpierre 1973; Du Plessis and Duncan, 1989). Daniels (2007) reported that asexual propagation by means of bulb cuttings and twin scaling was slow, but proved to be successful with *G. multifolia* and less effective with *G. villosa*. Scooping and scoring asexual propagation techniques were also reported successful for *G. multifolia* but is not recommended as a propagation method for increasing *G. villosa*. Further

asexual propagation studies by Daniels (2007) revealed that the fastest method for propagating both species would be by means of bulb division.

It was reported that *Gethyllis* plants require soil with excellent drainage and can be difficult to grow for those who like to water plants too frequently. It was also mentioned that they are poor vegetative producers but seed readily when cross-pollinated, and that a fairly large population is needed to ensure large seed quantities (Du Plessis and Duncan, 1989; Elvin, 2000). According to Du Plessis and Duncan (1989), the parent plants must not be allowed to dry out unnecessarily, and that they benefit from additional feeding during fruit formation. They also mentioned that full sun is required, and that the soil must be deep enough to accommodate the long cataphyll, bulb and fleshy roots. After the leaves have died down all watering must be stopped. Overhead shade is recommended in hot climates during the dormant stage. Watering can be carefully resumed after the leaves and/or berry appear at the onset of the new growing season. Apart from neck and root rot, is *Gethyllis* not susceptible to other serious pests and diseases (Buckly, 1999; Du Plessis and Duncan, 1989).

Buckly (1999) suggested a soil medium that consists of 6 parts sand, 1 part fine decomposed pine needles and 1 part fine peat, for growing *Gethyllis* species. It was also mentioned that all the *Gethyllis* species flowered and grew in this medium and none have ever rotted. A layer of small pebbles can be placed at the base of the pots over the drainage holes. Bone meal can be added to the soil medium and it is not necessary to sterilize the soil medium. Plants must be kept

out of the rain, especially during the dormant phase and a good watering must be given once every two weeks and in cooler climates every three weeks. The plants should not be left covered up during high humidity conditions as this will result in rotting and death of the plants. *Gethyllis* species thrive in areas with a good airflow and low relative humidity. A study by Daniels (2007) demonstrated that *G. multifolia* and *G. villosa* plants could be grown successfully by means of hydro culture, and that the recommended medium and system is leca clay pellets in a sub-irrigation system.

Du Plessis and Delpierre (1973) mentioned that seedlings must be kept moist (the winter rain is normally adequate) and overhead shade is advised. Young seedlings must also be protected against the harsh summer sun even though they are dormant. Young plants can be transferred to their permanent stations towards the beginning of the third growth cycle. It takes about six years or even longer before bulbs reach maturity and are able to flower and produce seeds. According to Snijman (2004) the cultivation of *Gethyllis* is best left to a bulb specialist and their use in gardens should be avoided.

2.1.9 *Gethyllis* as a threatened genus

The conservation of *Gethyllis* is crucial, in that this strange indigenous geophyte does not appear to have a bright future. The use of land for agricultural development has had a negative impact on the survival of this genus. Farmers are appealed to strictly protect and allow left over plants to fruit and seeds to germinate (Du Plessis and Delpierre, 1973). The use of land for agricultural

development could be one of the reasons why *Gethyllis multifolia* is classified as 'Vulnerable' in its natural habitat today (Hilton-Taylor, 1996; IUCN-World Conservation Union, 1998). This fact is further supported by the statement of Malan (2000) "I live in the southern Cape of South Africa, grow a lot of bulbous plants from seed and also collect a lot of things around here from building sites - where they tend to just bulldoze the lot before building!" According to Du Plessis & Delpierre (1973) and Van Wyk et al. (1997) the fragrance and taste of the berry, tempted children to gather the berries as part of a game. The child who found the most berries was regarded as the champion of the year and that this innocent game did not benefit the existence of the species.

Liltved (1992) reported that alien vegetation spread and agricultural and urban expansion are contributing factors to the destruction of natural habitats of genera such as *Gethyllis*. This was further supported through a study by Daniels (2007) that grazing domestic livestock, urban expansion (including agricultural extension) (Figures 2.13, 2.14, 2.15 and 2.16), invasive plants and in some cases, the lack of interest shown in our indigenous plant species, are some of the main factors influencing the decline in numbers of the *Gethyllis* genus. According to a report by Townsend and Viljoen (unpublished data - 1997), *G. multifolia* plants were collected by staff members of the Karoo Desert National Botanical Garden in 1997 from the Osplaas farm in De Doorns, because the land was required for agricultural development. Similarly, *G. villosa* plants were collected by the same staff in 1994 from the Hartebeesrivier farm (Fairway Heights) in Worcester,

because this land was required for urban expansion (Townsend and Viljoen - unpublished data, 1997).

Figure 2.13: Undisturbed natural habitat of *G. multifolia* and *G. villosa* (photo taken in 2005).

Figure 2.14: The same natural habitat of *G. multifolia* and *G. villosa* being destroyed for the laying of sewerage pipes.
(photo taken in 2006).

Figure 2.15: A different section of the natural habitat of *G. multifolia* and *G. villosa* (photo taken in 2005).

Figure 2.16: The same section under construction for the laying of more sewerage pipes (photo taken in 2006).

2.1.10 Conservation status of *G. multifolia* and *G. villosa*

Gethyllis multifolia is listed in the 'Vulnerable' category of the Red Data List of Southern African Plants (Hilton-Taylor, 1996). *G. villosa* grows in the same region and is not threatened at all. The term 'Vulnerable' (V) is defined as taxa believed likely to move into the 'Endangered' (En) category in the near future if the factors causing the decline, continue to exist. Included here are taxa of which most or all of the populations are decreasing because of over-exploitation, extensive destruction of habitat or other environmental disturbances; taxa with populations that have been seriously depleted and whose ultimate security has not yet been assured; and taxa with populations that are still abundant but are under threat from serious adverse factors throughout their range (Hilton-Taylor, 1996).

2.1.11 Justification for further research

The following statement was made by Saunders (2004): "many of us have quite extensive collections of *Gethyllis*, and none of us know what they are, due to the lack of literature on these plants". According to the literature survey conducted, it was observed that limited literature is available on the genus *Gethyllis*; therefore a serious need for further research on all aspects of this genus still exists. Liltved (1992) stated that much variability exists between members of the same species in different localities and that it is often not possible to truly verify the identity of species. It was also mentioned that it is feasible to assume that unidentified species do exist and that the identification thereof rests in the isolation and examination of genetic material of *Gethyllis*. Liltved (1992) also

mentioned that problems are associated with researching members of the genus *Gethyllis*, for the reason that the flowering period is extremely short-lived and that the plant remains dormant for almost half the year.

2.2 Plant physiology research

2.2.1 Background to the succulent Karoo biome

The succulent Karoo biome falls under one of the five Mediterranean-climate ecosystems in the world, the Cape Mediterranean zone of South Africa, which has the highest diversity of geophytes (Esler et al., 1999; Proches and Cowling, 2004) with 2100 species, of which 84% are endemic to the region. As mentioned previously, *G. multifolia* and *G. villosa* grows naturally in the succulent Karoo biome of South Africa (Snijman, 2004). This biome occurs mostly west of the western escarpment through the western belt of the Western Cape and inland towards the Little Karoo (Figure 2.17). This biome is characterized by an abundance of spring flowers, which for a few weeks each year, draw large numbers of tourists from all over the world. Succulent plant species with thick, fleshy leaves are commonly found in this biome and this diversity of specially adapted plant species is unparalleled anywhere else in the world. This, together with many geophytes (plants that survive by means of bulbs, tubers, tuberous roots, corms, etc. in times of unfavourable climatic conditions) and annual plants, makes the succulent Karoo unique and of international importance in terms of conservation (Lombard et al., 1999).

The succulent Karoo is primarily characterized by low to high winter rainfall and extreme summer aridity (Tabel 2.1). The rainfall varies between 20 and 290 mm per year and during summer the temperatures can be in excess of 40 ºC. These high summer temperatures and dry conditions can generally result in high transpiration rates, low photosynthetic activity, drought stress and eventually death of plants if survival strategies are not applied (Cowling et al., 1986).

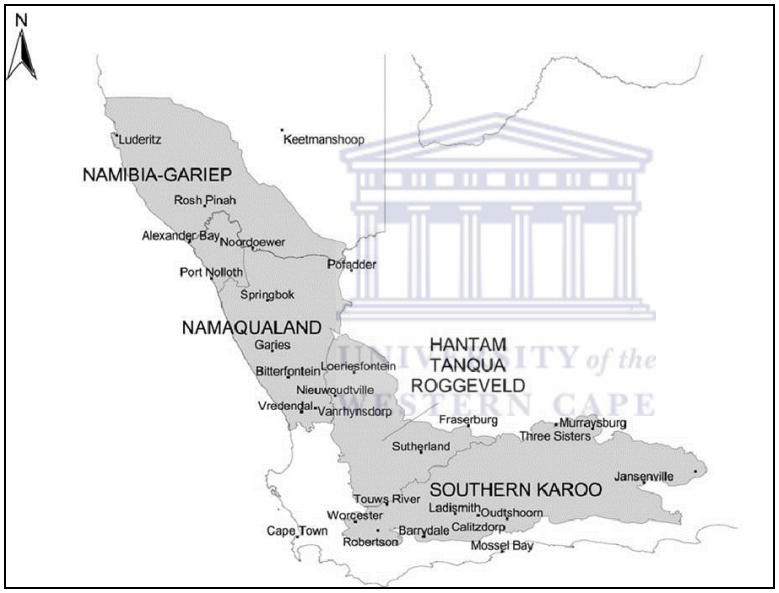


Figure 2.17: The succulent Karoo and adjacent biomes in the Western Cape of southern Africa, indicating the natural habitat of *G. multifolia* and *G. villosa*. (Environment, South Africa, 2010).

Table 2.1: The average rainfall (mm) and average daily minimum/maximum temperatures (ºC) for the Worcester area (Western Cape, South Africa) for the years 2006 and 2007. Each value was obtained by calculating the mean of the two years ± SE. The data were provided by the South African Weather Bureau and were recorded at the Worcester-AWS weather office.

MONTH	MEAN MIN.TEMP.	MEAN MAX. TEMP.	MEAN RAINFALL
January	17.5(±0.39)	33.1(±0.21)	0.4(±0.4)
February	17.3(±0.12)	32.1(±0.45)	4.4(±8.9)
March	13.6(±0.15)	29.8(±0.51)	3.2(±6.3)
April	12.3(±0.3)	25.8(±0.4)	19.9(±21.8)
May	8.8(±0.5)	21.7(±0.3)	41.9(±48.2)
June	6.2(±0.03)	19.3(±0.9)	25.2(±43.4)
July	5.7(±0.7)	18.2(±0.25)	60.2(±61.6)
August	6.7(±0.21)	18.1(±0.08)	39(±51.7)
September	8.5(±0.35)	22.3(±0.25)	4.8(±10.2)
October	10.9(±0.3)	25.6(±0.15)	22.9(±17.3)
November	13.2(±0.36)	27.2(±0.8)	61.7(±21.8)
December	15.8(±0.12)	29.7(±0.8)	18.4(±7.1)

2.2.2 Physiological responses of plants to the succulent Karoo environment

The flora of this biome is mainly composed of winter annuals, drought deciduous perennials with perennial below ground organs, and evergreen succulent dwarf shrubs. The survival strategy of geophytes and annuals is similar as both groups have to complete their life cycle within a short period of time, which is dependant on the availability of soil moisture (Von Willert et al., 1992). Geophytes also produce seeds or new daughter bulbs for propagation, but in the case of unfavourable conditions they do not produce seeds and rely on their subsoil storage organ for survival. Consequently, the survival strategy in this case is focusing on keeping the storage organ alive rather than producing flowers and seeds (Rossa and Von Willert, 1999).

Geophytes also seem to exhibit a high diversity of life cycles and the most obvious is the time of flowering and leaf production. Geophytes can flower prior to their vegetative stage (hysteranthous geophytes) supporting flowers from the storage organ, or they can flower during their vegetative stage (synanthous geophytes) and hence support flowers by the annual leaf production (Dafni et al., 1981). Some other adaptation strategies of bulbous geophytes from the succulent Karoo biome include hair on the leaves (including *Gethyllis*) of some species to trap moisture and prevents excessive transpiration (Liltved, 1992; Elvin, 2000). Geophytes also differ significantly in their growth-form where leaves may be pressed flat on the ground (geophyllic leaves), with various physiological benefits such as a reduction in water loss around the root zone and creating a CO_2 rich environment below the leaves (Eller and Gobbelaar, 1982; Esler et al., 1999). Geophytes also survive long periods of environmental stress such as summer drought by dying back to underground storage organs, only to re-sprout and growing vigorously the following growing season, as in the case with *Gethyllis* species (Esler and Rundel, 1998; Dafni et al., 1981).

2.2.3 General physiological responses of bulbous and other plants to drought

It has been reported that among the various abiotic stresses, drought is the major factor that limits crop productivity worldwide and activates various physiological and morphological changes in plants (Valliyodan and Nguyen, 2006). Liu et al. (2003) mentioned that drought stress increased the rate of pod abortion during the early stages of pod development in soybeans. According to

Pelleschi et al. (1997) and Kim et al. (2000), drought stress generally decreases the photosynthetic rate and disrupts carbohydrate metabolism in leaves and therefore could increase the rate of reproductive abortion in plants. In drought-stressed maize (*Zea mays* L.), loss of kernel set was observed due to a reduction in the photosynthetic rate and the photosynthate influx into kernels (Schussler and Westgate, 1991, 1995).

It has been suggested that the starch and sucrose levels in plant leaves are depleted under drought conditions, consequently affecting carbohydrate metabolism in plant reproductive organs (Chaves et al., 2002). The success of growth and reproduction in plants under stress conditions may be determined by their ability to control carbohydrate utilisation for metabolic energy and the performance of most food crops is evaluated by their ability to allocate enough nutrients to their seeds at the end of the season (Nielsen et al., 2001). Generally under drought stress conditions, restriction of lateral root development takes place and more resources are invested in the development of primary roots to search for moisture deeper down in the topsoil (Xiong et al., 2006). In addition to the restriction of lateral root development, the root hairs of certain plants become shortened and more tuberized or bulbous during drought (Schnall and Quatrano, 1992; Vartanian et al., 1994).

2.2.4 General physiological responses of bulbous and other plants to shade

Shade plants have a high specific leaf area (lamina area/leaf dry mass) for efficient irradiance capture, at the expense of a limited root system, but this

morphological adaptation results in a greater sensitivity to drought (Smith and Huston, 1989). Plants can acclimatize to their light environment by morphologically adapting any of its plant parts. Firstly, they can change the fraction of biomass invested in its leaves, stems and roots. Secondly, they are able to modulate the leaf area per unit biomass invested in leaves, by altering their anatomy. Thirdly, they can change the relative investment of nitrogen between photosynthetic components (Brouwer 1962; Poorter and Nagel, 2000). According to Rousset and Lepart (2000), moderate shade (down to 20-40% daylight) often improves plant performance during drought. Holmgren (2000) reported that shade reduced leaf and air temperatures, vapour-pressure deficit, and the oxidative stresses that can aggravate the impact of drought at higher irradiance levels.

According to the literature, flower morphology, flowering patterns and fruit formation under low light intensity conditions have not been observed or recorded for these two species. It is not known if and to what extent the application of shade would have a negative effect on the physiological processes and survival strategies of both species. *Gethyllis multifolia* and *G. villosa* plants were subjected to drought and shade stress conditions in this investigation to comparatively monitor their plant physiological responses to ascertain which of the two species have better survival strategies. This data could elucidate why *G. multifolia* is treatened in its natural habitat and *G. villosa* not, whilst both species are growing in the same habitats.

2.2.5 The relationship between carbon and nitrogen utilisation in plants

It is common knowledge that carbon, hydrogen and oxygen are utilised by plants in the production of their food through the process of photosynthesis. They obtain these elements from CO_2 in the atmosphere and water from the rhizosphere. Plants that are rapidly photosynthesizing and growing in a greenhouse in close proximity to one another may reduce the CO_2 concentration in the air to a very low level. Lower than normal levels of CO_2 is said to drastically reduce photosynthetic activity and plant growth (Preece and Read, 2005).

Nitrogen may be one of the most important nutrient elements for plants and is used in greater amounts than any of the other macronutrients and generally constitutes the largest percentage of a plants' dry weight. Nitrogen is a constituent of amino acids and thus, is found in all proteins including enzymes, and is one of the reasons why nitrogen deficiency may severely stunt a plant. The carbon:nitrogen (C:N) ratio refers to the interrelation of the carbonaceous and nitrogenous components of a plant (Preece and Read, 2005). Under conditions of drought or nutrient stress, roots capture very limited resources for the plant, but still respire significant amounts of carbon (C) to maintain their membrane and enzymatic activity (Eissenstat and Van Rees, 1994). It has also been reported by Crick and Grime (1987) that C resources in the soil may become limited at different times during a growing season, affecting the lifespan of fine roots. The onset of dormancy in certain plants is accelerated during dry conditions and when minerals such as nitrogen (N) are withheld (Preece and Read, 2005). N_2 fixation

has been noted to decline after flowering in some legume species, and this has been attributed to a reduced availability of C for nodules (Phillips, 1980). The carbon isotopic composition of plants varies, in a systematic manner, when there are differences in the growth environment and the biochemical pathways of photosynthesis (Maberly et al., 1992).

Nitrogen partitioning between proteins within leaves occur during the acclimation of plants to their growth irradiance (Evans, 2001). Primary N assimilation necessitates extensive co-operation between different cell compartments while changes in C and N status influence organ physiology and root/shoot relationships (Foyer and Bowsher, 2004). Considerable effort has been made to characterize the C and N isotopic composition of plants from different habitats (Currin et al., 1995), however, there have been limted scientific studies on the fluctuations in the C and N ratios in bulbous plants during their growth and dormant phases.

It has been reported by Ryan et al. (1997) and Amthor (2000) that the C use-efficiency in plants can vary greatly within species which is based on their respiratory needs for growth and maintenance. For this reason, a comparative C:N isotopic ratio analysis, C and N acquisition and utilisation rates of *G. multifolia* and *G. villosa* were some of the aims for this investigation, to elucidate why there is a difference in the conservation status of the two species.

2.2.6 Resource allocation of geophytes during the growth, reproductive, and dormant phases of their annual life cycle

Most geophytes avoid stress by growing vigorously during the rainy season, with the above-ground parts senescing during the summer-drought period (Dafni et al., 1981). An important life strategy of geophytes is characterized by allocation and re-allocation of reserves and nutrients between the leaves and the storage organ (Pate and Dixon, 1981; Ruiters, 1995). Translocation of reserves from the leaves to the bulb is an important survival strategy of geophytes, ensuring the economic utilisation of minerals, thus allowing geophytes to grow in nutrient-poor soils (Pate and Dixon, 1981; Witkowski, 1989). It has further been reported that in desert environments, geophyte growth is characterized by the "reserve-pulse" model (Noy-Meir, 1973), which describes the importance of stored reserves of resources for initial growth, which is triggered by the onset of rain. This is followed by periods of no growth during the adverse season. During the active pulse, leaf and root activity return resources to the bulb and root storage organs. Part of the available internal resources however, is used for reproduction, and does not flow back into the reserve stores.

Reproduction in geophyte or bulbous species from arid environments is directly limited by the plant's biomass budget, of which there is a relationship between reproductive biomass and below-ground storage of long-term as well as current biomass reserves. Thus, it is suggested that growth and reproduction in plants from arid environments is biomass-limited because low water availability restricts carbon assimilation (Noy-Meir, 1973; Orians and Solbrig, 1977; Bloom et al.,

1985). This suggestion was further supported by Boeken (1990), who found that two *Bellevalia* geophytic species from arid environments had limted biomass reserves before the start of the new growth season.

It is noteworthy that in one Iridaceous geophyte of Mediterranean-type environments in Chile, *Solenomelus peduncularis,* biomass invested in leaves and roots did not vary at all according to soil moisture availability (Jaksic and Montenegro, 1979). According to Rossa and Von Willert (1999) geophytes produce seeds or new daughter bulbs for propagation, and in the case of unfavourable weather conditions which do not allow for seed or daughter bulb production, the main focus is to keep this organ alive, rather than using its resources for propagation. Frontanier (1973) reported that in a natural unpredictable environment, geophytes must be more flexible in order to prevent flowering in a bad year, as a means of escaping starvation at the end of the season and to ensure the presence of reserves for the next vegetative phase.

Geophytes that are growing in a seasonal climate with a restricted growth period, must have an intelligent use of reserves in order to reduce risks. Furthermore, reproduction in geophytic species from arid environments is directly limited by the plant's biomass budget, of which there is a relationship between reproductive biomass and below-ground storage of long-term as well as current biomass reserves (Orians and Solbrig, 1977; Bloom et al., 1985).

2.2.7 Reported plant physiology responses of *Gethyllis*

According to Du Plessis and Duncan (1989), the process of seed maturation in *Gethyllis* exhausts the resources within the parent plants and they will die if allowed to set seed heavily for two consecutive years. It is further reported that *Gethyllis* produces fleshy seeds which have a viability period of only a few weeks, and if the distribution of seed is not followed by the winter rains, the seeds or seedlings will die. Du Plessis and Duncan (1989) and Manning et al. (2002) reported that young plants frequently produce flowers without styles and also with a reduced number of stamens. It was suggested that this is probably a protective mechanism to prevent the immature plants from being fertilised and use up all its resources to produce the berries. This phenomenon was also observed by Daniels (2007) where only *G. multifolia* and not *G. villosa* plants, produced flowers without a style and stigma under severe drought conditions. According to Du Plessis and Duncan (1989) and Elvin (2000) plants that are growing in large numbers and in close proximity to each other, yield more berries. This statement was further supported by Daniels (2007) who observed the same phenomenon which is most likely due to the dependence of *Gethyllis* on cross-pollination for seed and fruit formation.

A drought stress experiment in a hydro culture study conducted by Daniels (2007), revealed that when irrigation is withheld from *G. multifolia* and *G. villosa* plants at the onset of the new growing season, *G. multifolia* plants are approximately three to four weeks slower in initiating new leaves, compared to *G. villosa* plants. Du Plessis and Duncan (1989) and Elvin (2000) further reported

that the initiation of new leaves at the onset of the new growth season of *Gethyllis* species, is not dependant on moisture, but a drop in atmospheric pressure. An experiment by Daniels (2007) revealed that both *G. multifolia* and *G. villosa* plants did, however, produce new leaves with delayed irrigation at the onset of the new growing season, but the leaves were stunted, compared to other plants subjected to irrigation. Even though plants in the delayed irrigation experiment appeared stressed and stunted, this was gradually corrected as plants were irrigated. This experiment revealed that moisture speeds up the initiation of new leaves and improves the quality and number of leaves, but is not a requirement for leaf initiation. Thus, this experiment confirmed the statement made by Du Plessis and Duncan (1989) and Elvin (2000) that the initiation of new leaves at the onset of the new growing season of *Gethyllis* species, is not dependant on moisture but a drop in atmospheric pressure.

According to Daniels (2007) the reproductive phase of *G. multifolia* and *G. villosa* is from mid-November to early December, when both species produce flowers simultaneously. This normally happened on average two weeks after the first summer rains. It was further observed that when the summer rains were absent or delayed, the number and quality of flowers produced in *G. multifolia* plants were drastically reduced, compared to *G. villosa*. *Gethyllis multifolia* plants produced flowers which shrivelled up and died during the bud stage (Figure 2.18). This survival strategy could result in limited cross-pollination taking place and consequently the formation of a decreased number of seeds for the existence of populations and the species. The same study also revealed that, as the temperature increased from the end of August, leaves of *G. multifolia* and *G.*

villosa plants gradually started yellowing from the tips down to the cataphyll which indicates the onset of senescence. This normally lasted until the end of October. *Gethyllis multifolia* was always the first to react to this climatic impulse. A good example of this phenomenon is that when *G. multifolia*'s leaves have reached their final stages of senescence, *G. villosa*'s leaves were only at the beginning stages of senescence. Further observations by Daniels (2007) revealed that under shady conditions, both species produced thinner, longer and straighter leaves without the natural spiralling.

Figure 2.18: Abortion of flower buds of *G. multifolia* plants in its natural habitat during the absence of summer rains.

Gethyllis multifolia and *G. villosa* are two geophytes from the succulent Karoo biome in the Western Cape of South Africa and this study will investigate the comparative physiological responses during drought and shade as well as the utilisation of resources such as carbon and nitrogen during the growth,

reproductive and dormant phases of both species to possibly elucidate why there is difference in the conservation status of the two species.

2.3 Oxidative stress and antioxidants

2.3.1 Introduction

It is a known fact that oxygen is essential to human life and without it we cannot survive. Conversely, oxygen is also involved in toxic reactions and as such is a constant threat to the well-being of all living matter. It is also known that human beings can only tolerate oxygen because our evolutionary ancestors developed powerful defence systems to minimize its toxic effects. Without this protection, the by-products of the human body's metabolism would drastically shorten the lives of mankind (Langseth, 1995). The body's natural defences only limit the harm caused by oxygen but do not eliminate it completely. The body produces a wide range of antioxidants to neutralise the effects of free radicals but production decreases with age (Ames et al., 1993). It is revealed through literature that oxygen-induced damage to our body usually accumulates with time (Langseth, 1995). This damage is said to be a major contributor to ageing and also many of the degenerative diseases of ageing such as allergies, Alzheimer's, ALS (Amyotrophic lateral sclerosis), autoimmune diseases, cancers (including bladder, breast, cervical, colorectal, lung melanoma, ovarian/endometrial, prostate, stomach, and upper aerodigestive tract), cardiovascular and coronary heart disease, cataracts, cystic fibrosis, diabetes, diabetic neutrophy, glaucoma, Huntington's disease, macular degeneration, multiple sclerosis, muscular

dystrophy, pancreatitis, Parkinson's disease, rheumatoid arthritis, schizophrenia, and strokes (Rimm et al., 1996).

The past decade has shown major breakthroughs in trying to understand the link between oxygenated metabolites and human diseases. Researchers have learned that it may be possible to prevent, postpone or limit the severity of these diseases by enhancing the body's natural antioxidant defence mechanisms through improved nutrition (Packer and Glazer, 1990; Langseth, 1995). Previous studies confirm that people who consume a diet rich in fruit and vegetables have a lower risk of diseases associated with ageing, such as heart disease and many types of cancer. Researchers initially believed that this was due to the vitamins in these foods, but further studies using vitamin supplements failed to confirm the same health benefits for heart disease and cancer (Langseth, 1995).

Further research has uncovered other foods that are associated with longevity and good health. These include nuts, coffee, tea (green and black), red wine (moderate quantities only), extra-virgin olive oil and dark bitter chocolate. The common factor in all these products seems to be their range of antioxidants. Hence, scientists believe there's a link between eating antioxidant-rich foods and good health (Willcox et al., 2004). Further studies by Sesso et al. (2003) revealed no confirmation that dietary flavonoids have a positive effect on cardiovascular disease. Manach et al. (2004) further mentioned that clinical studies will be of great assistance if the markers linking polyphenols to the prevention of diseases, are reliable.

2.3.2 How do free radicals cause disease?

The molecules in the human body consist of atoms joined by chemical bonds, formed by the electrons in an atom's outer shell. Normally, these bonds maintain stability and if they do split, they do so in balanced reactions. When weak bonds split, however, they may do so unevenly, creating what scientists call free radicals, or loose, unbalanced atoms or molecules. These free radicals often come from oxygen atoms; thus the term "oxidation" which is very similar to the same process that causes metal to rust or apple pulp to turn brown (Packer and Glazer, 1990).

Free radicals are very unstable compounds without the proper number of electrons, and they tend to react quickly with neighbouring compounds, trying to "steal" electrons from other molecules in an attempt to regain stability. If they succeed, then the "victim" atom or molecule usually loses its electron balance and becomes a free radical itself. Even if the free radical is not able to steal the electron, it may still create such a pull as to disrupt the workings of neighboring atoms and molecules. This can start a chain reaction; and once the process is started, it can build exponentially (Aruoma et al., 1991).

Reactive oxygen species (ROS) are labeled as harmful byproducts generated during normal cell metabolism that can cause oxidative stress and serious damage to the body's natural defence system (Ou et al., 2002). Reactive nitrogen species (RNS) are a family of antimicrobial molecules derived from nitric oxide (·NO) and superoxide (O_2^-) which are produced via the enzymatic activity of inducible

nitric oxide synthase 2 (NOS-2) and NADPH oxidase, respectively (Iovine et al., 2008). RNS are also produced in plants as by-products of aerobic metabolism or in response to stress. RNS act together with ROS to damage cells, causing nitrosative stress and therefore, these two species are often collectively referred to as ROS/RNS (Nicolas et al., 2006). Oxidative stress is a condition arising from an imbalance between free radical production and antioxidant defences and is linked to damage of a wide range of molecular species including lipids, proteins, and nucleic acids. The damage caused by oxidative stress has been associated with many conditions such as atherosclerosis, certain inflammatory conditions, certain cancers, and the process of aging (Young and Woodside, 2001).

Regarding cancer, the danger comes from the damage free radicals cause when they react with important cellular components such as deoxyribonucleic acid (DNA) or the cell membrane. DNA is the reproductive map of a cell and if it changes, the cell may duplicate the change when it reproduces. These changes are known as "mutations" and they also start a chain reaction, leading to the development of mutated, cancerous tissue such as tumors and lesions (Packer and Glazer, 1990). Another modern disease caused by the damage of free radicals is atherosclerosis, which is the leading cause of morbidity and mortality among people who have adopted the western lifestyle (Borochov-Neuri et al., 2008). Atherosclerosis (degeneration of the arteries caused by fatty deposits) is mainly caused by the action of free radicals which results in the oxidation of low-density lipoproteins (LDL) cholesterol in the human body.

Antioxidants prevent the oxidation of LDL, hence the depletion of antioxidants in the artery walls might be a risk factor for various forms of cardiovascular disease (CVD) (Young and Woodside, 2001). Proteins are directly responsible for the various enzymatic processes, as well as structural support, necessary for cellular homeostasis in the human body (Sohal, 2002). Free radical damage to proteins is directly linked to the oxidative inactivation of several key metabolic enzymes in the body (Starke-Reed and Oliver, 1989). The oxidation of proteins is initiated by the interaction of proteins with reactive oxygen species which can be generated by ionizing radiation, metal ion-catalyzed reactions, photochemical processes and enzyme catalyzed redox reactions (Stadtman, 1995). The quantification of protein carbonyl content, as a measure of oxidative damage, can be used as a tool to help identify individuals at risk of developing diseases associated with oxidative damage to proteins (Fagan et al., 1999).

2.3.3 The role of antioxidants

An antioxidant can be defined as: “any substance that, when present in an oxidisable substrate, significantly delays or inhibits the oxidation of that substrate” (Diplock, 1991). Thus, the physiological role of antioxidants is to prevent damage to cellular components arising from chemical reactions involving free radicals (Young and Woodside, 2001). As a cancer preventative mechanism, antioxidants appear to neutralize free radicals by donating one of their own electrons or hydrogen atoms, thus making a free radical compound stable and ending the electron-"stealing" chain-reaction that causes damage to cells and DNA (Diplock, 1991). These antioxidant compounds are able to do this because

they do not become free radicals in the process and remain stable with or without their donated electrons. The human body naturally produce certain antioxidant enzymes (endogenous enzymes), but it can also absorb natural oxidants from various diets (exogenous antioxidants). The human body also has several mechanisms against the production of free radicals in the form of endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (Gpx), which decrease the concentration of the most harmful oxidants in the body (Langseth, 1995).

Glutathione is a small molecule made up of three amino acids, which exists in almost all cells of the human body. Glutathione is the body's most important natural antioxidant and is required to maintain the normal function of the immune system (Chaudru et al., 2009). Glutathione is primarily produced in the liver where it comes into contact with all the nutrients that we consume. Glutathione also allows the body to recycle its store of antioxidants and its function is most important as it is the main antioxidant that protects the brain (Gao et al., 2009). The best known exogenous antioxidants that can be obtained from food crops are beta-carotene, vitamins C and E, and the mineral selenium. Diet is believed to play an important role in the prevention of the following four major diseases/disorders (which are generally caused by the high intake of fats and sugars): cardiovascular disease, cancer, hypertension and obesity, which are generally found in advanced economies of the world (Silalahi, 2002). It must also be noted that more recent epidemiology studies show some conflicting reports on this subject in a sense that the mechanisms explaining this observation remains unclear. However, the American Heart Association still recommends a higher

intake of fruit and vegetables and other foods with high polyphenol contents (Vita, 2005).

Plants have evolved a wide range of enzymatic and non-enzymatic mechanisms to scavenge ROS and protect their cells against oxygen toxicity (Fridovich, 1975) and the production of these chemicals that scavenges ROS, could be useful for humans in the treatment of modern diseases. The active compounds in a variety of plant sources, such as cocoa shells, oats, tea, olives, garlic, ginger, red onion skin, grapes, apple cuticle, various spices and seed extractions continue to be of interest for use in complementary medicine supplements, because they contain important antioxidants such as vitamin E, vitamin C, carotene, flavonoids and other polyphenols (Aruoma, 2003).

The following lists (Tables 2.2, 2.3 and 2.4) of antioxidant-capacity and -content are based on similar assays as described in the methodology section of this thesis. The literature study revealed many reports containing valuable information on fruit and vegetable antioxidants, but different assays and different preparation methods for the plant material were used, and thus, were not used as comparable examples for this investigation. These tables can be used as a guide to compare antioxidant-capacity and -content of the fruits and flowers of *G. multifolia* and *G. villosa* with, what in general, are considered to be the most useful edible and medicinal parts of the plant.

Table 2.2: A list of fruits, which can be compared to the fruit of *Gethyllis*, with their respective polyphenol content and ORAC values (PR WEB, 2005; Thaipong et al., 2006; Wang et al., 1996; Wang and Lin, 2000).

Plant product	**Polyphenol content (mg GAE/g DW)**	**ORAC (umole TE/g DW)**
Raisins	28.30	*
Plums	9.49	79.1
Orange	7.50	51.7
Red grapes	7.39	36
White grapes	*	26.2
Blackberry	13.47	133.3
Black raspberry	15.35	136.2
Red raspberry	13.46	104.3
Blueberry	24.00	*
Strawberry	15.40	153.6
Kiwi fruit	*	36.5
Grapefruit (pink)	*	48.3
Apple	*	13.2
Melon	*	12.9
Guava (A. Safeda)	3.45 (FW)	25.5 (FW)
Guava (F. Retief)	3.01 (FW)	21.0 (FW)
Guava (R. Supreme)	1.70 (FW)	18.2 (FW)

Abbreviations: ORAC (µM TE/g DW), Oxygen radical absorbance capacity in micromoles Trolox equivalent per gram dry weight; mg GAE/g DW, milligram gallic acid equivalent per gram dry weight; FW, fresh weight; the * denotes data not available.

Table 2.3: A list of vegetables, which can be compared to the fruit of *Gethyllis*, with their respective polyphenol content, FRAP and ORAC values (Cao et al., 1996; Ou et al., 2002).

Vegetable	**Polyphenol content (mg GAE/g DW)**	**FRAP (µM TE/g FdW)**	**ORAC (µM TE/g DW)**
Spinach	17.70	64	29.6
Broccoli	8.80	41	*
Brussels sprout	9.80	*	38.5
Beetroot	8.40	86	36.0
Onion	4.50	17	4.1
Greenpepper	*	157	*
Cauliflower	*	61	13.6
Carrot	*	31	10.3
Tomato	*	56	37.8

Abbreviations: ORAC (µM TE/g DW), Oxygen radical absorbance capacity in micromoles Trolox equivalent per gram dry weight; FRAP (µM TE/g FdW) Ferric reducing antioxidant power in micromoles Trolox equivalent per gram freeze-dried weight; mg GAE/g DW, milligram gallic acid equivalent per gram dry weight; the * denotes data not available.

Table 2.4: A list of herbs and spices, which can be compared to the fruit of *Gethyllis*, with their respective polyphenol and ORAC values (Zheng and Wang, 2001).

Herbs and spices	**Polyphenol content (mg GAE/g FW)**	**ORAC (µM TE/g FW)**
Thyme	2.13	19.49
Basil	2.23	14.27
Aloe vera	0.23	1.88
Sage	1.31	11.55
Lavandula	1.5	16.2
Parsley	1.12	11.03
Rosemary	2.19	19.15
Chives	1.05	9.15
Peppermint	2.26	15.82
Coriander	3.09	22.3
Garlic	*	2.7
Celery	*	6.0

Abbreviations: ORAC (µM TE/g FW), Oxygen radical absorbance capacity in micromoles Trolox equivalent per gram fresh weight; mg GAE/g FW, milligram gallic acid equivalent per gram fresh weight; the * denotes data not available.

It must be noted that the flower and fruit of *Gethyllis* are the most used plant parts by traditional healers, but are the most elusive and difficult plant parts to find in nature, because of their dependence on rain, a definite drop in atmospheric pressure and the precise timing of the appearance of flowers and fruit after being exposed to the above-mentioned factors (Du Plessis and Duncan, 1989; Daniels, 2007). No antioxidant reports could be found on the flower and fruit of any of the *Gethyllis* species, thus, this investigation could make a valuable contribution to seek useful natural products derived from plants in the quest to win the battle against human ailments and diseases.

2.3.4 The influence of environmental stress factors on antioxidants

Plants possess different antioxidant properties, depending on their antioxidant molecule content, which is strongly affected by the plant's environmental conditions (Lin et al., 2006). According to Dixon and Paiva (1995), polyphenols are important secondary plant metabolites and their synthesis is affected by light, as plants growing in full sun conditions have been shown to contain higher levels of polyphenolic compounds than shade plants. Environmental stresses, such as shade, abnormal salt levels, high temperature and drought, may result in the generation of ROS in plants which in turn may cause oxidative stress when in excess. In plant cells, oxidative stress reactions are associated with the production of toxic free radicals (Price et al., 1989).

Wang et al. (2005) reported that in rice plants, oxidative stress caused by moisture deficiency, resulted in membrane damage which is a secondary effect of drought stress. Other toxic effects in plants due to environmental stresses include the generation of lesions in DNA, a reduction in protein synthesis and stability and a decrease in photosynthetic activity (Osmond and Grace, 1995). It was further reported by Sofo et al. (2004) that different irrigation schemes and different irradiance levels caused varying antioxidant enzyme levels in olive trees. In sweet potato plants, a reduction in the total polyphenol content was noticed during flooding and drought stresses (Lin et al., 2006).

Munné-Bosch' and Peñuelas (2004) reported that under severe drought stress, ascorbate levels increased significantly in *Arbutus unedo*. Similar findings were

reported by Herbinger et al. (2002) whereby severe drought stress caused an increase in α-tocopherol and glutathione concentrations in certain wheat cultivars. Another study by Munne-Bosch et al. (2001) revealed that as drought levels were increased, higher levels of rosmanol and isorosmanol were evident in the leaves of *Salvia officinalis* subs. *officinalis.* Similarly, a high ferric reducing antioxidant power (FRAP) value and higher 2,2'-azino-di-3-ethylbenzthiazoline sulphonate (ABTS) radical cation scavenging ability were recorded when sweet potato plants were subjected to drought stress (Lin et al., 2006). Conversely, a reduction in flavonoid levels was found in *Crataegus laevigata* and *C. monogyna* following drought stress (Kirakosyan et al., 2003) whereas Tattini et al. (2004) reported higher flavonoid concentrations in the leaves of *Ligustrum vulgare* which were exposed to high light intensities.

According to Manach et al. (2004) the polyphenol content in food vary according to genetic, environmental and technological factors and future research must focus on plant varieties that are richest in polyphenols, improving growing methods and limiting losses during industrial processing and cooking in general. In this study, *G. multifolia* and *G. villosa* were exposed to artificial drought and photo-stress conditions to investigate whether these environmental stresses result in increased antioxidant-capacity and -content in the different plant parts.

2.3.5 An overview of environmental stress on flavanoid biosynthesis and accumulation in plants

Plant adaptation to biotic and abiotic stresses is due to the production of protective secondary metabolites (Dixon et al., 1994). According to Brooker and Miller (1998) plants that were exposed to ozone responded with increased levels of enzymes in the phenylpropanoid and lignin pathways. Research reports by Zimmerman and Cohill (1991) revealed that an increase in a plant's temperature tolerance is related to the accumulation of phenolic metabolites and heat shock proteins that act as chaperons at hyperthermia. Certain plants, when infected, respond with an increase in phenolics and specific phenolic-like salicyclic acid levels, acting as defence compounds or serve as precursors for the synthesis of lignin, suberin and other polyphenolic barriers (Yalpani et al., 1994). Furthermore, it was mentioned that some of these antimicrobial phenolics called phytoalexins are synthesized around the infected wound as a protective mechanism during pathogen attack (Dixon and Paiva, 1995).

The levels of phenylpropanoid compounds such as flavonoids, isoflavonoids, anthocyanins and polyphenols are increased when certain plants are subjected to nutritional stress (Graham, 1991), cold stress (Christie et al., 1994) and high light intensities (Beggs et al., 1987). The activation of biotic and abiotic stress response arise from certain changes in the intracellular medium (Kurganova et al., 1997), which transmit the stress signal to cellular effector systems which in return result in changes in the cytosolic calcium concentration action potential (Retivin et al., 1997). In conclusion, it is mentioned by Shetty (2004) that phenolic

antioxidants resulting from such secondary stress responses in plants, can be targeted as a source of therapeutic and health-supporting ingredients for certain modern diseases.

2.3.6 Antioxidant content

2.3.6.1 Polyphenols

In plants, polyphenolic compounds are mainly responsible for the prevention of fungal and bacterial infection, protection against ultra-violet radiation, oxidative damage, the effect of insect infestation and the control of auxin levels and other plant regulators. They are also responsible for the colour, texture and taste of food crops (Jood et al., 1995). It is further reported that the total polyphenol content and antoxidant activity also varies in the different plant parts (leaves, branches, phloem, bark, cork and needles) of trees (Kähkönen et al., 1999; Zhishen et al., 1999). Many other factors such as plant species and variety, fruit ripeness, time of harvest, processing and storage may affect the polyphenol content of plants. Very specific environmental factors such as soil type, sun exposure and rainfall can significantly change the polyphenol content in certain plant species. Furthermore, cultural conditions such as greenhouse culture, field culture, hydroponic culture and fruit yield per tree can also considerably affect polyphenol concentrations (Macheix et al., 1990). It has also been reported that, during ripening of fruit, phenolic acid concentrations decrease and anthocyanin concentrations increase (Parr and Bolwell, 2000). Recent reports by Asami et al. (2003) revealed that the polyphenol content of vegetables grown by means of organic or sustainable methods, is higher than in vegetables grown without stress,

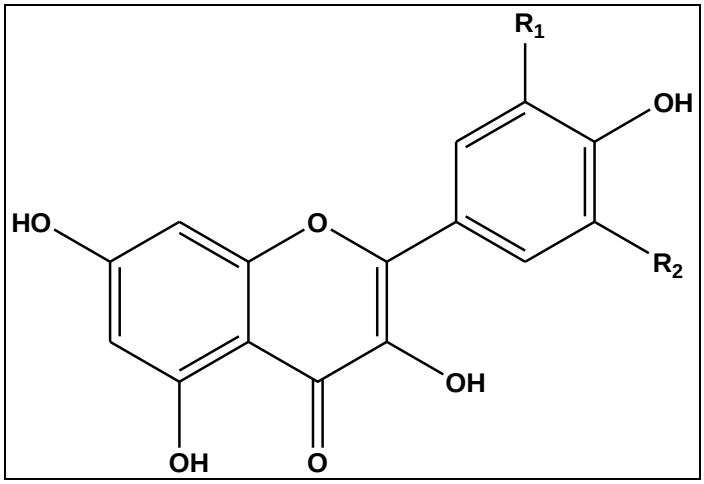
e.g. hydoponics or greenhouse culture. Earlier studies by Sosulski et al. (1982) concluded that storage of fruit or vegetables can also affect polyphenol content in a sense that it leads to colour changes and organoleptic characteristics. Culinary preparation such as peeling of fruit and vegetables, cooking, freezing, grinding of plant tissues (Clifford, 2000) and industrial processing in general can result in a significant reduction in the polyphenol content of food (Macheix and Fleuriet, 1998).

Asian and Mediterranean diets also contain a large quantity of polyphenols and have shown to have protective effects against most modern diseases and disorders such as cardiovascular disease, various cancers, osteoporosis, early ageing, inflammatory and immune disorders, nutritional deficiencies, etc (Cox et al., 2000; Strandhagen et al., 2000; Nakamura et al., 2003). More recent studies confirmed that our knowledge is still too limited for formulation of recommendations for the general population or for particular populations at risk of specific diseases. It was further mentioned that the use of flavonoids for a reduction in disease risk was considered "possible" for cardiovascular diseases and "insufficient" for cancers (WHO/FAO, 2003).

According to Manach et al. (2004) several thousand molecules having a polyphenol structure (hydroxyl groups on aromatic rings) have been identified in higher plants and several hundreds are found in edible plants. Polyphenols are subdivided into the following classes according to their structures: acetophenones,

benzofurans, chromones, coumarins, flavonoids, phenolic acids, phenylacetic acids, phenylpropanoids, quinones, stilbenes and xanthones (Waterhouse, 2005).

Flavonoids are a subclass of the polyphenols, which are characterized as containing two or more aromatic rings, each bearing at least one aromatic hydroxyl and connected with a carbon bridge. For flavonoids, this bridge consists of three carbons that combine with an oxygen and two carbons of one of the aromatic rings to form a third 6-member ring (Figure 2.19) (Clifford, 2001). Subclasses that are grouped under flavonoids according to their chemical structures are as follows: flavanols, flavonols, flavanones, flavones, isoflavones, and anthocyanidins (Beecher, 2003; Hertog et al., 1995; Rimm et al., 1996). Over 9000 flavonoid structures have been identified which are widely distributed in all higher plants throughout the plant kingdom (Martens and Mithöfer, 2005). The dietary intake of flavonoids has attracted increasing interest as a result of recent epidemiological, mechanistic, and human intervention studies suggesting potential benefits against cardiovascular diseases (Mazza et al., 1999). Recent epidemiological studies, however, show some conflicting reports on the subject of cardiovascular disease in a sense that the mechanisms explaining this observation remains unclear (Vita, 2005). The consumption of flavonoids in western countries is low compared to Asian countries and also the preparation of food results in a further decrease (50-90%) in the flavonoid content, hence, the need for a higher consumption of fresh vegetables and fruit to sustain a healthy lifestyle (Natuurlijkerwijs, 2010).



R_1 = H; R_2 = H: Kaempferol
R_1 = OH; R_2 = H: Quercetin
R_1 = OH; R_2 = OH: Myricetin
R_1 = OCH_3; R_2 = H: Isorhamnetin

Figure 2.19: General chemical structure and numbering pattern for common food flavonols (Clifford, 2001).

Flavonoids are synthesized via the phenylpropanoid pathway (Figure 2.20). Phenylalanine ammonia lyase (PAL) catalyzes the conversion of phenylalanine to cinnamate. PAL also shows activity with converting tyrosine to *p*-coumarate, albeit to a lower efficiency. The cinnamate 4-hydroxylase (C4H) catalyzes the synthesis of *p*-hydroxycinnamate from cinnamate and 4-coumarate:CoA ligase (4CL) converts *p*-coumarate to its coenzyme-A ester, activating it for reaction with malonyl CoA. The flavonoid biosynthetic pathway starts with the condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA, yielding naringenin chalcone. This reaction is carried out by the enzyme chalcone synthase (CHS). Chalcone is isomerised to a flavanone by the enzyme chalcone flavanone isomerase (CHI). From these central intermediates,

the pathway diverges into several side branches, each resulting in a different class of flavonoids. Flavanone 3-hydroxylase (F3H) catalyzes the stereospecific 3ß-hydroxylation of (2S)-flavanones to dihydroflavonols. For the biosynthesis of anthocyanins, dihydroflavonol reductase (DFR) catalyzes the reduction of dihydroflavonols to flavan-3,4-diols (leucoanthocyanins), which are converted to anthocyanidins by anthocyanidin synthase (ANS). The formation of glucosides is catalyzed by UDP glucose-flavonoid 3-o-glucosyl transferase (UFGT), which stabilize the anthocyanidins by 3-*O*-glucosylation (Harborne 1994).

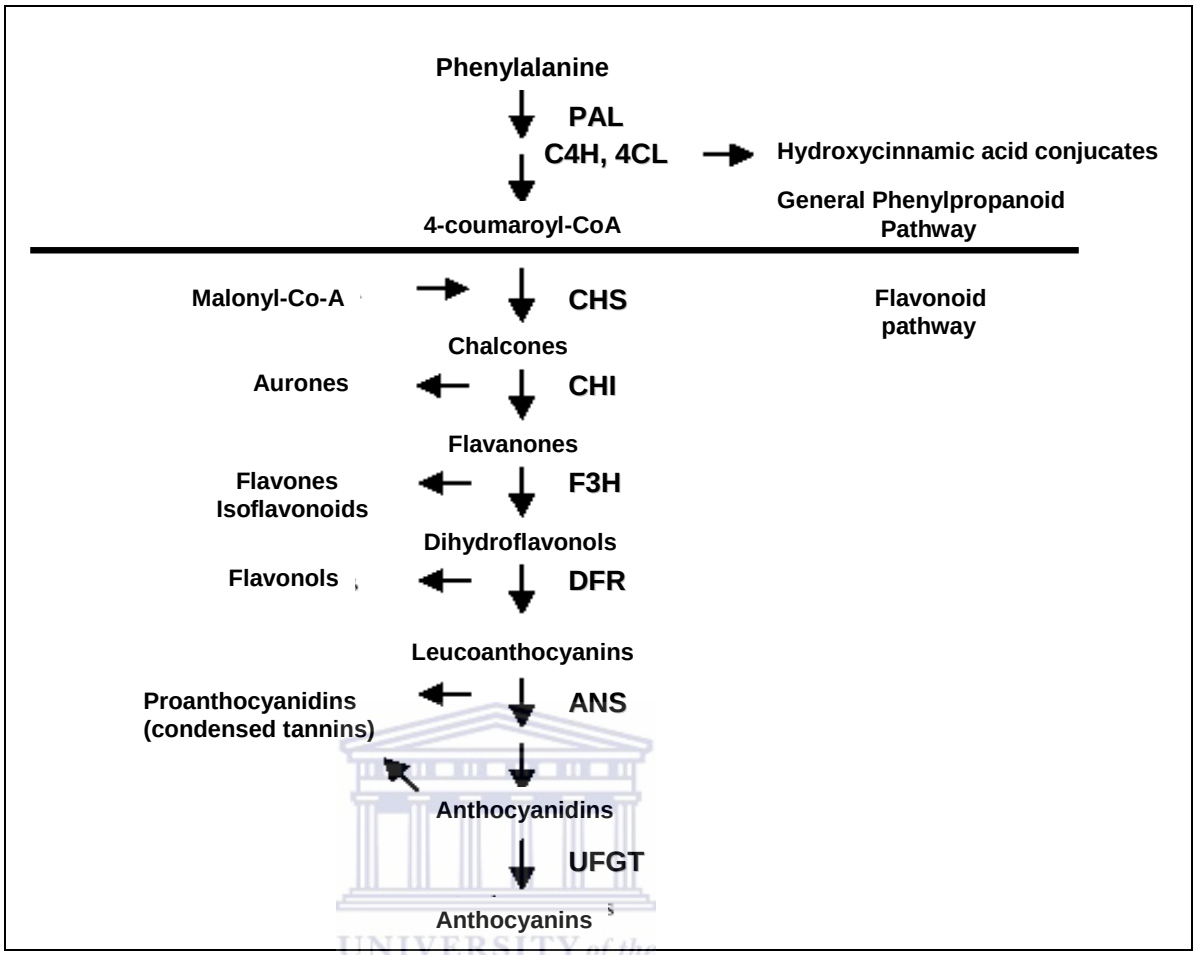


Figure 2.20: A schematic presentation of the flavonoid biosynthetic pathway. Enzyme abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, UDP glucose-flavonoid 3-o-glucosyl transferase (Stafford, 1991)

2.3.6.1.1 Flavonols

Flavonols (Figure 2.21) such as isorhamnetin, kaempherols, myricetin, and quercetin form one of the subclasses of flavonoids and are present in many plant foods such as fruits, plant leaves, vegetables and tea (Beecher, 2003; Hertog et al., 1995; Rimm et al., 1996). It has been reported that flavonols have positive effects on vascular health such as lowering blood pressure, improving blood flow to the brain and heart, reducing clotting of blood platelets, and a reduction in

cholesterol. Further health benefits of a high flavonol diet also include a reduction in the risk of pancreatic cancer (Nöthlings et al., 2008).

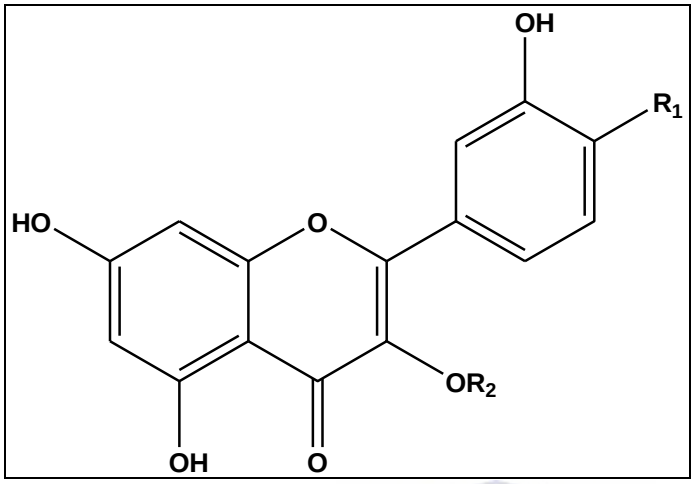


Figure 2.21: Chemical structure of the common flavonol (Manach et al., 2004).

2.3.6.1.2 Flavanols

Flavanols are most commonly found in the following food products: apples, blueberries, broccoli, chocolate, cranberry, green tea, black tea, red wine, and white wine (Beecher, 2003). Catechin, gallocatechin, epicatechin, epigallocatechin, epicatechin-3-gallate, epigallocatechin-3-gallate are some of the most common flavanols (Figure 2.22) found in plants. New research evidence has shown that foods rich in flavanols may improve the health of people with coronary artery disease, the western nations' leading cause of death. Clinical trials have shown that a flavanol-rich diet thins the plaque lining on the inside of blood vessels (endothelium), thus causing a reduction in blood pressure (UC Health, 2010).

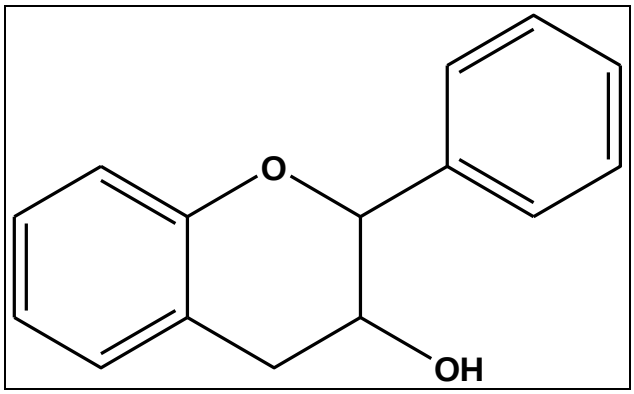


Figure 2.22: Chemical structure of the common flavan-3-ol (Manach et al., 2004).

2.3.6.1.3 Flavanones

Flavanones are commonly found in citrus fruits, tomatoes, peanuts and some herbs (mint, gaviota tarplant, yerba santa, and thyme). Some of the most common flavanones (Figure 2.23) found in plants include butin, eriodictyol, eriocitrin, eriodictyol, hesperetin, hesperidin, homoeriodictyol, isosakuranetin, narirutin, naringenin, naringin, neohesperidin, pinocembrin, poncirin, rhoifolin, sakuranetin, sakuranin, and sterubin (Tubaro et al., 1996). It has been reported that flavanones exhibit antiviral, antimicrobial and anti-inflammatory activities (Nakasugi and Komai, 1998).

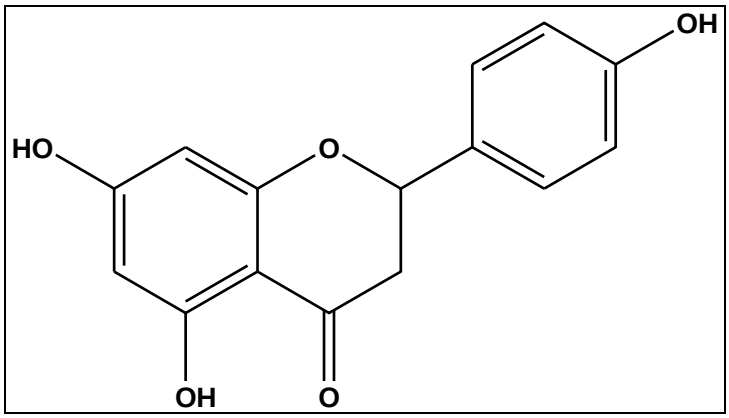


Figure 2.23: Chemical structure of the flavanone backbone (Fowler et al., 2011).

2.3.6.1.4 Flavones

Flavones (Figure 2.24) are polyphenolic crystalline compounds that are found in some seeds, leaves and in stems as a yellow pigment (Beecher, 2003). Apigenin and luteolin are some of the most common flavones found in plants. They occur in high concentrations in leafy and other crops such as parsley, celery, thyme, and hot peppers (Beecher, 2003). In recent years, scientific and public interest in flavones has grown enormously due to the purported beneficial effects against the development of atherosclerosis, osteoporosis, diabetes and certain cancers. The flavone intake in the form of dietary supplements and plant extracts has been steadily increasing because of their possibe health benefits (Sarda et al., 2006). It is also further reported by Nakasugi and Komai (1998) that flavones possess antimutagenic activity.

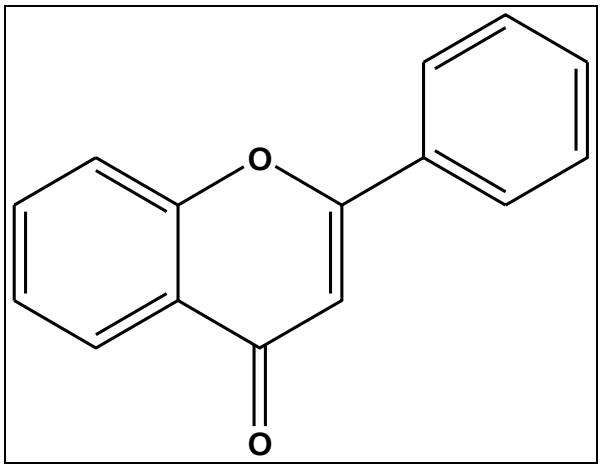


Figure 2.24: The basic chemical structure of a flavone (Quideau et al., 2011)

2.3.6.1.5 Isoflavones

Isoflavones have a limited distribution and the following are found in plants: daidzein, genistein, glycitein, biochanin A, and formononentin (Beecher, 2003). They are mostly found in soy food products such as soy beans, soy milk, tofu, and soy protein powder. Research has shown that isoflavones are effective in lowering blood cholesterol, reducing the risk of cancer, heart disease and osteoporosis (Lin et al., 1996). Recent studies have found that soy isoflavones can reduce menopause symptoms such as hot flushes and increase bone density in women, because the chemical structure of isoflavones (Figure 2.25) is very similar to that of the human body's estrogen. Further studies have also shown that in men, isoflavones slowed prostate cancer growth and caused prostate cancer cells to die (Jacobs et al., 2009).

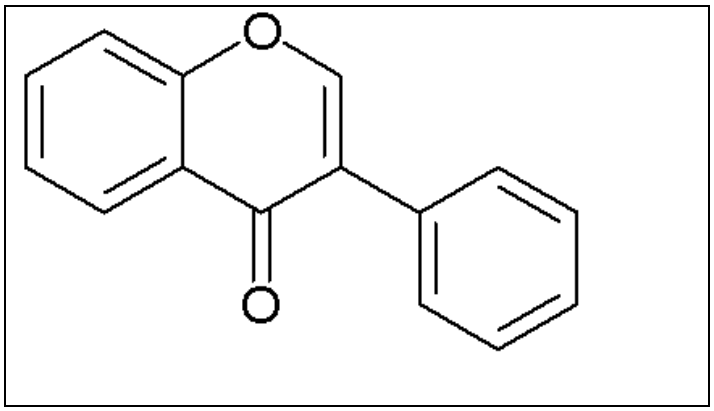


Figure 2.25: The basic chemical structure of isoflavones (Natuurlijkerwijs, 2011)

2.3.6.1.6 Anthocyanidins (anthocyanin aglycone)

Cyanidin, delphinidin, malvidin, pelargonidin, petunidin, and peonidin are some of the common anthocyanidins (Figure 2.26) found in plants (Beecher, 2003). Anthocyanin pigments are responsible for the attractive red, purple and blue colours of many fruits and vegetables. They are relatively unstable and often undergo degradative reactions during processing and storage (Francis, 1989). Food crops that contain high levels of anthocyanidins include apples, blueberries, broccoli, chocolate, orange juice, cranberry including red and white wine. In general, they are found in red, purple and blue berries (Beecher, 2003). They play a role in the reduction of coronary heart disease, increased eye health (visual sharpness), and also have carcinogenic properties. They have also been found to have considerable potential in the food industry as safe and effective food colourants (Francis, 1982).

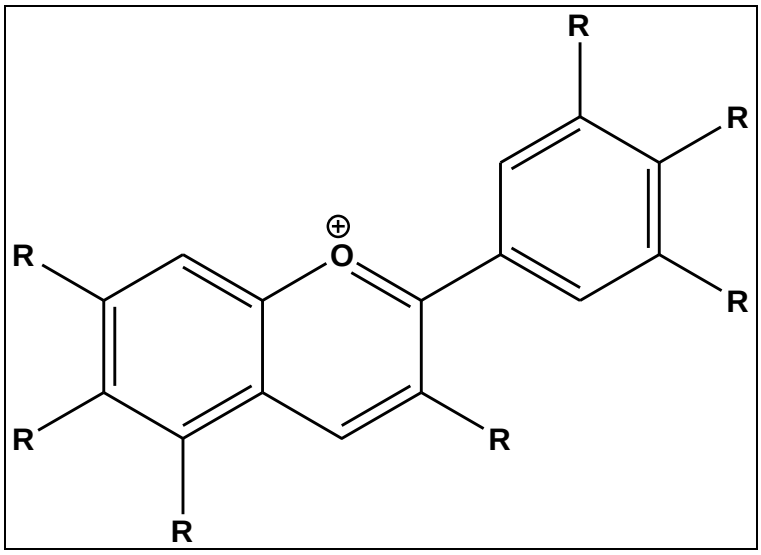


Figure 2.26: The basic chemical structure of anthocyanidin (Cancerquest, 2011)

2.3.6.1.7 Dihydrochalcones

Dihydrochalcones (Figure 2.27) belong to the family of the bicyclic flavonoids which are defined by the presence of two benzenoid rings joined by a three carbon bridge. The neohesperidin dihydrochalcone is the most familiar of these compounds and is known as an intense sweetener and permitted for commercial use as a food additive (Borrego et al., 1995). Nakamura et al. (2003) further reported that dihydrochalcones have some advantages for application as food additives because of their safety, stability and colorless property. Some dihydrochalcones (phloridzin and myrigalone B) have been found to occur naturally and to possess antioxidant activity (Mathiesen et al., 1997). Chalcones and dihydochalcones are also known to exert multiple biological activities such as anti-inflammatory, antioxidant and anticancer properties (Shah et al., 2008; Vogel et al., 2010).

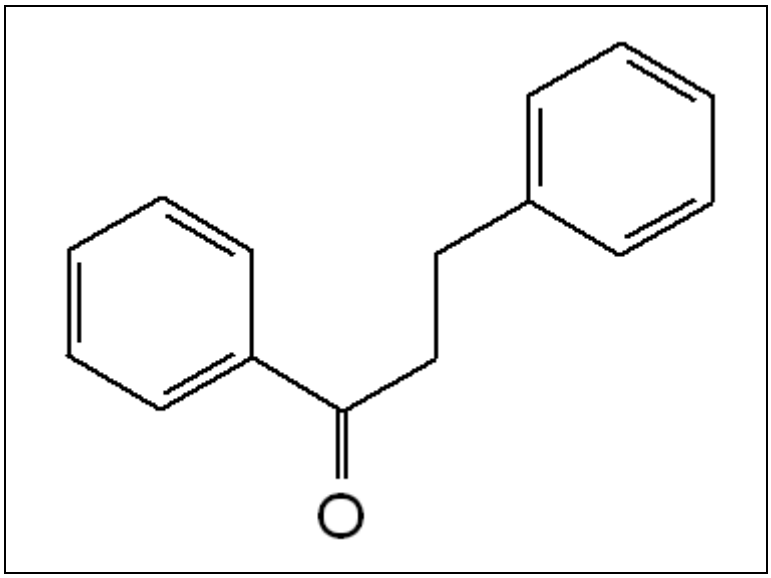


Figure 2.27: Chemical structure of the chalcone (Szliszka et al., 2010).

2.3.7 Antioxidant capacity determination

2.3.7.1 Introduction

According to Nakamura et al. (2003), phenolic compounds possess a wide range of biochemical activities such as antioxidant, antimutagenic, anticarcinogenic and the ability to modify gene expression. The chemistry and methodology involved in the antioxidant capacity assays are highlighted in reviews by Huang et al. (2005) and Prior et al. (2003), indicating three major methods for evaluating the antioxidant capacity. The methods include (1) oxygen radical absorption capacity (ORAC), (2) ferric reducing antioxidant power (FRAP), and (3) 2,2'-azino-di-3-ethylbenzthiazoline sulphonate (ABTS) or Trolox equivalent antioxidant capacity (TEAC).

2.3.7.2 ORAC assay

The ORAC assay is a simple, sensitive, and reliable way to measure the peroxyl radical absorbing capacity of antioxidants and serum or other biological fluids using the peroxyl radical generator, 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH) (Prior et al., 2003). The assay is different in a sense that the total antioxidant capacity of a sample is estimated by taking the oxidation reaction to completion. All of the non-protein antioxidants e.g. α-tocopherol, vitamin C, β-carotene, uric acid, etc. and the albumin in the sample are at that stage oxidized by the peroxyl radical. Results are then quantified by measuring the protection produced by antioxidants (Cao et al., 1993). This assay uses fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3-one) as the fluorescent probe.

An advantage of this assay, using fluorescein, is that it is less expensive, does not interact with other compounds, and is very stable. One disadvantage is that when the pH drops below 7.0, the intensity of fluorescein decreases drastically (Ou et al., 2001). Other shortcomings of this assay include the need for expensive equipment, variability across instruments, and lengthy analysis time (Zulueta et al., 2009). Above all, the determination of antioxidant capacity using the fluorescein-based ORAC assay is effective in many experimental models and conditions and is considered one of the best assays to measure antioxidant capacity (Powers et al., 2010). To determine the ORAC of herbal extracts, Trolox is used as a standard and fluorescein as the substrate, with the fluorescence

conditions set at a 485 nm excitation wavelength and a 530 nm emission wavelength (Prior et al., 2003).

2.3.7.3 FRAP assay

The FRAP assay uses an oxidation/reduction reaction to measure the ability of a sample to reduce Fe^{III} to Fe^{II} at a low pH of 3.4. An antioxidant donates electrons in the same manner as a reductant in oxidation/reduction reactions, so it is assumed that the FRAP assay is a method for evaluating antioxidant capacity. However, it does not directly measure the antioxidant capacity of a potential antioxidant. Also, since there are no free radicals introduced into the system, there is no way of comparing the antioxidant capacity towards different kinds of radicals.

The FRAP assay was developed by Benzie and Strain and generates fast, reproducible results with plasma, with single antioxidants in pure solution, and with mixtures of antioxidants in aqueous solution (Benzie and Strain, 1999). Furthermore, this assay is inexpensive and easy to perform and thus has been commonly used in research to evaluate *in vitro* antioxidant capacity. Nonetheless, there are several problems with the FRAP assay. Some of the drawbacks of this assay are that it is also based on a single electron transfer and therefore, cannot measure the total capacity of antioxidants such as chain-breaking antioxidant activity. Another disadvantage of this assay is that it is carried out at a very low and non-physiological pH of 3.4, compared to the neutral pH used in the TEAC assay (Huang et al., 2005). In addition, the absorption at 593 nm of some

antioxidant polyphenols such as caffeic acid, tannic acid, ferulic acid, ascorbic acid and quercetin, does not stop during the 4-min reaction time of the FRAP assay. Instead, it slowly increases for several hours, thus making it inadequate because it does not measure the true antioxidant capacity of a sample (Pulido et al., 2000). Furthermore, the FRAP assay does not detect the activity of important thiol antioxidants such as glutathione. Therefore, because of these multiple drawbacks, the FRAP antioxidant assay is less than ideal (Powers et al., 2010).

2.3.7.4 ABTS (TEAC) assay

The 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonate] (ABTS) radical cation scavenging assay was first developed by Miller et al., (1993) for the determination of the total antioxidant status (TAS) of body fluids. This assay is based on the scavenging of the ABTS radical by antioxidants present in a sample. During this assay a biological sample is added to the free-radical-generating system, and inhibition of the free-radical reaction is proportional to the antioxidant capacity of that sample (Cao and Prior, 1998). The antioxidants quench the ABTS radical cation, resulting in a loss of colour and thus, a decrease in absorbance. The change in absorbance corresponds to the concentration of antioxidants present in the system.

The ABTS radical cation has a maximum absorbance value of 734 nm at 25 ºC and Trolox is used as a standard (Re et al., 1999). Some of the advantages of this assay include that it is inexpensive, fast, easy to perform, and stable to changes in pH. This assay, however, has several limitations i.e. the ABTS radical cation is

relatively unstable, and different TEAC-assay methods makes it difficult to compare TEAC results from studies using these different variations of the same assay. Furthermore, the TEAC assay is based on a one-electron transfer and can therefore not measure chain-breaking antioxidant activity. Due to the limitations of this assay some authors have concluded that this assay should be used with caution (Frankel and Meyer, 2000).

In conclusion, there is no single assay that is capable of reliably measuring total antioxidant capacity and the ORAC assay using fluorescein appears to be one of the best single antioxidant-capacity assays to use. Nevertheless, to comprehensively measure total antioxidant capacity, specific assays are needed in addition to ORAC. Indeed, a total antioxidant-capacity assay that measures the antioxidant capacity against one ROS species is not biologically realistic (Huang et al., 2005). Therefore, when assessing total antioxidant capacity of tissues, it is sensible to evaluate the antioxidant potential of tissues after exposure to a wide variety of biologically relevant ROS (Coombes et al., 2000).

2.4 Medicinal uses, natural compound extraction and characterization of *Gethyllis*

2.4.1 Introduction to traditional medicine

Botanical drugs are defined by the Food and Drug Administration as those drugs containing ingredients from fresh or dried plants, plant parts, isolated or combined chemical components of plant origin, algae, macroscopic fungi or

combinations thereof (Goldman, 2001; Liu and Wang, 2008). According to the World Health Organisation (WHO) the use of plants for medicinal purposes (as traditional medicine) is now internationally recognized as a building block for primary health care (WHO, 2005). It was further reported by Van Wyk and Gericke (2000) that mankind have been aware of the healing power of herbs since creation and the use of plants as traditional medicine, is one of the oldest practiced professions. It is reported that India has the biggest repository of medicinal plants in the world with nearly 15 000 plant species being used as sources of medicine (Singh et al., 2003).

In South Africa the use of traditional medicine is widespread, where it is estimated that 80% of the black population consult traditional healers. The more available traditional healthcare system offers a cheap and more culturally acceptable alternative to the costly allopathic system (Eastman, 2005). It was further reported that about 80% of the world's population depends mainly on traditional medicine derived from plant extracts (WHO, 2005). This practice is generally found in rural areas where synthetic drugs are not available and also not affordable. In light of this, there exists an urgent need for a systematic study of our medicinal plants to isolate their active compounds, before they are lost forever (Cox, 1991; Ogungbamila, 1993; Van Wyk et al., 2008).

The Cape Floral Kingdom or Cape Floristic Region is widely recognized as one of the richest floras of the world (Good, 1974; Goldblatt and Manning, 2000). This region is also part of the traditional home of the Khoikhoi (Hottentot) and San (Bushman) and these two cultural groups are collectively referred to as the Khoi-

San people (Shapera, 1930). In the second half of the 17th century, the region became inhabited by Europeans and the interactions between Khoi-San and Cape Europeans resulted in a distinct but poorly studied and documented healing culture, known as Khoi-San or Cape Dutch medicine (Van Wyk et al., 2008). Very little has been published on the medicinal uses of plants from these regions and numerous publications on the use of plants for food and water (Story, 1964), utility items (Tanaka, 1978) and hunting poisons (Neuwinger, 1996) were available. According to Dold and Cocks (2002), the use and trade of plants for medicine is no longer confined to traditional healers but has spread to the informal and formal entrepreneurial sectors of the South African economy because of unemployment, resulting in an increase in the number of herbal gatherers and traders. Consequently, the trade in traditional medicines is now greater than ever before and a complex biodiversity management issue is facing conservation authorities in South Africa.

2.4.2 Medicinal value and other uses of *Gethyllis* species

Several authors (Rood, 1994; Van Wyk et al., 1997; Watt and Breyer-Brandwijk, 1962) reported that 'Koekemakranka' brandy (Figures 2.28 and 2.29), made of the fruit of *G. afra* and *G. ciliaris*, is one of the early Cape remedies for colic and indigestion and that the edible fruit was highly valued for perfuming rooms and linen. The fruit of this plant is sweet and juicy, pleasantly aromatic and good to eat (Fox and Norwood Young, 1983). Some authors (Rood, 1994; Watt and Breyer-Brandwijk, 1962) reported that the early Cape colonists used an alcoholic infusion of the fruit of *G. linearis* and *G. spiralis* as a remedy for

digestive disturbances. For children, the gathering of the inconspicuous fruit (which they refer to as "Koekemakrankies"), is a special occasion, and success depends to a large extent on a good sense of smell (Smith, 1966).

In more recent times a diluted infusion of the flower has been used for teething troubles and the skin of the fruit as a local application on boils, bruises and insect bites. Du Plessis and Delpierre (1973) reported that the club-shaped fruit was used to perfume rooms and was often dried in newspaper for use as a bookmarker. It was further reported that in the past, the odourous dried fruits were often used to scent handkerchiefs and cupboards (Van Wyk and Gericke, 2000). It was also mentioned by Rood (1994), that the fruit was boiled by the Khoi and used as an aphrodisiac. Van der Walt (2003) reported that *G. ciliaris* was used for fatigue and gave a recipe for the 'Koekemakranka' brandy. Traditionally, an alcoholic infusion (Figure 2.28) or tincture is made from a few ripe fruits (Dic. Nat. Prod., 1996), which are presumed to contains some oils and esters of low molecular weight alcohols (Watt and Breyer-Brandwijk, 1962). Van Wyk et al. (1997) reported that no published information on the chemistry of *Gethyllis* existed and that preliminary tests on the fruit indicated slight analgesic effects, but no details were available. Further studies by Elgorashi and Van Staden (2004) confirmed that the anti-inflammatory and antibacterial activities found in *G. multifolia*, *G. villosa* as well as other *Gethyllis* species, were in line with their uses as traditional medicines.

Figure 2.28: Alcohol infusion of the fruit of *G. afra* (Photo taken in 2005 at the Rondeberg Nature Reserve, Westcoast, Western Cape).

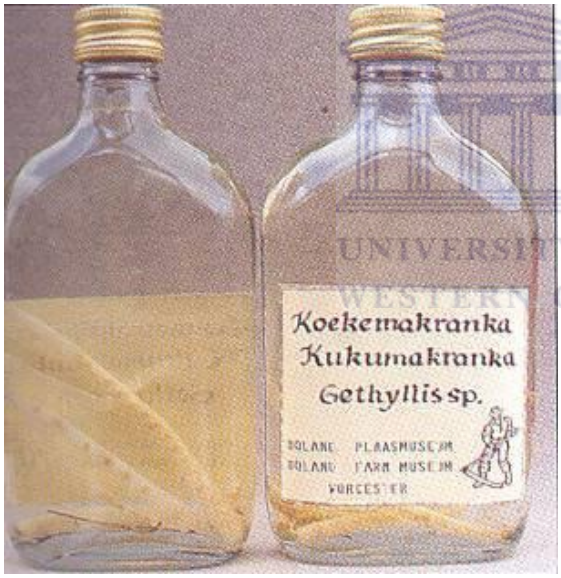


Figure 2.29: Fruit of *Gethyllis* being used to make 'Kukumakranka' brandy (Photo courtesy of Van Wyk et al. (1997).

2.4.3 Major chemical constituents of *Gethyllis* species

Pharmaceutical studies by Elgorashi et al. (2007) on the bulbs and roots *G. ciliaris* using a cyclooxygenase-1 enzyme assay, resulted in the isolation of a

dihydroxydimethylbenzopyran-4-one (isoeugenitol) (Figure 2.30) and a 90% methanolic extract yielded an isoeugenitol glycoside (Figure 2.30) and 9Z-octadec-9-enamide (Figure 2.31).

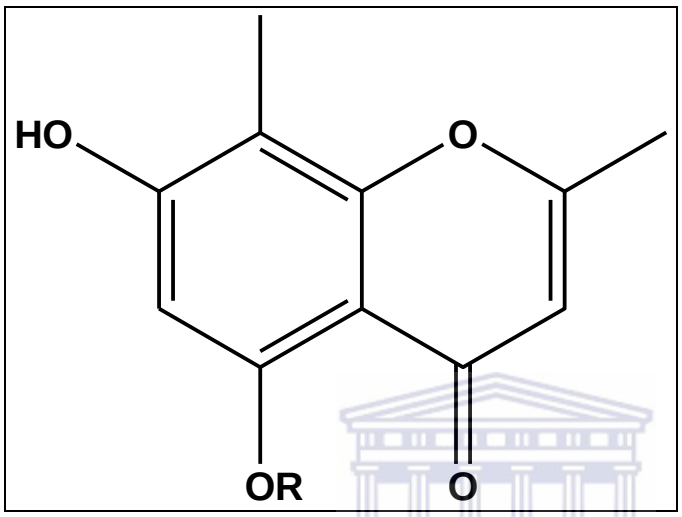


Figure 2.30: (a) R=H, Isoeugenitol and **(b) R=Glucosyl**, its glycoside as isolated from bulbs and roots of *G. ciliaris* plants (Elgorashi et al., 2007).

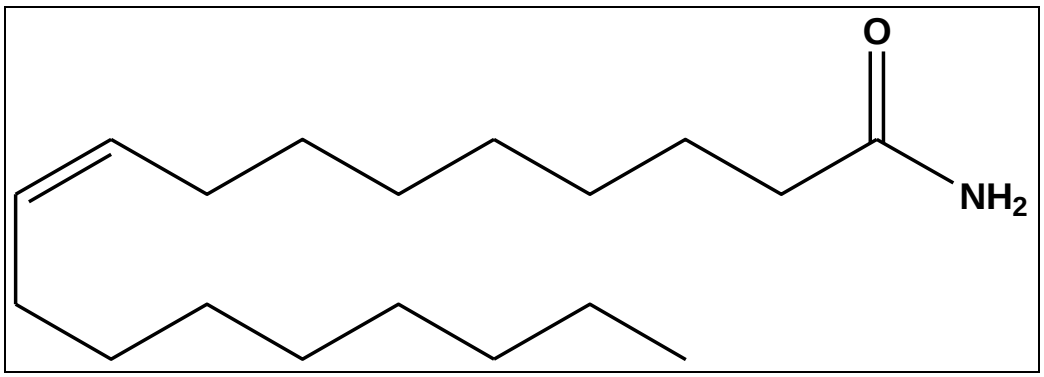


Figure 2.31: 9Z-octadec-9-enamide isolated from bulbs and roots of *G. ciliaris* plants (Elgorashi et al., 2007).

In a study by Kamatou et al. (2008), 29 volatile organic compounds were identified in the fruit of *Gethyllis ciliaris* and 43 compounds from the fruit of *G. afra*. The major compounds isolated from the fruit of *G. ciliaris* were as follows: pentacosane; ethyl octanoate; ethyl isovalerate; ethyl hexanoate and ethyl benzoate. According to the authors, these compounds may be contributing to the fruity-sweet odour of *G. ciliaris*. The following compounds were isolated from the fruit of of *G. afra*: α-pinene, n-butyl n-butyrate, isoamyl acetate, β-pinene and 2-methylbutyl butyrate. Similarly, these compounds could be contributors to the banana/piney/fruity odours found in the fruit of *G. afra*.

The majority of compounds detected in the Amaryllidaceae family are mostly alkaloids. It must be mentioned that alkaloids were not detected in either the dichloromethane or 90% methanolic extracts of *G. ciliaris* using Dragendorff's reagent as a conventional method of detection. On the contrary, the following compound classes: tannins, flavonoids, phenolics, saponins, anthraquinones, glycosides and essential oils were present in all the extracts (Fennell and Van Staden, 2001). Specific alkaloids, which are unique to the Amaryllidaceae family consistently indicated antitumour potential and amongst other characteristics, showed *in vivo* activity against various human viruses (Duri et al., 1994; Hutchings et al., 1996). In addition to alkaloids, bulbs from this plant family (including *Gethyllis*) also contain flavonols, organic acids, carbohydrates and soluble nitrogen compounds. However, the fact that alkaloids were not detected in *G. ciliaris*, does not necessarily mean that this species does not contain alkaloids. More emphasis, in future research, needs to be placed on the ontogenic

and environmental factors that affect the production of alkaloids (Viladomat et al., 1997).

2.4.4 Reported biological activities of *Gethyllis multifolia* and *G. villosa* and other *Gethyllis* species

Gethyllis multifolia and *G. villosa* species showed high anti-inflammatory activity and virtually no antibacterial activities whereas the leaves and roots of *G. ciliaris* showed similar anti-inflammatory activity but increased bacteriostatic activity in a pharmaceutical study conducted by Elgorashi and Van Staden (2004). A brine shrimp lethality assay indicates toxicology levels of bioactive compounds which is based on three bands: inactive (LC_{50} > 700), active (LC_{50} < 700) and very active (LC_{50} < 10) (Babajide et al., 2008; McLaughlin et al., 1991). According to Babajide et al. (2010), the brine shrimp lethality assay revealed that *G. multifolia* methanolic (6.20) and water extracts (139.55) and only the *G. villosa* methanolic extract (4.23) were both active and very active, whereas the *G. gregoriana* methanolic extract (0.22) was the most active when compared to the other two species. This is an indication of the presence of good antimicrobial and antiviral agents, since the lethality test of a substance to brine shrimp nauplii indicates the ability of that substance to kill cancer cells (antitumor activity) as well as having pesticidal and antibacterial activities. It may thus be deduced that all the "very active" samples, in this case *G. gregoriana*, *G. multifolia* and *G. villosa* methanolic extracts, can be considered potentially useful for such applications (Babajide et al., 2010).

2.4.5 Chemical constituents of other Amaryllidaceae species

Plant species belonging to the Amaryllidaceae family, are herbaceous perennials that grow from bulbous geophytes. The family consists of about sixty genera, with eight hundred species that are widely distributed in many countries around the world. They are cultivated as ornamental plants for their beautiful flowers and for the production of volatile oil (Louw et al., 2002). More than 100 compounds have been isolated from various Amaryllidaceae species since the isolation of the compound lycorine in 1877 from *Narcissus pseudonarcissus* (Cook and Loudon, 1952). The medicinal value of species that are members of the Amaryllidaceae family had been known for centuries but only in the past few decades were the active cytotoxic principles isolated and clinically tested as promising anti-tumor agents (Collins et al., 2010). Recent studies revealed that it is likely that isocarbostyril constituents of the Amaryllidaceae family, such as narciclasine, pancratistatin and their congeners, are the most important metabolites in the folk medical treatment of cancer (Kornienko and Evidente, 2008). Furthermore, Collins et al. (2010) confirmed with new scientific evidence that pancratistatin and narciclasine emerged as the most active compounds, with the corresponding 7-deoxy congeners, 7-deoxypancratistatin and lycoricidine, as being less active**.** According Piozzi et al. (1969) *Narcissus poeticus* L., as used by the ancient Greek physicians, is now known to contain 0.12 g of narciclasine per kg of fresh bulbs.

It has been reported by Heinrich and Teoh (2004) that in recent years, galanthamine was isolated from several members of the Amaryllidaceae

(*Leucojum* spp., *Narcissus* spp., and *Galanthus* spp.) and has become an important therapeutic option to slow down the process of neurological degeneration in Alzheimer's disease. The use of galanthamine was traced back to the Caucasus Mountains (southern Russia) and eastern European countries (esp. Bulgaria) in the treatment of poliomyelitis and ultimately to the recent introduction in the treatment of Alzheimer's disease. It was further reported by Lamoral-Theys et al. (2010) that lycorine displays very promising anti-tumor properties and that the lycorine family of compounds highlights the existence of various potential leads for the development of novel anticancer agents.

Other species such as *Ammocharis coronica* are known to contain biochemicals such as alkaloids and triterpenoids in its bulbs, which are toxic to humans. Instead of oral administration, fresh, wet scales of these bulbs are cooked and used as enemas for blood cleansing or applied topically to open wounds or boils (Louw et al., 2002). A root infusion of *Clivia miniata* is used to treat snakebites and wounds while the leaves are taken by South African women during pregnancy and child birth to induce labor. Bulb decoctions are also used against infertility and urinary complaints (Louw et al., 2002).

Bulbous plants, even though being studied less intensively than herbs and trees for medicinal potential, have proven to contain a unique range of active compounds. Medicinal uses include their analgesic, anticancer, antimutagenic, immuno-stimulatory, anti-infective, antimalarial, cardiovascular and respiratory system effects (Fennel and Van Staden, 2001; Scott, 1993). Traditional uses of some

bulbous species, mainly belonging to the Amaryllidaceae and Hyacinthaceae plant families, could provide useful leads in novel pharmaceutical developments and the biochemical content and pharmacological action of many of these species still remains poorly understood (Hutchings, 1989).

Previous studies revealed anti-inflammatory, antibacterial and antifungal properties in certain plant parts of *G. multifolia* and *G. villosa* and other literature reports endlessly on the traditional uses of plant parts of the genus *Gethyllis*. In this investigation, extracts of natural compounds were prepared from plant parts, purified and characterized, and this data could create a good platform for further research in an attempt to understand the chemistry behind the claims made by traditional healers of the medicinal potential of the genus *Gethyllis*, in specific, *G. multifolia* and *G. villosa*.

REFERENCES

Books, Journal articles, Seminars and Conference proceedings

Ames, B.N., Shigenaga, M.K., Hagen, T.M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences (USA)*, 90: 7915-7922.

Amthor, J.S. (2000). The McCree, De Wit, Penning, De Vries & Thornley Respiration paradigms: 30 years later. *Annals of Botany*, 86: 1-20.

Aruoma, O.I. (2003). Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. *Mutation Research*, 523: 9-20.

Aruoma, O.I., Kaur, H., Halliwell, B. (1991). Oxygen free radicals and human diseases. *Journal of the Royal Society of Health*, 111: 172-177.

Asami, D.K., Hong, Y.J., Barrett, D.M., Mitchell, A.E. (2003). Comparison of the total phenolic and ascorbic acid content of freeze-dried and air-dried marionberry, strawberry and corn grown using conventional, organic and sustainable agricultural practices. *Journal of Agricultural and Food Chemistry*, 51: 1237-1241.

Babajide, J.O., Babajide, O.O., Daramola, A.O., Mabusela, W.T. (2008). Flavonols and an oxychromonol from *Piliostigma reticulatum*. *Phytochemistry*, 690: 2245-2250.

Babajide, J.O. (2009). Chemical and biological investigation into some selected African indigenous medicinal plants. Doctoral thesis, University of the Western Cape, Bellville, South Africa: pp. 218-225.

Babajide, J.O., Mabusela, W.T., Green, I.R., Ameer, F., Weitz, F., Iwuoha, E.I. (2010). Phytochemical screening and biological activity studies of five South African indigenous medicinal plants. *Journal of Medicinal Plants Research*, 4: 1924-1932.

Beecher, G.R. (2003). Overview of dietary flavonoids: Nomenclature, occurrence and intake. *Journal of Nutrition*, 133: 3248-3254.

Beggs, C.J., Kuhn, K., Bocker, R., Wellmann, E. (1987). Phytochrome-induced flavonoid biosynthesis in mustard *Sinapsis alba* L. cotyledons: enzymatic control and differential regulation of anthocyanin and quercitin formation. *Planta*, 172: 121-126.

Benzie, I.F.F., Strain, J.J. (1999). Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods in Enzymology*, 299: 15-27.

Bloom, A.J., Chapin, F.S., Moony, H.A. (1985). Resource limitation in plants - an economic analogy. *Annual Review of Ecology and Systematics*, 16: 363-392.

Boeken, B. (1990). Life histories of two desert species of the bulbous genus, *Bellevalia*: The relation between biomass partitioning and water availability. *Oecologia*, 82: 172-179.

Bolus, L. (1929). Novitates Africanae. *Journal of Botany*, 67: 132-139.

Borrego, F., Canales, I., Lindley, M. (1995). Neohesperidin dihydrochalcone: state of knowledge review. Zeitschrift für Lebensmittel-Untersuchung und - Forschung, 200: 32-37.

Borochov-Neori, H., Judeinstein, S., Greenberg, A., Fuhrman, B., Attias, J., Volkova, N., Hayek, T., Aviram, M. (2008). Phenolic antioxidants and anti-atherogenic effects of marula (*Sclerocarrya birrea* Subsp. *caffra*) fruit juice in healthy humans. *Journal of Agricultural and Food Chemistry*, 56: 9884-9891.

Brooker, F.L., Miller, J.E. (1998). Phenylpropanoid metabolism and phenolic composition of soybean [*Glycine max* (L.) Merr.] leaves following exposure to ozone. *Journal of Experimental Botany*, 49: 1191-202.

Brouwer, R. (1962). Distribution of dry matter in the plant. *Netherlands Journal of Agricultural Sciences*, 10: 361-376.

Cao, G., Alessio, H.M., Cutler, R.G. (1993). Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biology & Medicine*, 14: 303-311.

Cao, G., Prior, R.L. (1998). Comparison of different analytical methods for assessing total antioxidant capacity of human serum. *Clinical Chemistry*, 44: 1309-1315.

Cao, G., Sofic, E., Prior, R.L. (1996). Antioxidant capacity of tea and common vegetables. *Journal of Agricultural and Food Chemistry*, 44: 3426-3431.

Chaudru, V., Lo, M.T., Lesueur, F., Marian, C., Mohamdi, H., Laud, K., Barrois, M., Chompret, A., Avril, M.F., Demenais, F., Paillerets, B.B. (2009). Protective effect of copy number polymorphism of glutathione S-transferase T1 gene on melanoma risk in presence of CDKN2A mutations, MC1R variants and host-related phenotypes. *Familial Cancer*, 112: 1134-1142.

Chaves, M.M., Pereira, J.S., Maroco, J., Roderigues, M.L., Ricardo, C.P.P., Oso´rio, M.L., Carvalho, I., Faria, T., Pinheiro, C. (2002). How plants cope with water stress in the field: Photosynthesis and growth. *Annals of Botany*, 89: 907-916.

Christie, P.J., Alfenito, M.R., Walbot, V. (1994). Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: Enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. *Planta*, 194: 541-549.

Clifford, M. (2000). Chlorogenic acid and other cinnamates – nature, occurrence, dietary burden, absorbtion and metabolism. *Journal of Science and Food Agriculture*, 80: 1033-1043.

Clifford, M. (2001). A nomenclature for phenols with special reference to tea. *Critical Reviews in Food Science and Nutrition*, 41: 393-397.

Collins, J., Rinner, U., Moser, M., Hudlicky, T. (2010). Chemo-enzymatic synthesis of amaryllidaceae constituents and biological evaluation of their C-1 analogs. The next generation synthesis of 7-deoxypancratistatin and dihydrolycoricidine. *Journal of Organic Chemistry*, 75: 3069-3084.

Cook, J.W., Loudon, J.D. (1952). *The alkaloids*, 1st ed. Academic Press, New York, USA: pp. 67.

Coombes, J.S., Powers, S.K., Hamilton, K.L., Demirel, H.A., Shanely, R.A., Zergeroglu, M.A. (2000). Improved cardiac performance after ischemia in aged rats supplemented with vitamin E and alpha-lipoic acid. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*, 279: 2149-2155.

Cowling, R.M., Richardson, D.M., Pierce, S.M. (1986). *Vegetation of southern Africa*, 1st ed. Cambridge University Press, Cambridge, UK: pp. 89.

Cox, B.D., Whichelow, M.J., Prevost, A.T. (2000). Seasonal consumption of salad vegetables and fresh fruit in relation to the development of cardiovascular disease and cancer. *Public Health Nutrition*, 3: 19-29.

Cox, P.A. (1991). *Islands, plants and polynesians: An introduction to Polynesian ethnobotany*. Dioscorides Press, Oregon, USA: pp. 7-9.

Crick, J.C., Grime, J.P. (1987). Morphological plasticity and mineral nutrient capture in two herbaceous species of contrasted ecology. *New Phytologist*, 107: 403-414.

Currin, C.A., Newell, S.Y., Paerl, H.W. (1995). The role of standing dead *Spartina alterniflora* and benthic microalgae in salt marsh food webs: Considerations based on multiple stable isotope analysis. *Marine Ecology Programme Service*, 121: 99-116.

Dafni, A., Cohen, D., Noy-Meir, I. (1981). Life-cycle variation in geophytes. *Annals of the Missouri Botanical Gardens*, 68: 652-660.

Daniels, C.W. (2007). *A comparative study of the propagation and cultivation of Gethyllis multifolia and G. villosa.* Masters thesis, Cape Peninsula University of Technology, Bellville, South Africa: pp. 42-57.

Dixon, R.A., Harrison, M.J., Lamb, C.J. (1994). Early events in the activation of plant defense responses. *Annual Review of Phytopathology*, 32: 479-501.

Dixon, R., Paiva, N. (1995). Stress-induced phenylpropanoid metabolism. *The Plant Cell*, 7: 1085-1097.

Dictionary of natural products, (1996). Release 4:2 (CD289 ROM), Chapman and Hall, London, UK.

Diplock, A.T. (1991). Antioxidant nutrients and disease prevention: An overview. *American Journal of Clinical Nutrition*, 53: 189-193.

Dold, A.P., Cocks, M.L. (2002). The trade in medicinal plants in the eastern Cape Province, South Africa. *South African Journal of Science*, 98: 589-596.

Drewes, F.E., Van Staden, J. (1994). In vitro propagation of *Gethyllis linearis* L. Bol, a rare indigenous bulb. *South Africa Journal of Botany*, 60: 295-296.

Du Plessis, N., Delpierre, G. (1973). Blommeprag uit eie bodem: Koekemakranka. *Landbouweekblad*, 21 Aug: 37-39.

Du Plessis, N., Duncan, G. (1989). *Bulbous plants of southern Africa.* Tafelberg Publishers, Cape Town, South Africa: pp. 104-109.

Duri, Z.J., Scovill, J.P., Huggins, J.W. (1994). Activity of a methanolic extract of Zimbabwean *Crinum macowanii* against exotic RNA viruses in vitro. *Phytotherapy Research*, 8: 121-122.

Eastman, R. (2005). Epilepsy in South Africa. *Acta Neurolica Scandinavica*, 112: 8-11.

Eissenstat, D.M., Van Rees, K.C.J. (1994). The growth and function of pine roots. *Ecology Bulletin*, 43: 76-91.

Elgorashi, E.E., Coombes, P.H., Mulholland, D.A., Van Staden, J. (2007). Isoeugenitol, a cyclo-oxygenase-1 inhibitor from *Gethyllis cilliaris*. *South African Journal of Botany*, 73: 156-158.

Elgorashi, E.E., Van Staden, J. (2004). Pharmacological screening of six Amaryllidaceae species. *Journal of Ethnopharmacology*, 90: 27-32.

Eller, B.M., Grobbelaar, N. (1982). Geophylly: Consequences for *Ledebouria ovatifolia* in its natural habitat. *Journal of Experimental Botany*, 33: 366-375.

Elvin, M. (2000). *Gethyllis.* University of California, Irvine. *Arboretum*, 3: 1-2.

Esler, K.J., Rundel, P.W., Vorster, P. (1999). Biogeography of prostrate-leaved geophytes in semi-arid South Africa: Hypotheses on functionality. *Plant Ecology*, 142: 105-120.

Esler, K.J., Rundel, P.W. (1998). Unusual geophytes of the succulent Karoo: How form can relate to function. *Veld & Flora*, 84: 6-7.

Evans, R.D., (2001). Physiological mechanisms influencing plant nitrogen isotope composition. *Trends in Plant Science*, 6: 121-126.

Fagan, J.M., Bogdan, G., Sleczka, I.S. (1999). Quantitation of oxidative damage to tissue proteins. *The International Journal of Biochemistry & Cell Biology*, 31: 751-757.

Fennell, C.W., Van Staden, J. (2001). *Crinum* species in traditional and modern medicine. *Journal of Ethnopharmacology*, 78: 15-26.

Fowler, Z.L., Shah, K., Panepinto, J.C., Jacobs, A., Koffas, M. (2011) Development of non-natural flavanones as antimicrobial agents. *PLoS ONE* 6: 25681-25686.

Fox, F.W., Norwood-Young, M.E. (1983). *Food from the veld: Edible wild plants of southern Africa.* National Book, Cape Town, South Africa: pp. 67.

Foyer, C.H., Bowsher, C. (2004). Preface to plant carbon-nitrogen interactions from rhizosphere to planet. *Journal of Experimental Botany*, 55: 4-4.

Francis, F.J. (1982). *Analysis of anthocyanins: In anthocyanins as food colors* (P. Markakis, ed.), Academic Press, New York, USA: pp. 182-205.

Francis, F.J. (1989). Food colorants: Anthocyanins. *Critical Reviews in Food Science and Nutrition*, 28: 273-314.

Frankel, E.N., Meyer, A.S. (2000). The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *Journal of the Science of Food and Agriculture*, 80: 1925-1941.

Fridovich, I. (1975). Superoxide dismutase. *Annual Review of Biochemistry*, 44:147-59.

Frontanier, E.J. (1973). Reviewing the length of the generation period and its shortening, particularly in tulips. *Scientia Horticulturae*, 1: 107-116.

Gao, P., Yang, X., Xue, Y.W., Zhang, X.F., Wang, Y., Liu, W.J., Wu, X.J. (2009). Promoter methylation of glutathione S-transferase pi1 and multidrug resistance gene-1 in bronchioloalveolar carcinoma and its correlation with DNA methyltransferase-1 expression. *Cancer*, 115: 3222-3232.

Goldblatt, P., Manning, J. (2000). *Cape plants: A conspectus of the Cape flora of southern Africa*. ABC Press, Epping, Cape Town, South Africa: 25-26.

Goldman, P. (2001). Herbal medicines today and the roots of modern pharmacology. *Annals of Internal Medicine*, 135: 594-600.

Good, R. (1974). *The geography of flowering plants*, 4th ed. Longman, London, UK: pp. 129-133.

Graham, T.L. (1991). Flavonoid and isoflavonoid distribution in developing soybean seedling tissue and in seed and root exudates. *Plant Physiology*, 95: 594-603.

Harborne, J.B. (1994). *The flavonoids, advances in research since 1986*. Chapman & Hall, London, UK: pp. 14-17.

Heinrich, M., Teoh, H.L. 2004. Galanthamine from snowdrop - the development of a modern drug against Alzheimer's disease from local Caucasian knowledge. *Journal of Ethnopharmacology*, 92: 147-162.

Herbinger, K., Tausz, M., Wonisch, A., Soja, G., Sorger, A., Grill, D. (2002). Complex interactive effects of drought and ozone stress on the antioxidant defence systems of two wheat cultivars. *Plant Physiology and Biochemistry* 40: 691-696.

Hertog, M.G.L., Kromhout, D., Aravanis, C. (1995). Flavonoid intake and long-term risk of coronary-heart-disease and cancer in the 7 countries study. *Archives of Internal Medicine*, 155: 381-386.

Hilton-Taylor, C. (1996). *Red data list of southern African plants.* National Biodiversity Institute, Cape Town, South Africa: pp. 57.

Holmgren, M. (2000). Combined effects of shade and drought on tulip poplar seedlings: trade-off in tolerance or facilitation? *Oikos*, 90: 67-78.

Horstmann, A. (1999). The genus *Gethyllis*, a leaf to leaf, or spiral to spiral, account. *The Indigenous Bulb Association of South Africa*, 48: 33-37.

Huang, D., Ou, B., Prior, R.L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*, 53: 1841-1856.

Hutchings, A. (1989). A survey and analysis of traditional medicinal plants as used by the Zulu, Xhosa and Sotho. *Bothalia*, 19: 111-123.

Iovine, N.M., Pursnani, S., Voldman, A., Wasserman, G., Blaser, M.J., Weinrauch, Y. 2008. Reactive nitrogen species contribute to innate host defense against *Campylobacter jejuni*. *Infection and Immunity*, 76: 986-993.

IUCN, 1998. Red list of threatened plants. IUCN, World Conservation Union, Cambridge, UK, pp. 617.

Jacobs, A., Wegewitz, U., Sommerfeld, C., Grossklaus, R., Lampen, A. (2009). Efficacy of isoflavones in relieving vasomotor menopausal symptoms: A systematic review. *Molecular Nutrition and Food Research*, 53: 1084-1097.

Jaksic, F.M., Montenegro, G. (1979). Resource partitioning of Chilean herbs in response to climatic and microclimatic factors. *Oecologia*, 40: 81-89.

Jood, S., Kapoor, A.C., Singh, R. (1995). Polyphenol and phytic acid contents of cereal grains as affected by insect infestation. *Journal of Agricultural and Food Chemistry*, 43: 435-438.

Kähkönen, M.P., Hopia, A.I., Vuorela, H.J., Rauha, J.P., Pihlaja, K., Kujala, T.S., Heinonen, M. (1999). Antioxidant activity of plant extracts containing phenolic compounds. *Journal of Agricultural and Food Chemistry*, 47: 3954-3962.

Kamatou, G.P.P., Viljoen, A.M., Őzek, T., Başer, K.H.C., (2008). Head-space volatiles of *Gethyllis afra* and *G. ciliaris* fruits ("kukumakranka"). *South African Journal of Botany*, 74: 768-770.

Kim, J.Y., Mahe´, A., Brangeon, J., Prioul, J.L. (2000). A maize vacuolar invertase, IVR2, is induced by water stress: Organ/ tissue specificity and diurnal modulation of expression. *Plant Physiology*, 124: 71-84.

Kirakosyan, A., Seymor, E., Kaufman, P.B., Warber, S., Bolling, S., Chang, S.C. (2003). Antioxidant capacity of polyhenolic extracts from leaves of *Crataegus laevigata* and *C. monogyna* (hawthorn) subjected to drought and cold stress. *Journal of Agricultural and Food Chemistry*, 51: 3973-3976.

Kornienko, A., Evidente, A. (2008). Chemistry, biology, and medicinal potential of narciclasine and its congeners. *Chemical Reviews*, 108: 1982-2014.

Kurganova, L.N., Veselov, A.P., Goncharova, T.A., Sinitsyna, Y.V. (1997). Lipid peroxidation and antioxidant system protection against heat shock in pea (*Pisum sativum* L.) chloroplasts. *Russian Journal of Plant Physiology*, 44: 725-30.

Lamoral-Theys, D., Decaestecker, C., Mathieu, V., Dubois, J., Kornienko, A., Kiss, R., Evidente, A., Pottier, L. (2010). Lycorine and its derivatives for anticancer drug design. *Mini-Reviews in Medicinal Chemistry*, 10: 41-50.

Langseth, L., (1995). *Oxidants, antioxidants and disease prevention*. International Life Sciences Institute, ILSI Press, Washington, D.C., USA: pp. 7-8.

Lighton, C. (1992). The kukumakranka. *Veld & Flora*, 78: 100-103.

Liltved, W.R. (1992). The kukumakranka, past and present. *Veld & Flora*, 78 (4): 104-106.

Lin, K., Chao, P., Yang, C., Cheng, W., Lo, H., Chang, T. (2006). The effects of flooding and drought stress on the antioxidant constituents in sweet potato leaves. *Botanical Studies*, 47: 417-426.

Lin, R.C., Guthrie, S., Xie, C.Y., Mai, K., Lee, D.Y.W., Lumeng, L., Li, T.K. (1996). Isoflavonoid compounds extracted from *Pueraria lobata* suppress alcohol preference in a pharmacogenetic rat model of alcoholism. *Alcoholism: Clinical and Experimental Research*, 20: 659-663.

Liu, F., Andersen, M.N., Jensen, C.R. (2003). Loss of pod set caused by drought stress is associated with water status and ABA content of reproductive structures in soybean. *Functional Plant Biology*, 30: 271-280.

Liu, Y., Wang, M.W. (2008). Botanical drugs: challenges and opportunities - Contribution to Linnaeus Memorial Symposium 2007. *Life Sciences*, 82: 445-449.

Lombard, A.T., Hilton-Taylor, C., Rebelo, A.G., Pressey, R.L., Cowling, R.M. (1999). Reserve selection in the succulent Karoo, South Africa: Coping with high compositional turnover. *Plant Ecology*, 142: 35-55.

Louw, C.A.M., Regnier, T.J.C., Korsten, L., (2002). Medicinal bulbous plants of South Africa and their traditional relevance in the control of infectious diseases. *Journal of Ethnopharmacology*, 82: 147-154.

Maberly, S.C., Raven, J.A., Johnston, A.M. (1992). Discrimination between ^{12}C and ^{13}C by marine plants. *Oecologia*, 91: 481-492.

Macheix, J.J., Fleuriet, A. (1998). *Phenolic acids in fruit*. In: Rice-Evans, C., Packer. L., eds. *Flavonoids in health and disease*. Marcel Dekker Inc., New York, USA, pp. 35-59.

Macheix, J.J., Fleuriet, A., Billot, J. (1990). *Fruit phenolics*. CRC Press, Boca Raton, Florida, USA.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., Jiménez, L. (2004). Polyphenols: Food sources and bioavailability. *American Society of Clinical Nutrition*, 79: 727-747.

Manning, J., Goldblatt, P., Snijman, D. (2002). *The color encyclopedia of cape bulbs*. Timber Press, Cambridge, UK: pp. 181-185.

Martens, S., Mithöfer, A. (2005). Flavones and flavone synthases. *Phytochemistry*, 66: 2399-2407.

Mathiesen, L., Malterud, K.E., Sund, R.B. (1997). Hydrogen bond formation as basis for radical scavenging activity: A structure activity study of C-methylated dihydrochalcones from *Myrica gale* and structurally related acetophenones. *Free Radical Biology and Medicine*, 22: 307-311.

Mazza, G., Fukumoto, L., Delaquis, P., Girard, B., Ewert, B. (1999). Anthocyanins, phenolics and color of Cabernet Franc, Merlot and Pinot Noir wines from British Columbia. *Journal of Agriculture and Food Chemistry*, 47: 4009-4017.

McLaughlin, J.L., Chang, C.J. and Smith, D.L. (1991). *Bench – top bioassay for the discovery of bioactive natural products. An update.* Attamra (Ed), *Studies in natural product chemistry*, Elsevier science publishers, Amsterdam, pp. 383 - 409.

Miller, J.N., Rice-Evans, C.A., Davies, M.J., Gopinathan, V., Milner, A. (1993). A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical Science*, 84: 407-412.

Müller-Doblies, D. (1986). Enumeration. *Willdenowia,* 15: 465-471.

Munne-Bosch, S., Mueller, M., Schwarz, K., Alegre, L. (2001). Diterpenes and antioxidative protection in drought stressed *Salvia officinalis* plants. *Plant Science,* 158: 1431-1437.

Munne-Bosch, S., Penuelas, J. (2004). Drought-induced oxidative stress in strawberry tree (*Arbutus unedo* L.) growing in mediterranean field conditions. *Plant Science*, 166: 1105-1110.

Nakamura, Y., Watanabe, S., Miyake, N., Kohno, H., Osawa, T.J. (2003). Dihydrochalcones: Evaluation as novel radical scavenging antioxidants. *Journal of Agricultural and Food Chemistry*, 51: 3309-3312.

Nakasugi, T., Komai, K. (1998). Antimutagens in the Brazilian folk medicinal plant Carqueja (*Baccharis trimera* Less.). *Journal of Agricultural and Food Chemistry*, 46: 2560-2564.

Neuwinger, H.D. (1996). *African ethnobotany: Poisons, drugs, chemistry, pharmacology and toxicology*. Chapman and Hall, Germany: pp. 69-73.

Nicolas, P., Pucciariello, C., Mandon, K., Innocenti, G., Jamet, A., Baudouin, E., Hérouart, D., Frendo, P., Puppo, A. (2006). Reactive oxygen and nitrogen species and glutathione: key players in the legume - *Rhizobium* symbiosis. *Journal of Experimental Botany*, 57: 1769-76.

Nielsen, K.L., Eshel, A., Lynch, J.P. (2001). The effect of phosphorus availability on the carbon economy of contrasting common bean (*Phaseolus vulgaris* L.) genotypes. *Journal of Experimental Botany*, 52: 329-339.

Nöthlings, U., Murphy, S., Wilkens, R., Boeing, H., Schulze, M., Bueno-de-Mesquita, H., Michaud, D., Roddam, A., Rohrmann, S., Tjønneland, A., Clavel-Chapelon, F., Trichopoulou, A., Sieri, S., Rodriguez, L., Ye, W., Jenab, M., Kolonel, L. (2008). A food pattern that is predictive of flavonol intake and risk of pancreatic cancer [1,2,3]. *American Journal of Clinical Nutrition*, 88: 1653-1662.

Noy-Meir, I, (1973). Desert ecosystems: environment and producers. *Annual Review Ecology and Systematics*, 4: 25-51.

Ogungbamila, F.O. (1993). *Traditional healing methods in the control and treatment of infectious diseases*. Proceedings of the National Workshop, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria, 21-23 January: pp. 100.

Orians, G.H., Solbrig, O.T. (1977). A cost-income model of leaves and roots with special reference to arid and semi-arid areas. *American Midrand Naturalist*, 111: 677-690.

Osmond, C.B., Grace, S.C. (1995). Perspectives on photo-inhibition and photorespiration in the field: quintessential inefficiencies of the light and dark reactions of photosynthesis? *Journal of Experimental Botany*, 46: 1351-62.

Ou, B., Hampsch-Woodill, M., Prior, R.L. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry*, 49: 4619-4626.

Ou, B., Huang, D., Hampsch-Woodill, M., Flanagan, J.A., Deemer, E.K. (2002). Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) Assays: A comparative study. *Journal of Agricultural and Food Chemistry*, 50: 3122-3128.

Packer, L., Glazer, A.N. (1990). *Methods in enzymology: oxygen radicals and antioxidants* (Vol. 186, Part B). Academic Press, San Diego, California, USA: pp. 136.

Parr, A.J., Bolwell, G.P. (2000). Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *Journal of the Science of Food and Agriculture*, 80: 985-1012.

Pate, J.S., Dixon, K.W. (1981). Plants with fleshy underground storage organs - A Western Australian survey. Pate, J.S., McComb, A.J. (eds.), *The biology of Australian plants*. University of Western Australia Press: 181-215.

Pelleschi, P., Rocher, J.P., Prioul, J.L. (1997). Effect of water restriction on carbohydrate metabolism and photosynthesis in mature maize leaves. *Plant, Cell and Environment*, 20: 493-503.

Phillips, D.A. (1980). Efficiency of symbiotic nitrogen fixation in legume. *Annual Review of Plant Physiology*, 31: 29-49.

Piozzi, F., Marino, L., Fuganti, C., Di Martino. A. (1969). Occurrence of non-basic metabolites in amaryllidaceae. *Phytochemistry*, 8: 1745-1748.

Poorter, H., Nagel, O.W. (2000). The role of biomass allocation in the growth response of plants to different levels of light, CO_2, nutrients and water: A quantitative review. *Australian Journal of Plant Physiology*, 27: 595-607.

Powers, S.K., Smuder, A.J., Kavazis, A.N., Hudson, M.B. (2010). Experimental guidelines for studies designed to investigate the impact of antioxidant supplementation on exercise performance. *International Journal of Sport Nutrition and Exercise Metabolism*, 20: 2-14.

Preece, J.E., Read, P.E. (2005). *The biology of Horticulture - An introductory textbook*, 2nd ed. John Wiley & Sons, New Jersey, USA: pp. 265-266**.**

Price, A.H., Atherton, N., Hendry, G.A.F. (1989). Plants under drought stress generated activated oxygen. *Free Radical Research Communications*, 8: 61-66.

Prior, R.L., Hoang, H., Gu, L., Wu, X., Bacchiocca, M., Howard, L., Hampsch-Woodill, M., Huang, D., Ou, B., Jacob, R. (2003). Assays for hydrophilic and lipophilic antioxidant capacity ($ORAC_{FL}$) of plasma and other biological and food samples. *Journal of Agricultural and Food Chemistry*, 51: 3273-3279.

Proches, S., Cowling, R.M. (2004). *Cape geophytes: Putting the pieces together*. Proceedings of the 10th MEDECOS Conference. Millpress, Rotterdam, Netherlands: pp. 1-10.

Pulido, R., Bravo, L., Saura-Calixto, F. (2000). Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *Journal of Agricultural and Food Chemistry*, 48: 3396-3402.

Quideau, S., Deffieux, C., Douat-Casassus, D., Pouységu, L. (2011). Plant polyphenols: chemical properties, biological activities, and synthesis. *Angewandte Chemie International Edition*, 50: 586-621.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans C. (1999). Antioxidant activity applying an improved ABTS radical cation assay. *Free Radical Biology & Medicine*, 26: 1231-1237.

Retivin, V., Opritov, V., Fedulina, S.B. (1997). Generation of action potential induces preadaptation of *Cucurbita pepo* L. stem tissues to freezing injury. *Russian Journal of Plant Physiology*, 44: 499-510.

Rimm, E.B., Katan, M.B., Ascerio, A. (1996). Relation between intake of flavonoids and risk of coronary heart disease in male health professionals. *Annals of Internal Medicine*, 125: 384-389.

Rood, B. (1994). *Uit die veldapteek*. Tafelberg Publishers, Cape Town, South Africa: pp. 4.

Rossa, B., Von Willert, D.J. (1999). Plant form and function: Physiological characteristics of geophytes in semi-arid Namaqualand, South Africa. *Plant Ecology*, 142: 121-132.

Rousset, O., Lepart, J. (2000). Positive and negative interactions at different life stages of a colonizing species (*Quercus humilis*). *Journal of Ecology*, 88: 401-412.

Ruiters, C. (1995). Biomass and recource allocation patterns within the bulb of the perennial geophyte *Haemanthus pubescens* L. subsp. *pubescens* (amaryllidaceae) in a periodic arid environment of lowland fynbos, South Africa. *Journal of Arid Environments*, 31: 311-323.

Ryan, M.G., Lavigne, M.B., Gower, S.T. (1997). Annual carbon cost of autotrophic respiration in boreal forest ecosystems in relation to species and climate. *Journal of Geophysical Research*, 102: 28871-28883.

SANBI. (2009). *Red list of South African plants*. Strelitzia 25, South African National Biodiversity Institute, Claremont, Cape Town, South Africa.

Sarda, S.R., Pathan, M.Y., Paike, V.V., Pachmase, P.R., Jadhav, W.N., Pawar, R.P. (2006). A facile synthesis of flavones using recyclable ionic liquid under microwave irradiation. *Arkivoc*, 16: 43-48.

Schnall, J.A., Quatrano, R.S. (1992). Abscisic acid elicits the water-stress response in root hairs of *Arabidopsis*. *Plant physiology*, 100: 216-218.

Schussler, J.R., Westgate, M.E. (1991). Maize kernel set at low water potential. II. Sensitivity to reduced assimilates at pollination. *Crop Science*, 31: 1196-1203.

Schussler, J.R., Westgate, M.E. (1995). Assimilate flux determines kernel set at low water potential in maize. *Crop Science*, 35: 1126-1134.

Scott, G. (1993). Medicinal and aromatic plants. Healthcare economics and conservation in South Africa. *Veld & Flora*, 79: 84-87.

Sesso, H.D., Paffenbarger, R.S., Oguma, Y., Lee, I.M. 2003. Lack of association of tea and cardiovascular disease in college alumni. International Journal of Epidemiology, 32: 527-533.

Shah, A., Khan, A.M., Qureshi, R., Ansari, F.L., Nazar, M. F., Shah, S.S. (2008). Redox behavior of anticancer chalcone on a glassy carbon electrode and evaluation of its interaction parameters with DNA. *International Journal of Molecular Sciences*, 9: 1424-1434.

Shapera, I. (1930). *The khoisan peoples of South Africa: Bushmen and hottentots*. Routledge and Kegan Paul Ltd., London, UK: pp. 24.

Shetty, K. (2004). Role of proline-linked pentose phosphate pathway in biosynthesis of plant phenolics for functional food and environmental applications: A review. *Process Biochemistry*, 39: 789-803.

Silalahi, J. (2002). Anticancer and health protective properties of citrus fruit components. *Asia Pacific Journal of Clinical Nutrition*, 11: 79-84.

Singh, J., Singh, A.K., Pravesh, R. (2003). *Production and trade potential of some important medicinal plants: An overview.* In: Proceedings of the First National Interactive Meeting on medicinal and aromatic plants, CIMAP, Lucknow, India, pp. 50-58.

Smith, C.A. (1966). *Common names of South African plants. Memoirs of the botanical survey of South Africa*, vol. 35. Department of Agricultural and Technical Services, The Government Printer, Pretoria: pp. 44.

Smith, T., Huston, M., (1989). A theory of the spatial and temporal dynamics of plant communities. *Vegetatio*, 83: 49-69.

Sofo, A., Dichio, B., Xiloyannis, C., Masia, A. (2004). Effects of different irradiance levels on some antioxidant enzymes and on malondialdehyde content during re-watering in olive trees. *Plant Science*, 166: 293-302.

Sohal, R.S. (2002). Role of oxidative stress and protein oxidation in the aging process. *Free Radical Biology and Medicine*, 33: 37-44.

Sosulski, F., Krygier, K., Hogge, L. (1982). Free esterified, and insoluble-bound phenolic acids: Composition of phenolic acids in cereal and potato flour. *Journal of Agricultural and Food Chemistry*, 30: 337-340.

Stadtman, E.R. (1995). Role of oxidized amino acids in protein breakdown and stability. *Methods in Enzymology*, 258: 379-393.

Starke-Reed, P., Oliver, C.N. (1989). Protein oxidation and proteolysis during aging and oxidative stress. *Archives of Biochemistry and Biophysics*, 257: 559-567.

Story, R. (1964). Plant lore of the Bushmen. Davies, D.H.S. (ed.), *Ecological studies in Southern Africa*. W. Junk, The Hague: pp. 87-99.

Strandhagen, E., Hansson, P.O., Bosaeus, I., Isaksson, B., Eriksson, H. (2000). High fruit intake may reduce mortality among middle-aged and elderly men: The study of men born in 1913. *European Journal of Clinical Nutrition*, 54: 337-341.

Szliszka, E., Czuba, Z., Mazur, B., Paradysz, A., Krol, W. (2010). Chalcones and dihydrochalcones Augment TRAIL-Mediated Apoptosis in prostate cancer cells. *Molecules*, 15: 5336-5353.

Tanaka, J. (1978). A study of the comparative ecology of African gatherer-hunters with special reference to San (Bushman-speaking people) and Pygmies. *Senri Ethnological Studies*, 1: 189-212.

Tattini, M., Galardi, C., Pinelli, P., Massai, R., Remorini, D., Agati, G. (2004). Differential accumulation of flavonoids and hydroxycinnamates in leaves of *Ligustrum vulgare* under excess light and drought stress. *New Phytologist*, 163: 547-561.

Thaipong, K., Boonprakop, U., Crosby, K., Cisneros-Zevallos, L., Byrne, D.H. (2006). Comparison of ABTS, DPPH, FRAP and ORAC assays for estimating antioxidant activity from guava fruit extracts. *Journal of Food Composition and Analysis*, 19: 669-675.

Townsend, R., Viljoen, D. (1997). *Collection of Gethyllis multifolia and G. villosa bulbs from their natural habitats*. Karoo Desert National Biodiversity Garden, Worcester, South Africa, Unpublished data: pp. 4.

Tubaro, F., Micossi, E., Urini, F. (1996). The antioxidant capacity of complex mixture by kinetic analysis of crocin bleaching inhibition. *Journal of the American Oil Chemists Society*, 73: 567-574.

Valliyodan, B., Nguyen, H.T. (2006). Understanding regulatory networks and engineering for enhanced drought tolerance in plants. *Plant Biology*, 9: 189-195.

Van der Walt, P. (2003). Koekemakranka. *Wild en Jag*, 4: 16-17.

Van Reenen, C.J. (1975). *Morphological and embryological studies of the genus Gethyllis*. Unpublished Masters thesis, University of Stellenbosch, Stellenbosch, South Africa: pp. 63.

Van Wyk, B., De Wet, H., Van Heerden, F.R. (2008). An ethnobotanical survey of medicinal plants in the south-eastern Karoo, South Africa. *South African Journal of Botany*, 74: 696-704.

Van Wyk, B., Gericke, N., (2000). *People's plants: A guide to useful plants of Southern Africa*, 1st ed. Briza Publications, Pretoria, South Africa: pp. 169.

Van Wyk, B., Van Oudtshoorn, B., Gericke, N. (1997). *Medicinal plants of South Africa*. Briza Publications, Pretoria, South Africa: pp. 220.

Vartanian, N., Marcotte, L., Ciraudat, J. (1994). Drought rhizogenesis in *Arbidopsis thaliana*, different responses of hormonal mutants. *Plant Physiology*, 104: 761-767.

Viladomat, F., Bastida, J., Codina, C., Nair, J.J., Campbell, W. (1997). Alkaloids of the southern African amaryllidaceae. *Recent Research Development in Phytochemistry*, 1: 131-171.

Vita, J.A. (2005). Polyphenols and cardiovascular disease: Effects on endothelial and platelet function. *American Journal of Clinical Nutrition*, 81: 292-297.

Vogel, S., Barbic, M., Jurgenliemk, G., Heilmann, J. (2010). Synthesis, cytotoxicity, anti-oxidative and anti-inflammatory activity of chalcones and influence of A-ring modifications on the pharmacological effect. *European Journal of Medicinal Chemistry*, 45: 2206-2213.

Von Willert, D.J., Eller, B.M., Werger, M.J.A., Brinckmann, E., Ihlenfeldt, H.D. (1992). *Life strategies of succulents in deserts*. Cambridge University Press, Cambridge, UK: pp. 69.

Vosa, C. (1986). Chromosome studies in the genus *Gethyllis* (Amaryllidaceae). *Caryologia*, 39: 251-257.

Wang, H., Cao, G., Prior, R.L., (1996). Total antioxidant capacity of fruits. *Journal of Agricultural and Food Chemistry*, 44: 701-705.

Wang, S.Y., Lin, H. (2000). Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. *Journal of Agricultural and Food Chemistry*, 48: 140-146.

Wang, F.Z., Wang, Q.B., Kwon, S.Y., Kwak, S.S., Su, W.A. (2005). Enhanced drought tolerance of transgenic rice plants expressing a pea manganese superoxide dismutase. *Journal of Plant Physiology*, 162: 465-472.

Waterhouse, A. (2005). *Folin-Ciocalteau method for total phenol in wine*. Department of Viticulture & Enology, University of California, Davis, USA.

Watt, J.M., Breyer-Brandwijk, M.G. (1962). *The Medicinal and poisonous plants of southern and eastern Africa*, 2nd ed. Livingstone, London, UK: pp. 32.

WHO/FAO. (2003). *WHO Technical report series: Joint WHO/FAO expert consultation on diet, nutrition, and the prevention of chronic diseases, 28 January - 1 February 2002*. WHO Publications, Geneva, Switzerland.

Willcox, J.K., Ash, S.L., Catignani, G.L. (2004). Antioxidants and prevention of chronic disease. *Critical Reviews in Food Science and Nutrition*, 44: 275-295.

Witkowski, E.T.F. (1989). Response to nutrient additions by the plant growth forms of sand-plain lowland fynbos, South Africa. *Vegetatio*, 79: 89-97.

Xiong, L., Wang, R., Mao, G., Koczan, J.M. (2006). Identification of drought tolerance determinants by genetic analysis of root response to drought stress and abscisic acid. *Plant Physiology*, 142: 1065-1074.

Yalpani, N., Enyedi, A.J., Leon, J., Raskin, I. (1994). Ultraviolet light and ozone stimulate accumulation of salicylic acid pathogenesis-related proteins and virus resistance in tobacco. *Planta*, 193: 372-376.

Young, I.S., Woodside, J.V. (2001). Antioxidants in health and disease. *Journal of Clinical Pathology*, 54: 176-186.

Zheng, W., Wang, S.Y. (2001). Antioxidant activity and phenolic compounds in selected herbs. *Journal of Agricultural and Food Chemistry*, 49: 5165-5170.

Zhishen, J., Mengcheng, T., Jianming, W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry*, 64: 555-559.

Zimmerman, Y.Z., Cohill, P.R. (1991). Heat shock and thermotolerance in plant and animal embryogenesis. *New Biology*, 3: 641-50.

Zulueta, A., Esteve, M., Frigola, A. (2009). ORAC and TEAC assays comparison to measure the antioxidant capacity of food products. *Food Chemistry*, 114: 310-316.

Internet references

Buckley, G. (1999). *Confronting God and talking about Gethyllis.* [Online]. Available http://www.suite101.com/article.cfm/3061/15638 [Accessed 16 February 2008]

Cancerquest. (2011). *The basic chemical structure of anthocyanins*. [Online]. Available http://www.cancerquest.org/cancer-prevention-anthocyanin.html [Accessed 21 April 2011]

Environment, South Africa, (2010). *The succulent Karoo and adjacent biomes.* [Online]. Available http://www.ekapa.ioisa.org.za/biomes/succulentkaroo.html [Accessed 13 June 2011]

Malan, K. (2000). *Where can I find Ammocharis, Gethyllis, Lycoris, Polyxena and Whiteheadia?* IBS Discussion Forums: Swap Column. [Online]. Available http://www.bulbsociety.org/Forum/messages/1/84.html [Accessed 3 July 2007]

Natuurlijkerwijs. (2010). *Alles over kruiden, voedingscomponenten en het metabolisme*. [Online] Available http://www.natuurlijkerwijs.com/html [Accessed 15 March 2011]

Natuurlijkerwijs. (2011). *The basic chemical structure of isoflavones*. [Online]. Available http://www.natuurlijkerwijs.com/english/flavonoids/htm [Accessed 25 June 2011]

Pacific Bulb Society. (2004). *Gethyllis*. [Online]. Available http://www.pacificbulbsociety.org/pbswiki/index/php/gethyllis/html [Accessed 24 August 2007]

PR WEB. (2005). Jobelyn, African wonder herb set to rule the world of natural plant antioxidants and phenolics. [Accessed February 29, 2008] http://www.prweb.com/pdfdownload/312415.pdf

Saunders, R. (2004). *Gethyllis-TOW*. [Online]. Available http://www.lists.ibiblio.org/pipermail/pbs/2004-july/007480.html [Accessed 17 March 2007]

Snijman, D. (2004). *Gethyllis*. [Online]. Available http://www.plantzafrica.com/plantefg/gethyllis.htm [Accessed 25 January 2007]

UC Health. (2010). *The good drug guide*. California: The University of California. [Online]. Available http://www.universityofcalifornia.edu/sites/uchealth/html

World Health Organisation. (2005). *World malaria report*. [Online]. Available http://rbm.who.int/wmr2005/pdf/adve.pdf [Accessed 25 October 2010]

CHAPTER 3: RESEARCH PAPER 1 - A comparative drought- and -shade tolerance analysis of two semi-arid desert bulb species of *Gethyllis* (Kukumakranka)

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RESEARCH PAPER 1

A comparative drought- and -shade tolerance analysis of two semi-arid desert bulb species of *Gethyllis* (Kukumakranka)

CW Daniels[a], WT Mabusela[b], JL Marnewick[c], AJ Valentine[d]

[a]Department of Horticultural Sciences, Cape Peninsula University of Technology, P.O. Box 1906, Bellville 7535, South Africa.

[b]South African Herbal Science and Medicine Institute, University of the Western Cape, Private Bag X17, Bellville 7535, South Africa.

[c]Oxidative Stress Research Centre, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, P.O. Box 1906, Bellville 7535, South Africa.

[d]Department of Botany and Zoology, University of Stellenbosch, Private Bag X1, Matieland 7602, Stellenbosch, South Africa.

Abstract

Gethyllis multifolia and *G. villosa* are winter-growing, summer-blooming, deciduous and bulbous geophytes that grow naturally in the semi-arid succulent Karoo biome of South Africa. *Gethyllis multifolia* is threatened in its natural habitat and is listed in the 'Vulnerable' category of the 'Red Data List of Southern

African Plants'. Observations in a previous investigation of these two species suggested that *G. multifolia* suffers more during drought stress than *G. villosa* and that both species adopted certain morphological changes in their leaves under shade conditions. Both species have adapted to the wet and cold winters and summer aridity of this biome but current models are showing that this biome is exposed to increasingly drier conditions and shade from invasive plant species due to global warming and ecosystem change, and as a consequence is losing its current distribution of flora.

In this investigation, experiments were conducted on the photosynthetic rate (P_{max}), stomatal conductance (G_s), transpiration rate (E), photosynthetic water-use efficiency (PWUE), specific leaf mass (SLM) and light compensation points (LCP), to elucidate responses of both species to ecosystem change. It was found that during drought stress, *G. villosa* had a better photosynthetic performance than *G. multifolia* which appears not to be related to foliar adaptations such as specific leaf mass (SLM), but to *G. villosa*'s leaves maintaining their stomatal conductance (G_s), photosynthetic light compensation (LCP) and photon yields. This study further revealed a better performance for *G. villosa* under low light conditions whereby *G. villosa* did not change its photosynthetic light compensation point (LCP) during shade and was able to synthesize more chlorophyll *b* for light harvesting than *G. multifolia*, hence, a higher net carbon gain. It can be concluded that the inability of *G. multifolia* to adapt to a wider range of environmental extremes, may be a contributing factor to its threatened conservation status.

Keywords: *Drought tolerance; Gethyllis; Kukumakranka; Photosynthesis; Shade tolerance; Succulent Karoo biome.*

1. Introduction

The genus *Gethyllis* (family: Amaryllidaceae), indigenous to South Africa, consists of 37 currently accepted species and subspecies (Müller-Doblies, 1986). *Gethyllis* species have medicinal properties which range from cures for colic, digestive disturbances, teething problems, fatigue, boils, bruises and insect bites, to being used as an aphrodisiac (Liltved, 1992; Elgorashi and Van Staden, 2003). The plants have four distinct growth phases, thrive under full sun conditions, and are classified as winter-growing, summer-blooming, deciduous and bulbous geophytes (Du Plessis and Delpierre, 1973, Manning et al., 2002). *Gethyllis multifolia* L. Bolus and *G. villosa* Thunb. grow naturally in the succulent Karoo biome of South Africa, which is primarily characterized by low to high winter rainfall and extreme summer aridity. The rainfall varies between 20 and 290 mm per year and during summer the temperatures can be in excess of 40 ºC. *Gethyllis multifolia* is threatened in its natural habitat and is listed in the 'Vulnerable' category of the 'Red Data List of Southern African Plants' and 'World Conservation Union List of Plants' (Hilton-Taylor, 1996; IUCN-World Conservation Union, 1998), whilst *G. villosa* is not threatened in the same habitats. In their natural habitat, both these species encounter environmental limitations such as increasing drought stress (Rutherford et al., 1999; Midgeley et

al., 2002; Von Maltitz et al., 2006) and light restrictions due to shading caused by encroaching indigenous shrubs (Daniels, 2007).

It has been observed that both *G. multifolia* and *G. villosa* are limited by drought stress and this limiting factor appears to have a more significant effect on *G. multifolia* (Daniels, 2007). According to preliminary work by Daniels (2007), *G. multifolia* has impaired leaf and flower development during the growth and reproductive phases when exposed to dry conditions (Daniels, 2007). These features are part of a survival strategy during harsh environmental changes (Du Plessis and Duncan, 1989). This concurs with studies on soybeans that drought stress increases the rate of pod abortion during the early stages of pod development (Liu et al., 2003). The success of plants under stress conditions may be determined by their ability to control carbohydrate utilisation for metabolic energy and their ability to allocate enough materials to their reproductive phase (Nielsen et al., 2001). According to Pelleschi et al. (1997) and Kim et al. (2000), drought stress generally decreases the photosynthetic rate and disrupts carbohydrate metabolism in leaves, and therefore could increase the rate of reproductive abortion.

In addition to drought stress, *Gethyllis* plants may also encounter light stress in the form of shading from encroaching indigenous shrubs (Daniels, 2007). This encroachment from the invasive shrub, *Galenia africana*, is also posing a growing threat in the natural habitat of *G. multifolia*, where many plants are found growing in the shade of this 1-1.5 meter tall, shrubby species (Daniels, 2007; Klaasen et

al., 2009). A previous investigation (Daniels, 2007) revealed that under controlled shade conditions both species produced thinner leaves with no natural spiralling, which is a departure from the natural characteristic of *Gethyllis* species (Esler and Rundel, 1998). The survival and growth of *Gethyllis* species in a changing light environment may be dependent on their shade-tolerance levels, as found for other plants (Daniels et al., 1979; Lorimer, 1983). This concurs with previous work that shade stressed plants can respond to low irradiance via altered leaf morphology for more efficient irradiance capture (Givnish, 1988; Smith and Huston, 1989; Oliver and Larson, 1996) and enhanced photosynthetic investment (Brouwer 1962; Poorter and Nagel 2000).

Since both bulbous species occur in a semi-arid area, which is being threatened by increasing aridity and shading from invasive species, the aim of this investigation was therefore to investigate the capacity for photosynthetic adaptation of both species to these environmental changes.

2. Materials and methods

2.1. Plant materials

Mature *G. multifolia* and *G. villosa* bulbs of equal size were identified and obtained with permission from the Karoo National Biodiversity Garden (KNBG) (Worcester, Western Cape, South Africa) after their winter growth phase (March to mid-August), from their natural habitat (Worcester), where new roads and sewerage lines were planned through part of an existing population. *Gethyllis multifolia* is threatened in its natural habitat, as already mentioned, and for

conservation purposes the exact location of these plants are omitted from this investigation. The bulbs of both species (n=10 per species per treatment) were potted up in 15 cm nursery pots in sandy, clay soil (pH 4.3-4.4) from the natural habitat. The plants were grown under outdoor conditions for 12 months which included one dormant phase (6 months - spring and summer) and one growth phase (6 months - autumn and winter) at the nursery of the Department of Horticultural Sciences, Cape Peninsula University of Technology (CPUT), Cape Town. Table 1 indicates the average rainfall and daily temperatures for the Cape Town area where plants were grown and also indicates the higher rainfall and lower temperatures for the growth phase and lower rainfall and higher temperatures for the dormant phase. Weather data for the Cape Town area were supplied by the South African Weather Service.

Table 1

The average rainfall (mm) and average daily minimum/maximum temperatures (ºC) for the Cape Town area (Western Cape, South Africa) for the years 2006 and 2007. Each value was obtained by calculating the mean of the two years ± SE. The data were provided by the South African Weather Service and were recorded at the Cape Town weather office.

MONTH	MEAN MIN.TEMP.	MEAN MAX. TEMP.	MEAN RAINFALL
January	17.3(±0.35)	27.9(±0.25)	0.3(±0.2)
February	16.4(±0.15)	27.1(±0.65)	20.2(±14.2)
March	14.2(±0.05)	25.9(±0.55)	11.7(±8.2)
April	12.4(±0.4)	23.4(±0.6)	47.9(±33.8)
May	9.5(±0.2)	20.5(±0.6)	108.9(±77.0)
June	8.1(±0)	19.0(±1.1)	78.8(±55.6)
July	7.9(±0.9)	17.3(±0.35)	111.5(±78.8)
August	8.1(±0.15)	17.8(±0.05)	78.9(±55.8)
September	9.8(±0.55)	20.4(±0.55)	19.1(±13.5)
October	11.7(±0.4)	22.9(±0.45)	27.9(±19.8)
November	13.4(±0.55)	23.4(±1.2)	39.3(±27.8)
December	15.6(±0.15)	25.7(±0.65)	14.3(±10.1)

2.2. Environmental stresses

Plant samples which represented the control (n=10 per species) were grown under full sun and irrigated by the ambient rainfall of the Western Cape (Table 1). The mean photosynthetic photon flux density (PPFD) (converted from lux to PPFD) on cloudless days at 12h00 was 1825±63 µmol $m^{-2}.s^{-1}$ during the winter growth period of both species. Temperatures around the plant samples varied from 8 to 24 ºC and the relative humidity from 36 to 100%. The PPFD for all treatments was measured three times per day with a Toptronic T630 digital lux meter (Spraytech, Bellville, Western Cape, South Africa) and the temperatures and relative humidity were measured with a Majortech MT669 digital relative humidity/temperature meter (Spraytech, Bellville, Western Cape, South Africa).

Plants in the drought stress experiment (n=10 per species) were subjected to full sunlight and covered with a 6 mm clear glass sheet, placed 300 mm above the plants. The PPFD, temperature and relative humidity environmental conditions were similar to those of the control. The drought stressed plants were irrigated at a rate of 30% field capacity once a month with de-ionized water (Mortimer et al., 2003).

Plants in the shade stress experiment (n=10 per species) were grown under a shade structure covered with 80% neutral black shade cloth (Alnet, Epping, Western Cape, South Africa), which has a neutral effect on light quality (Yates, 1989; Duan et al., 2005). During the experimental period, the mean PPFD on cloudless days at 12h00 was 365±26 µmol m^{-2} s^{-1} and was approximately 20% of full sunlight. The temperature around the shade stressed plant samples was ~1-2 ºC lower than that of the control, and the relative humidity 2-4% higher than that of the control. The plants in the shade stress treatment were also irrigated by the ambient rainfall of the Western Cape (Table 1). Readings of the environmental conditions under all treatments were taken daily at the following time intervals: 09h00, 12h00 and 15h00.

2.3. Physiological responses

An infra-red gas analyzer (Licor, Li-6400 Portable photosynthesis system, Lincoln, Nebraska, USA) coupled to a leaf chamber, was used to measure the photosynthetic rates (P_{max}), leaf dark respiration rates (D_r), stomatal conductance

(G_s) and transpiration rates (E) of the plant samples during the growth phase. The readings were taken on fully expanded leaves at the peak of the growth season during the month of June. *Gethyllis villosa* plants have flat leaves (± 5 mm in diameter) and a section of one leaf blade per plant was used for the readings. *Gethyllis multifolia* plants consist of thin needle-like leaves (± 1-2 mm in diameter) and 6-8 leaf sections per plant were used for the readings. More (6-8) leaves for *G. multifolia* had to be used to equate the same leaf diameter of *G. villosa*. The infra-red gas analyzer was set to take the net photosynthetic rate readings at the following light photosynthetic photon flux densities (PPFD): 0, 50, 150, 350, 500, 750, 950, 1200 and 1500 μmol m^{-2} s^{-1}. Six readings were taken per PPFD and readings were recorded from four plants per species per treatment. The corresponding temperature for the photosynthetic rate readings in the leaf chamber was set at 25 ºC and the relative humidity at 55-75%. Linear regression analysis was performed on data within the light-limited part of the light response curve to calculate the apparent photon yield. Photosynthetic water-use efficiency (PWUE) was calculated as P_{max}/E.

2.4. Chlorophyll determination

Fresh leaf material (100-150 mg) of both species was pulverised in a mortar and pestle and the chlorophyll was extracted using 80% ammoniacal acetone (Arnon, 1949; Lichtenthaler and Wellburn, 1983). The procedure was carried out at 4 ºC in an ice-bucket and the resulting extract was centrifuged at 2500 revs, 545 g for 5 min. (Model Sigma-16, Wirsam Scientific, South Africa). The absorbence of chlorophyll *a* and *b* was measured at 663 and 710 nm respectively on a

spectrophotometer (Model Pharmacia LKB - Ultrospec 11 E, Wirsam Scientific, South Africa). The chlorophyll concentration, expressed per unit fresh mass of plant material (Arnon, 1949), was calculated using the equations of Hendry and Grime (1993).

2.5. Statistical analysis

Significant differences of the means for each species were separately tested under drought and shade stress. The means were separated using a *post hoc* Fishers's Protected LSD, multiple comparison test (SuperANOVA, version. 6.11 for Macintosh Abacus Concepts, USA). Different letters indicate significant differences between treatments ($P \leq 0.05$, $n=4$), and superscript numbers indicate the comparisons for each species only (1=*G. multifolia*; 2=*G. villosa*).

3. Results

During shade (Figure 1a) and drought (Figure 1b) stress, *G. multifolia* had a reduction in its light saturated photosynthetic rates (P_{max}), whilst the P_{max} for *G. villosa* remained unchanged. These photosynthetic patterns of both species, concur with their respective stomatal conductance (G_s) (Figure 2a,b) and transpiration rates (E) (Figure 3a,b).

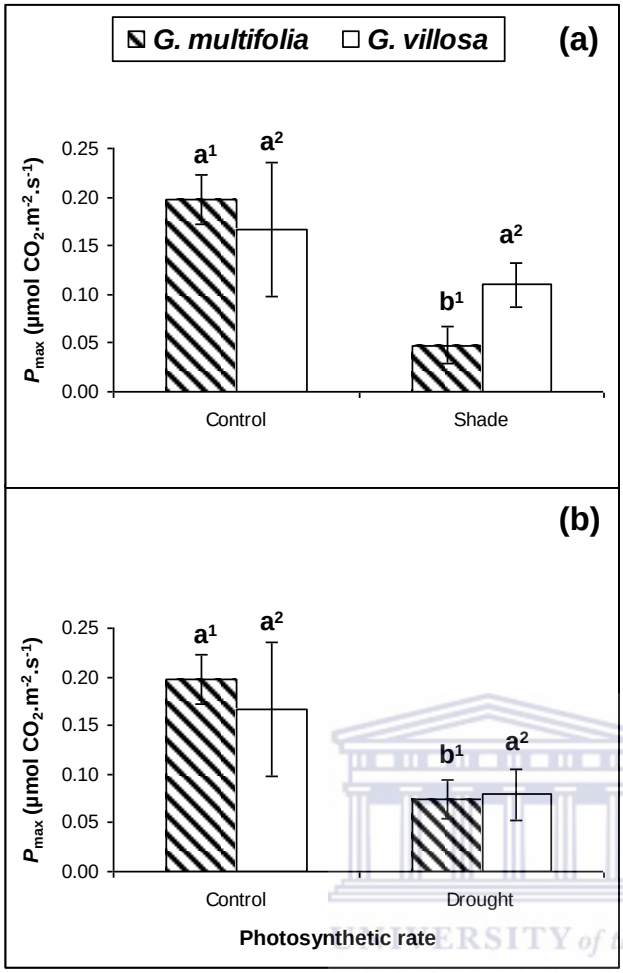

Fig. 1: Leaf photosynthetic rates (P_{max}) in µmol $CO_2.m^{-2}.s^{-1}$ of *Gethyllis multifolia* and *G. villosa* during drought and shade stress over an experimental period of six months. Readings were taken at full leaf emergence during the peak of the growth phase (June). Different letters indicate significant differences between means ± SE of treatments (P≤0.05, n=4), and superscript numbers indicate the comparisons for each species only (1=*G. multifolia*; 2=*G. villosa*).

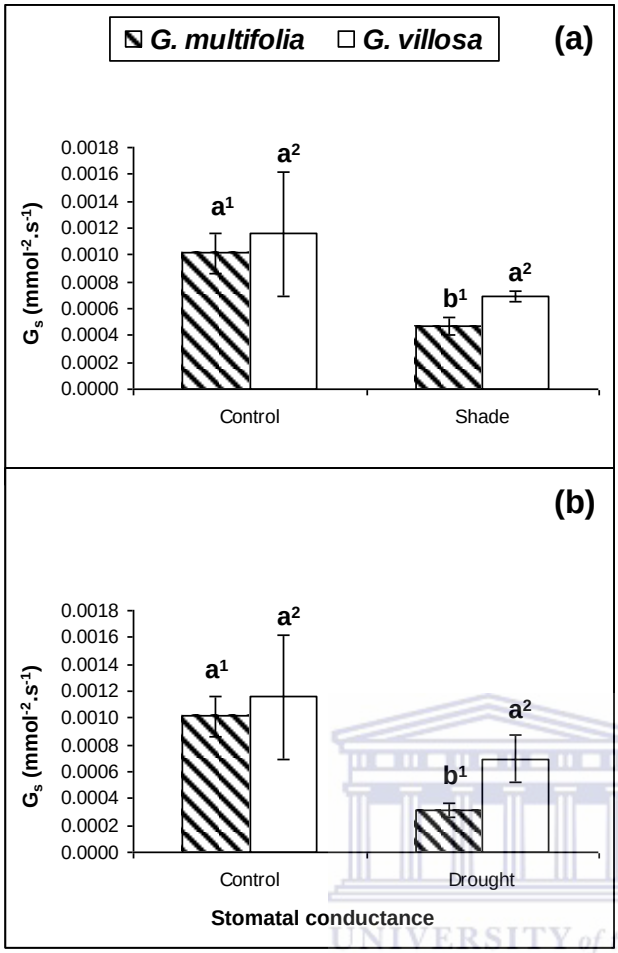

Fig. 2: Stomatal conductance (G_s) in $mmol^{-2}.s^{-1}$ of *Gethyllis multifolia* and *G. villosa* during drought and shade stress over an experimental period of six months. Readings were taken at full leaf emergence during the peak of the growth phase (June). Different letters indicate significant differences between means ± SE of treatments ($P \leq 0.05$, n=4), and superscript numbers indicate the comparisons for each species only (1=*G. multifolia*; 2=*G. villosa*).

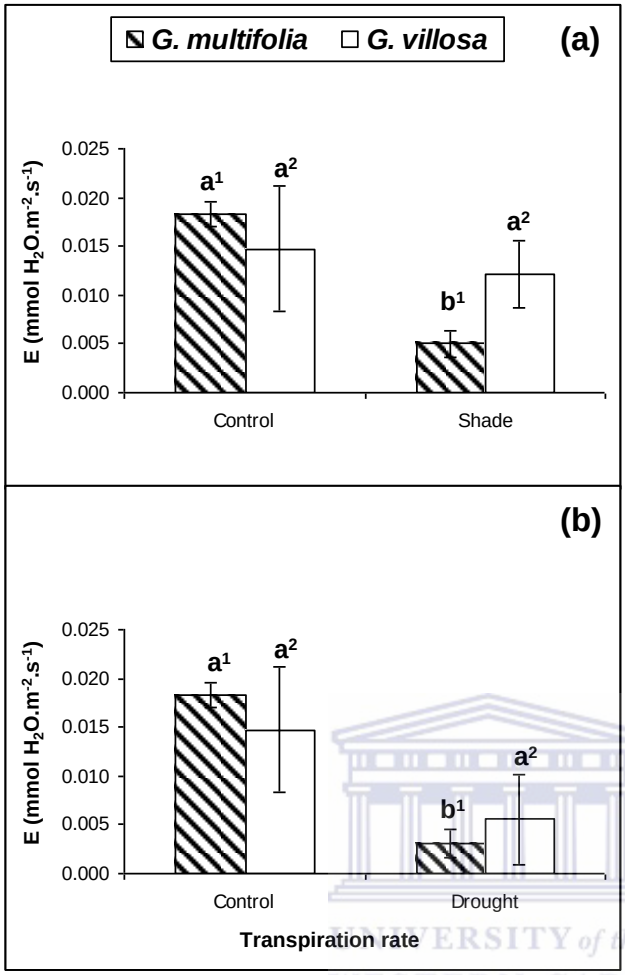

Fig. 3: Transpiration rates (E) in mmol $H_2O.m^{-2}.s^{-1}$ of *Gethyllis multifolia* and *G. villosa* during drought and shade stress over an experimental period of six months. Readings were taken at full leaf emergence during the peak of the growth phase (June). Different letters indicate significant differences between means ± SE of treatments ($P \leq 0.05$, n=4), and superscript numbers indicate the comparisons for each species only (1=*G. multifolia*; 2=*G. villosa*).

In spite of the differences in photosynthetic shade responses in *G. multifolia* and *G. villosa*, the photosynthetic water-use efficiencies (PWUE) remained unchanged in both species during shade stress (Figure 4a). However, *G. multifolia* had an

increase in PWUE under drought stress, whilst *G. villosa* remained unchanged (Figure 4b).

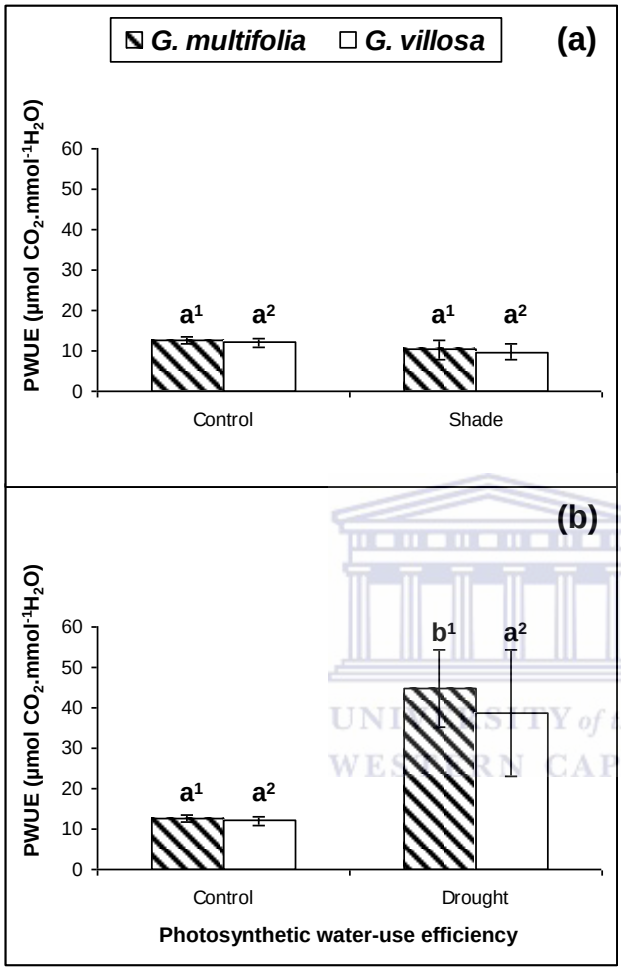

Fig. 4: Water-use efficiency (PWUE) in µmol CO_2.mmol^{-1} H_2O of *Gethyllis multifolia* and *G. villosa* during drought and shade stress over an experimental period of six months. Readings were taken at full leaf emergence during the peak of the growth phase (June). Different letters indicate significant differences between means ± SE of treatments ($P \leq 0.05$, n=4), and superscript numbers indicate the comparisons for each species only (1=*G. multifolia*; 2=*G. villosa*).

The decline in the P_{max} of *G. multifolia* in response to drought and shade stress is not related to leaf morphological adaptations such as specific leaf mass (SLM) (Figure 5a,b), but rather to leaf photochemistry and the associated pigments. In this regard, *G. multifolia* plants had an increase in the light compensation point (LCP) under drought and shade stress, whereas the LCP of *G. villosa* remained unchanged (Figure 6a,b). Furthermore, the apparent photon yield of *G. multifolia* declined sharply during shade and drought stress, whilst *G. villosa* remained unaffected (Figure 7a,b). These responses were not associated with an alteration in the dark respiratory costs of *G. multifolia* and *G. villosa* leaves (Figure 8a,b). However, the ratio of photosynthesis to dark respiration indicates that although there were no changes in *G. villosa*, there was a significant decline in *G. multifolia* during shade and drought stress (Figure 8c,d).

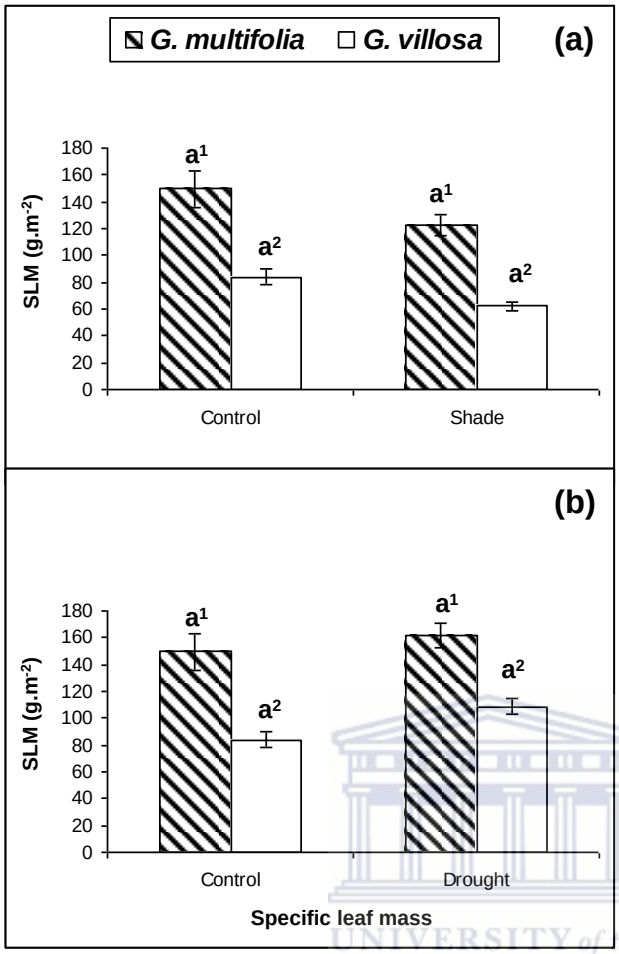

Fig. 5: Specific leaf mass (SLM) in g.m^{-2} of *G. multifolia* and *G. villosa* during drought and shade stress over an experimental period of six months. Readings were taken at full leaf emergence during the peak of the growth phase (June). Different letters indicate significant differences between means ± SE of treatments ($P \leq 0.05$, n=4), and superscript numbers indicate the comparisons for each species only (1=*G. multifolia*; 2=*G. villosa*).

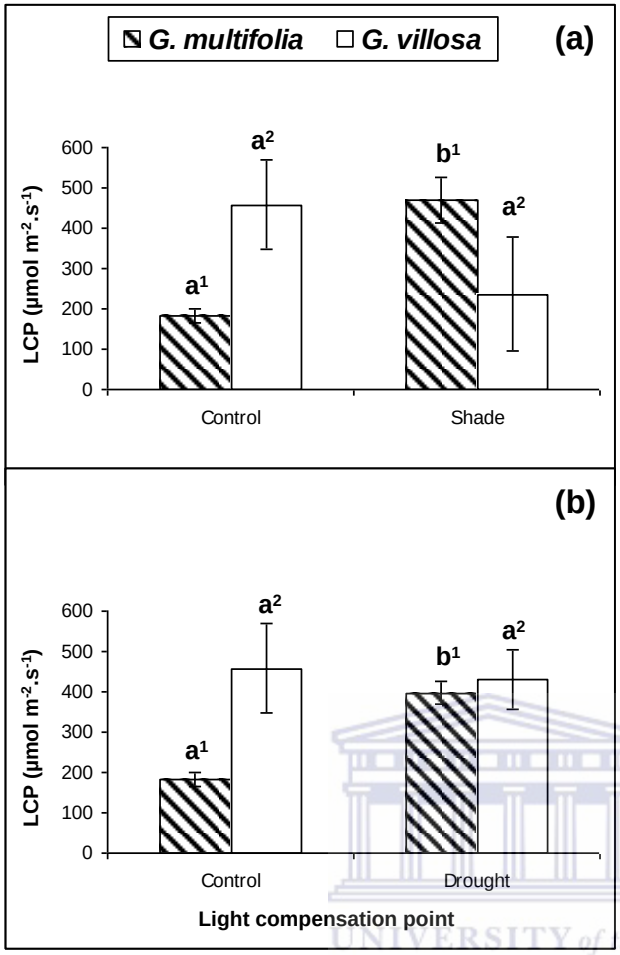

Fig. 6: Light compensation points (LCP) in µmol m-^{2}.s-^{1} of *Gethyllis multifolia* and *G. villosa* during drought and shade stress over an experimental period of six months. Readings were taken at full leaf emergence during the peak of the growth phase (June). Different letters indicate significant differences between means ± SE of treatments (P≤0.05, n=4), and superscript numbers indicate the comparisons for each species only (1=*G. multifolia*; 2=*G. villosa*).

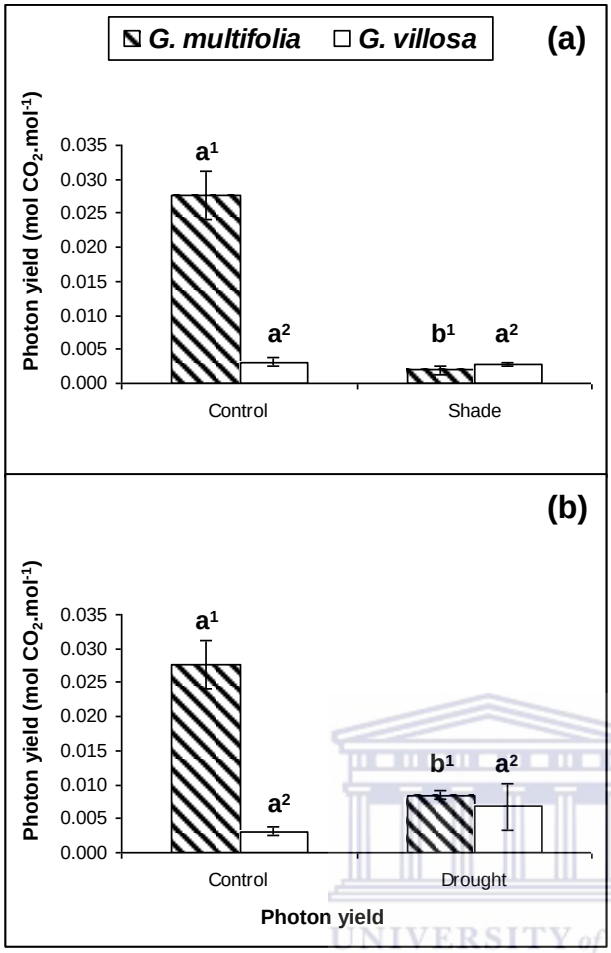

Fig. 7: Leaf photon yield in mol $CO_2.mol^{-1}$ of *G. multifolia* and *G. villosa* during drought and shade stress over an experimental period of six months. Readings were taken at full leaf emergence during the peak of the growth phase (June). Different letters indicate significant differences between means ± SE of treatments ($P \leq 0.05$, n=4), and superscript numbers indicate the comparisons for each species only (1=*G. multifolia*; 2=*G. villosa*).

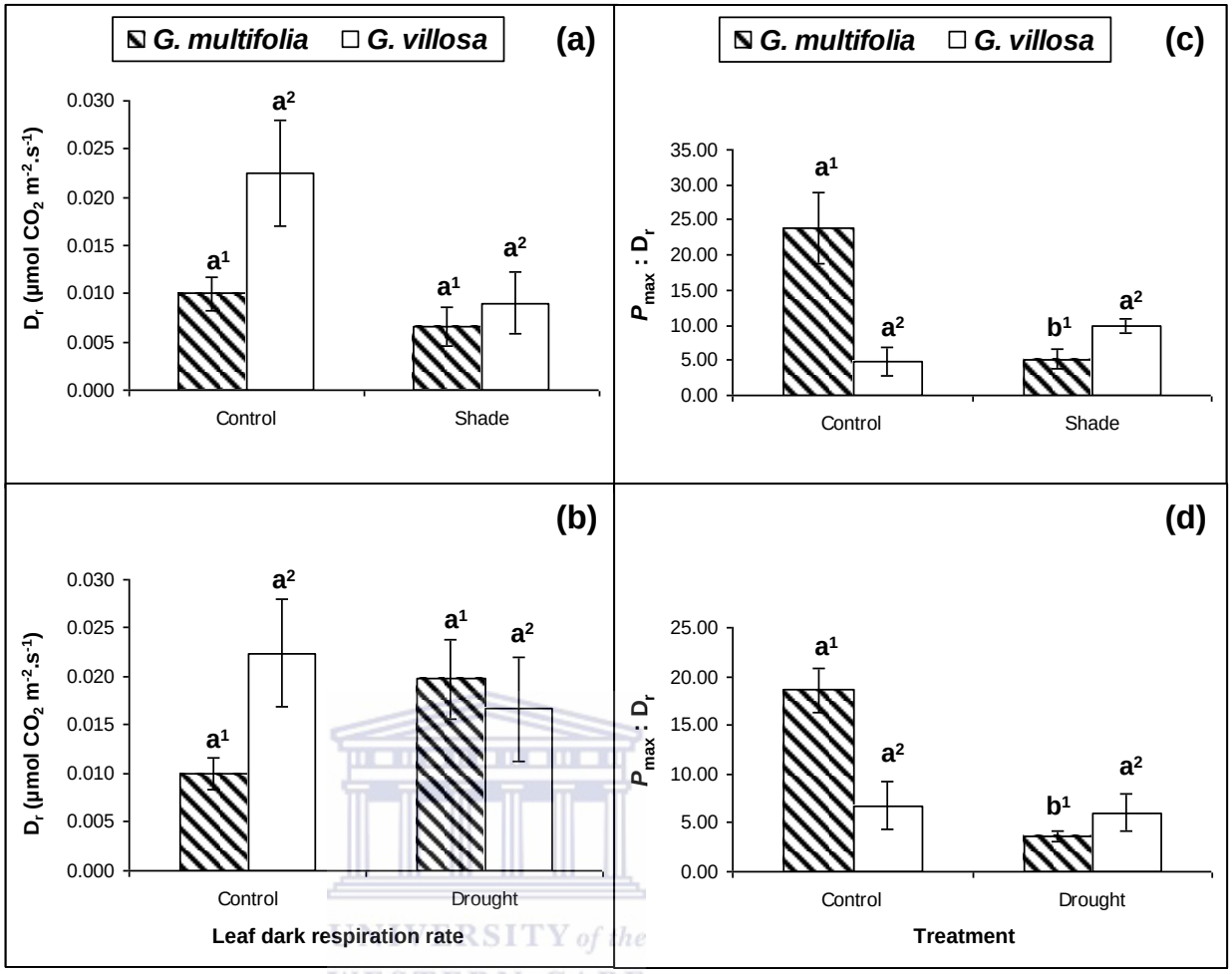

Fig. 8: Leaf dark respiration rates (D_r) **(a)** and **(b)** in µmol CO_2 $m^{-2}.s^{-1}$ and ratio of photosynthesis (P_{max}) to dark respiration (D_r) **(c)** and **(d)** of *Gethyllis multifolia* and *G. villosa* during drought and shade stress over an experimental period of six months. Readings were taken at full leaf emergence during the peak of the growth phase (June). Different letters indicate significant differences between means ± SE of treatments ($P \leq 0.05$, n=4), and superscript numbers indicate the comparisons for each species only (1=*G. multifolia*; 2=*G. villosa*).

The chlorophyll analyses indicated that although *G. multifolia* maintained the chlorophyll levels under drought (Figure 9a) and increased them during shade stress (Figure 9b), there was no change in the ratio of chlorophyll *a*:*b* (Figure 9c,d). However, for *G. villosa*, there was a change in chlorophyll levels during

drought and shade, with a ratio that shifted towards more synthesis of chlorophyll *b* (Figure 9c,d).

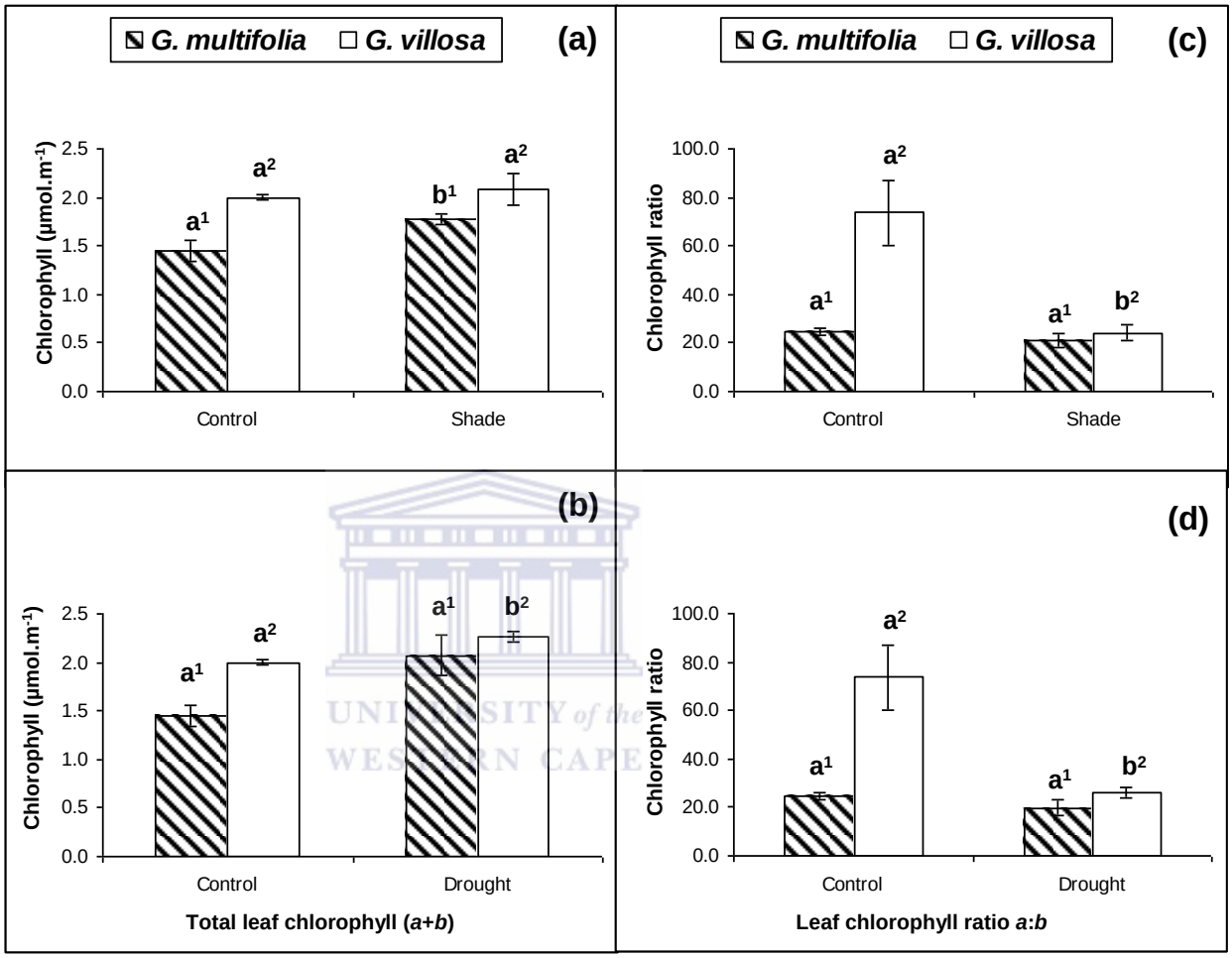

Fig. 9: Total leaf chlorophyll **(a)** and **(b)** in µmol.m^{-1} and leaf chlorophyll ratio **(c)** and **(d)** of *G. multifolia* and *G. villosa* during drought and shade stress over an experimental period of six months. Readings were taken at full leaf emergence during the peak of the growth phase (June). Different letters indicate significant differences between means ± SE of treatments (P≤0.05, n=4), and superscript numbers indicate the comparisons for each species only (1=*G. multifolia*; 2=*G. villosa*).

4. Discussion

Both species responded to drought and low light stress conditions by alterations in their photosynthesic performance.

During drought stress, the ability of leaf photosynthesis to adapt to dry conditions depends on a suite of alterations relating to leaf morphology, stomatal control and photochemistry. Under drought stress, *G. villosa* had a better photosynthetic performance than *G. multifolia*, which appears not to be related to foliar adaptations such as specific leaf mass (SLM), but to *G. villosa*'s leaves maintaining their stomatal conductance (G_s), photosynthetic light compensation (LCP) and photon yields during the dry periods. Stomatal control during photosynthesis is a well-known adaptation, as documented in previous research work done in various ecosystems (Winter and Schramm, 1986; Duan et al., 2005; Valliyodan and Nguyen, 2006). The maintenance of LCP and photon yields may be related to the increases in the light harvesting pigment, chlorophyll *b*. The role of chlorophyll in photosynthetic drought adaptations have been previously demonstrated by Musil et al. (2009) for a geophytic plant species and Sanchez et al. (1982) for maize plants.

The inability of *G. multifolia* plants to maintain their photosynthetic performance under drought conditions, is underpinned by both stomatal and photochemical factors. In *G. multifolia* plants, the increase in photosynthetic LCP and the decline in apparent photon yield under drought conditions indicated that these leaves were not able to efficiently utilise light energy for photosynthesis. According to Tabaeizadeh (1998), drought-induced responses in plant cells are characterized by a reduction in photosynthetic activity due to a series of effects including the reduced activity of photosynthetic enzymes. Although the chlorophyll concentrations of *G. multifolia* were unaffected by drought, the LCP and photon

yields indicate that the efficiencies of the pigment-related photochemical reactions may have been impaired.

The decline in G_s, concurs with previous findings to that of stomatal closure in order to conserve water (Tabaeizadeh, 1998; Duan et al., 2005). In *G. multifolia*, the G_s declined by 70% of the control, whereas P_{max} declined by 62%, indicating that G_s may serve as a major adaptive measure for leaves under drought stress. This relationship between G_s and photosynthesis is congruent with previous work (Winter and Schramm, 1986; Duan et al., 2005; Valliyodan and Nguyen, 2006) and in particular with findings from mediterranean-type climates where the decrease in stomatal conductance caused the stomatal limitation on photosynthesis to increase by as much as 70% (Galmés et al., 2007). In spite of the decrease in photosynthesis for *G. multifolia* under drought stress, the increase in photosynthetic water-use efficiency (PWUE) was a consequence of a greater decline in transpiration rate than photosynthesis. Similar responses were reported in *Castanea dentata* plants where an increase in irradiance and not necessarily drought, resulted in an increase in the PWUE (Wang et al., 2006).

During shade stress, leaf photosynthesis can adapt to low light conditions via a suite of alterations relating to leaf morphology, phytochemistry and photochemistry. *Gethyllis villosa* had a better photosynthetic performance in low light, compared to *G. multifolia*. Compared to the P_{max} decline of *G. multifolia*, *G. villosa* maintained its photosynthetic rate under low light. This was possible in *G. villosa*, by not changing the photosynthetic LCP in the shade. The underlying

cause of this adaptation in *G. villosa* is probably due to its capacity to synthesize relatively more chlorophyll *b*, which is used for light harvesting. Additionally, this improved chlorophyll *b* investment of *G. villosa* may also underpin its unchanged photon yield during shade, whereas *G. multifolia* had a decline. It was reported by Adamson et al. (1991) that the chlorophyll *a*:*b* ratio decreases with decreased irradiance, and this adaptive strategy was reflected in *G. villosa*. Similarly, a decrease in the chlorophyll *a*:*b* ratio during shade was reported for *Tradescantia fluminensis*, a problem plant in New Zealand forests (Maule et al., 1995).

In the case of *G. multifolia*, the inability to shift its chlorophyll ratios during shade, may have led to its increase in LCP and its decline in photon yield. Furthermore, the decline in the ratio of photosynthesis to dark respiration in *G. multifolia*, indicates a significant decline in *G. multifolia*'s capacity to maintain a higher net carbon gain during shade. These findings indicate that *G. multifolia*'s inability to acclimate to shade is drastically compounded by the lower net gain in carbon during shade. Similarly, in *Tradescantia albiflora* (which is closely related to *T. fluminensis*), no difference was reported in the chlorophyll *a*:*b* ratio during shade, which reflected a reduction in the photosynthetic rate and consequently a lower net carbon gain (Chow et al., 1991).

5. Conclusion

These findings indicate that *G. villosa* plants are able to adapt their P_{max} to drought and shade conditions. The inability of *G. multifolia* to adapt to a wider range of environmental extremes such as drought and shade, may be a contributing factor underpinning its threatened conservation status.

References

Adamson, H.Y., Chow, W.S., Anderson, J.M., Vesk, M., Sutherland, M.W., 1991. Photosynthetic acclimation of *Tradescantia albiflora* to growth irradiance: morphological, ultrastructural and growth responses. Physiol. Plant. 82, 353-359.

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts: polyphenoloxidases in *Beta vulgaris*. Plant Physiol. 24, 1-15.

Brouwer, R., 1962. Distribution of dry matter in the plant. Netherlands J. Agric. Sc. 10, 361-376.

Chow, W.S., Adamson, H.Y., Anderson, J.M., 1991. Photosynthetic acclimation of *Tradescantia albiflora* to growth irradiance: lack of adjustment of light-harvesting components and its consequences. Physiol. Plant. 81, 175-182.

Daniels, C.W., 2007. A study of the propagation and cultivation of *Gethyllis multifolia* and *G. villosa*. MTech dissertation, Cape Peninsula University of Technology, Bellville, Cape Town, South Africa, pp. 65-71.

Daniels, T.W., Helms, J.A., Baker, F.S., 1979. Principles of silviculture, 2nd ed. McGraw-Hill, New York, pp. 119.

Du Plessis, N., Delpierre, G., 1973. Blommepraguiteiebodem: Koekemakranka. Landbouweekblad 21 Aug., 37-39.

Du Plessis, N., Duncan, G., 1989. Bulbous plants of southern Africa. Tafelberg Publishers, Cape Town, South Africa, pp. 104.

Duan, B., Lu, Y., Yin, C., Junttila, O., Li, C., 2005. Physiological responses to drought and shade in two contrasting *Picea asperata* populations. Physiol. Plant. 124, 476-484.

Elgorashi, E.E., Van Staden, J., 2003. Pharmacological screening of six amaryllidaceae species. J. Ethnopharmacol. 90, 27-32.

Esler, K.J., Rundel, P.W., 1998. Unusual geophytes of the succulent Karoo: How form can relate to function. Veld & Flora 84, 6-7.

Galmés, J., Medrano, H., Flexas, J., 2007. Photosynthetic limitations in response to water stress and recovery in mediterranean plants with different growth forms. New Phytol. 175, 81–93.

Givnish, T.J., 1988. Adaptation to sun and shade: a whole-plant perspective. Aust. J. Plant Physiol. 15, 63-92.

Hendry, G.A.F., Grime, J.P., 1993. Methods in comparative plant ecology. Chapman & Hall, London, UK.

Hilton-Taylor, C., 1996. Red data list of southern African plants. National Biodiversity Institute. Cape Town, South Africa, pp. 57.

IUCN., 1998. Red list of threatened plants. IUCN, World Conservation Union, Cambridge, UK, pp. 617.

Kim, J.Y., Mahe´, A., Brangeon, J., Prioul, J.L., 2000. A maize vacuolar invertase, IVR2, is induced by water stress. Organ/tissue specificity and diurnal modulation of expression. Plant Physiol. 124, 71-84.

Klaasen, J., Knoetze, H., Van Wyk, G., Mali, T., Knowles, C., 2009. Biofuels from indigenous energy crops: A feasibility study. African Crop Science Conference Proceedings Vol. 9, pp. 735-738.

Lichtenthaler, H.K., Wellburn, A.R., 1983. Determinations of total carotenoids and chlorophylls *a* and *b* in leaf extracts in different solvents. Biochem. Soc. Trans. 11, 591-592.

Liltved, W.R., 1992. The Kukumakranka, past and present. Veld & Flora 78, 104-106.

Liu, F., Andersen, M.N., Jensen, C.R., 2003. Loss of pod set caused by drought stress is associated with water status and ABA content of reproductive structures in soybean. Funct. Plant Biol. 30, 271-280.

Lorimer, C., 1983. A test of the accuracy of shade-tolerance classification based on physiognomic and reproductive traits. Can. J. Bot. 61, 1595-1598.

Manning, J., Goldblatt, P., Snijman, D., 2002. The color encyclopedia of Cape bulbs. Timber Press, Cambridge, UK, pp. 147.

Maule, H.G., Andrews, M., Morton, J.D., Jones, A.V., Daly, G.T. 1995. Sun/shade acclimation and nitrogen nutrition of *Tradescantia fluminensis*, a problem weed in New Zealand native forest remnants. New Zealand J. Ecol. 19, 35-46.

Midgeley, G.F., Hannah, L., Millar, D., Rutherford, M.C., Powrie, L.W., 2002. Assessing the vulnerability of species richness to anthropogenic climate change in a biodiversity hotspot. Global Ecol. Biodiv. 11, 445-451.

Mortimer, P., Swart, J.C., Valentine, A.J., Jacobs, G., Cramer, M.D., 2003. Does irrigation influence the growth, yield and water use efficiency of the protea hybrid 'Sylvia' (*Protea susannae X Protea eximia*)? S. Afr. J. Bot. 69, 1-9.

Müller-Doblies, D., 1986. Enumeration. Willdenowia 15, 465-471.

Musil, C.F., Arnolds, J.L., Van Heerden, P.D., Kgope, B.S., 2009. Mechanisms of photosynthetic and growth inhibition of a southern African geophyte *Tritonia crocata* (L.) Ker. Gawl. by an invasive European annual grass *Lolium multiflorum* Lam. Environ. Exp. Bot. 66, 38-45.

Nielsen, K.L., Eshel, A., Lynch, J.P., 2001. The effect of phosphorus availability on the carbon economy of contrasting common bean (*Phaseolus vulgaris* L.) genotypes. J. Exp. Bot. 52, 329-339.

Oliver, C.D., Larson, B.C., 1996. Forest stand dynamics (Upd. ed.). JohnWiley and Sons, New York, NY, USA, pp. 137.

Pelleschi, P., Rocher, J.P., Prioul, J.L., 1997. Effect of water restriction on carbohydrate metabolism and photosynthesis inmature maize leaves. Plant Cell Environ. 20, 493-503.

Poorter H., Nagel O.W., 2000. The role of biomass allocation in the growth response of plants to different levels of light, CO_2, nutrients and water: A quantitative review. Aus. J. Plant Physiol. 27, 595-607.

Rutherford, M.C., Midgeley, G.F., Bond, W.J., Powrie, L.W., Roberts, R., Allsopp, L., 1999. Plant biodiversity: Vulnerability and adaptation assessment. South African country study on climate change. Cape Town, South Africa: National Biodiversity Institute, Cape Town, South Africa, pp. 34.

Sanchez, R.A., Hall, A.J., Trapani, N., Cohen de Hunau, R., 1982. Effects of water stress on the chlorophyll content, nitrogen level and photosynthesis of leaves of two maize genotypes. Photosyn. Res. 4, 35-47.

Smith, T., Huston, M., 1989. A theory of the spatial and temporal dynamics of plant-communities.Vegetatio 83, 49-69.

Tabaeizadeh, Z., 1998. Drought-induced responses in plant cells. Int. Rev. Cytol. 182, 193-247.

Valliyodan, B., Nguyen, H.T., 2006. Understanding regulatory networks and engineering for enhanced drought tolerance in plants. Plant Biol. 9, 189-195.

Von Maltitz, G.P., Scholes, R.J., Erasmus, B., Letsoalo, A., 2006. Adapting conservation strategies to accommodate impacts of climate change in southern Africa. AIACC Working Paper 35, 2-3.

Wang, G.G., Bauerle, W.L., Mudder, B.T., 2006. Effects of light acclimation on the photosynthesis, growth, and biomass allocation in American chestnut (*Castanea dentata*) seedlings. Forest. Ecol. Manag. 226, 173-180.

Winter, K., Schramm, M.J., 1986. Analysis of stomatal and nonstomatal components in the environmental control of CO_2 exchange in leaves of *Welwitschia mirabilis*. Plant Physiol. 82, 173-178.

Yates, D.J., 1989. Shade factors of a range of shade cloth materials. Acta Hort. 257, 201-218.

CHAPTER 4: RESEARCH PAPER 2 - The carbon costs associated with the growth, reproductive and dormant phases of two semi-arid desert bulb species of *Gethyllis* (Kukumakranka)

CHAPTER 4

RESEARCH PAPER 2

The carbon costs associated with the growth, reproductive and dormant phases of two semi-arid desert bulb species of *Gethyllis* (Kukumakranka)

CW Daniels[a], WT Mabusela[b], JL Marnewick[c], AJ Valentine[d]

[a]Department of Horticultural Sciences, Cape Peninsula University of Technology, P.O. Box 1906, Bellville 7535, South Africa.

[b]South African Herbal Science and Medicine Institute, University of the Western Cape, Private Bag X17, Bellville 7535, South Africa.

[c]Oxidative Stress Research Centre, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, P.O. Box 1906, Bellville 7535, South Africa.

[d]Department of Botany and Zoology, University of Stellenbosch, Private Bag X1, Matieland 7602, Stellenbosch, South Africa.

Abstract

Gethyllis multifolia L. Bolus and *G. villosa* Thunb. are winter-growing, summer-blooming, deciduous and bulbous geophytes and grow in the succulent Karoo biome of South Africa. *G. multifolia* is threatened in its natural habitat and is

listed in the 'Vulnerable' category of the Red Data List of Southern African Plants. *Gethyllis villosa* grows in the same natural habitats, has smaller bulbs and therefore fewer reserves but has the same flower production as *G. multifolia*. The aim of this study was to determine, how efficiency of growth and flower production costs over the four phases of growth, senescence, reproduction and dormancy, has enabled *G. villosa* to achieve this. The dry weights, nitrogen and carbon concentrations and relative growth rate for the roots, bulbs and leaves were measured over the four phases to determine which of the two species uses its available natural resources more efficiently. This investigation revealed that with a significantly smaller bulb structure, *G. villosa* was able to produce more leaves per bulb mass, invested more resources back into the bulb during senescence and had a higher flower to bulb ratio during the reproduction phase. These physiological responses of *G. villosa* suggest that it is more efficient at resource utilisation as a survival strategy and this could be a contributing factor to why *G. multifolia* is threatened in its natural habitat and *G. villosa* not.

Keywords: Carbon; *Gethyllis* species; Nitrogen; Relative growth rate; Specific carbon acquisition rate; Specific nitrogen aquistion rate

1. Introduction

The genus *Gethyllis* (family: Amaryllidaceae) consists of 37 currently accepted species and subspecies (Müller-Doblies, 1986). *Gethyllis villosa* occurs more frequently throughout South Africa whilst *G. multifolia* is threatened in its natural habitat and is listed in the 'Vulnerable' category of the 'Red Data List of Southern African Plants' (Hilton-Taylor, 1996), which is the list of endangered plants in

southern Africa. Both species are winter-growing, summer-blooming, deciduous, bulbous geophytes (Du Plessis and Delpierre, 1973). *Gethyllis* species, in general, thrive under full sun conditions and their habitat ranges from the succulent Karoo, coastal areas and the inland plateau of South Africa (Snijman, 2004). *Gethyllis multifolia* and *G. villosa* grow naturally in the succulent Karoo biome (Figure 1) of South Africa, which is primarily characterized by low to high winter rainfall and extreme summer aridity. The rainfall varies between 20 and 290 mm per year and during summer the temperatures can be in excess of 40 ºC (Figure 3). These high summer temperatures and dry conditions can generally result in high transpiration rates, low photosynthetic activity, drought stress and eventually death of plants if efficient survival strategies are not being applied (Cowling et al., 1986).

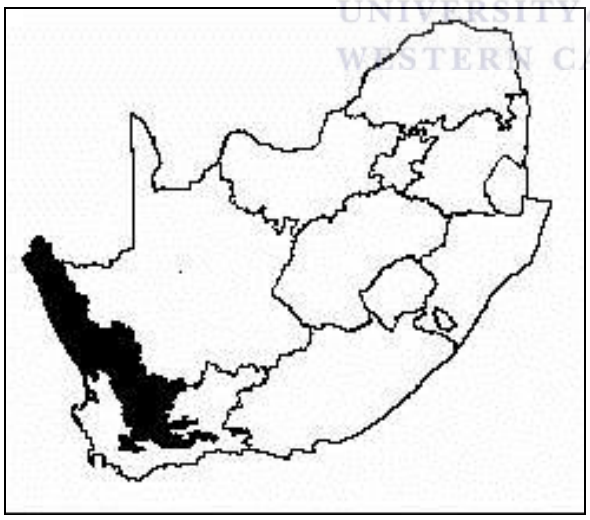

Figure 1: Succulent Karoo biome (north-western Cape, South Africa) which represents the natural habitat of *Gethyllis multifolia* and *G. villosa* (National State of the Environment Report, 2010).

Some of the adaptation strategies of bulbous geophytes from the succulent Karoo biome include hair on the leaves (including *Gethyllis* species) of some species to trap moisture, prevents excessive transpiration and helps to protect the bulbs from the sun (Liltved, 1992; Elvin, 2000). Geophytes also survive long periods of environmental stress such as summer drought by dying back to underground storage organs, only to re-sprout and growing vigorously the following growing season, as in the case with *Gethyllis* species (Esler and Rundel, 1998; Dafni et al., 1981). Reproduction in geophyte or bulbous species from arid environments is also directly limited by the plant's biomass budget, of which there is a relationship between reproductive biomass and below-ground storage of long-term as well as current biomass reserves (Orians and Solbrig 1977; Bloom et al., 1985).

Both *G. multifolia* and *G. villosa* suffer under drought stress and this phenomenon was found to be more significant on *G. multifolia* (Daniels, 2007). It was observed that under drought stress *G. multifolia* plants have a delayed production of leaves at the onset of the new growing season. Leaves are normally stunted and during the flowering season, no flowers are produced or flowers are aborted during the bud stage. In some cases the flowers are produced without a style and stigma or with a reduced number of stamens to prevent the flower from being fertilized and consequently seed being produced (Daniels, 2007; Du Plessis and Duncan, 1989).

Considerable effort has been made to characterize the carbon (C) and nitrogen (N) isotopic composition of plants from different habitats (Currin et al., 1995), however, there have been few, if any scientific studies on the fluctuations in the

C:N concentrations of bulbous plants during their growth and dormant phases. It has been reported by Ryan et al. (1997) and Amthor (2000) that the carbon-use efficiency can vary greatly among and within species based on the respiratory needs for growth and maintenance.

Gethyllis multifolia plants are 200 mm in height (when fully grown), with twisted, lightly hairy, needle-like leaves and white to cream flowers in early December. The bulb structure of *G. villosa* is significantly smaller than that of *G. multifolia* (Figure 2). Mature plants of *G. villosa* are 30-150 mm in height with flat, loosely spiraled leaves covered with soft, white, T-shaped hairs and white to pink flowers (same size as *G. multifolia*) also in early December (Hortsmann, 1999). In spite of having smaller bulbs and therefore fewer reserves, *G. villosa* has the same flower production as *G. multifolia*.

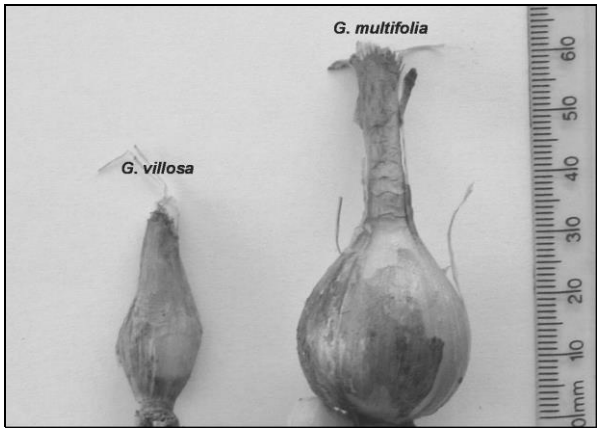

Figure 2: The bulb structures of *Gethyllis multifolia* and *G. villosa* indicating the significant size difference in the two structures.

According to Ruiters et al. (1993a), few studies of resource allocation in geophytes of the Cape fynbos, which experiences a dry summer from October to March (Figure 3), have been conducted. The aim of this study was to determine, over the four phases of growth, senescence, reproduction and dormancy, how efficiency of growth and flower production costs, have enabled *G. villosa* to achieve this. This investigation could elucidate the survival strategies of these two species and provide answers as to why *G. multifolia* is threatened and *G. villosa* not, even though both are growing in the same areas. The literature review revealed no data on the physiological adaptations of these two species and this investigation could provide much needed data, which could contribute to the future conservation and existence of the threatened and indigenous *G. multifolia* plant.

2. Materials and methods

2.1. Plant materials

Mature *G. multifolia* and *G. villosa* plants were identified and obtained with permission from the Karoo Desert National Biodiversity Garden (KDNBG) (Worcester, Western Cape, South Africa) towards the end of their winter growth phase (September), from their natural habitat (Worcester), where new roads and sewerage lines were planned through existing populations. *Gethyllis multifolia* is threatened in its natural habitat, as already mentioned, and for conservation purposes the exact location of this species is omitted. The plants (with fleshy roots) of both species (n=10 per species per growth phase) were potted up in 15

cm nursery pots in sandy, clay soil (pH 4.3-4.4) obtained from the natural habitat of the two species (Table 1). The plants were grown under outdoor conditions for 18 months which included a dormancy and reproductive phase (6 months- spring and summer), a growth and senescence phase (6 months- autumn and winter) and a second dormancy and reproductive phase (6 months- spring and summer) at the nursery of the Department of Horticultural Sciences, Cape Peninsula University of Technology (CPUT), Cape Town, Western Cape, South Africa.

2.2. Environmental conditions

Temperatures around the growing plants during the winter growth phase ranged from 8-24ºC (Figure 3) and the relative humidity from 36-100% (measured with a Majortech CE Digital relative humidity/temperature meter, Spraytech, Bellville, Western Cape, South Africa). The photosynthetic photon flux density (PPFD) (measured with a Toptronic T630 digital lux meter, Spraytech, Bellville, Western Cape, South Africa) and converted from lux to PPFD, averaged 1825 ± 63 μmol $m^{-2}.s^{-1}$. All readings were taken at the following time intervals: 09h00 am, 12h00 pm and 15h00 pm daily. The PPFD for the dormant phase was not measured since no growth took place over this period. The plants were irrigated by the ambient rainfall of the Western Cape (Figure 3) (weather data provided by the South African Weather Service). During the senescent and dormant phases these plants receive very little or no rain in their natural habitat, therefore, no irrigation was applied during the senescence and dormant phases of the experimental period.

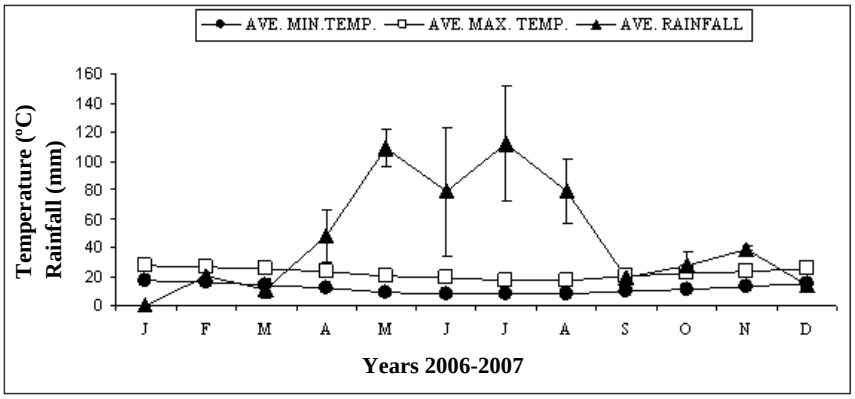

Figure 3: The average rainfall (mm) and average daily minimum/maximum (ºC) temperatures for the Cape Town area (Western Cape, South Africa) for the years 2006 and 2007. Each value was obtained by calculating the mean for the two years ±SE. The data was provided by the South African Weather Bureau and was recorded at the Cape Town weather office.

Soil analysis were done on soil samples (n=4) which were collected from four pots in which *G. multifolia* and *G. villosa* plants were growing (Table 1). These soil samples were originally collected from the natural habitat of both species, as previously mentioned. The soil samples were analyzed for total N, P, K, Na, Ca, Mg, Cu, Zn, Mn, B, and C using ICP-MS analysis and a LECO-nitrogen analyzer with suitable standards (BemLab, Van der Berg Crescent, Strand, South Africa).

Table 1: Soil sample analysis (n=4) of soil from the natural habitat of *Gethyllis multifolia* and *G. villosa* plants (Worcester, Western Cape, South Africa). Plants were grown in the same soil in 15 cm pots in a north-facing nursery (Cape Town, Western Cape, South Africa) during April-July (growth phase of both species). Plants were exposed to the ambient rainfall, irradiance and temperature of the area during this period.

Parameter	Mean±SE
Soil type	Sandy/clay
pH (KCl)	4.33±0.025
Stone (Vol. %)	26.25±2.84
H+ (cmol/kg)	0.89±0.025
P (mg/kg)	16.75±1.79
K (cmol(+)/kg)	0.32±0.01
Ca (cmol(+)/kg)	3.61±0.04
Mg (cmol(+)/kg)	9.54±0.32
Na (cmol(+)/kg)	2.23±0.06
Mn (mg/kg)	2.35±0.05
Cu (mg/kg)	0.16±0.01
Zn (mg/kg)	1.80±0.43
B (mg/kg)	0.32±0.05
C (%)	0.40±0.03

2.3. Harvesting and chemical analyses

The leaf, bulb, root, and flower carbon and nitrogen concentrations were measured to determine their fluctuation over the growth, senescence, dormancy and reproductive phases. Ten plants per growth phase of each species were originally planted and eight of the healthiest plants were selected for harvesting. The first harvesting (phase 1 - fresh leaves, bulbs and roots) was done during new biomass emergence, 30 days after planting (dap) at the onset of the growth phase (April).

The second harvesting (phase 2 - fresh leaves, bulbs and roots) was done at full emergence (pre-senescence) at 130 dap, at the peak of the growth phase (August). The third harvesting (phase 3 - dry leaves, fresh bulbs and fresh roots) was done at 161 dap, after yellowing and drying of the leaves (senescence) at the end of the growth phase (October). The fourth harvesting (phase 4 - fresh flowers, bulbs and roots) was done at 246 dap, during the reproductive phase (early December).

Gethyllis multifolia and *G. villosa* plants were excised into leaves, bulbs and roots. All the excised plant parts and later the flowers, were dried in a fan-drying laboratory oven (Memmert, Laboratory & Scientific, Cape Town, South Africa) at 50 ºC for 48 hours while the bulbs took five days to dry. Individual plant parts were ground to a powder in a portable spice grinder using a 0.5 mm mesh (Krups 75 model F203, Hecho En Mexico, Mexico City, Mexico). Between 2.6 mg and 3.0 mg of each sample was weighed into 8 mm by 5 mm tin capsules (Elemental Microanalysis Ltd., Devon, U.K.) on a Sartorius microbalance (Goettingen, Germany). Dried samples were sent to the Archeometry Department of the University of Cape Town, Observatory, Western Cape, South Africa for further analysis according to the procedures that follows. The samples were combusted in a Fisons NA 1500 (Series 2) CHN analyser (Fisons Instruments SpA, Milan, Italy). The carbon (C) and nitrogen (N) values for the C and N gases released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyser by a Finnigan MAT Conflo control unit. Three standards were used to correct the

samples for machine drift; two in-house standards (Merck Gel and Nasturtium) and one IAEA (International Atomic Energy Agency) standard- $(NH_4)_2SO_4$.

2.4. Carbon and nitrogen cost calculations

The specific utilisation and acquisition rates of C and N were calculated according to Mortimer et al. (2008), following the adaptions made to the formula of Nielson et al., (2001). Specific C absorption rate (SCAR) (µg $C.g^{-1}$ root dry weight (DW).d^{-1}) is the calculation of the net C absorption rate per unit root DW (Nielson et al., 2001):

$$SCAR = (M_2 - M_1) / (t_2 - t_1) \times (\log_e R_2 - \log_e R_1) / (R_2 - R_1)$$

Where M is the C content per plant and R is the root DW.

Specific C utilisation rate (SCUR) (g DW.$\mu g^{-1}C.d^{-1}$) is a measure of the DW gained for the C taken up by the plant (Nielson et al., 2001):

$$SCUR = (W_2 - W_1) / (t_2 - t_1) \times (\log_e M_2 - \log_e M_1) / (M_2 - M_1)$$

Where M is the C content of the plant and W is the plant DW.

The specific N utilisation rates (SNUR) and N absorption rates (SNAR) were adapted from the above equations to include N instead of C.

Relative growth rate (RGR) = Plant DW (Phase 2) – Plant DW (Phase 1) / Plant DW (Phase 2) / number of days between (Phases 1 and 2) x 1000.

2.5. Statistical analysis

Significant differences of the means were separated using a *post hoc* Fishers's Protected LSD, multiple comparison test (SuperANOVA, version. 6.11 for Macintosh Abacus Concepts, USA). Different letters indicate significant differences ($P \leq 0.05$) between species per developmental phase (n= 8).

3. Results

3.1. Pre-senescent vegetative bulb, leaf and root growth

Gethyllis multifolia initially started with larger bulbs (Figure 2) during leaf emergence (PH1), which increased by 80% in the full leaf emergence phase (PH2) (Figure 4b). At 30 days after planting (dap) there was an increase in dry matter accumulation by both plant bulbs over this period (Figure 4b). In contrast to *G. multifolia*, *G. villosa* initially started with smaller bulbs (Figure 2) in PH1 and increased its mass by 10% over the same period to PH2 (Figure 4b). *Gethyllis multifolia* also started with larger roots during PH1 and increased their growth to a greater extent than that of *G. villosa* (Figure 4a). During the reproductive phase (PH4) this increase in *G. multifolia* root dry weight (DW) was significantly higher than that of *G. villosa* (Figure 4a). Interestingly, the high root DW of *G. multifolia* corresponded with a loss in the bulb N concentration over the same period (Figure 6b).

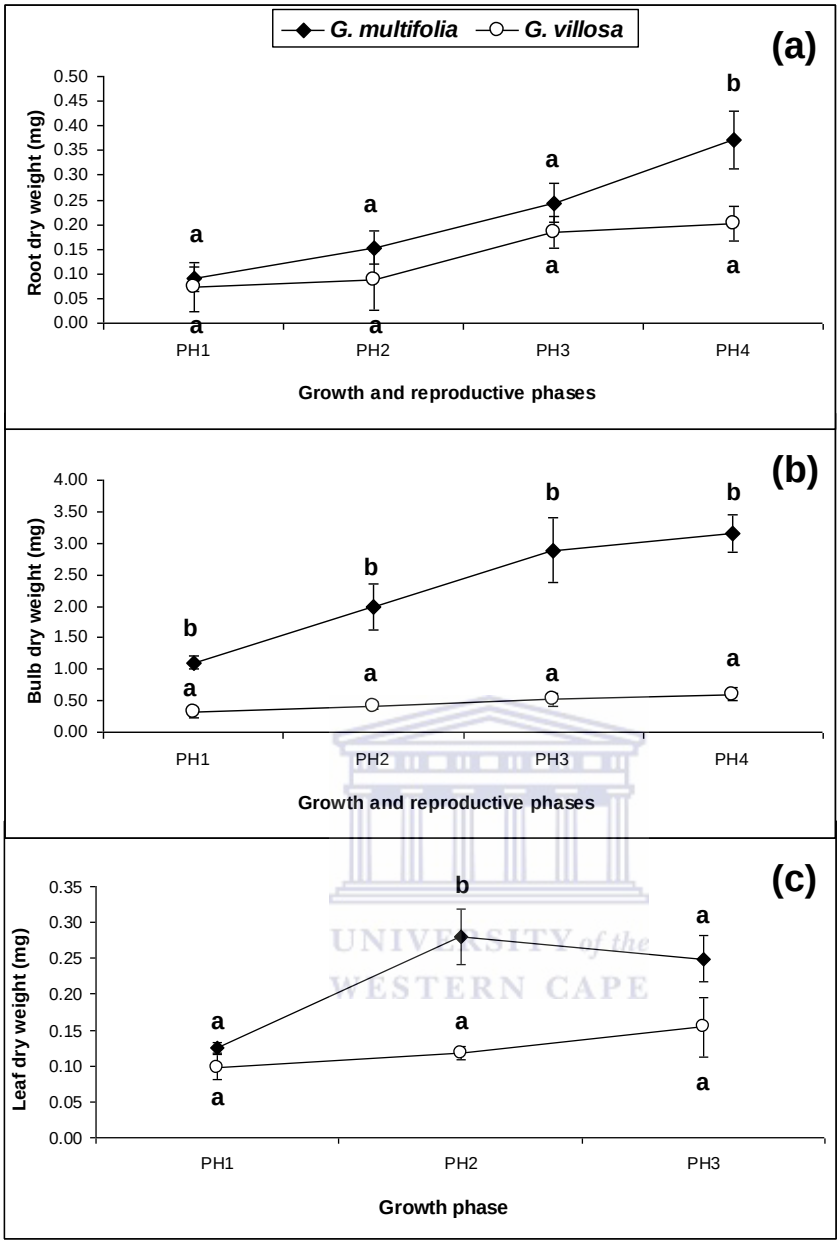

Figure 4: Dry weights of **(a)** roots **(b)** bulbs and **(c)** leaves of *Gethyllis multifolia* and *G. villosa.* Plants were cultivated in soil from their natural habitat and harvested at 4 phases. PH1=leaf emergence; PH2=leaf full emergence; PH3=leaf senescence; PH4= reproductive. Values are presented as means (n=8) with standard error bars. Different letters indicate significant differences between *G. multifolia* and *G. villosa* at each phase (P≤0.05).

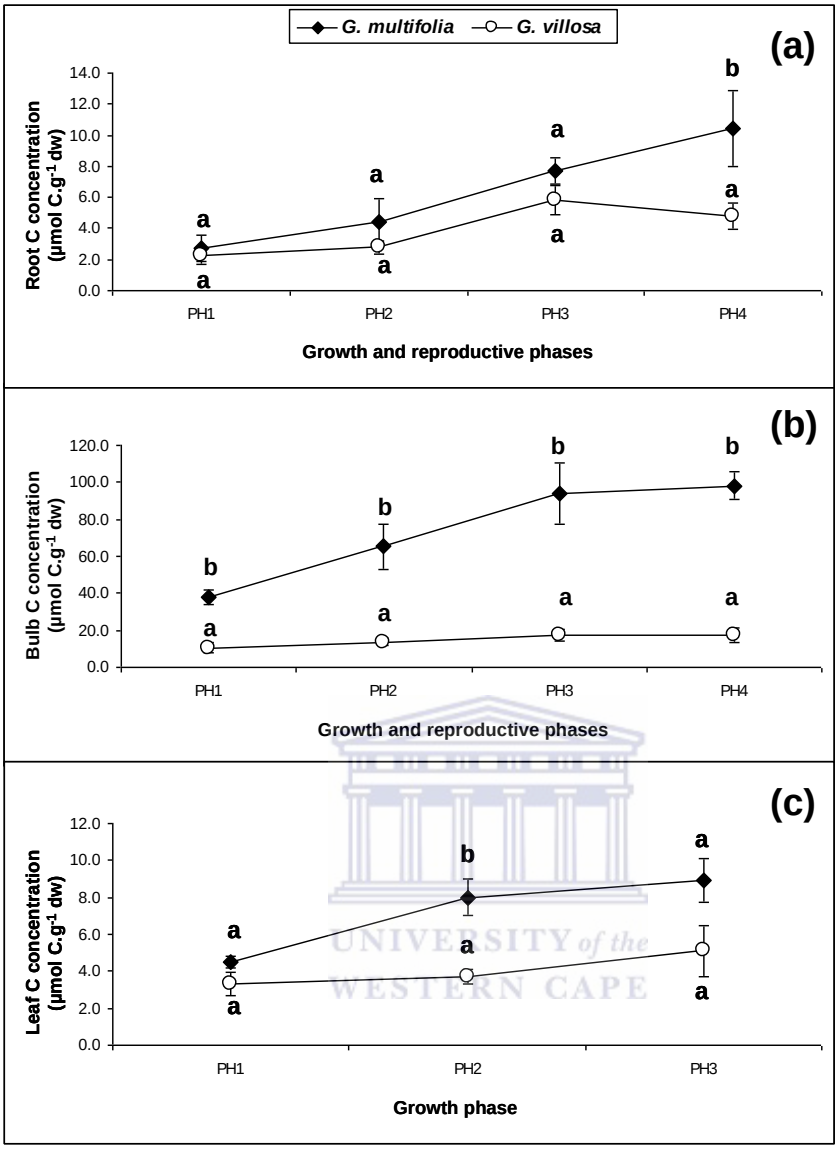

Figure 5: Carbon (C) concentrations of **(a)** roots **(b)** bulbs and **(c)** leaves of *Gethyllis multifolia* and *G. villosa.* Plants were cultivated in soil from their natural habitat and harvested at 4 phases. PH1=leaf emergence; PH2=leaf full emergence; PH3=leaf senescence; PH4= reproductive. Values are presented as means (n=8) with standard error bars. Different letters indicate significant differences between *G. multifolia* and *G. villosa* at each phase (P≤0.05).

G. multifolia had faster leaf growth during PH1 to PH2 (Figure 4c; Figure 7c) but it also had a lower leaf:bulb ratio than *G. villosa* (Figure 7d). This greater proportion of leaf:bulb growth of *G. villosa* compared to *G. multifolia* (Figure 7d), corresponds with *G. villosa* not depleting its bulb C and N reserves (Figures 5b;6b).

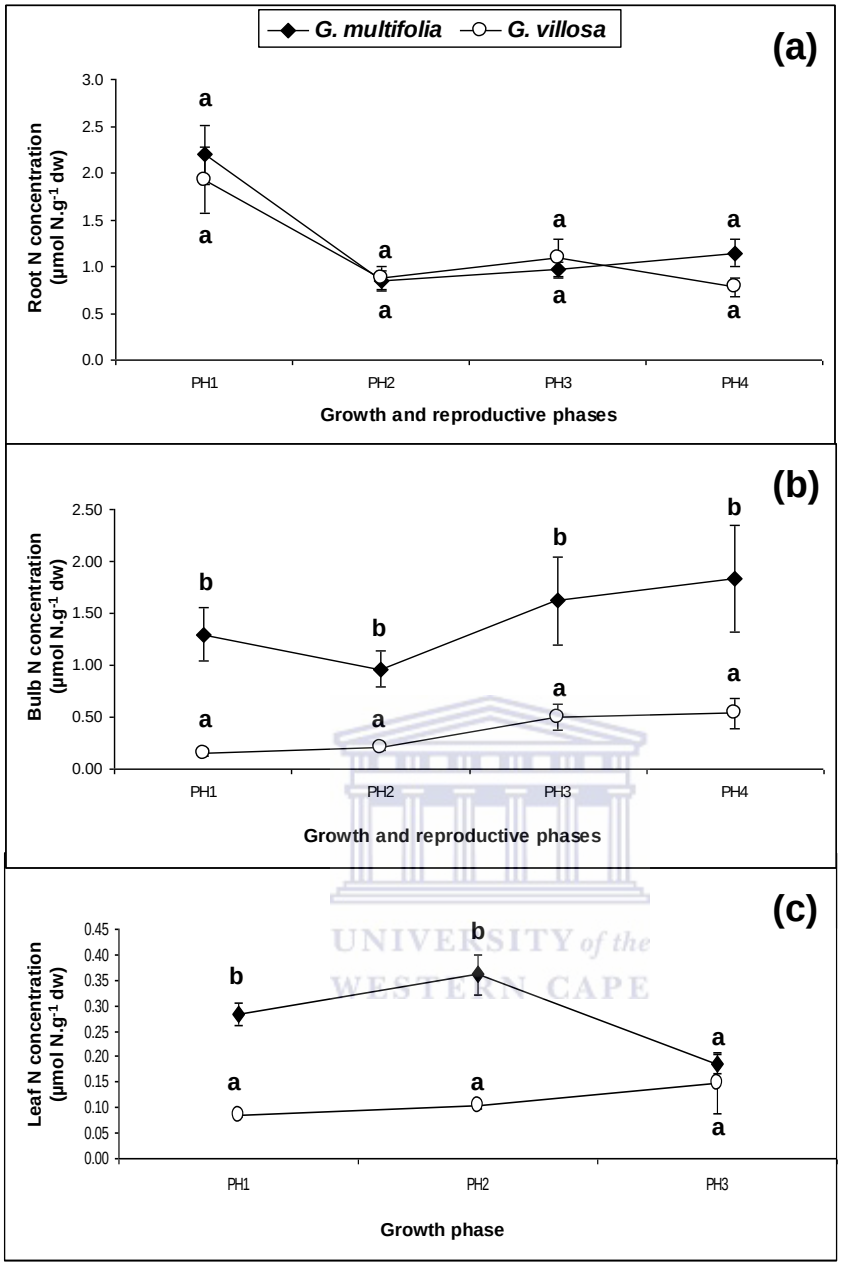

Figure 6: Nitrogen (N) concentrations of **(a)** roots **(b)** bulbs and **(c)** leaves of *Gethyllis multifolia* and *G. villosa.* Plants were cultivated in soil from their natural habitat and harvested at 4 phases. PH1=leaf emergence; PH2=leaf full emergence; PH3=leaf senescence; PH4=reproductive. Values are presented as means (n=8) with standard error bars. Different letters indicate significant differences between *G. multifolia* and *G. villosa* at each phase (P≤0.05).

3.2. Leaf maturity and senescence

During the stages of initial leaf emergence (PH1 or phase 1) and full leaf emergence (PH2), *G. villosa* produced more leaves per bulb mass (Figure 7d). During the leaf senescent stage (PH2 to PH3), *G. multifolia* had a greater loss of N than *G. villosa* over the same period, while *G. villosa* had no significant change in leaf N concentrations during PH2 to PH3 (Figure 6c).

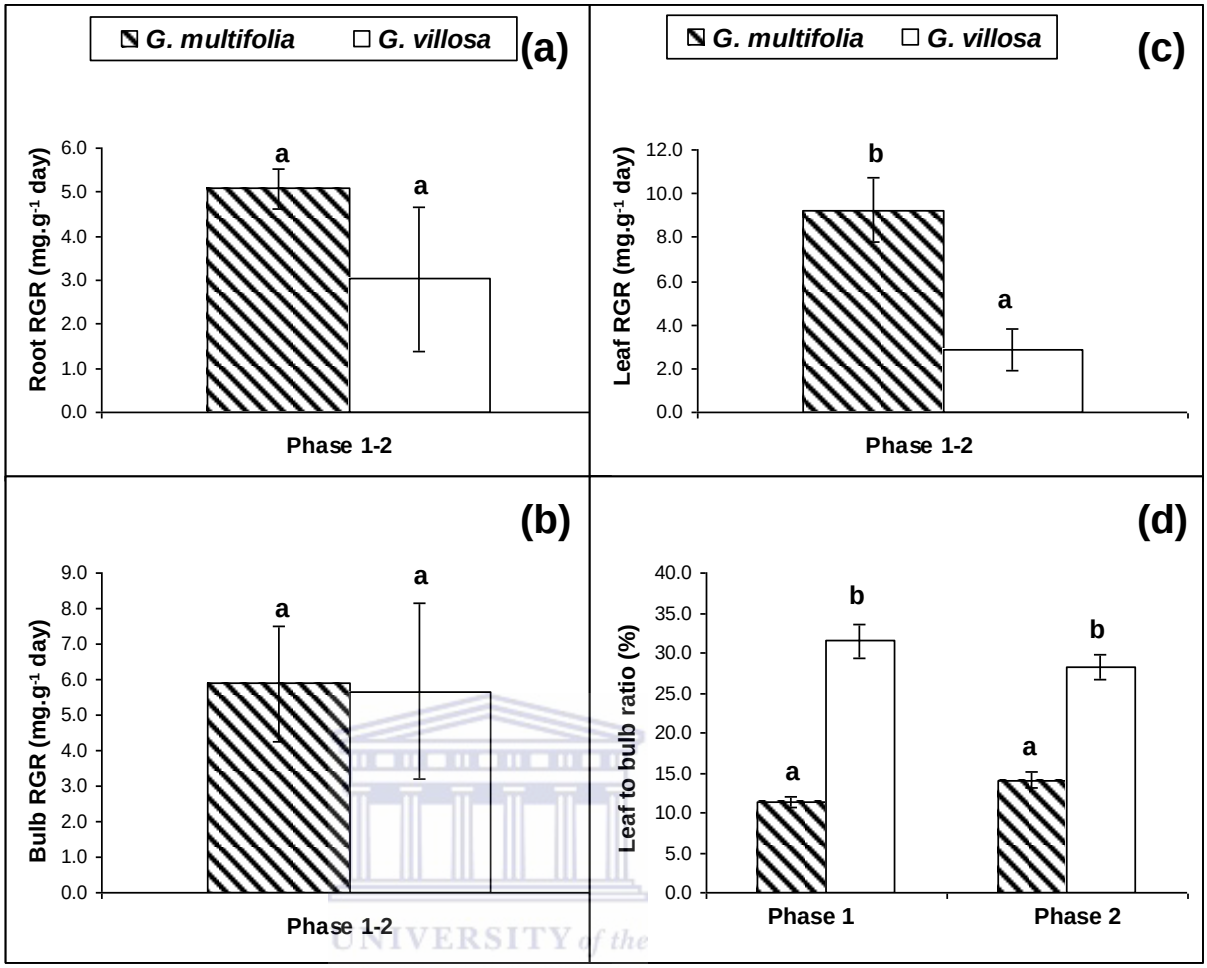

Figure 7: Relative growth rate (RGR) of **(a)** roots **(b)** bulbs**(c)** leaves and **(d)** leaf to bulb ratio of *Gethyllis multifolia* and *G. villosa.* Plants were cultivated in soil from their natural habitat and harvested at 2 phases. PH1=leaf emergence; PH2=leaf full emergence. Values are presented as means (n=8) with standard error bars. Different letters indicate significant differences between *G. multifolia* and *G. villosa* at each phase (P≤0.05).

3.3. Leaf post-senescence and flowering

In spite of having started the growth season with smaller bulbs, the flower production of *G. villosa* was similar to that of *G. multifolia*, which started with larger bulbs (Figures 2; 8a). This represented a significantly higher percentage of flower dry weight per bulb in *G. villosa*, compared to *G. multifolia* (Figure 8b).

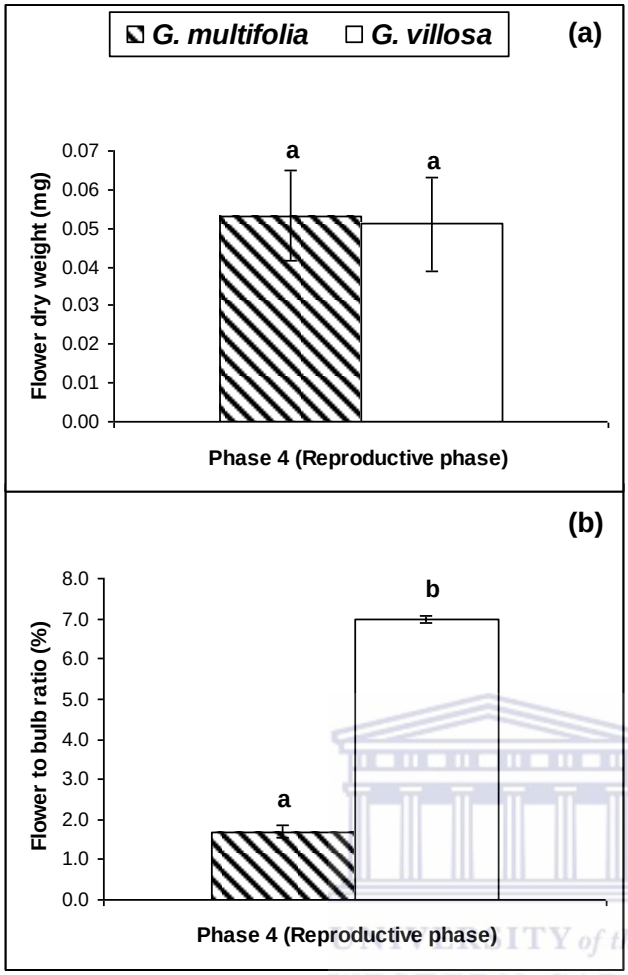

Figure 8: Dry weight of flowers **(a)** and flower to bulb ratio **(b)** for the reproductive phase of *Gethyllis multifolia* and *G. villosa.* Plants were cultivated in soil from their natural habitat and flowers and bulbs were harvested at the reproductive phase (PH4). Values are presented as means (n=8) with standard error bars. Different letters indicate significant differences between *G. multifolia* and *G. villosa* at each phase (P≤0.05).

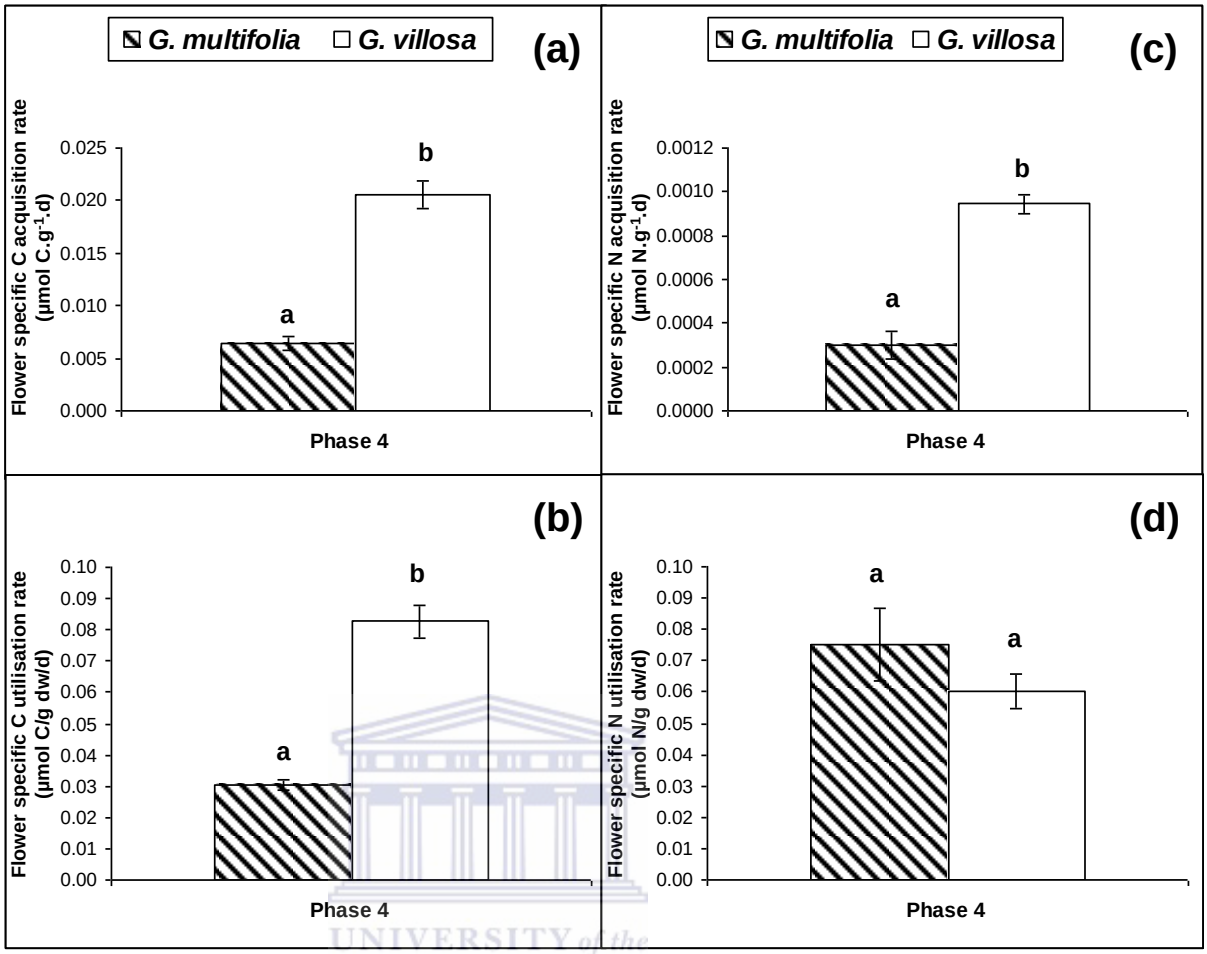

Figure 9: Specific carbon (C) acquisition rate (SCAR) (a), specific C utilisation rate (SCUR) (b), specific nitrogen (N) acquisition rate (SNAR) (c) and specific N utilisation rate (SNUR) (d) for the reproductive phase of *Gethyllis multifolia* and *G. villosa.* Plants were cultivated in soil from their natural habitat and flowers were harvested at the reproductive phase (PH4). Values are presented as means (n=8) with standard error bars. Different letters indicate significant differences between *G. multifolia* and *G. villosa* at each phase (P≤0.05).

4. Discussion

Although *G. multifolia* produced more growth in all its vegetative stages, the flower production of the smaller *G. villosa*, was strikingly similar in size. Our findings show that *G. villosa* had higher allocation of nutrients to flower production and an increase in the efficiency of its nutrient re-location.

4.1. Pre-senescent vegetative bulb, leaf and root growth

Owing to a larger initial bulb mass, *G. multifolia* increased its bulb size to a greater percentage during the leaf emergent phases than *G. villosa,* which had a smaller initial bulb size. This concurs with previous work on two desert bulb species of *Bellevalia,* where it was found that *B. eigii,* which has a larger size bulb than *B. desertorum,* invested more dry weight into its roots, bulbs and leaves at the onset of the growth season (Boeken, 1990). It was furthermore found that this initial investment of new growth was not determined by water availability, but by initial bulb mass (Boeken, 1990). This implies that storage material in the initial bulb may be an important factor in subsequent bulb success.

Similarly, *G. multifolia* also increased its larger initial root size to a greater percentage than *G. villosa*. This phenomenon is supported by Noy-Meir (1973); Orians and Solbrig (1977) and Bloom et al. (1985) who suggested that leaf and root growth in plants from arid environments is limited to the available biomass. To support the high root growth of *G. multifolia,* there was a loss in the bulb N concentration, possibly to supply nutrients to the new growing roots. This occurrence is mirrored in *Pinguicula alpina,* a perennial geophyte, where 40% of the N reserves in the bulbs were invested in root and leaf development during the actively growing season (Chapin, 1980; Karlsson, 1988). It was also found in *Curcuma alismatifolia,* which has two underground storage organs, a rhizome and storage roots, that the N accumulated in both these organs was rapidly depleted after the first 10 weeks of vegetative growth during the growing season (Khuankaew et al., 2010). In *G. multifolia,* this allocation of N from bulb to roots

may have been supported by the initial high N concentrations in bulbs, relative to *G. villosa* bulbs. Similar results were found in a study of the seasonal allocation of biomass and resources in *Sparaxis grandiflora,* where the highest concentration of N was found in daughter corms towards the end of the growing season. This N allocation was acquired from the absorption by other plant parts and indicates a very efficient process of N storage in the daughter corms for this plant species (Ruiters and Mckenzie, 1994). Similar findings were observed in the 'weedy gladiolus' (*Gladiolus caryophyllaceus*), where N that was accumulated by the leafy shoot during the growth phase, redistributed this resource with 70% efficiency to the other plant parts (Hocking, 1993).

Leaf growth is an essential part of the vegetative growth stage, to enable the generation of photosynthate for sustaining growth of other organs. Leaves of spring ephemerals take advantage of the high light conditions in deciduous forests during the winter period and as a consequence have high photosynthetic rates and absorb moisture efficiently over the entire growth stage to supply the energy demands of all actively growing meristems (Lapointe, 2001). In spite of the faster leaf growth of *G. multifolia* during PH1 to PH2, it had a lower leaf:bulb ratio than *G. villosa,* which may indicate the efficiency of *G. villosa* in producing leaves as a proportion of its existing bulb reserves. This may be an apparent benefit of *G. villosa* not depleting its bulb reserves to support the greater proportion of leaf:bulb growth, compared to *G. multifolia.* This suggests that *G. villosa*'s proportionally greater investment in the leaves may have been to increase its photosynthetic capacity in order to drive resources to the bulb for storage before leave

senescence. Similar physiological adaptations were observed in the spring ephemeral, *Erythronium americanum*, which evolved a characteristic phenology that allows them to take advantage of the high light intensities whilst growing on the forest floor, prior to canopy closure during spring. Growth of this species is initiated in autumn and is characterized by the development of new roots and the start of shoot growth (Lapointe and Lerat, 2006).

4.2. Leaf maturity and senescence

Although *G. villosa* produced more leaves per bulb mass, as an indication of photosynthetic investment, the ability to extract as much reserves from these leaves at the end of their life cycle, may also be a distinct advantage for resource-limited plants (Boeken, 1990). Since the senescent phase is followed by the flowering phase in both *G. multifolia* and *G. villosa*, the efficient resource recovery may benefit the plants during flower production. Similar physiological responses were observed in the bulbous geophyte *Haemanthus pubescens* L. subsp. *pubescens* where substantial quantities of N, soluble carbohydrates and starch were moved into the bulb components with the onset of unfavourable conditions (summer drought). This response indicates that the levels of macronutrients, in particular nitrogen and carbohydrate storage in the bulb structure, appeared to be added determinants for flowering in this species (Ruiters, 1995). The greater loss of N in the leaves of *G. multifolia*, compared to *G. villosa* during the senescent phase, may have been to replenish below-ground N in bulbs and roots. This is an interesting possibility for *G. multifolia*, since its leaf production relied heavily on bulb N reserves during the leaf emergent phases.

4.3. Leaf post-senescence and flowering

Although *G. villosa* has smaller bulbs than *G. multifolia*, the similar flower production in the species is an indication of higher resource allocation to flowering in *G. villosa.* This high resource allocation may be underpinned by the efficient utilisation of C and N in *G. villosa* to support such a high ratio of flowers and leaves to bulb size. This was most likely due to the significantly higher C acquisition (SCAR) and utilisation (SCUR) rates of flowers from bulbs in *G. villosa*, compared to *G. multifolia* (Figure 9a;b). The N acquisition (SNAR) and utilisation (SNUR) of flowers from bulb reserves are also significantly higher in *G. villosa* than in *G. multifolia*. Similar findings were reported by Kamenetsky (1994) where *Allium rothii*, a desert geophyte, which lacks specific xerophytic adaptations such as sclerophyllous or succulent leaves, survives drought stress. *Allium rothii* successfully survives in desert environments with nutrient poor soils, due to its effective life strategy, reproductive mechanisms and life cycle specifically adapted to these environments (Chapin et al., 1990; Ruiters et al.. 1993b).

5. Conclusion

It has been reported that resource allocation in plants is the result of both their genotype and environment, and that intra- and interspecific variation has been exhibited by plants for both biomass and nutrient allocation (Chmielewski and Ringius, 1987; Fitter and Setters, 1988; Zimmerman and Whigham, 1992). This investigation found that *G. villosa* has more efficient C and N utilisation during

vegetative and reproductive growth stages than *G. multifolia,* as evidenced by the high leaf and flower production per unit bulb mass and C and N bulb re-allocation during senescence. This may contribute to the less vulnerable conservation status of *G. villosa* relative to *G. multifolia* in their natural semi-arid environment.

References

Amthor, J.S., 2000. The McCree–de Wit-Penning de Vries–Thornley Respiration paradigms: 30 years later. Annals of Botany 86, 1-20.

Boeken, B., 1990. Life histories of two desert species of the bulbous genus *Bellevalia*: The relation between biomass partitioning and water availability. Oecologia 82, 172-179.

Bloom, A.J., Chapin, F.S., Moony, H.A., 1985. Resource limitation in plants - an economic analogy. Annual Review of Ecology and Systematics 16, 363-392.

Chapin, F.S., 1980. The mineral nutrition of wild plants. Annual Review of Ecology and Systematics 11, 233-260.

Chapin, F.S., Schulze, E.D., Mooney, H.A., 1990. The ecology and economics of storage in plants. Annual Review of Ecology and Systematics 21, 423-447.

Chmielewski, J.G., Ringuis, G.S., 1987. Biomass allocation patterns in populations of *Trillium erectum* and *T. grandiflora* in southern Ontario. Canadian Journal of Botany 64, 1671-1675.

Cowling, R.M., Richardson, D.M., Pierce, S.M., 1986. Vegetation of southern Africa. First ed. Cambridge University Press, Cambridge, pp. 89-97.

Currin, C.A., Newell, S.Y., Paerl, H.W., 1995. The role of standing dead *Spartina alterniflora* and benthic microalgae in salt marsh food webs: Considerations based on multiple stable isotope analysis. Marine Ecology Programme Service 121, 99-116.

Dafni, A., Cohen, D., Noy-Meir, I., 1981. Life-cycle variation in geophytes. Annals of the Missouri Botanical Gardens 68, 652-660.

Daniels, C.W., 2007. A study of the propagation and cultivation of *Gethyllis multifolia* and *G. villosa*. MTech dissertation, Cape Peninsula University of Technology, Bellville, Cape Town, pp. 93-98.

Du Plessis, N., Delpierre, G., 1973. Blommeprag uit eie bodem: Koekemakranka. Landbouweekblad Aug, 37-39.

Du Plessis, N., Duncan, G., 1989. Bulbous plants of southern Africa. First ed. Tafelberg Publishers, Cape Town, pp. 67-78.

Elvin, M., 2000. *Gethyllis*. University of California, Irvine. Arboretum 3, 1-2.

Esler, K.J., Rundel, P.W., 1998. Unusual geophytes of the succulent Karoo: How form can relate to function. Veld & Flora 84, 6-7.

Fitter, A.H., Setters, N.L., 1988. Vegetative and reproductive allocation of phosphorus and potassium in relation to biomass in six species of *Viola*. Journal of Ecology 76, 617-636.

Hilton-Taylor, C., 1996. Red data list of southern African plants. First ed. National Botanical Institute, Cape Town, pp. 119-122.

Hocking, P.J., 1993. Seasonal dynamics of the accumulation, distribution and redistribution of dry matter and mineral nutrients in a weedy species of Gladiolus (*Gladiolus caryophyllaceus*). Annals of Botany 71, 495-509.

Horstmann, A., 1999. The genus *Gethyllis*, a leaf to leaf, or spiral to spiral, account. The Indigenous Bulb Association of South Africa 48, 33-37.

Kamenetsky, R., 1994. Life cycle, flower initiation, and propagation of the desert geophyte *Allium rothii*. International Journal of Plant Science 155, 597-605.

Karlsson, S.P., 1988. Seasonal patterns of nitrogen, phosphorus and potassium utilization by three *Pinguicula* species. Functional Ecology 2, 203-209.

Khuankaew, T., Ruamrungsri, S., Ito, S., Sato, T., Ohtake, N., Sueyoshi, K., Ohyama, T., 2010. Assimilation and translocation of nitrogen and carbon in *Curcuma alismatifolia* Gagnep. Plant Biology 12, 414-423.

Liltved, W.R., 1992. The kukumakranka, past and present. Veld & Flora 78, 104-106.

Lapointe, L., 2001. How phenology influences physiology in deciduous forest spring ephemerals. Physiologia Plantarum 113, 151-157.

Lapointe, L., Lerat, S., 2006. Annual growth of the spring ephemeral *Erythronium americanum* as a function of temperature and mycorrhizal status. Canadian Journal of Botany 84, 39-48.

Mortimer, P.E., Perez-Fernandez, M.A., Valentine, A.J. 2008. The role of arbuscular mycorrhizal colonization in the carbon and nutrient economy of the tripartite symbiosis with nodulated *Phaseolus vulgaris.* Soil Biology & Biochemistry 40, 1019-1027.

Müller-Doblies, D.,1986. Enumeration. Willdenowia 15, 465-471.

National State of the Environment Report, 2010. The succulent Karoo biome. http://www.environment.gov.za/enviro-info/nat/biome.htm [9 October].

Nielson, K.L., Amram, E., Lynch, J.P., 2001. The effect of phosphorous availability on the carbon economy of contrasting common bean (*Phaseolus vulgaris* L.) genotypes. Journal of Experimental Botany 52, 329-339.

Noy-Meir, I., 1973. Desert ecosystems: environment and producers. Annual Reviews of Ecology and Systematics 4, 25-51.

Orians, G.H., Solbrig, O.T., 1977. A cost-income model of leaves and roots with special reference to arid and semi-arid areas. American Midland Naturalist 111, 677-690.

Ruiters, C., 1995. Biomass and resource allocation patterns within the bulb of the perennial geophyte *Haemanthus pubescens* L. subsp. *pubescens* (Amaryllidaceae) in a periodic arid environment of lowland fynbos, South Africa. Joumal of Arid Environments 31, 31 l-323.

Ruiters, C., Mckenzie, B., 1994. Seasonal allocation and efficiency of biomass and resources in the perennial geophyte *Sparaxis grandiflora* subsp. *fibriata* (Iridaceae) in lowland coastal fynbos, South Africa. Annals of Botany 74, 33-646.

Ruiters, C., McKenzie, B., Aalbers, J., Raitt, L.M., 1993a. Seasonal allocation of biomass and resources in the geophytic species *Haemanthus pubescens* subs. *pubescens* in lowland coastal fynbos, South Africa. South African Journal of Botany 59, 251-258.

Ruiters, C., McKenzie, B., Raitt, L.M., 1993b. Life history studies of the perennial bulbous geophyte species *Haemanthus pubescens* subsp. *pubescens* (Amaryllidaceae) in lowland coastal fynbos, South Africa. International Journal of Plant Sciences 154, 441-449.

Ryan, M.G., Lavigne, M.B., Gower, S.T., 1997. Annual carbon cost of autotrophic respiration in boreal forest ecosystems in relation to species and climate. Journal of Geophysical Research 102, 871-883.

Snijman, D., 2004. *Gethyllis.* http://www.plantzafrica.com/plantefg/gethyllis.html [19 November].

Zimmerman, J.K, Whigham, D.F., 1992. Ecological functions of carbohydrates stored in corms of *Tipularia discolor* (Orchidaceae). Functional Ecology 6, 575-581.

CHAPTER 5: RESEARCH PAPER 3 - Comparative antioxidant-capacity and -content of leaves, bulbs, roots, flowers and fruit of *Gethyllis multifolia* L. Bolus and *G. villosa* Thunb. species

CHAPTER 5

RESEARCH PAPER 3

Comparative antioxidant-capacity and -content of leaves, bulbs, roots, flowers and fruit of *Gethyllis multifolia* L. Bolus and *G. villosa* Thunb. species

C. W. Daniels[a], F. Rautenbach [b], W. T. Mabusela [c], A. J. Valentine [d], J. L. Marnewick [e]

[a]Department of Horticultural Sciences, Cape Peninsula University of Technology, P.O. Box 1906, Bellville 7535, South Africa.

[c]South African Herbal Science and Medicine Institute, University of the Western Cape, Private Bag X17, Bellville 7535, South Africa.

[b,e]Oxidative Stress Research Centre, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, P.O. Box 1906, Bellville 7535, South Africa.

[d]Department of Botany and Zoology, University of Stellenbosch, Private Bag X1, Matieland 7602, Stellenbosch, South Africa

Abstract

The oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP), the ABTS radical cation scavenging ability, total polyphenol, flavonol/

flavone and flavanone contents were measured in the leaves, bulbs, roots, flowers and fruit (dry weight) of two natural populations of *Gethyllis multifolia* (Kukumakranka) and *G. villosa*. The flowers and fruit of *G. multifolia* and *G. villosa* showed higher, and in some cases significantly ($P<0.05$) higher antioxidant activities and total polyphenol content when compared to the leaves, bulbs and roots. The flavonol/ flavone content of the fruit and flowers of *G. multifolia* was also found to be higher than the other plant parts. The fruit and flower flavonol/ flavone content for *G. villosa* was lower than the other plant parts, whereas the flavanone content in the fruit and flowers for both species were found to be lower than the other plant parts. The total polyphenol content in the fruits of *G. multifolia* (21.54 mg GAE/g) and *G. villosa* (27.64 mg GAE/g) were found to be in agreement with those of raisins (28.30 mg GAE/g) and blueberries (24 mg GAE/g). The FRAP values of *G. multifolia* flowers (76.66 µmole AAE/g) and fruit (91.51 µmole AAE/g) were found to be significantly ($P<0.05$) higher than those of the other plant parts (16.76 to 39.08 µmole AAE/g). On the other hand, the flowers (590.23 µmole TE/g) and fruit (741.16 µmole TE/g) of *G. villosa* revealed a significantly ($P<0.05$) higher ORAC when compared to the other plant parts (251.25 to 410.60 µmole TE/g). A strong correlation was evident in the fruit of both species between the total polyphenols and FRAP ($r=0.95$), ORAC ($r=0.95$) and flavonol content ($r=0.79$). No flavanols were detected in the leaves, bulbs, roots, flowers and fruit of both species.

Key words: ABTS; Antioxidant; flavanones; flavonols; FRAP; *Gethyllis*; Kukumakranka; ORAC; polyphenols.

1. Introduction

The medicinal value of plants has become more evident during the past few decades owing largely to the discovery of extracts from plants that contain a diverse array of secondary metabolites with antioxidant potential (Akinmoladun et al., 2007). Free radicals, as associated with exposure to environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods as well as physical stress, may cause depletion of antioxidants which are implicated in the protection of the immune system (Halliwell, 1994; Kühnau, 1976; Kumpulainen and Salonen, 1999; Younes, 1981). These free radicals also contribute to many disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of various tissues, central nervous system injury, gastritis, cancer and AIDS (Kumpulainen, 1999; Cook and Samman, 1996). Recently there has been an upsurge of interest in the therapeutic potentials of medicinal plants in reducing such free radical-induced tissue injury (Schuler, 1990).

Fruits and vegetables contain high levels of polyphenolic compounds with antioxidant activity and can reduce the risk of chronic inflammation in humans (Finley, 2004). These findings have inspired the widespread screening of plants for possible medicinal and antioxidant properties, and the development and utilisation of antioxidants of natural origin (Jayaprakasha et al., 2001). Recent antioxidant studies further confirmed that our knowledge is still too limited for formulation of

recommendations for the general population or for particular populations at risk of specific diseases (WHO/FAO, 2003).

Gethyllis multifolia L. Bolus and *Gethyllis villosa* Thunb. (family: Amaryllidaceae) are deciduous, winter-growing, summer-blooming, bulbous geophytes indigenous to South Africa (Du Plessis and Delpierre, 1973). The genus *Gethyllis* (more commonly known as "Kukumakranka" in South Africa), consists of 37 currently accepted species and subspecies (Müller-Doblies, 1986) and is one of the most extraordinary and poorly researched of all southern African genera (Liltved, 1992). *Gethyllis* species grow under full sun conditions and their natural habitats range from sandy soils to mountainous rocky terrains. The genus have four growth phases: a winter (cold and wet) growing phase, leaf senescence towards spring, flowering during the hot and dry summer months (when no leaves are present) and fruit formation in autumn, which is the start of a new growing season (Du Plessis and Duncan, 1989). The medicinal uses of this genus range from cures for colic, digestive disturbances, teething problems, fatigue, boils, bruises and insect bites, to being used as an aphrodisiac (Du Plessis and Delpierre, 1973; Louw et al., 2002; Van Wyk et al., 2009). Apart from its medicinal properties, many members of this genus (including *G. multifolia*) have a highly fragrant, edible fruit, which was used in the past to perfume rooms and linen (Lighton, 1992).

Studies by Babajide et al. (2010) and Elgorashi & Van Staden (2003) revealed anti-inflammatory and anti-bacterial activity for both *G. multifolia* and *G. villosa*.

Babajide (2009) further reported higher polyphenolic content, flavonoid content and antioxidant activity in whole plants for *G. multifolia* when compared to *G. villosa*. Although, according to Rood (1994), the fruit and flowers are the most medicinally useful parts of *Gethyllis* species, there has however, to our knowledge, been no published literature reporting on scientific studies based on these plant parts. Previously, the following compounds, the dihydroxydimethylbenzopyran-4-one: isoeugenitol, its 5-*O*-glycoside and 9Z-octadec-9-enamide, have been isolated from the roots and bulbs of *G. ciliaris* (Elgorashi et al., 2007). It is also proposed, through photographic studies of the fruit of *G. multifolia* and *G. villosa*, that the dark red colour in the fruit of *G. multifolia* may be due to the presence of colour pigments i.e. carotenoids or anthocyanins that are known to have antioxidant activity (Daniels, 2007). Antioxidant studies by Babajide (2009) found the presence of anthocyanins in water and methanol extracts of whole plants of *G. multifolia* while none was detected in similar extracts of *G. villosa*.

In South Africa, there is a reluctance amongst traditional healers to use medicinal plants from cultivated sources, although some would be willing to test their potency (Dold and Cocks, 2002). *Gethyllis multifolia* is threatened in its natural habitat and conservation methods to prevent traditional healers from plundering the natural biodiversity have largely been unsuccessful (Dold and Cocks, 2002). *Gethyllis multifolia* and many other members of this genus have a highly fragrant and tasty fruit with claimed medicinal uses by traditional healers and old folk. The genus *Gethyllis* is poorly researched and there are many reports of its medicinal uses in the

past which have not been scientifically justified. This study reports on the comparative investigation of antioxidant-capacity and -content of the leaves, bulbs, roots, flowers and fruit of natural populations of *G. multifolia* and *G. villosa*.

2. Materials and methods

2.1. Plant materials

Gethyllis multifolia and *G. villosa* bulbs were obtained, with permission from the Karoo National Biodiversity Garden, in 2006 after their winter growth phase (July to mid-August), from their natural habitat (Worcester, Western Cape, South Africa). Mature plants of the same height and diameter were excised into leaves, bulbs and roots (n=10) and dried in a laboratory oven (Memmert, Laboratory & Scientific, Cape Town, South Africa) at 50 ºC for 48 hours. The bulbs of both species took approximately five days to dry. Individual plant parts were ground to a powder in a portable spice grinder (Krups 75 model F203, Hecho En Mexico, Mexico City, Mexico). From the same population, flowers (n=10) of both species were collected at the beginning of December 2006 (summer), while the fruit (n=7) were collected towards the end of March 2007 (autumn). The same drying and grinding procedures as for the leaves and roots were followed.

2.2. Sample preparation

Crude extracts of the leaves, bulbs, roots, flowers and fruit of both species were prepared without delay, by stirring the various dried, powdered plant materials (0.05 g of each) in 80% (v/v) ethanol (50 mL) (EtOH) (Saarchem, South Africa) whereafter

it was centrifuged at 4000 rpm for 5 min. The supernatants were used for all analyses. The same sample preparation technique was followed for all assays and all analyses were performed in triplicate.

2.3. Total polyphenol, flavonol/ flavone and flavanone content

The total polyphenol content of the various crude extracts was determined by the Folin Ciocalteu method (Singleton et al., 1999). Using a 96-well clear (visible range) microplate, 25 µL of sample was mixed with 125 µL Folin-Ciocalteu reagent (Merck, South Africa), diluted 1:10 with distilled water. After 5 min., 100 µL (7.5%) aqueous sodium carbonate (Na_2CO_3) (Sigma-Aldrich, South Africa) was added to the well. The plates were incubated for 2 hours at room temperature before the absorbance was read at 765 nm using a Multiskan plate reader (Thermo Electron Corporation, USA). The standard curve was prepared using 0, 20, 50, 100, 250 and 500 mg/L gallic acid in 10% EtOH and the results were expressed as mg gallic acid equivalents per g dry weight (mg GAE/g DW).

The flavonol content was determined using quercetin (0, 5, 10, 20, 40, 80 mg/L) in 95% ethanol (Sigma-Aldrich, South Africa) as standard. This assay measures both flavonols and flavones since both groups absorb ultra-violet light maximally around 360 nm. In the sample wells, 12.5 µL of the crude sample extracts was mixed with 12.5 µL 0.1% HCl (Merck, South Africa) in 95% ethanol, 225 µL 2% HCl and incubated for 30 min. at room temperature. The absorbance was read at 360 nm at a

temperature of 25 ºC (Mazza et al., 1999). The results were expressed as mg quercetin equivalents per g dry weight (mg QE/g DW).

The flavanone content was determined using an adapted version of the method as described by Kosalek et al. (2004). This method was adapted with minor modifications such as reducing assay volumes for the 96-well plates. Briefly, 100 µL of sample was mixed with 200 µL 1% 2,4-dinitrophenylhydrazine (DNPH) (2% H_2SO_4 in methanol (MeOH). After incubation at 50 ºC for 50 min., 700 µL of 10% potassium hydroxide (KOH) in 70% MeOH was added. The samples were centrifuged and 30 µL of the resulting supernatant mixed with 270 µL MeOH in a 96-well clear plate (visible range) and the absorbance read at 495 nm. A linear standard curve using 0, 0.2, 0.5, 1.0, 1.5, 2.0 mg/mL naringenin (Sigma-Aldrich, South Africa) in methanol was included. The results were expressed as mg naringenin equivalents per g dry weight (mg NE/g DW).

2.4. Antioxidant capacity

2.4.1. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed using the method of Benzie and Strain (1999). In a 96-well clear microplate (visible range), 10 µL of the crude sample extract was mixed with 300 µL FRAP reagent [0.3 M acetate buffer, pH 3.6 (Saarchem, South Africa), 10 mM 2, 4, 6-tripyridyl-*s*-triazine (TPTZ) in 0.1 M HCl (Sigma-Aldrich, South Africa), 20 mM iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) (Sigma-Aldrich, South Africa), 6.6 mL distilled water] and incubated for 30 min. at 37 ºC in the plate reader.

Absorbance was measured at 593 nm. L-Ascorbic acid (Sigma-Aldrich, South Africa) was used as a standard with concentrations varying between 0 and 1 000 µM. The results were expressed as µM ascorbic acid equivalents per g dry weight (µM AAE/g DW).

2.4.2. 2,2 -azino-di-3-ethylbenzthiazoline sulphonate (ABTS) assay

The ABTS assay was performed following the method of Re et al. (1999). The stock solutions included 7 mM ABTS and 140 mM potassium-peroxodisulphate ($K_2S_2O_8$) (Merck, South Africa) solutions. The working solution was then prepared by adding 88 µL $K_2S_2O_8$ solution to 5 mL ABTS solution. The two solutions were mixed well and allowed to react for 24 h at room temperature in the dark. Trolox (6-hydrox-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the standard with concentrations ranging between 0 and 500 µM. The ABTS mix solution was diluted with ethanol to read the start-up absorbance (control) of approximately 2.0 (±0.1). Crude sample extracts (25 µL) were allowed to react with 300 µL ABTS in the dark at room temperature for 30 min. before the absorbance was read at 734 nm at 25 ºC in a plate reader. The results were expressed as µM Trolox equivalents per g dry weight (µM TE/g DW).

2.4.3. Oxygen radical absorbance capacity (ORAC) assay

The H-$ORAC_{FL}$ values were determined according to the methods of Prior et al. (2003) and Wu et al. (2004). A stock standard solution of Trolox (500 µM) was

diluted in phosphate buffer (75 mM, pH 7.4) to provide calibration standards ranging from 5 to 25 µM. The Fluoroskan ascent plate reader (Thermo Fisher Scientific, Waltham, USA) equipped with an incubator was set at 37 ºC. Fluorescence filters with an excitation wavelength of 485 nm and emission wavelength of 538 nm were used. A fluorescein stock solution was prepared in phosphate buffer and further diluted to provide a final concentration of 14 µM per well. The peroxyl generator, 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH) (25 mg/ mL in phosphate buffer), was added with a multichannel pipette to give a final AAPH concentration of 4.8 mM in each well. The fluorescence from each well, containing 12 µL diluted hydrophilic extract, was read every 5 min. for 2 hours. The final $ORAC_{FL}$ values were caculated using the regression equation $y = ax^2 + bx + c$ between the Trolox concentration (µM) and the area under the curve. The results were expressed as µM Trolox equivalents per g dry weight (µM TE/g DW).

2.5. Statistical analysis

The statistical significance between antioxidant activity values of the various crude plant extracts was determined by an analysis of variance (ANOVA) where $P<0.05$ was considered to be statistically significant. The computer program employed for the statistical analysis was Medcalc version 9.4.2.0 (Medcalc, Belgium). Microsoft Office Excel 2006, version 12 (Microsoft Corporation, USA) was employed to determine the correlation between antioxidant contents and activity.

3. Results

3.1. Total polyphenol, flavonol/ flavone and flavanone content

The total polyphenol content in the leaves and bulbs of *Gethyllis villosa* were found to be significantly ($P<0.05$) higher than those of *G. multifolia* (Table 1). On the contrary, the polyphenol content of the roots of *G. multifolia* was significantly ($P<0.05$) higher than that of *G. villosa*. No significant difference ($P>0.05$) was observed between the flowers of both species, but a significantly ($P<0.05$) higher polyphenolic content of 28% was recorded for the fruit of *G. villosa*, when compared to *G. multifolia*. The total polyphenol content in the flowers and fruit of *G. multifolia* were found to be significantly ($P<0.05$) higher than the other plant parts. In *G. villosa*, only the total polyphenol content in the fruit were found to be significantly ($P<0.05$) higher than the other plant parts.

In *Gethyllis villosa*, the flavonol contents of the leaves and bulbs were significantly ($P<0.05$) higher than the leaves and the bulbs of *G. multifolia* (Table 1). On the other hand, the flavonol contents of the flowers in *G. multifolia* were found to be significantly ($P<0.05$) higher than the flowers of *G. villosa*. The flavonol contents in the roots were 16% higher ($P>0.05$) for *G. multifolia* when compared to the roots of *G. villosa*. The flavonol value for the fruit of *G. villosa* was found to be 12% higher ($P<0.05$) than that of *G. multifolia*. In *G. multifolia*, the flavonol contents were found to be higher ($P>0.05$) in the flowers and fruit compared to the other plant parts. In *G. villosa*, the flavonol content was higher ($P>0.05$) in the leaves and fruit compared to

the other plant parts. The flavonol contents were higher in *G. villosa* for the leaves, bulbs and fruit when compared to *G. multifolia*.

Table 1: The total polyphenol (mg GAE/g dry weight), flavonol/ flavone (mg QE/g dry weight) and flavanone (mg NE/g dry weight) content of the leaves, bulbs, roots, flowers and fruit of *Gethyllis multifolia* and *G. villosa* plants.

Plant parts	Total polyphenols	Flavonols/ flavones	Flavanones
G. multifolia			
Leaves	12.36±0.57a	2.60±0.30a	3.27±0.19a
Bulbs	7.67±1.30c	1.20±0.58b	3.02±0.45a
Roots	14.32±1.52b	3.70±0.78c	4.12±0.87b
Flowers	20.86±2.58de	7.12±1.38d	2.09±0.36ce
Fruit	21.54±11.45e	5.70±4.32eg	2.26±1.18ce
Fruit (FW)	1.74±0.31	*	*
G. villosa			
Leaves	20.81±1.95de	6.74±0.61dg	4.42±1.11bd
Bulbs	17.84±8.16d	5.55±3.25e	5.15±2.17d
Roots	12.04±2.44a	3.20±1.46a	4.05±0.98b
Flowers	22.23±5.46e	4.76±1.65f	2.50±0.37e
Fruit	27.64±8.57f	6.38±5.25g	2.99±0.81ae
Fruit (FW)	2.73±0.39	*	*

Values represent the mean ± SD (leaves, bulbs, roots and flowers n=10; fruit n=7). Means in the same column with different letters are significantly ($P<0.05$) different. GAE: gallic acid equivalents; QE: quercetin equivalents and NE: naringenin equivalents; FW: Fresh weight.

In *Gethyllis villosa*, the flavanone contents of the leaves and bulbs were significantly ($P<0.05$) higher than those of the leaves and bulbs of *G. multifolia* (Table 1), but the flavanone contents in the roots of both species were very similar (less than 2%). There was no significant ($P>0.05$) difference in the flavanone content of the flowers and fruit of *G. villosa* when compared to those of *G. multifolia* (Table 1). The flavanone contents in the flowers and fruit of *G. multifolia* were significantly ($P<0.05$) lower compared to the other plant parts. *Gethyllis villosa* revealed a similar trend with significantly ($P<0.05$) lower flower and fruit flavanone contents compared to the other plant parts. The flavanone contents for the leaves and bulbs of *G. villosa* were higher than that of *G. multifolia*. In contrast to the total polyphenols and flavonols, the flavanone content in the flowers and fruit of both species were found to be lower than the other plant parts.

3.2. Antioxidant capacity

The FRAP values of *Gethyllis villosa* were found to be significantly ($P<0.05$) higher in the leaves and bulbs than those of *G. multifolia*. The average flower and fruit FRAP values of *G. villosa* were not significantly higher ($P>0.05$) than those of *G. multifolia*. The flower and fruit FRAP values of *G. multifolia* were found to be significantly ($P<0.05$) higher than those of the other plant parts (Table 2). *G. villosa* displayed a similar trend with higher FRAP values in the flower and fruit when compared to the leaves and significantly higher ($P<0.05$) compared to the bulbs and roots.

Only the ORAC value of the roots of *Gethyllis multifolia* was found to be significantly ($P<0.05$) higher (109%) compared to the roots of *G. villosa* (Table 2). The ORAC values of the other plant parts of *G. villosa* were not significantly ($P>0.05$) different from those of *G. multifolia*. The ORAC value of the flowers and fruit of *G. multifolia* were found to be higher ($P>0.05$) than the roots and significantly ($P<0.05$) higher than that of the leaves and bulbs. *G. villosa* revealed a similar trend with significantly ($P<0.05$) higher ORAC values in the flowers and fruit when compared to the other plant parts. A strong correlation was also evident in the fruit of both species between the ORAC and FRAP ($r=0.94$), and ORAC and flavonol content ($r=0.77$).

Table 2: The ferric reducing antioxidant power (FRAP) (µM AAE/g dry weight), oxygen radical absorbance capacity (ORAC) (µM TE/g dry weight) and ABTS radical cation scavenging ability (ABTS) (µM TE/g dry weight) of the leaves, bulbs, roots, flowers and fruit of *Gethyllis multifolia* and *G. villosa* plants.

Plant parts	ORAC	FRAP	ABTS
G. multifolia			
Leaves	375.29±26.99ad	38.53±2.78a	28.96±2.00a
Bulbs	322.98±54.45ae	16.76±4.08b	14.35±2.65b
Roots	525.17±75.61bg	39.08±6.60a	29.76±4.10a
Flowers	627.48±55.38cg	76.66±6.11c	105.48±6.33c
Fruit	672.67±329.00ch	91.51±36.97ce	103.09±35.51c
Fruit (FW)	86.65±17.56	*	*
G. villosa			
Leaves	410.60±50.88d	87.90±9.68ce	76.83±3.81d
Bulbs	287.12±115.56ef	72.21±37.16cd	62.82±19.89e
Roots	251.25±39.82f	52.91±11.81da	54.53±6.92e
Flowers	590.23±156.58g	92.68±33.28ce	107.57±21.97c
Fruit	741.16±140.50h	103.45±17.81e	117.21±22.43c
Fruit (FW)	94.90±19.11	*	*

Values represent the means ± SD (leaves, bulbs, roots and flowers n=10; fruit n=7). Means in the same column with different letters are significantly ($P<0.05$) different. AAE: ascorbic acid equivalents; TE: Trolox equivalents; FW: Fresh weight.

The ABTS radical cation scavenging ability of the leaves, bulbs and roots of *G. villosa* were found to be significantly ($P<0.05$) higher than those of *G. multifolia* (Table 2). Results for the flowers and fruit were similar to those of *G. multifolia*. The ABTS radical cation scavenging ability of the flowers and fruit of *G. multifolia* were significantly ($P<0.05$) higher than those of the other plant parts. Furthermore a strong correlation was evident in the fruit of both species between the total polyphenols and FRAP ($r=0.95$), and ORAC ($r=0.95$) and the flavonol content ($r=0.79$).

4. Discussion and conclusion

According to Prior et al. (2005), it was recommended, through evaluation of the literature and data presented at the 'First International Congress on Antioxidant Methods in 2004', that three methods be considered for standardization of antioxidant capacity and total polyphenol determination in food and dietary supplements. Method 1: the oxygen radical absorbance capacity (ORAC) assay which represents a hydrogen atom transfer (HAT) reaction mechanism. Method 2: the trolox equivalent antioxidant capacity (TEAC/ABTS) assay represents a single electron transfer (SET) -based method which indicates reducing capacity. Method 3: the ferric reducing/antioxidant power (FRAP) assay which is also a SET-based assay and a direct test of the total antioxidant power of a biological sample. It is further suggested that experimental work on a series of assays gives a better understanding of the antioxidant capacity of a sample (Prior et al., 2005; Prior and Cao, 1999).

Babajide (2010) recorded a higher total polyphenol content for *G. multifolia* where whole plants of *G. multifolia* and *G. villosa* were compared. The present study, however, only found a higher total polyphenol content in the roots of *G. multifolia* compared to higher values for the leaves, bulbs, flowers and fruit of *G. villosa*. In addition to a higher total polyphenol content, the flavonol content for *G. multifolia* whole plants was also found to be higher than that of *G. villosa*, compared to varying values for different plant parts of both species in this study. A similar trend was observed in a comparison of the flavanone content of the two species when results in this study were compared to those of Babajide (2010).

Furthermore, in this study, the flowers and fruit of *G. multifolia* and *G. villosa* generally showed higher and in some cases significantly higher total polyphenol and antioxidant capacity than the leaves, bulbs and roots. The polyphenol content in the fruits of *G. multifolia* (21.54 mg GAE/g) and *G. villosa* (27.64 GAE) on a dry basis were found to be in line with other commercial fruits such as raisins (28.30 GAE), blueberries (24.00 GAE), strawberries (15.40 GAE), plums (9.49 GAE), oranges (7.50 GAE) and red grapes (7.39 GAE) (PR WEB, 2005).

The fruit pulp of the amarula tree (*Sclerocarya birrea*) has a higher polyphenol value than the fruit of *G. multifolia* but a lower value than the fruit of *G. villosa* (Table 1), when fresh weights were compared (Ndhlala et al., 2007). The flavonol content in the fruit of *G. multifolia* and *G. villosa* was found to be higher than that of raw beehive propolis samples (Kosalek et al., 2004). When compared to some commercial vegetables, the polyphenol content of the fruit and flowers of both

species, as well as the leaves of *G. villosa* was higher than that of spinach (17.70 GAE) broccoli (8.80 GAE), brussels sprouts (9.80 GAE), beetroot (8.40 GAE) and onions (4.50 GAE). Of all the vegetables mentioned, only the polyphenol content of spinach was found to be higher than the leaves of *G. multifolia* (12.36 GAE) (PR WEB, 2005). The polyphenol content of certain guava cultivars were found to be similar to the fruit of *G. multifolia* and *G. villosa* (Table 1) on a fresh weight basis (Thaipong et al., 2006).

The ORAC values of the flowers and fruit of *G. multifolia* were found to be significantly higher than the fruit of the caneberry range (red raspberry, black raspberry, boysenberry, Marion blackberry and evergreen blackberry) (Bushman et al., 2004; Wang and Lin, 2000) while values reported for kiwi fruit, banana, apple, tomato and orange (Wang et al., 1996) were found to be significantly lower than the fruit of the *Gethyllis* species in this study. ORAC values for the fresh weights of the fruit of both *G. multifolia* and *G. villosa* (Table 2) were also found to be higher than the fresh weights of the following herb species: *Thymus vulgaris*, *Aloe vera*, *Lavandula angustifolia*, *Petroselinum crispum* and *Rosmarinus officinalis* (Zheng and Wang, 2001). A study by Babajide (2010) revealed similar ORAC values for *G. multifolia* and *G. villosa* when whole plants of both species were compared. This study, however, revealed varying results for *G. multifolia* and *G. villosa* when the ORAC values of leaves, roots, bulbs, flowers and fruit were compared. Furthermore, Babajide (2010) reported higher FRAP values for *G. multifolia* (whole plants), which is contradictory to findings

in this study, in which higher FRAP values were recorded for all the plant parts of *G. villosa* when compared to those of *G. multifolia*.

Commercialization of southern African medicinal plants is a process that has rapidly been gaining momentum during the last 10 years (Van Wyk, 2008). The genus *Gethyllis* has not been grown commercially before and some of its species (including *G. multifolia*) appear on 'The Red Data List of Southern African Plants' as endangered, threatened or vulnerable (Hilton-Taylor, 1996). Thus, a serious need still exist for the propagation, cultivation, conservation and pharmaceutical research on members of this genus. This *in vitro* study has shown that the flowers and fruit of both species may be used as good natural sources of dietary antioxidants. Further studies are also needed to identify individual active components responsible for the antioxidant activity shown here and to investigate the powerful fragrance and taste of the fruit of *G. multifolia* for possible use in the food and beverages industry.

Acknowledgements

The authors acknowledge the Cape Peninsula University of Technology (CPUT) for financial assistance, the Department of Horticultural Sciences (CPUT) for the use of the nursery facilities and laboratory facilities at the Antioxidative Stress Research Centre (CPUT). The authors further acknowledge the Karoo National Biodiversity Garden for permission to harvest *G. multifolia* and *G. villosa* plants from their natural habitat. Gratitude is expressed to Naomi Daniels for the preparation of the plant samples.

References

Akinmoladun, A.C., Ibukun, E.O., Afor, E., Akinrinlola, B.L., Onibon, T.R., Akinboboye, A.O., Obuotor, E.M., Farombi, E.O., 2007 Chemical constituents and antioxidant activity of *Alstonia boonei*. African Journal of Biotechnology 6, 1197-1201.

Babajide, J.O. (2009). Chemical and biological investigation into some selected african indigenous medicinal plants. Doctoral thesis, University of the Western Cape, Bellville, South Africa: pp. 218-225.

Babajide, J.O., Mabusela, W.T., Green, I.R., Ameer, F., Weitz, F., Iwuoha, E.I. (2010). Phytochemical screening and biological activity studies of five South African indigenous medicinal plants. *Journal of Medicinal Plants Research*, 4: 1924-1932.

Benzie, F.F., Strain, J.J., 1999. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods in Enzymology 299, 15-27.

Bushman, B., Phillps, B., Isbell, T., Ou, B., Crane, J., Knapp, S., 2004. Chemical composition and antioxidant potential of the seeds and oils of caneberry (*Rubus spp*.). Journal of Agricultural and Food Chemistry 52, 7982-7987.

Cook, N.C., Samman, S., 1996. Flavonoids- chemistry, metabolism, cardioprotective effects, and dietary sources. Journal of Nutritional Biochemistry 7, 66-76.

Dold, A.P., Cocks, M.L., 2002. The trade in medicinal plants in the eastern Cape Province, South Africa. South African Journal of Science 98, 589-596.

Du Plessis, N., Delpierre, G., 1973. Blommeprag uit eie bodem: Koekemakranka. Landbouweekblad, August, pp. 37-39.

Du Plessis, N., Duncan, G., 1989. Bulbous plants of southern Africa, Tafelberg Publishers, Cape Town, South Africa, pp. 104.

Elgorashi, E.E., Coombes, P.H., Mulholland, D.A., Van Staden, J., 2007. Isoeugenitol, a cyclooxygenase-1 inhibitor from *Gethyllis ciliaris*. South African Journal of Botany 73, 156-158.

Elgorashi, E.E., Van Staden, J., 2003. Pharmacological screening of six amaryllidaceae species. Journal of Ethnopharmacology 90, 27-32.

Finley, J.W., 2004. Phenolic antioxidants and prevention of chronic inflammation. Food Technology, 58, 42-46.

Halliwell, B., 1994. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? Lancet 344, 721-724.

Hilton-Taylor, C., 1996. Red data list of southern African plants. South African National Biodiversity Institute, Cape Town, South Africa, pp. 57.

Jayaprakasha, G.K., Singh, R.P., Sakariah, K.K., 2001. Antioxidant activity of grape seed (*Vitrus vinifera*) extracts on peroxidation models *in-vitro*. Food Chemistry 73, 285-290.

Kosalek, I., Bakmaz, M., Pepeljnjak, S., Vladimir-Knezevic, S., 2004. Quantitative analysis of the flavanoids in raw propolis from northern Croatia. Acta Pharmaceutica 54, 65-72.

Kühnau, J., 1976. The flavonoids. A class of semi-essential food components; their role in human nutrition. World Review of Nutrition and Dietetics 24, 117-191.

Kumpulainen, J.T., Salonen, J.T., 1999. Natural antioxidants and anticarcinogens in nutrition, health and disease, The Royal Society of Chemistry, UK, pp. 178-187.

Lighton, C., 1992. The kukumakranka. Veld & Flora, December, pp. 100-103.

Liltved, W.R., 1992. The kukumakranka, past and present. Veld & Flora, December, pp. 104-106.

Louw, C.A., Regniers, T.C., Kortsen, L., 2002. Medicinal bulbous plants of South Africa and their traditional relevance in the control of infectious diseases. Journal of Ethnopharmacology 82, 147-154.

Mazza, G., Fukumoto, L., Delaquis, P., Girard, B., Ewert, B., 1999. Anthocyanins, phenolics and color of Cabernet Franc, Merlot and Pinot Noir wines from British Columbia. Journal of Agricultural and Food Chemistry 47, 4009-4017.

Müller-Doblies, 1986. Enumeration. Willdenowia 15, 465-471.

Ndhlala, A.R., Kasiyamhuru, A., Mupure, C., Chitindingo, K., Benhura, M.A., Muchuweti, M., 2007. Phenolic composition *of Flacourtia indica, Opuntia megacantha* and *Sclerocarya birrea*. Food Chemistry 103, 82-87.

PR WEB. (2005). Jobelyn, African wonder herb set to rule the world of natural plant antioxidants and phenolics. [Accessed February 29, 2008] http://www.prweb.com/pdfdownload/312415.pdf.

Prior, R.L., Cao, G., 1999. In vivo total antioxidant capacity: Comparison of different analytical methods. Free Radical Biology & Medicine 27, 1173-1181.

Prior, R.L., Hoang, H., Gu, L., Wu, X., Bacchiocca, M., Howard, L., Hampsch-Woodill, M., Huang, D., Ou, B., Jacob, R., 2003. Assays for hydrophilic and lipophilic antioxidant capacity ($ORAC_{FL}$) of plasma and other biological and food samples. Journal of Agricultural and Food Chemistry 51, 3273-3279.

Prior, R.L., Wu, X., Schaich, K., 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. Journal of Agricultural and Food Chemistry 53, 4290-4302.

Re, R., Pellegrini, N., Proteggente, A, Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation assay. Free Radical Biology & Medicine 26, 1231-1237.

Rood, B. Uit die veldapteek., 1994. Tafelberg Publishers, Cape Town, South Africa, pp. 4.

Schuler, P., 1990. Natural antioxidants exploited commercially, In food antioxidants, Hudson BJF (ed.). Elsevier, London, UK, pp 99-170.

Singleton, V.L., Orthofer, R., Lamuela-Raventos, R.M., 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods in Enzymology 299, 152-178.

Thaipong, K., Boonprakop, U., Crosby, K., Cisneros-Zevallos, L., Byrne, D.H., 2006. Comparison of ABTS, DPPH, FRAP and ORAC assays for estimating antioxidant activity from guava fruit extracts. Journal of Food Composition and Analysis 19, 669-675.

Van Wyk, B., 2008. A broad review of commercially important southern African medicinal plants. Journal of Ethnopharmacology 119, 342-355.

Van Wyk, B., Van Oudtshoorn, B., Gericke, N., 2009. Medicinal plants of South Africa. 2nd Ed. Briza Publications, Pretoria, South Africa, pp. 151-152.

Wang, H., Cao, G., Prior, R.L., 1996. Total antioxidant capacity of fruits. Journal of Agricultural and Food Chemistry 44, 701-705.

Wang, S.Y., Lin, H., 2000. Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. Journal of Agricultural and Food Chemistry 48, 140-146.

Watt, J.M., Breyer-Brandwijk, M.G., 1962. The medicinal and poisonous plants of southern and eastern Africa. 2nd ed. Livingstone, London, UK, pp. 32.

WHO/FAO. (2003). WHO Technical report series: Joint WHO/FAO expert consultation on diet, nutrition, and the prevention of chronic diseases, 28 January - 1 February 2002. WHO Publications, Geneva, Switzerland.

Wu, X., Beecher, G.R., Holden, J.M., Haytowitz, D.B., Gebhardt, S.E., Prior, R.L., 2004. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. Journal of Agricultural and Food Chemistry 52, 4026-4037.

Younes, M., 1981. Inhibitory action of some flavonoids on enhanced spontaneous lipid peroxidation following glutathione depletion. Planta Medica 43, 240-245.

Zheng, W., Wang, S.Y., 2001. Antioxidant activity and phenolic compounds in selected herbs. Journal of Agricultural and Food Chemistry 49, 5165-5170.

CHAPTER 6: RESEARCH PAPER 4 - Comparative antioxidant-capacity and -content of *Gethyllis multifolia* L. Bolus and *G. villosa* Thunb. species under drought and photo-stress

CHAPTER 6

RESEARCH PAPER 4

Comparative antioxidant-capacity and -content of *Gethylli*s *multifolia* L. Bolus and *G. villosa* Thunb. species under drought and photo-stress

C.W. Daniels [a], F. Rautenbach [b], W.T. Mabusela [c], A.J. Valentine [d], and J.L. Marnewick [b]

[a] *Department of Horticultural Sciences, Cape Peninsula University of Technology, P.O. Box 1906, Bellville 7535, South Africa.*

[c] *South African Herbal Science and Medicine Institute, University of the Western Cape, Private Bag X17, Bellville 7535, South Africa.*

[b] *Oxidative Stress Research Centre, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, P.O. Box 1906, Bellville 7535, South Africa.*

[d] *Department of Botany and Zoology, University of Stellenbosch, Private Bag X1, Matieland 7602, Stellenbosch, South Africa*

Abstract

The total polyphenol, flavonol/ flavone and flavanone contents, oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP) and radical cation scavenging ability (ABTS) were measured in the leaves, bulbs and

roots (dry weight) of *Gethyllis multifolia* (Kukumakranka) and *Gethyllis villosa* under photo- and drought stress. The antioxidant activity and content in the bulbs of both species were relatively low and consistent compared to significant fluctuations in the leaves and roots subjected to the two environmental stress factors. In general, significantly ($P<0.05$) higher antioxidant activity was observed in *G. multifolia* plants compared to *G. villosa*. Significantly ($P<0.05$) higher antioxidant activity was observed in the roots of both species under drought stress when compared to the photo-stressed roots and the control. The highest total polyphenol level was observed in the drought-stressed roots of *G. multifolia* (15.85 mg GAE/g) and the lowest in photo-stressed roots of *G. villosa* (7.66 mg GAE/g). A strong correlation was evident between the total polyphenol level and FRAP ($r=0.91$), ABTS ($r=0.80$) and flavonol content ($r=0.91$) for both species. No flavanols were detected in the leaves, bulbs and roots of the control, photo-stress and drought stress treatments of both species.

***Key words*:** ABTS; Environmental stress; Flavanones; Flavonols/ flavones; FRAP; *Gethyllis multifolia*; *Gethyllis villosa*; Kukumakranka; ORAC; Polyphenols.

1. Introduction

Gethyllis multifolia L. Bolus and *G. villosa* Thunb. (Family: Amaryllidaceae) are deciduous, winter-growing, summer-blooming and bulbous geophytes indigenous to South Africa (Du Plessis and Delpierre, 1973). *Gethyllis* grows under full sun conditions and their natural habitats range from sandy soils to mountainous rocky

terrains (Du Plessis and Duncan, 1989). The medicinal uses of this genus range from cures for colic, digestive disturbances, teething problems, fatigue, boils, bruises, insect bites to an aphrodisiac (Du Plessis and Delpierre, 1973). Apart from this genus' medicinal properties, many species (including *G. multifolia*) have a highly fragrant, tasty and edible fruit which has been used in the past to perfume rooms and linen (Lighton, 1992).

Plants possess different antioxidant properties, depending on their antioxidant molecule content, which is strongly affected by the plant's environmental conditions (Lin et al., 2006). Environmental stress factors such as shade, abnormal salt levels, high temperature and drought, may result in the generation of reactive oxygen species (ROS) in plants which in turn may cause oxidative stress when in excess. In plant cells, oxidative stress reactions are associated with the production of toxic free radicals (Price et al., 1989). Different irrigation schemes and different irradiance levels caused varying antioxidant enzyme levels in olive trees (Sofo et al., 2004). In sweet potato plants, a reduction in the total polyphenol content was noticed during flooding and drought stresses (Lin et al., 2006). In rice plants, oxidative stress caused by moisture deficiency resulted in membrane damage, a secondary effect of drought stress (Wanga et al., 2005). Other toxic effects in plants subjected to environmental stress factors, include the generation of lesions in DNA, reduction in protein synthesis and stability and a decrease in photosynthetic activity (Osmond and Grace, 1995). Plants have evolved a wide range of enzymatic and non-enzymatic mechanisms to scavenge ROS and protect its cells against oxygen toxicity (Fridovich, 1975). According to Di Carlo et al.

(2001) the relationship between plant stress acclimation and human health comprises a broad array of metabolites some of which possess ‘‘desirable’’ pharmacological properties. Many examples can be found in nature as in the case of hyperforin, which is the active ingredient in St. John’s wort (*Hypericum perforatum*) and is known for alleviating mild depression. When St. John’s wort plants are subjected to heat stress it substantially increases hyperforin concentration in shoots (Zobayed et al., 2005).

The phytochemical antioxidant compounds that plants produce protect them against harmful ROS activities and, when ingested, may also protect human cells against oxidative damage caused by an overproduction of free radicals and an impaired antioxidant defense system (Namiki, 1990). When in excess, these free radicals may contribute to the development of many debilitating diseases such as atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS (Kumpulainen and Salonen, 1999). Certain flavonoids have been employed to treat heart disease and moderate depression (Cook and Samman, 1996). It has been also reported that the major polyphenols in green tea are known for their antioxidative (Chen and Ho, 1995), antitumor and antimutagenic activities in humans (Susanne et al., 1996). Polyphenols in hawthorn is widely used in Europe as a tonic for the treatment of chronic heart disease and high blood pressure (Kirakosyan et al., 2003). Recent epidemiology studies show some conflicting reports on the subject of antioxidants and cardiovascular disease in a sense that the mechanisms explaining this relationship remains unclear (Vita, 2005) while the World Health Organization

(WHO) reported that the use of flavonoids for a reduction in disease risk was considered "possible" for cardiovascular diseases but "insufficient" for cancers (WHO/FAO, 2003).

Gethyllis multifolia species is threatened in its natural habitat (Lighton, 1992), which stresses the need for future cultivation of this species by pharmaceutical companies, traditional healers and farmers. Should certain environmental stresses increase the antioxidant content or activity of these two species, it can be incorporated in future cultivation practices to induce increased antioxidant levels in essential plant parts during production. To date no published data are available on how important biological properties, such as antioxidant activity of these two *Gethyllis* species, are affected by environmental stress factors. Thus, the aim of this study was to investigate the changes in the antioxidative capacity and levels in the leaves, bulbs and roots of *G. multifolia* and *G. villosa* during controlled photo- and drought environmental stresses over one growth season.

2. Materials and methods

2.1. Plant materials

Gethyllis multifolia and *G. villosa* bulbs were identified and obtained with permission from the Karoo National Biodiversity Garden (Worcester, Western Cape, South Africa) towards the end of their winter growth phase (March to mid-August). *Gethyllis multifolia* is threatened in its natural habitat, and for conservation purposes the exact location of this species is omitted. The plants of both species were transferred into 15 cm nursery pots in sandy, clay soil (pH 4.3-

4.4) obtained from the natural habitat. The plants were grown under full sunlight conditions for 12 months, which included one dormant phase (6 months - spring and summer) and one growth phase (6 months - autumn and winter) at the nursery of the Department of Horticultural Sciences, Cape Peninsula University of Technology (CPUT), Cape Town. Figure 1 shows the average rainfall and daily temperatures for the Cape Town area where plants were grown and also indicates the higher rainfall and lower temperatures for the growth phase and lower rainfall and higher temperatures for the dormant phase. Weather data for the Cape Town area was supplied by the South African Weather Service. Plant samples which represented the control (n=10 per species) were grown under full sun and irrigated by the ambient rainfall of the Western Cape (Figure 1). The mean photosynthetic photon flux density (PPFD) (converted from lux to PPFD) on cloudless days at 12h00 was 1825±63 µmol $m^{-2}.s^{-1}$ (measured with a Toptronic T630 digital lux meter Spraytech, Bellville, Western Cape, South Africa). Temperatures around the plant samples varied from 8–24 ºC and the relative humidity from 36–100% (measured with a Majortech MT669 digital relative humidity/ temperature meter, Spraytech, Bellville, Western Cape, South Africa).

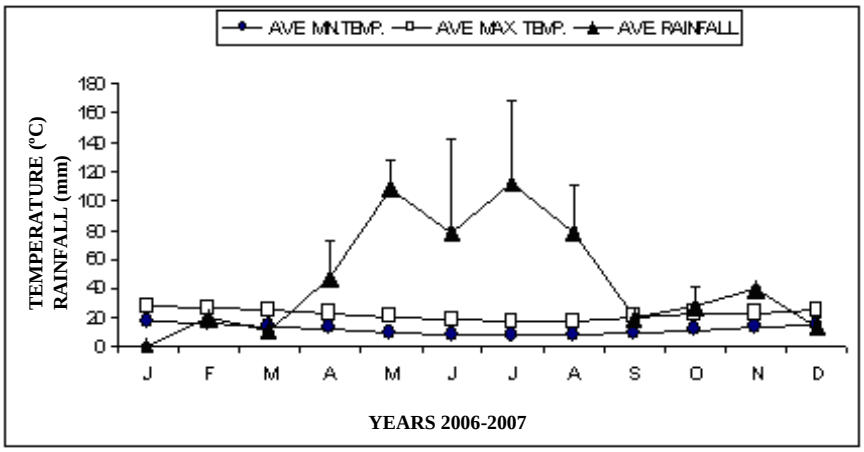

Figure 1: The average rainfall (mm) and the average daily minimum/maximum temperature (ºC) for the Cape Town area (Western Cape, South Africa) for the years 2006 and 2007. Each value was obtained by calculating the mean of the two years ±standard deviation. The data provided by the South African Weather Service was recorded at the permanent Cape Town weather office.

Plants which represented the drought stressed samples (n=10 per species) were grown under full sunlight and covered with a 6 mm clear glass sheet, placed 300 mm above the plants. The PPFD, temperature and relative humidity environmental conditions were similar to those of the control. The drought stressed plants were irrigated at a rate of 30% field capacity once a month with de-ionized water (Mortimer et al., 2003). Plants representing the photo-stressed samples (n=10 per plant species) were grown under a shade structure covered with 80% neutral black shade cloth (Alnet, Epping, Western Cape, South Africa), which has a neutral effect on light quality (Yates, 1989; Duan et al., 2005). During the experimental period, the mean PPFD on cloudless days at 12h00 was 365±26 µmol $m^{-2}.s^{-1}$ which was approximately 20% of full sunlight. The temperature around the photo-stressed plant samples was ~1–2 ºC lower than that of the control, and the relative humidity 2–4% higher than that of the control. The

readings of all the environmental conditions under all treatments were taken daily at the following time intervals: 09h00, 12h00 and 15h00.

2.2. Sample preparation

All plants were excised into leaves, flowers, bulbs and roots and dried in a fan-drying laboratory oven (Memmert, Laboratory & Scientific, Cape Town, South Africa) at 50 ºC for 48 hours. The bulbs of both species took approximately five days to dry. Individual plant parts were ground to a powder in a portable spice grinder using a 0.5 mm mesh (Krups 75 model F203, Hecho En Mexico, Mexico City, Mexico). Crude extracts of the leaves, bulbs and roots of both species were prepared without delay, by stirring the various dried, powdered plant materials (0.05 g of each) in 80% (v/v) ethanol (50 mL) (EtOH) (Saarchem, South Africa) whereafter it was centrifuged at 4 000 rpm for 5 min. The supernatants were used for all analyses. The same sample preparation technique was followed for all assays and all analyses were performed in triplicate.

2.3. Total polyphenol, flavonol/ flavone and flavanone content

The total polyphenol content of the various crude extracts were determined by the Folin Ciocalteu method (Singleton et al., 1999). Using a 96-well clear microplate (visible range), 25 µL of sample was mixed with 125 µL Folin-Ciocalteu reagent (Merck, South Africa), diluted 1:10 with distilled water. After 5 min., 100 μL (7.5%) aqueous sodium carbonate (Na_2CO_3) (Sigma-Aldrich, South Africa) was added to the well. The plates were incubated for 2 hours at room temperature before the absorbance was read at 765 nm using a Multiskan plate reader (Thermo

Electron Corporation, USA). The standard curve was prepared using 0, 20, 50, 100, 250 and 500 mg/L gallic acid in 10% EtOH and the results were expressed as mg gallic acid equivalents per g dry weight (mg GAE/g DW).

The flavonol content was determined using quercetin (0, 5, 10, 20, 40, 80 mg/L) in 95% ethanol (Sigma-Aldrich, South Africa) as standard. This assay measures both flavonols and flavones since both groups absorb ultra-violet light maximally around 360 nm. In the sample wells, 12.5 µL of the crude sample extracts was mixed with 12.5 µL 0.1% HCl (Merck, South Africa) in 95% ethanol, 225 µL 2% HCl and incubated for 30 min. at room temperature. The absorbance was read at 360 nm, at a temperature of 25 ºC (Mazza et al., 1999). The results were expressed as mg quercetin equivalents per g dry weight (mg QE/g DW).

The flavanone content was determined using an adapted version of the method as described by Kosalek et al. (2004). This method was adapted with minor modifications such as reducing assay volumes for the 96-well plates. Briefly, 100 µL of sample was mixed with 200 µL 1% 2,4-dinitrophenylhydrazine (DNPH) (2% H_2SO_4 in methanol (MeOH). After incubation at 50 ºC for 50 min., 700 µL of 10% potassium hydroxide (KOH) in 70% MeOH was added. The samples were centrifuged and 30 µL of the resulting supernatant mixed with 270 µL MeOH in a 96-well clear plate (visible range) and the absorbance read at 495 nm. A linear standard curve using 0, 0.2, 0.5, 1.0, 1.5, 2.0 mg/mL naringenin (Sigma-Aldrich, South Africa) in methanol was included. The results were expressed as mg naringenin equivalents per g dry weight (mg NE/g DW).

2.4. Antioxidant capacity

2.4.1. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed using the method of Benzie and Strain (1999). In a 96-well clear microplate (visible range), 10 µL of the crude sample extract was mixed with 300 µL FRAP reagent [0.3 M acetate buffer, pH 3.6 (Saarchem, South Africa), 10 mM 2, 4, 6-tripyridyl-*s*-triazine (TPTZ) in 0.1 M HCl (Sigma-Aldrich, South Africa), 20 mM iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) (Sigma-Aldrich, South Africa), 6.6 mL distilled water] and incubated for 30 min. at 37 ºC in the plate reader. Absorbance was measured at 593 nm. L-Ascorbic acid (Sigma-Aldrich, South Africa) was used as a standard with concentrations varying between 0 and 1 000 µM. The results were expressed as µM ascorbic acid equivalents per g dry weight (µM AAE/g DW).

2.4.2. 2,2 -azino-di-3-ethylbenzthiazoline sulphonate (ABTS) assay

The ABTS assay was performed following the method of Re et al. (1999). The stock solutions included 7 mM ABTS and 140 mM potassium-peroxodisulphate ($K_2S_2O_8$) (Merck, South Africa) solutions. The working solution was then prepared by adding 88 µL $K_2S_2O_8$ solution to 5 mL ABTS solution. The two solutions were mixed well and allowed to react for 24 h at room temperature in the dark. Trolox (6-hydrox-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the standard with concentrations ranging between 0 and 500 µM. The ABTS mix solution was diluted with ethanol to read the start-up absorbance (control) of approximately 2.0 (±0.1). Crude sample extracts (25 µL) were allowed to react with 300 µL ABTS in the dark at room temperature for 30 min.

before the absorbance was read at 734 nm at 25 ºC in a plate reader. The results were expressed as µM Trolox equivalents per g dry weight (µM TE/g DW).

2.4.3. Oxygen radical absorbance capacity (ORAC) assay

The H-ORAC$_{FL}$ values were determined according to the methods of Prior et al. (2003) and Wu et al. (2004). A stock standard solution of Trolox (500 µM) was diluted in phosphate buffer (75 mM, pH 7.4) to provide calibration standards ranging from 5 to 25 µM. The Fluoroskan ascent plate reader (Thermo Fisher Scientific, Waltham, USA) equipped with an incubator was set at 37 ºC. Fluorescence filters with an excitation wavelength of 485 nm and emission wavelength of 538 nm were used. A fluorescein stock solution was prepared in phosphate buffer and further diluted to provide a final concentration of 14 µM per well. The peroxyl generator, 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH) (25 mg/mL in phosphate buffer), was added with a multichannel pipette to give a final AAPH concentration of 4.8 mM in each well. The fluorescence from each well, containing 12 µL diluted hydrophilic extract, was read every 5 min. for 2 hours. The final ORAC$_{FL}$ values were caculated using the regression equation $y = ax^2 + bx + c$ between the Trolox concentration (µM) and the area under the curve. The results were expressed as µM Trolox equivalents per g dry weight (µM TE/g DW).

2.5. Statistical analysis

The statistical significance between antioxidant content and activity values of the various crude plant extracts were determined by an analysis of variance

(ANOVA) where $P<0.05$ was considered to be statistically significant. The computer program employed for the statistical analysis was Medcalc version 9.4.2.0 (Medcalc, Belgium). The computer program, Microsoft Office Excel 2006, version 12 (Microsoft Corporation, USA) was employed to determine the correlation between antioxidant contents and activity.

3. Results

3.1. Total polyphenol, flavonol/ flavone and flavanone content

In *G. multifolia* there was no significant ($P>0.05$) difference in the total polyphenols of the leaves of the control and the leaves subjected to the two stress treatments (Table 1). The roots of *G. multifolia* responded with a significant ($P<0.05$) increase in the total polyphenols for both the photo-stress (9%) and drought stress (26%) treatments, when compared to the control (Figure 2). In contrast to the leaves of *G. multifolia*, *G. villosa*'s leaves showed a significant ($P<0.05$) decrease (51%) in the total polyphenols for the leaves under drought stress when compared to the control. No significant difference in the total polyphenol levels was observed in the leaves of *G. villosa* between the control and photo-stress treatment. Furthermore, none of the environmental stresses had any significant ($P>0.05$) effect on the total polyphenol content in the roots of *G. villosa*. The highest total polyphenol content was recorded in the roots of *G. multifolia* under drought stress (Table 1). In general, the drought stress treatment brought about an increase in the total polyphenols in the roots of both species.

Irrespective of treatment, no significant (P>0.05) difference was found in the flavonol content of the leaves and roots of *G. multifolia* (Table 1). A similar trend was noticed in the roots of *G. villosa* where none of the treatments had any significant effect (P>0.05) on the flavonol content. The leaves of *G. villosa*, however, showed a significant (P<0.05) decrease in the flavonol content when subjected to both the photo-stress (41%) and drought stress (47%) treatments (Figure 2).

No significant (P>0.05) differences were evident in the flavanone content of the leaves and roots under photo-stress and drought stress treatments (Table 1). When comparing the three treatments the flavanone content was found to be highest in the *G. multifolia* control plants. The flavanone contents of the leaves and roots of plants under the photo-stress and drought stress treatments were not significantly (P>0.05) different. When comparing the three treatments, significantly (P<0.05) higher flavanone levels were evident in the control plants of *G. villosa*. Amongst the two species, the highest flavanone content was found in the leaves of the control of *G. multifolia.*

Table 1: The total polyphenol (mg GAE/g dry weight), flavonol/ flavone (mg QE/g dry weight) and flavanone (mg NE/g dry weight) content of the leaves, bulbs and roots of *Gethyllis multifolia* and *G. villosa* plants under different environmental stresses.

	Total polyphenols		
G. multifolia			
Plant parts	**Control**	**Photo-stress**	**Drought stress**
Leaves	13.44±3.37ed	12.43±2.19d	13.39±2.24ed
Bulbs	6.69±2.26abc	7.31±2.90bc	7.84±2.34bc
Roots	12.54±1.38d	13.65±1.87e	15.85±2.75f
G. villosa			
Leaves	13.59±3.09ed	11.31±2.31dc	9.00±1.52c
Bulbs	4.49±4.67a	5.25±2.96ab	5.84±2.32ab
Roots	8.14±4.53c	7.66±2.22bc	9.26±1.36c
	Flavonols/ Flavones		
G. multifolia			
Plant parts	**Control**	**Photo-stress**	**Drought stress**
Leaves	2.93±0.93bc	3.02±0.49c	3.17±0.71c
Bulbs	0.91±0.56a	1.61±0.55a	1.64±0.85ab
Roots	2.83±0.54bc	3.57±1.11c	3.61±0.70c
G. villosa			
Leaves	4.45±0.91d	3.18±0.82c	3.02±0.70c
Bulbs	0.88±1.24a	0.72±0.67a	0.75±0.53a
Roots	1.62±1.68a	1.49±0.86a	1.35±0.39a
	Flavanones		
G. multifolia			
Plant parts	**Control**	**Photo-stress**	**Drought stress**
Leaves	4.18±0.91g	1.62±0.63abc	1.21±0.31a
Bulbs	3.28±0.80f	1.29±0.56ab	1.08±0.39a
Roots	2.31±0.61ce	1.21±0.51a	1.56±0.54ab
G. villosa			
Leaves	2.85±1.03ef	1.66±0.52abc	1.04±0.51a
Bulbs	1.99±0.16bc	1.79±0.46abc	1.64±0.54abc
Roots	2.80±0.89ef	1.96±0.50bc	1.63±0.67abc

Values represent the means ± SD for the leaves, bulbs and roots (n=10). Means with different letters in the same columns, rows and under the same assays are significantly ($P<0.05$) different. GAE: gallic acid equivalents; QE: quercetin equivalents and NE: naringenin equivalents.

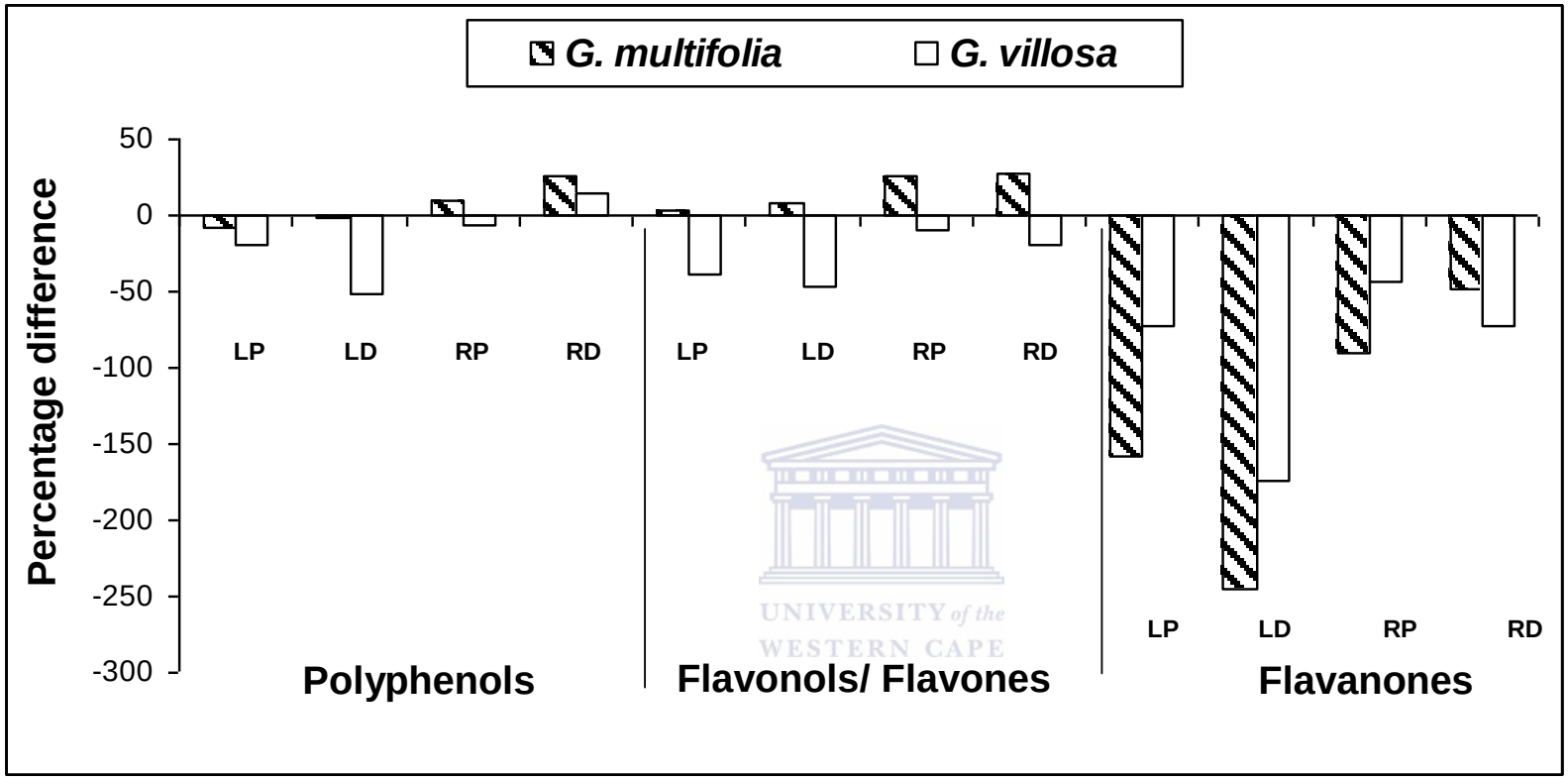

Figure 2: Percentage difference of the total polyphenol, flavonol/ flavone and flavanone content for the leaves and roots of *Gethyllis multifolia* and *G. villosa*. This graph represents the percentage difference between the control and photo-stress and the control and drought stress for the two species**. LP** = Leaves photo-stress; **LD** = Leaves drought stress; **RP** = Roots photo-stress; **RD** = Roots drought stress.

3.2. Antioxidant capacity

No significant (P>0.05) difference was found in the FRAP value for the leaves of *G. multifolia* when the photo-stress and drought stress treatments were compared to the control (Table 2). *G. multifolia* showed a significant (P<0.05) increase in the FRAP value for the roots under both photo-stress (22%) and drought stress (32%) when compared to the control (Figure 3). The leaves of *G. villosa* showed a significant (P<0.05) decrease in the FRAP value for both photo-stress (24%) and drought stress (36%) treatments when compared to the control (Figure 3). No significant (P>0.05) differences were observed in the FRAP value for the roots of *G. villosa* when both treatments were compared to the control. The highest FRAP value overall, was recorded in the leaves of the control plants of *G. villosa* (Table 2).

The leaves of *G. multifolia* showed a significant (P<0.05) decrease in the ORAC value when the photo-stress (18%) and drought stress (21%) treatments were compared to the control (Figure 3). In contrast to this response of the leaves, the roots of *G. multifolia* showed an increase in the ORAC value in the photo-stress treatment and a more significant (P<0.05) increase in the drought stress (57%) treatment when both stresses were compared to the control (Figure 3). For *G. villosa*, a significant (P<0.05) increase was observed in the ORAC value for the leaves under drought stress, when compared to the control. Similarly, a significant (P<0.05) increase (84%) was noticed in the ORAC value of the roots of *G. villosa* under drought stress when compared to the control. The highest ORAC value overall, was recorded in the roots of *G. multifolia* under drought stress (Table 2).

The photo-stress and drought stress treatments had a significant ($P<0.05$) effect on the leaves of *G. multifolia* with a decrease of (34%) and (39%) in the ABTS value for both treatments, respectively, when compared to the control. The photo-stress treatment did not have a significant effect on the roots of *G. multifolia*, but the opposite was true for the drought stress treatment with a significant ($P<0.05$) increase in the ABTS value of 34% when compared to the control. Similarly (to leaves of *G. multifolia*), the ABTS value in the leaves of *G. villosa* decreased significantly ($P<0.05$) under both treatments (28%) when compared to the control (Figure 3). In the case of the roots of *G. villosa*, neither of the treatments affected the ABTS value significantly ($P>0.05$). The highest ABTS value overall, was recorded in the control of *G. villosa* (Table 2).

Table 2: The ferric reducing antioxidant power (FRAP) (µM AAE/g dry weight), oxygen radical absorbance capacity (ORAC) (µM TE/g dry weight) and ABTS radical cation scavenging ability (ABTS) (µM TE/g dry weight) of the leaves, bulbs and roots of *Gethyllis multifolia* and *G. villosa* plants under different environmental stresses.

	FRAP		
G. multifolia			
Plant parts	**Control**	**Photo-stress**	**Drought stress**
Leaves	40.92±10.81cd	37.80±5.52bc	40.68±6.37cd
Bulbs	18.64±5.29a	19.95±8.91a	21.60±4.90a
Roots	39.41±4.48bc	48.09±6.32de	51.51±4.91e
G. villosa			
Leaves	55.78±9.66e	44.81±6.61d	41.14±8.70cd
Bulbs	18.35±18.04a	18.96±8.46a	19.24±4.80a
Roots	38.51±21.41bc	32.58±9.34b	37.96±5.50bc
	ORAC		
G. multifolia			
Plant parts	**Control**	**Photo-stress**	**Drought stress**
Leaves	719.83±95.99f	610.67±116.00e	594.05±93.48e
Bulbs	444.98±91.52d	556.64±98.82ae	583.46±103.46e
Roots	497.55±93.15de	705.83±91.58ef	779.48±72.23f
G. villosa			
Leaves	311.08±49.60bc	356.53±66.05c	459.17±86.18d
Bulbs	129.68±77.83a	169.62±62.50a	357.84±103.38c
Roots	264.77±106.60b	281.55±67.32b	486.00±101.98de
	ABTS		
G. multifolia			
Plant parts	**Control**	**Photo-stress**	**Drought stress**
Leaves	32.85±9.30fg	24.55±4.40c	23.67±4.76c
Bulbs	11.24±6.19a	9.76±7.32a	11.68 ±5.39a
Roots	23.73±3.15c	27.27±0.89cde	31.79±3.50f
G. villosa			
Leaves	34.27±10.36g	26.79±5.88cd	26.84±6.00cd
Bulbs	10.67±14.83a	12.71±9.46a	18.68±8.26b
Roots	30.23±15.84ef	28.26±6.46de	33.57±5.56fg

Values represent the means ± SD for the leaves, bulbs and roots (n=10). Means with different letters in the same columns, rows and under the same assays are significantly ($P<0.05$) different. AAE: ascorbic acid equivalents; TE: Trolox equivalents.

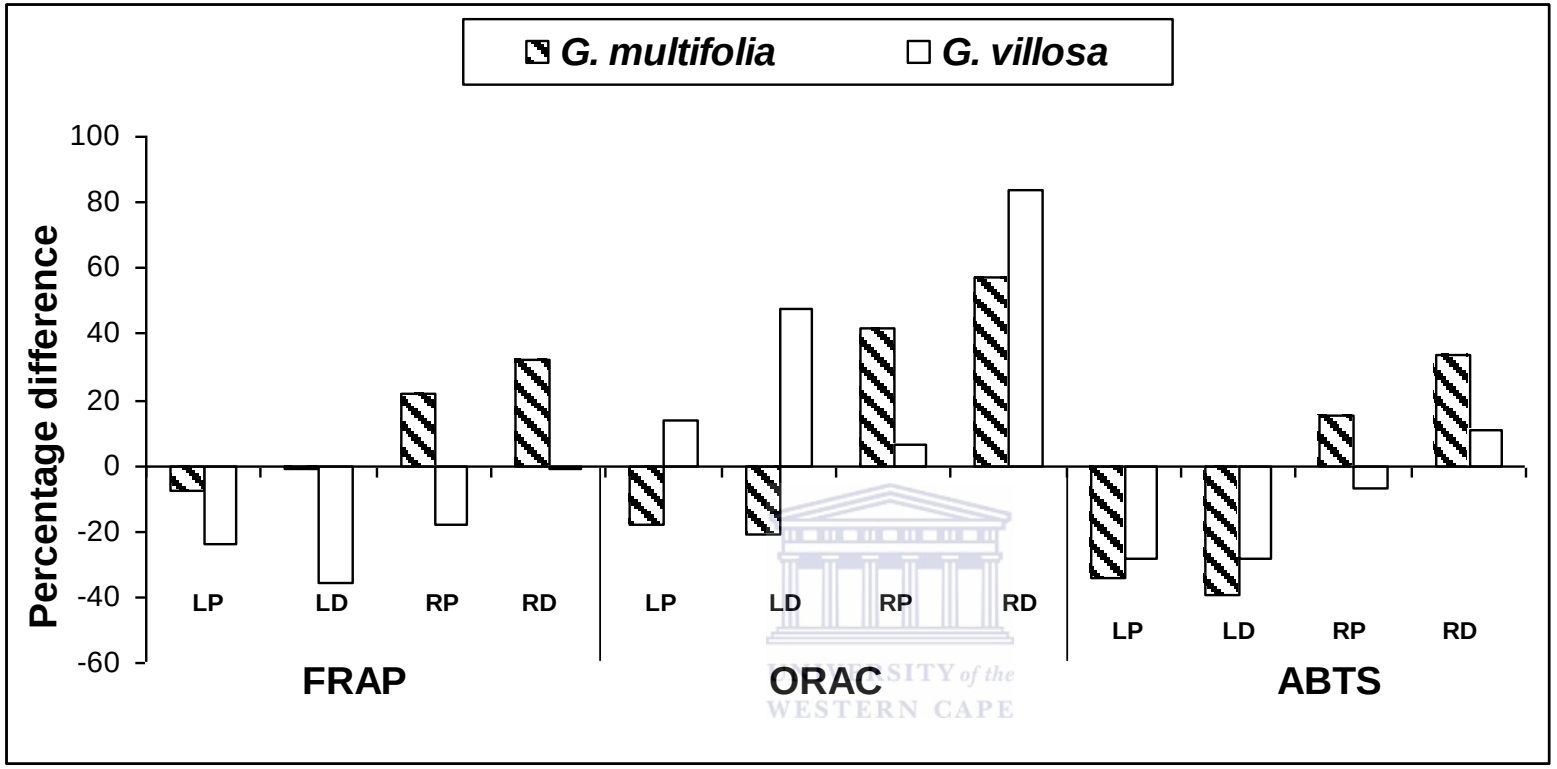

Figure 3: Percentage difference of the ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC) and ABTS radical cation scavenging ability (ABTS) for the leaves and roots of *Gethyllis multifolia* and *G. villosa*. This graph represents the percentage difference between the control and photo-stress and the control and drought stress for the two species.

LP = Leaves photo-stress; **LD** = Leaves drought stress; **RP** = Roots photo-stress; **RD** = Roots drought stress.

It must be noted that the photographic evidence of this study revealed that the leaves of plants in both species under the photo-stress treatment appeared green and vigorous but without the natural spiral (Figure 4B,C). Under the drought stress treatment, the number of leaves of both species was drastically reduced, appeared short and stunted with reduced chlorophyll pigments, but maintained the natural spiral (Figure 4A,B). This observation also revealed that when plants were subjected to artificial drought stress, the biomass yield (leaves, bulbs, roots, flower and fruit) was drastically reduced, especially in the case of *G. multifolia*.

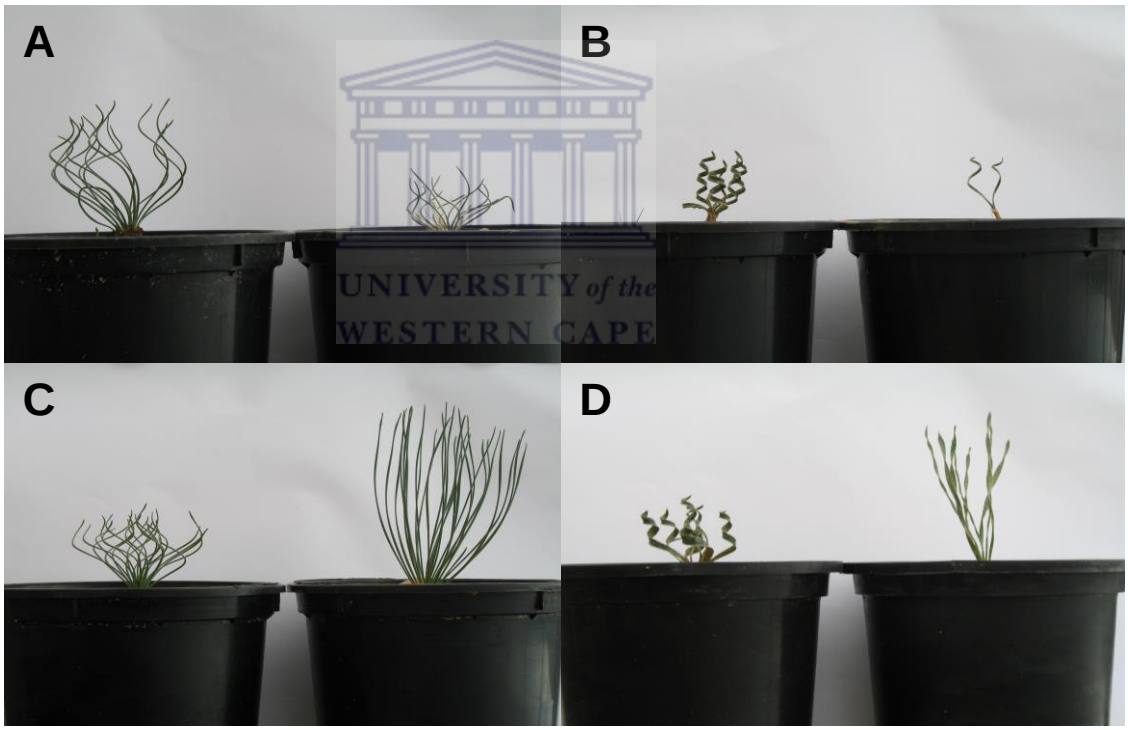

Figure 4: Comparative images on the effect of the artificial drought and photo-stress treatments on the growth of *Gethyllis multifolia* and *G. villosa*. Plants on the left side of all images represent the controls in both species. Drought stress on *G. multifolia* **(A)**; Drought stress on *G. villosa* **(B)**; Photo-stress on *G. multifolia* **(C)**; Photo-stress on *G. villosa* **(D)**.

3. Discussion and conclusion

It was recommended, through evaluation of the literature and data presented at the 'First International Congress on Antioxidant Methods in 2004', that three methods be considered for standardization of antioxidant capacity and total polyphenol determination in food and dietary supplements (Prior et al., 2005). Method 1: the oxygen radical absorbance capacity (ORAC) assay which represents a hydrogen atom transfer (HAT) reaction mechanism, which is most relevant to human biology. Method 2: the trolox equivalent antioxidant capacity (TEAC/ABTS) assay represents a single electron transfer (SET) -based method which indicates reducing capacity. Method 3: the ferric reducing/antioxidant power (FRAP) assay which is also a SET-based assay and a direct test of the total antioxidant power of a biological sample. It is further suggested that experimental work on a series of assays gives a better understanding of the antioxidant capacity of a sample (Prior et al., 2005; Prior and Cao, 1999).

The antioxidant-capacity and -content fluctuated in the leaves and roots of *G. multifolia* and *G. villosa* when subjected to both environmental stresses. Conversely, the antioxidant-capacity and -content in the bulbs were relatively low and consistent, and were therefore not compared and discussed in this investigation. In comparison to the control, it was evident in this study that the total polyphenols increased significantly in the roots of *G. multifolia* when the plants were subjected to the drought stress. Though not significant, higher flavonol levels were also recorded in both the leaves and roots of *G. multifolia* when subjected to the drought stress treatment. Similarly, it was reported that in

Arbutus unedo plants, severe drought stress resulted in significantly higher ascorbate levels in plant parts (Munné-Bosch and Peñuelas, 2004). Furthermore, a study by Herbinger et al. (2002) reported that under drought stress, α-tocopherol and glutathione concentrations increased in certain wheat cultivars. In contrast to the above reports, a study by Kirakosyan et al. (2003) reported that drought stress effected a reduction in the flavonoid levels of *Crataegus laevigata* and *C. monogyna* plants. The increase in the total polyphenols and flavonol content of *G. multifolia* when subjected to drought stress, could suggest that this species produces higher levels of total polyphenol antioxidants than *G. villosa*, to possibly serve as a protective measure or adaptive strategy to cope with this specific environmental stress.

In this study, *G. villosa* decreased its total polyphenol levels and flavonol content in the leaves and roots when the plants were subjected to the photo-stress (deep shade) treatment. In comparison to the control, significantly lower flavanone contents were also evident in the leaves and roots of both species under the photo-stress treatment. Evidence of similar plant responses to light was mentioned in a study by Tattini et al. (2004) where high light intensities effected an increase in the flavonoid concentrations in the leaves of *Ligustrum vulgare*. Furhermore, Heuberger et al. (2004) reported that under chronic UV-exposure conditions the ascorbate levels in certain plant species increased, which reflected the stress acclimation process. In support of the above observations, Dixon and Paiva (1995) reported that plants which are subjected to full-sun conditions have been shown to contain higher levels of polyphenolic compounds than shade plants.

Both the photo-stress and drought stress treatments did not have an effect on the FRAP value of the leaves of *G. multifolia*, but the FRAP value decreased significantly when the leaves of *G. villosa* were subjected to the same treatments. Conversely, the leaves of sweet potatoe plants increased its FRAP values when subjected to drought stress, as reported in a study by Lin et al. (2006). The ORAC values increased significantly in the leaves of *G. villosa* after exposure to drought stress. Similarly, increased ORAC values were recorded in the leaves of Sushu 18 and Simon 1 when these two sweet potato cultivars were subjected to a drought stress treatment (Lin et al., 2006). In contrast to this response, *G. multifolia* leaves showed a significant decrease in the ORAC values for both photo-stress and drought stress treatments. This study, however, revealed that under drought stress the ORAC and ABTS radical cation scavenging ability is increased in the root system of both species, which could form part of the acclimation process or adaptive strategy to this environmental stress factor.

The photographic evidence in this study (Figure 4A,B) and a horticultural study by Daniels (2007) confirmed that during natural and artificial drought stress the biomass yield (leaves, bulbs, roots, flower and fruit) is drastically reduced, especially in the case of *G. multifolia*. Conversly, the leaves of plants of both species under the photo-stress treatment appeared green and vigorous but without the natural spiral (Figure 4B,C). However, observations of natural populations over a period of four years (Daniels, 2007), indicated higher flower production and consequently fruit production where individual plants were growing in close

proximity to one another (to ensure cross-pollination) under no environmental stress. Furthermore, various irrigation delay tactics can also be applied as to not interfere with flower production and consequently fruit production of the species.

It can be concluded that the antioxidant activities in the mentioned plant parts under drought stress may be a protective and acclimation mechanism against drought stress, which is found to be more significant in *G. multifolia*. The responses of plants in this study have also given a good indication as to how the different species and plant organs respond to different environmental stresses by increasing and decreasing their secondary metabolites as possible protective mechanisms. The findings in the current study is echoed by this statement: "responses to environmental stresses are species and/or cultivar specific, and depend on the intensities and the dynamics of stress exposure, as well as other environmental parameters which could influence the production of nutritionally important plant metabolites" (Jansen et al., 2008). Results from this study could have a significant impact on how traditional healers, pharmaceutical companies and farmers choose conducive environmental conditions for the cultivation of the two *Gethyllis* species in order to ensure enhanced polyphenolic content and antioxidant activities in the relevant plant "parts" that are traditionally used in medicinal practices. Future research is needed on the fruit and flowers of both species to further elucidate other biological properties of these two endemic plant species but also to confirm the antioxidant activity *in vivo.*

Acknowledgements

The authors acknowledge the Cape Peninsula University of Technology (CPUT) for financial assistance, the Department of Horticultural Sciences (CPUT) for the use of the nursery facilities and laboratory facilities at the Antioxidative Stress Research Centre (CPUT). The authors further acknowledge the Karoo National Biodiversity Garden for permission to harvest *G. multifolia* and *G. villosa* plants from their natural habitat. Gratitude is expressed to Naomi Daniels for the preparation of the plant samples.

References

Benzie IFF, Strain JJ (1999). Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol. 299:15-27.

Chen C, Ho C (1995). Antioxidant properties of polyphenols extracted from green and black teas. J. Food Lipids. 2:35-46.

Cook NC, Samman S (1996). Flavonoids- chemistry, metabolism, cardioprotective effects, and dietary sources. Nutr. Biochem. 7:66-76.

Daniels CW (2007). A comparative study of the propagation and cultivation of *Gethyllis multifolia* and *G. villosa*. MTech dissertation 2007, Cape Peninsula University of Technology, Bellville, South Africa.

Di Carlo F, Borrelli F, Ernst E, Izzo A (2001). St. John's wort: prozac from the plant kingdom. Trends Pharmacol. Sci. 22:292-297.

Dixon R, Paiva N (1995). Stress-induced phenylpropanoid metabolism. The Plant Cell 7:1085-1097.

Duan B, Lu Y, Yin C, Junttila O, Li C (2005). Physiological responses to drought and shade in two contrasting *Picea asperata* populations. Physiol. Plant. 124:476-484.

Du Plessis N, Delpierre G (1973). Blommeprag uit eie bodem: Koekemakranka. Landbouweekblad, pp. 37-39. August.

Du Plessis N, Duncan G (1989). Bulbous plants of southern Africa. Tafelberg Publishers, Cape Town, South Africa, pp.104.

Fridovich I (1975). Superoxide dismutase. Ann. Rev. of Biochem. 44:147-59.

Herbinger K, Tausz M, Wonisch A, Soja G, Sorger A, Grill D (2002). Complex interactive effects of drought and ozone stress on the antioxidant defence systems of two wheat cultivars. Plant Physiol. Biochem. 40:691-696.

Heuberger H, Praeger U, Georgi M, Schitrrmacher G, Grassmann J, Schnitzler WH (2004). Precision stressing by UV-B radiation to improve quality of spinach under protected cultivation, Acta Hort. 659:201-206.

Jansen M, Hectors K, O'Brien NM, Guisez Y, Potters G (2008). Plant stress and human health: Do human consumers benefit from UV-B acclimated crops? Plant Sci. 175:448-458.

Kirakosyan A, Seymor E, Kaufman PB, Warber S, Bolling S, Chang SC (2003). Antioxidant capacity of polyhenolic extracts from leaves of *Crataegus laevigata* and *C. monogyna* (Hawthorn) subjected to drought and cold stress. J. Agric. Food Chem. 51:3973-3976.

Kosalek I, Bakmaz M, Pepeljnjak S, Vladimir-Knezevic S (2004). Quantitative analysis of the flavanoids in raw propolis from northern Croatia. Acta Pharm. 54:65-72.

Kumpulainen JT, Salonen JT (1999). Natural antioxidants and anticarcinogens in nutrition, health and disease. The Royal Society of Chemistry, UK, pp.178-187.

Lighton C (1992). The kukumakranka. Veld & Flora 78, pp.100-103. December.

Lin K, Chao P, Yang C, Cheng W, Lo H, Chang T (2006). The effects of flooding and drought stress on the antioxidant constituents in sweet potato leaves. Bot. Studies 47:417-426.

Mazza G, Fukumoto L, Delaquis P, Girard B, Ewert B (1999). Anthocyanins, phenolics and color of Cabernet Franc, Merlot and Pinot Noir wines from British Columbia. J. Agric. Food Chem. 47:4009-4017.

Mortimer P, Swart JC, Valentine AJ, Jacobs G, Cramer MD (2003). Does irrigation influence the growth, yield and water use efficiency of the protea hybrid 'Sylvia' (*Protea susannae* X *Protea eximia*)? S. Afr. J. Bot. 69:1-9.

Munne-Bosch S, Penuelas J (2004). Drought-induced oxidative stress in strawberry tree (*Arbutus unedo* L.) growing in Mediterranean field conditions. Plant Sci. 166:1105-1110.

Namiki M (1990). Antioxidant/antimutagens in food. CRC critical review. Food Sci. Nutr. 29:273-300.

Osmond CB, Grace SC (1995). Perspectives on photo-inhibition and photorespiration in the field: Quintessential inefficiencies of the light and dark reactions of photosynthesis? J. Exp. Bot. 46:1351-1362.

Price AH, Atherton N, Hendry GAF (1989). Plants under drought stress generated activated oxygen. Free Radical Res. Commun. 8:61-66.

Prior RL, Cao G (1999). In vivo total antioxidant capacity: comparison of different analytical methods. Free Radical Biol. Med. 27:1173-1181.

Prior RL, Hoang H, Gu L, Wu X, Bacchiocca M, Howard L, Hampsch-Woodill M, Huang D, Ou B, Jacob R (2003). Assays for hydrophilic and lipophilic antioxidant capacity ($ORAC_{FL}$) of plasma and other biological and food samples. J. Agric. Food Chem 51:3273-3279.

Prior RL, Wu X, Schaich K (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J. Agric. Food Chem. 53:4290-4302.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999). Antioxidant activity applying an improved ABTS radical cation assay. Free Radical Biol. Med. 26:1231-1237.

Sharp R.E, Poroyko V, Hejlek LG, Spollen WG, Springer GK, Bohnert HJ, Nguyen HT (2004). Root growth maintenance during water deficits: physiology to functional genomics. J. Exp. Bot. 55:2343-2352.

Singleton VL, Orthofer R, Lamuela-Raventos RM (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of the Folin-Ciocalteu reagent. Methods Enzymol. 299:152-178.

Sofo A, Dichio B, Xiloyannis C, Masia A (2004). Effects of different irradiance levels on some antioxidant enzymes and on malondialdehyde content during re-watering in olive trees. Plant Sci. 166:293-302.

Susanne V, Barbara T, Davids A, Gerald W, Mary K, Juli W, Jose G (1996). Inhibitory effect of six green tea catechins and caffeine on the growth of four selected human tumor cell lines. Anti-Cancer Drugs 7:461-468.

Tattini M, Galardi C, Pinelli P, Massai R, Remorini D, Agati G (2004). Differential accumulation of flavonoids and hydroxycinnamates in leaves of *Ligustrum vulgare* under excess light and drought stress. New Phytol. 163:547-561.

Vita JA (2005). Polyphenols and cardiovascular disease: Effects on endothelial and platelet function. Am. J. Clin. Nutr. 81:292-297.

Wanga FZ, Wanga QB, Kwonb SY, Kwakb SS, Sua WA (2005). Enhanced drought tolerance of transgenic rice plants expressing a pea manganese superoxide dismutase. J. Plant Physiol. 162:465-472.

WHO/FAO. (2003). WHO Technical report series: Joint WHO/FAO expert consultation on diet, nutrition, and the prevention of chronic diseases, 28 January - 1 February 2002. WHO Publications, Geneva, Switzerland.

Wu X, Beecher GR, Holden JM, Haytowitz DB, Gebhardt SE, Prior RL (2004). Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. J. Agric. Food Chem. 52:4026-4037.

Yates DJ (1989). Shade factors of a range of shade cloth materials. Acta Hort. 257:201-218.

Zobayed S, Afreen F, Kozai T (2005). Temperature stress can alter the photosynthetic efficiency and secondary metabolite concentration in St. John's wort. Plant Physiol. Biochem. 43:977-984.

CHAPTER 7: RESEARCH PAPER 5 - Phytochemical studies of *Gethyllis multifolia* L. Bolus (Kukumakranka) and *G. villosa* Thunb.

CHAPTER 7

RESEARCH PAPER 5

Phytochemical studies of *Gethyllis multifolia* L. Bolus (Kukumakranka) and *G. villosa* Thunb.

[a]C. W. Daniels, [b]O. J. Babajide, [c]A. J. Valentine, [d]J. L. Marnewick, [e]W. T. Mabusela

[a]Department of Horticultural Sciences, Cape Peninsula University of Technology, P.O. Box 1906, Bellville 7535, South Africa.

[b]Department of Chemistry, University of the Western Cape, Private Bag X17, Bellville 7535, South Africa.

[c]Department of Botany and Zoology, University of Stellenbosch, Private Bag X1, Matieland 7602, Stellenbosch, South Africa.

[d]Oxidative Stress Research Centre, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, P.O. Box 1906, Bellville 7535, South Africa.

[e]South African Herbal Science and Medicine Institute, University of the Western Cape, Private Bag X17, Bellville 7535, South Africa.

Abstract

Phytochemical investigation of the leaves, bulbs and roots of *Gethyllis multifolia* L. Bolus and *Gethyllis villosa* Thunb. revealed the presence of tannins, flavonoids, phenolics, saponins, glycosides (phenolic and terpenoid) as well as essential oils, while the test for alkaloids was negative. Further in depth studies on the roots of *G. multifolia* led to the isolation of three known flavonoids, of which one was also isolated as its endogenously acetylated derivative. Their structures were elucidated by chemical and spectroscopic methods as 2,3-dihydro-7-

hydroxy-2-phenyl-4*H*-1-benzopyran-4-one (**1**), (1-[2.4-dihydroxyphenyl]-3-phenylpropan-1-one) (**2**)**,** 2,3-dihydro-5,7-dihydroxy-2-phenyl-4*H*-1-benzopyran-4-one or pinocembrin (**3**) and 5,7-diacetoxy-2,3-dihydro-2-phenyl-4*H*-1-benzopyran-4-one (**4**).

Key words: characterization; *Gethyllis*; Kukumakranka; isolation; natural compounds; phytochemistry.

1. Introduction

Botanical medicine is known as one of the oldest practiced professions by mankind (Van Wyk and Gericke, 2000; Iwu, 1993) and the use of plants as traditional medicine is now recognized by the World Health Organisation (WHO) as a building block for primary health care (Akerele, 1988; WHO, 2005). It has been estimated that 25% of prescribed medicines today are derived from plants (Hamburger and Hostettmann, 1991). According to WHO about 80% of the world's population depends mainly on traditional medicine and the traditional treatment involves mainly the use of plant extracts (WHO, 2005). This practice commonly exists in rural areas where synthetic drugs are not available or, where available, are not affordable.

The Cape Floristic Region forms part of the traditional home of the Khoikhoi (Hottentot) and San (Bushman) peoples and these two cultural groups are collectively referred to as the Khoi-San people (Shapera, 1930; Boonzaier et al., 1996). Very little has been published on the medicinal uses of plants from this

region and numerous publications report on the use of plants for food and water (Story, 1964), utility items (Tanaka, 1978) and hunting poisons (Neuwinger, 1996) are available. According to Dold and Cocks (2002), the use and trade of plants for medicine is no longer confined to traditional healers, but has spread to the informal and formal entrepreneurial sectors of the South African economy because of unemployment, resulting in an increase in the number of herbal gatherers and traders. Consequently, the trade in traditional medicines is now greater than ever before and a complex biodiversity management issue is facing conservation authorities in South Africa.

The genus *Gethyllis* belongs to the plant family Amaryllidaceae and is better known as "Kukumakranka" by the Khoi-San people. The genus comprises 37 currently accepted species in Southern Africa (Du Plessis and Duncan, 1989), among which many are considered to be endangered. Presently, very little is known about the chemical composition and bioactivities of this genus (Van Wyk et al., 1997). The word "Kukumakranka" is described by farmers as meaning "goed vir my krank maag" in Afrikaans, one of South Africa's eleven languages, which translates to "cure for my upset stomach" in English (Van der Walt, 2003). *Gethyllis multifolia* L. Bolus and *G. villosa* Thunb. are winter-growing, summer-blooming, deciduous, bulbous geophytes (Du Plessis and Delpierre, 1973) with four distinct growth phases. *Gethyllis multifolia* has a highly fragrant and tasty, edible fruit, and this species is threatened in its natural habitat (Lighton, 1992). Watt and Breyer-Brandwijk, (1962) reported that "Kukumakranka brandy", which is made from the fruit of *Gethyllis afra* and *Gethyllis ciliaris*, is believed to

contain oils and esters of low molecular weight, and is an old Cape remedy that was used for colic and indigestion.

According to Rood (1994) the early Cape colonialists used an alcoholic infusion of the fruit of *G. linearis* and *G. spiralis* as a remedy for digestive disturbances. In more recent times, a diluted infusion of the flower has been used for teething problems, and the skin of the fruit as a local application on boils, bruises and insect bites. Further reports by Rood (1994) indicated that the fruit was boiled by the Khoi-San and used as an aphrodisiac, while Van der Walt (2003) mentioned that *G. ciliaris* was used as a tonic for fatigue. Further pharmaceutical studies by Elgorashi and Van Staden (2004) revealed some anti-inflammatory and antibacterial activities in certain *Gethyllis* species and reported that the findings were in agreement with its uses as a traditional medicine. Previously, the following compounds: dihydroxydimethylbenzopyran-4-one, isoeugenitol, its 5-*O*-glycoside and 9Z-octadec-9-enamide, had been isolated from the roots and bulbs of *G. ciliaris* (Elgorashi et al., 2007). During head-space analysis of the volatiles from the fruits of *Gethyllis afra* and *G. ciliaris*, the following major compounds were characterized for *G. afra*: α-pinene, n-butyl n-butyrate, isoamyl acetate, β-pinene and 2-methylbutyl butyrate and for *G. ciliaris*: pentacosane; ethyl octanoate; ethyl isovalerate; ethyl hexanoate and ethyl benzoate. It was further reported that these compounds are responsible for the sweet/banana/piney odours of the fruit of these two species (Kamatou et al., 2008). An antioxidant capacity-and -content study of plant parts of *G. multifolia* and *G. villosa* revealed higher polyphenol content and antioxidant activity in the roots of *G. multifolia*

than in *G. villosa* (Chapter 5). This study further revealed high total polyphenols and antioxidant activity in the fruits and flowers of both species, which is comparable to blueberries, strawberries and raisins (Daniels et al., 2011). According to Babajide et al. (2008) and McLaughlin et al. (1991), the brine shrimp lethality assay, which indicates toxicology levels of bioactive compounds, revealed that methanolic extracts of both *G. multifolia* (LC_{50} 6.2) and *G. villosa* (LC_{50} 4.2) indicated a high potential for antimicrobial and antiviral activities (Babajide et al., 2010).

In the current investigation, phytochemical screening was undertaken and some natural compounds present in the dried leaves, bulbs and roots of *G. multifolia* and *G. villosa,* were isolated and characterized, in an attempt to understand the chemistry behind the claimed medicinal values of the "Kukumakranka".

2. Materials and methods

2.1. General methods

All laboratory grade solvents (Merck, South Africa) were distilled prior to use and all spectroscopic grade solvents were used as such. Cleaning up of crude isolates was performed using Sephadex LH-20 (Sigma-Aldrich, South Africa). Preparative thin layer chromatography (TLC) (Sigma-Aldrich) was performed using Merck Silica gel 60 PF_{254} on glass plates (20 cm x 20 cm) with a thickness of 0.5 mm. Analytical TLC was conducted on normal-phase Merck Silica gel 60 PF_{254} pre-coated aluminium plates. Separated compounds on TLC were visualized under ultra-violet (UV) light at (254 and 360 nm) and spraying of the plates where

required, was carried out using 2% vanillin in H_2SO_4, followed by heating at 120 °C for 3 - 4 min. All extracts were concentrated on a rotary evaporator (Buchi Rotavator R-114, Germany) at ≤ 45 °C. Column chromatography was performed using Merck Silica gel 60 H (0.040 - 0.063 mm particle size, Merck). Melting points (m.p) were determined on a Fisher-John's melting point apparatus (Fisher-Scientific). Ultra-violet spectra (UV) of some of the isolated compounds (dissolved in methanol), were obtained with a Unicam UV 4 -100 UV/Vis Recording Spectrophotometer. Infra-red (IR) spectra were recorded on a Perkin Elmer Universal ATR (Precisely) Spectrum 100 series FT-IR spectrometer. Mass spectrometry (MS) was performed on a Waters Synapt G2API Q-TOF Ultima LC-MS-ESI instrument in the positive mode, while nuclear magnetic resonance (NMR) spectra were recorded on a Varian Inova 600 MHz NMR spectrometer in MeOH-d_4, using the solvent signals as internal reference.

2.2. Plant material

Gethyllis multifolia and *G. villosa* bulbs were identified and obtained with permission from the Karoo Desert National Biodiversity Garden (Worcester, Western Cape, South Africa) towards the end of their winter growth phase (mid-August to September). *Gethyllis multifolia* is threatened in its natural habitat, and for conservation purposes the exact location of this species is omitted. Mature plants of the same height and diameter were washed in distilled water and allowed to dry at room temperature in a well-ventilated room. The plants were then excised into leaves, bulbs and roots (n=10) and dried in a laboratory oven (Memmert, Laboratory & Scientific, Cape Town, SA) at 50 ºC. Roots and leaves

of both species took two days to dry while the bulbs took between five and seven days. Individual plant parts were ground to a fine powder in a portable spice grinder (Krups 75 model F203, Hecho En Mexico, Mexico City, Mexico) and stored in air-tight stoppered glassware prior to analysis.

2.3. Extraction of the constituents

The dried, powdered parts of the plants, namely the leaves, bulbs and roots were separately extracted for both *G. multifolia* and *G. villosa.* The dry weights (DW) for excised plant parts were as follows: *G. multifolia* leaves (7.0 g), bulbs (10.0 g) and roots (9.9 g) and *G. villosa* leaves (5.3 g), bulbs (10.0 g) and roots (9.9 g). Extraction was carried out sequentially using hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH). Pant material was not dried inbetween treatments and was left with some residual of the different solvents. Exraction was done under the ambient light conditions of the laboratory facility. By means of occasional stirring using a mechanical stirrer, each portion of plant material was macerated twice in 250 ml of each solvent at room temperature (25 °C) for 24 h, and the extracts were evaporated on a rotary evaporator at ≤ 45°C. The two extracts in each case were pooled and concentrated. Each extract was screened for the presence of tannins, flavonoids, phenolics, saponins, glycosides (phenolic and terpenoid), alkaloids, steroids, essential oils and terpenes according to the method of Wagner and Bladt (2001). Since the chromatographic profiles of the leaves, bulbs and roots of both species were similar, while higher recoveries were indicated in the roots of *G. multifolia* (data not shown), more in depth

investigation of the natural product content was thus conducted on the roots of *G. multifolia*.

2.4. Column chromatography

The height of the column was 750 mm with an internal column diameter of 25 mm. The flow rate of the eluent was measured 2 ml/ min. and the volumes collected were 10 ml in Pyrex test tubes. The ethyl acetate extract (5.0 g) of the root of *G. multifolia* was adsorbed on silica gel 60 and chromatographed using the solvent mixtures:- 100 ml of toluene and then 50 ml each of the following mixtures, toluene - EtOAc (90 : 10), (80 : 20), (60 : 40), (20 : 80) and (10 : 90). This was followed by EtOAc (50 ml) and 50 ml each of the following mixtures, EtOAc - MeOH (90: 10), (80: 20), (60: 40), (20: 80), (10: 90). Finally the column was washed with 70 ml of MeOH. Fractions (10 ml) collected were analyzed by TLC using toluene: EtOAc: MeOH (5: 4: 3). Fractions showing the same TLC profile were pooled and concentrated *in vacuo*. Three distinct major fractions were selected and coded A - C. Fraction B (1.32 g) was rechromatographed using the same solvent mixtures, column diameter and flow rate as described above. Out of the four fractions collected (on the basis of TLC profiles), fraction B (iii) was chromatographed on Sephadex LH 20 using toluene - MeOH (7: 3), to yield a brownish-yellow powder, which upon further purification by preparative TLC using toluene: EtOAc: MeOH (5: 4: 3), afforded compound **1** (GT1) (22 mg). Fraction C (0.5 g) was rechromatographed using the same solvent mixtures as shown above. Successive chromatography, followed by preparative TLC of the fraction obtained using toluene - MeOH (7: 3), afforded compound **2** (GT2) (12

mg) as an off-white powder. Although this isolate (compound **2**) appeared as a single spot on TLC, MS and NMR subsequently revealed that it was a mixture of two compounds.

The methanol extract of the root (5.0 g) of *G. multifolia* was chromatographed using the same solvent mixtures as for the EtOAc extract, except that the final volume of MeOH was 80 ml. Sixty five (10 ml) fractions were analyzed by TLC and those showing the same profile were pooled. Five fractions coded D - H, were obtained. One of the fractions F (0.9 g) was rechromatographed using the same solvent mixtures. Repeated preparative TLC afforded compound **3** (GT3) (11.1 mg) as a dull, yellow powder. Compound **3** (as indicated here) becomes compound **4** in the 'Results' and 'Discussion sections' because the isolated compound **2** is a mixture of two different compounds, and was discussed as such.

3. Results

The preliminary phytochemical screening results indicated the presence of tannins, flavonoids, phenolics, saponins, glycosides as well as essential oils, while alkaloids were absent in all plant parts screened (data pooled). The results are summarized in Table 1.

Table 1: Preliminary phytochemical screening results for leaves, bulbs and roots (pooled) of *Gethyllis multifolia* and *G. villosa* indicating the presence of some phytochemical compounds. **Tann** = tannins, **Phen** = phenolics, **Glyc** = glycosides, **Sapo** = saponins, **Flav** = flavonoids, **Alka** = alkaloids, **Esse** = essential oils, **GMM** = *G. multifolia* MeOH extract; **GVM** = *G. villosa* MeOH extract; **+** equals positive; **–** equals negative.

Test materials	**Tann**	**Phen**	**Glyc**	**Sapo**	**Flav**	**Alka**	**Esse**
GMM	+	+	+	+	+	-	+
GVM	+	+	+	+	+	-	+

The following compounds were isolated from *G. multifolia* roots by means of column chromatography:

Compound 1 (2,3-dihydro-7-hydroxy-2-phenyl-4*H*-1-benzopyran-4-one)

A brownish-yellow powder (22 mg) with m.p. 203 - 205 °C.

UV λ_{max} nm (log ε): 261 , 365; 262, 366; 280, 443; 268, 412.

IR (ν cm^{-1}) pronounced peaks: 3400, 2931, 1656, 1615 and 1556.

ESI-MS:*m/z* 241.08 ($[C_{15}H_{12}O_3 + H]^+$); ^{1}H NMR δ 7.73 (1H, d, J8.2Hz, H-5), 7.49 (2H, m, H-2',6'), 7.40 (2H, m, H-3',5'), 7.35 (1H, m, H-4'), 6.51 (1H, dd, J8.2Hz and 2.0Hz, H-6), 6.39 (1H, d, J2.0Hz, H-8), 5.48 (1H, dd, J12.8 and 3.0Hz, H-2), 3.02 (1H, dd, J17.0 and 12.8Hz, H-3a), 2.75 (1H, dd, J17.0 and 3.2Hz, H-3a); ^{13}C NMR δ 193.0 (C-4), 166.8 (C-7), 165.4 (C-8a), 140.7 (C-4'), 129,9 (C-5), 129.7 (C-1'), 129.5 (C-3',5'), 127.3 (C-2',6'), 115.1 (C-4a), 111.9 (C-6), 103.9 (C-8), 81.0 (C-2), 49.4 (C-3) (Addendum 1).

Compounds 2 (1-[2.4-dihydroxyphenyl]-3-phenylpropan-1-one) and 3 (2,3-dihydro-5,7-dihydroxy-2-phenyl-4*H*-1-benzopyran-4-one or pinocembrin)

An off-white powder (12 mg) with m.p. 225 - 227 °C.

IR (ν cm^{-1}) pronounced peaks: 3385, 2828, and 1690.

2; ESI-MS:*m/z* 243.10 ($[C_{15}H_{14}O_3 + H]^+$); ^{1}H NMR δ 12.70 (1H, s, 2'-OH), 7.67 (1H, d, J9.6Hz, H-6'), 7.23 (5H, m, H-Aryl), 7.14 (1H, d, J2.1Hz, H-3'), 6.32 (1H, dd, J9.6 and 2.1Hz, H-5'), 3.20 (2H, t, J7.1Hz, H-2), 2.98 (2H, t, J7.1Hz, H-3); ^{13}C NMR δ 205.2 (C-1), 133.7 (C-6'), 129.4 (C-Aryl), 127.1 (C-3'), 109.1 (C-5'), 40.5 (C-2), 31.6 (C-3) (Addendum 2).

3; ESI-MS:*m/z* 257.08 ($[C_{15}H_{12}O_4 + H]^+$); ^{1}H NMR δ 12.05 (1H, s, 5-OH), 7.47 (2H, dd, J7.5 and 1.5Hz, H-2',6'), 7.39 (2H, m, H-3',5'), 7.34 (1H, m, H-4'), 6.24 (1H, d, J2.2Hz, H-8), 5.91 (1H, d, J2.2Hz, H-6), 5.41 (1H, dd, J12.8 and 3.2Hz, H-2), 3.05 (1H, dd, J12.8 and 17.2Hz, H-3a), 2.74 (1H, dd, J17.2 and 3.2Hz, H-3e); ^{13}C NMR δ 197.3 (C-4), 129.7 (C-3',5'), 129.6 (C-4'), 127.3 (C-2',6'), 103.7 (C-8), 97.2 (C-6), 80.4 (C-2), 44.2 (C-3) (Addendum 2).

Compound 4 (5,7-diacetoxy-2,3-dihydro-2-phenyl-4*H*-1-benzopyran-4-one)

A dull, yellow powder (11.1 mg) with m.p. 215 - 217 °C.

IR (ν cm^{-1}) pronounced peaks: 3500 - 3400, 1652, 1613, 1568, 1556.

ESI-MS:*m/z* 341.10 ($[C_{19}H_{16}O_6 + H]^+$) and m/z 363.08 ($[C_{19}H_{16}O_6 + Na]^+$); ^{1}H NMR δ 7.45 (2H, dd, J7.3 and 1.5Hz, H-2',6'), 7.41 (2H, m, H-3',5'), 7.36 (1H,

m, H-4'), 6.81 (1H, d, J1.7Hz, H-6), 6.60 (1H, d, J1.7Hz, H-8), 5.56 (1H, dd, J13.2 and 2.8Hz, H-2), 3.10 (1H, dd, J16.7 and 13.2Hz, H-3a), 2.77 (1H, dd, J16.7 and 2.8Hz), 2.31 (3H, s, 5-CH_3CO), 2.27 (3H, s, 7-CH_3CO), ^{13}C NMR δ 191.1 (C-4), 170.9 and 169.7 (2X C=O, CH_3CO), 129.8 (C-3',4',5'), 127.4 (C-2',6'), 111.7 (C-8), 110.3 (C-6), 81.0 (C-2), 45.9 (C-3) (Addendum 3).

4. Discussion and conclusion

The molecular formula of compound **1** (Figure 1) was determined as $C_{15}H_{12}O_3$ by ESI-MS, on the basis of the pseudomolecular ion peak at *m/z* 241.08 $[M + H]^+$. The flavanone characteristics were evident from the presence of an ABX spin system due to the protons H-3e, H-3a and H-2, along with the typical coupling constants. Further evidence for the structural assignment came from both 1-D and 2-D NMR measurements (COSY, HSQC, HMBC and NOESY). H-5 appeared as the most deshielded proton at δ 7.73 due to its occupancy of a β position in an α-β unsaturated carbonyl system. This observation, along with the complete absence of the familiar chelated OH-5, characteristic of most naturally occurring flavonoids, was indicative of the fact that this was one of those unusual flavonoids. This flavonoid has recently been reported as isolated from *Zuccagnia punctata*, (Zampini et al., 2012), *Spatholobus suberectus* (Shim, 2011) and *Dalbergia cochinchinensis* (Shirota et al., 2003), among many other sources. Its synthesis has also been reported for the purpose of crystallographic and conformational studies (Kendi et al., 1995).

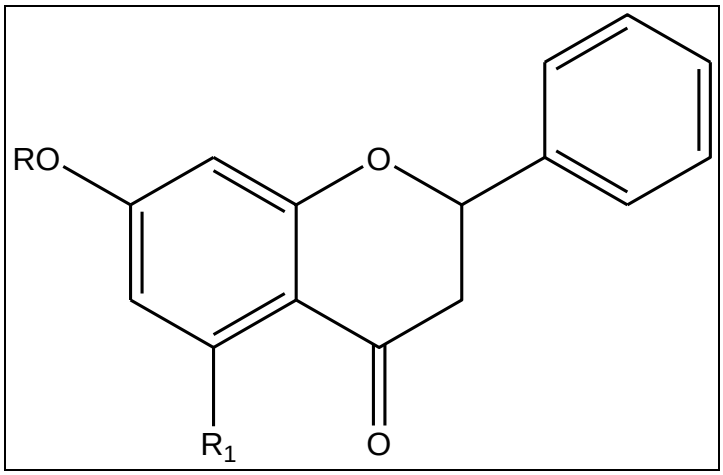

Fig. 1: Compound **1**; R, R_1 = H

(2,3-dihydro-7-hydroxy-2-phenyl-4*H*-1-benzopyran-4-one)

Compound **3**; R = H, R_1 = OH

(2,3-dihydro-5,7-dihydroxy-2-phenyl-4*H*-1-benzopyran-4-one or pinocembrin)

Compound **4**; R = Ac, R_1 = OAc

(5,7-diacetoxy-2,3-dihydro-2-phenyl-4*H*-1-benzopyran-4-one)

Compound **2** (Figure 2) displayed a molecular formula of $C_{15}H_{14}O_3$ as determined by ESI-MS, {pseudomolecular ion peak at *m/z* 243.10 ($[M + H]^+$). It also displayed the characteristic AB spin system usually observed for dihydrochalcones. This dihydrochalcone, which was also shown to possess a chelated OH group (δ 12.70) was assigned the proposed structure on the basis of 1-D and 2-D NMR spectral analysis. This compound has previously been reported both as a synthetic (Nakamura et al., 2003) as well as a natural (Vries et al., 2005) product.

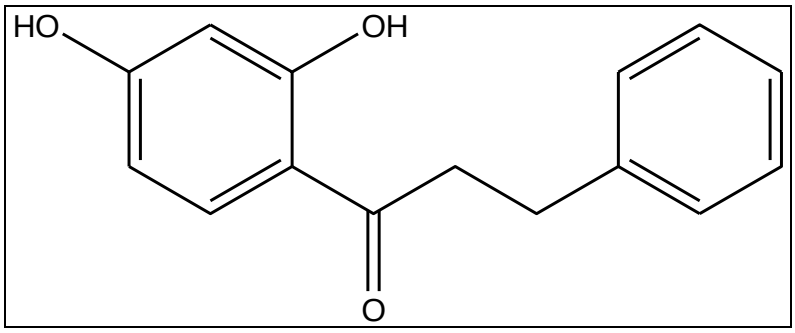

Fig. 2: Compound 2

(1-[2,4-dihydroxyphenyl]-3-phenylpropan-1-one)

The molecular formula of compound **3** (Figure 1) was determined as $C_{15}H_{12}O_4$ by ESI-MS, on the basis of the pseudomolecular ion peak at *m/z* 257.08 ($[M + H]^+$). NMR spectroscopy revealed features of a flavanone structure, as well as a chelated OH. Comparison with literature data (Wollenweber, 1982), suggested the structure to be that of a well-known flavanone, pinocembrin. Pinocembrin has been shown to be a flavonoid constituent in several plants, such as litchi (Wang et al., 2011) and has reportedly displayed antibacterial and antifungal activity *in vitro* (Bremner and Meyer, 1998). Furthermore, Ahn et al. (2009) reported that pinocembrin exhibited very low antioxidant activity but possessed a considerable degree of antiangiogenic activities. Jaganathan and Manda (2009) revealed in a recent study that pinocembrin has evolved as a promising pharmacological agent in the treatment of cancer.

Compound **4** (Figure 1) had a molecular formula of $C_{19}H_{16}O_6$ as determined by ESI-MS, {pseudomolecular ion peaks at *m/z* 341.10 ($[M + H]^+$) and 363.08 ($[M + Na]^+$)}. The NMR spectroscopic features of **4** were very similar to those of **3**, except that **4** carried two acetyl groups at O positions 5 and 7 (δ2.27 and 2.31),

and of course showed no evidence for a chelated OH. The presence of the acetyl groups was also evident from the significant downfield shift observed for H-6 and H-8, as well as the appearance of two carbonyl ^{13}C signals at δ 170.9 and 169.7, in addition to δ 191.1 for C-4. Deacetylation of **4** with sodium methoxide in methanol yielded a mixture of **3** and the partially deacetylated product (5-acetoxy-2,3-dihydro-7-hydroxy-2-phenyl-4*H*-1-benzopyran-4-one).

The occurrence of the latter compound as a by-product may be attributed to some degree of hindrance from the C-4 carbonyl group to the approach of the methoxide anion. Furthermore, acetylation of pinocembrin gave a product whose spectral data was identical to that of **4**. The occurrence of pinocembrin as its diacetylated form in nature has, to our knowledge, not been reported previously. However, based on the existence of a broad spectrum of acetyl transferases in plants (see for example Hampel et al, 2006), it is possible that similar enzymes may be responsible for the production of this diacetate. On the other hand, that this compound may be an artifact cannot be ruled out, especially given that ethyl acetate was a prominent solvent constitutent of the eluent during column chromatography. The acetyl group has been shown to migrate between hydroxyl groups in the presence of a suitable catalyst, such as certain brands of silica gel.

According to Fennell and Van Staden (2001), the majority of compounds found in the Amaryllidaceae family are usually alkaloids. These authors also reported that alkaloids were not detected in either the dichloromethane or 90% methanolic extracts of *G. ciliaris*, using the Dragendorff's reagent; only tannins, flavonoids,

phenolics, saponins, anthraquinones, glycosides and essential oils tested positively in all the extracts. However, the fact that alkaloids were not detected in *G. ciliaris*, does not necessarily imply that this species does not contain Amaryllidaceae alkaloids; but perhaps more attention in future research needs to be paid to the ontogenic and environmental factors that could influence the production and concentrations of alkaloids (Viladomat et al., 1997). A study conducted by Babajide et al. (2010) also revealed the presence of the same phytochemical compounds, and the absence of alkaloids from the methanol and water extracts of *G. multifolia* and *G. villosa* whole plants. The latter study also revealed the absence of saponins in the water extracts for both species, an observation that was further confirmed in all the plant parts in this study (data not shown) Table 1. Earlier reports by Viladomat et al. (1997) had revealed that the bulbs of *Gethyllis* species also contained flavonols, organic acids, carbohydrates and soluble nitrogen compounds.

Although the phenolic group is considered to be the most fundamental structural feature essential for antioxidant activity, the multiple presence of this functionality, as well as their specific locations on the flavonoid skeleton relative to other functional groups, have been shown to be critical for the enhancement of antioxidant activity in selected flavonoids. Thus, the presence of one or more of the following features in a flavonoid is known to contribute to enhanced antioxidant activity:- **(1)** the pyrogallol group; **(2)** the catechol group; **(3)** the 2,3-double bond in conjugation with a 4-oxo group and a 3-hydroxyl group; **(4)** and additional resonance-effective substituents (Janeiro and Brett, 2004). Since the

flavonoids reported in this paper lack these key features, while they will contribute to the total polyphenolic content, they may not be considered to be the key contributing compounds towards the antioxidant activities which have been reported previously for this plant species (Daniels et al., 2011).

Acknowledgements

The authors are grateful to the South African National Research Foundation (SANRF) for financial assistance and the Department of Chemistry, University of the Western Cape, Bellville, South Africa, for the use of the laboratory facilities. We are also grateful to the staff at The Central Analytical Facility of the University of Stellenbosch, Stellenbosch, South Africa for the NMR and MS analysis.

References

Ahn, M., Kunimasa, K., Kumazawa, S., Nakayama, T., Kaji, K., Uto, Y., Hori, H., Nagasawa, H., Ohta, T. (2009). Correlation between antiangiogenic activity and antioxidant activity of various components from propolis. Mol. Nutr. Food Res. 53: 643-651.

Akerele, O. M. (1988). Medicinal plants and primary health care: An agenda for action. Fitoterapia LIX 59: 355-363.

Babajide, J. O., Babajide, O. O., Daramola, A. O., Mabusela, W. T. (2008). Flavonols and an oxychromonol from *Piliostigma reticulatum*. Phytochemistry 69: 2245-2250.

Babajide, O. J., Mabusela, W. T., Green, I. R., Ameer, F., Weitz, F., Iwuoha, E. I. (2010). Phytochemical screening and biological activity studies of five SouthAfrican indigenous medicinal plants. J. Med. Plants Res. 4: 1924-1932.

Boonzaier, E., Malherbe, C., Smith, A., Berens, P. (1996). The cape herders: A history of the khoikhoi of southern Africa. David Phillips Publishers, Cape Town, South Africa.

Bremner, P. D., Meyer, J. J. (1998). Pinocembrin chalcone: an antibacterial compound from *Helichrysum trilineatum*. Planta Med. 64: 777-784.

Daniels, C. W., Rautenbach, F., Mabusela, W. T., Valentine, A. J., Marnewick, J. (2011). Comparative antioxidant capacity and content of leaves, bulbs, roots, fruit and flowers of *Gethyllis multifolia* L. Bolus and *G. villosa* Thunb. species. S. Afr. J. Bot. 77: 711-717.

Dold, A. P., Cocks, M. L. (2002). The trade in medicinal plants in the eastern Cape Province, South Africa. S. Afr. J. Sci. 98: 589-596.

Du Plessis, N., Delpierre, G. (1973). Blommeprag uit eie bodem: Koekemakranka. Landbouweekblad 21 Aug: 37-39.

Du Plessis, N., Duncan, G. (1989). Bulbous plants of southern Africa. Tafelberg Publishers, Cape Town, South Africa.

Elgorashi, E. E., Van Staden, J. (2004). Pharmacological screening of six Amaryllidaceae species. J. Ethnopharmacol. 90: 27-32.

Elgorashi, E. E., Coombes, P. H., Mulholland, D. A., Van Staden, J. (2007). Isoeugenitol, a cyclo-oxygenase-1 inhibitor from *Gethyllis cilliaris*. S. Afr. J. Bot. 73: 156-158.

Fennell, C. W., Van Staden, J. (2001). *Crinum* species in traditional and modern medicine. J. Ethnopharmacol. 78: 15-26.

Hamburger, M., Hostettmann, K. (1991). Bioactivity in plants. The link between phytochemistry and medicine. Phytochemistry 30: 3874-3879.

Hampel, D., Mosandl, A., Wuest, M. (2006). Biosynthesis of mono- and sesquiterpenes in strawberry fruits and foliage: 2H labeling studies. J. Agric. Food Chem. 54: 1473-1478.

Iwu, M. M. (1993). Handbook of african medicinal plants. C.R.C. Press, Boca Raton, Florida, USA.

Jaganathan, S. K., Mandal, M. (2009). Antiproliferative effects of honey and of its polyphenols: A review. J. Biomed. Biotech. (Journal details unknown - Article ID 830616).

Janeiro, P., Ana Maria Oliveira Brett, A. O. (2004). Catechin electrochemical oxidation mechanisms. Anal. Chim. Acta. 518: 109-115.

Kamatou, G. P. P., Viljoen, A. M., Őzek, T., Başer, K. H. C. (2008). Head-space volatiles of *Gethyllis afra* and *G. ciliaris* fruits ("kukumakranka"). S. Afr. J. Bot. 74: 768-770.

Kendi, E., Özbey, S., Ide, S., Fun, H. K., Yip, B. C. (1995). The phenylhydrazone form of 2-(4-chlorophenylazo)-1,3-indandione. Acta Cryst. 51: 1144-1146.

Lighton, C. (1992). The kukumakranka. Veld & Flora 78: 100-103.

McLaughlin, J. L., Chang, C. J., Smith, D. L. (1991). Bench - top bioassay for the discovery of bioactive natural products. An update: Studies in natural product chemistry, Attamra (ed). Elsevier Science Publishers B.V, Amsterdam, Netherlands: 383-409.

Nakamura, Y., Watanabe, S., Miyake, N., Kohno, H., Osawa, T.J. (2003). Dihydrochalcones: Evaluation as novel radical scavenging antioxidants. J. Agric. Food Chem. 51: 3309-3312.

Neuwinger, H. D. (1996). African ethnobotany: Poisons, drugs, chemistry, pharmacology and toxicology. Chapman and Hall, Weinheim, Germany.

Rood, B. (1994). Uit die veldapteek. Tafelberg Publishers, Cape Town, South Africa.

Shapera, I. (1930). The khoisan peoples of South Africa: Bushmen and hottentots. Routledge and Kegan Paul Ltd., London, UK.

Shim, S. H. (2011). 20S Proteasome inhibitory activity of flavonoids isolated from *Spatholobus suberectus*. Phytother. Res. 25: 615-618.

Shirota, O., Pathak, V., Sekita, S., Satake, M., Nagashima, Y., Hirayama, Y., Hakamata, Y., Hayashi, T. (2003). Phenolic constituents from *Dalbergia cochinchinensis*. J. Nat. Prod. 66: 1128-1131.

Story, R. (1964). Plant lore of the bushmen: Ecological studies in southern Africa. W. Junk Publishers, The Hague, Netherlands.

Tanaka, J. (1978). A study of the comparative ecology of african gatherer- hunters with special reference to San (Bushman-speaking people) and Pygmies. Senri Ethnol. Studies 1: 189-212.

Van der Walt, P. (2003). Koekemakranka. Wild en Jag 4**:** 16-17.

Van Wyk, B. E., Gericke, N. (2000). People's plants: A guide to useful plants of Southern Africa (1st edition). Briza Publications, Pretoria, South Africa.

Van Wyk, B., Van Oudtshoorn, B., Gericke, N. (1997). Medicinal plants of South Africa. Briza Publications, Pretoria, South Africa.

Viladomat, F., Bastida, J., Codina, C., Nair, J. J., Campbell, W. (1997). Alkaloids of the southern African amaryllidaceae. Recent research developments in phytochemistry, Vol. 1, Research Signpost, Trivandrum, India: 131-171.

Vries, Y. P., Atmadja, R. D., Hornstra, L. M., De Vos, W. M., Abee, T. (2005). Influence of glutamate on growth, sporulation, and spore properties of *Bacillus cereus* ATCC 14579 in a defined medium. Appl. Environ. Microbiol. 71: 3248-3254.

Wagner, H., Bladt, S. (2001). Plant drug analysis: A thin layer chromatography atlas (2nd edition). Springer, New York, USA.

Wang, Q., Xu, W., Dai, S., Song, Y. (2011). Development and application of ice bank precooling equipment used in litchi producing area. In Luo, X., Du, R., Wang, X. (eds.) 2011 International conference on new technology of agricultural engineering, Zibo, China, 27-29 May, IEEE, Piscataway, NJ, USA: pp 1116-1120.

Watt, J. M., Breyer-Brandwijk, M. G. (1962). The medicinal and poisonous plants of southern and eastern Africa (2nd edition). Livingstone, London, UK.
World Health Organisation, (2005). World malaria report
http://www.rbm.who.int/wmr2005/pdf/ adv e.pdf.

Wollenweber, E. (1982). Flavones and flavonols. In: The flavonoids: Advances in research. Harborne, J.B. and Marbry, T. J. (Eds). Chapman and Hall, London.

Zampini, I. C., Villena, J., Salva, S., Herrera, M., Isla, M. I., Alvarez, S. (2012). Potentiality of standardized extract and isolated flavonoids from *Zuccagnia punctata* for the treatment of respiratory infections by *Streptococcus pneumoniae*: In vitro and in vivo studies. J. Ethnopharmacol. 140: 287-292.

CHAPTER 8: FINAL DISCUSSION AND CONCLUSION

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Introduction

Gethyllis multifolia L. Bolus is threatened in its natural habitat and is listed in the 'Vulnerable' category of the 'Red Data List of Southern African Plants' and the 'IUCN-World Conservation Union List of Plants' while *G. villosa* Thunb. is not threatened although growing in the same habitats (Hilton-Taylor, 1996; IUCN-World Conservation Union, 1998). The habitats of both species are being exposed to drier conditions (Von Maltitz et al., 2006) and is further threatened by the encroachment of invasive indigenous plant species (Daniels, 2007). Currently it is not known to what extent these factors can pose a threat to the existence of the two species. Furthermore, medicinal uses for the genus *Gethyllis* (including *G. multifolia* and *G. villosa*) have been reported, ranging from cures for colic, digestive disturbances, teething problems, fatigue, boils, bruises and insect bites, to being used as an aphrodisiac (Du Plessis and Delpierre, 1973), but very little research have been done to understand the chemistry behind the reported medicinal uses of this genus.

The first objective of this investigation was to determine the costs of vegetative and reproductive growth during the seasonal life cycle of the plant, using carbon and nitrogen as a physiological currency. The second objective was to elucidate a functional basis to explain the difference in the conservation status of both species

in their natural habitat. Both species were subjected to environmental drought and shade stresses and leaf gas exchange physiology was assessed. The third objective of the study was to evaluate the antioxidant-content and -capacity of natural populations and plant samples that were exposed to artificial photo- and drought stresses. This investigation also attempted to isolate and characterize, by means of thin-layer chromatography (TLC) and column chromatography (CC), natural compounds from both species to create a better understanding of the chemistry behind the medicinal uses of the genus, *Gethyllis*. Herewith is a discussion and conclusion of the main findings of the experimental chapters.

Carbon and nitrogen costs of growth and reproduction

The study of the carbon (C) and nitrogen (N) costs associated with *G. multifolia* and *G. villosa*'s growth and reproductive stages revealed significant functional alterations in the above-ground organs. At the onset of the growth season, *G. multifolia*, which morphologically has a bigger bulb structure than *G. villosa*, had a larger biomass and nutrient concentration associated with leaf production during the growth phase but had a lower leaf:bulb ratio than *G. villosa***.** During the reproductive phase, *G. villosa*, which has a smaller bulb structure, had a flower production that was similar to that of *G. multifolia*. This represented a significantly higher percentage of flower dry weight per bulb in *G. villosa*, compared to *G. multifolia*. The implications and underlying mechanism of this altered above-ground investment were further studied using the photosynthetic gas exchange of leaf material.

Photosynthesis, stomatal conductance and respiration

The photosynthetic gas exchange data showed that the above-ground non-reproductive organs experienced more stress from drought than from shading. Under drought stress, *G. villosa* had a better photosynthetic performance than *G. multifolia*, which appears not to be related to foliar adaptations such as specific leaf mass (SLM), but to *G. villosa*'s leaves maintaining their stomatal conductance (G_s), photosynthetic light compensation (LCP) and photon yields better than *G. multifolia*. In *G. multifolia* plants, the increase in photosynthetic LCP and the decline in apparent photon yield under drought stress indicate that these leaves are not able to efficiently utilise light energy for photosynthesis. This indicates that *G. multifolia* was more severely affected by drought, owing to its inability to adapt to increasing drought.

Physiological outcomes

The functional mechanisms of growth and reproductive nutrient costs during the normal growth phases of *Gethyllis* concur with the above-ground photosynthetic physiology, where *G. multifolia* was less able to compete with *G. villosa*. The functional plasticity of *G. villosa* and the inability of *G. multifolia* to adapt to the environmental changes may be a contributing factor underpinning its 'Vulnerable' conservation status. Stomatal control of photosynthesis is a well-known adaptation strategy during drought, as reported in previous work for various ecosystems (Valliyodan and Nguyen, 2006). Furthermore, the survival of *G. villosa* is also enhanced by its more successful reproductive survival strategy than *G. multifolia*. This reproductive strategy may be suited for nutrient-limiting

environments such as the succulent Karoo biome, to ensure flowering for reproduction status. These findings are confirmed by reports on *Haemanthus* species, which successfully survives in semi-desert environments with nutrient poor soils due to its effective life strategy, reproductive mechanisms, and life cycle specifically adapted to these environments (Ruiters et al., 1993).

Although these physiological studies elucidate why *G. multifolia* may be more threatened in its natural habitat than *G. villosa*, it also show the need to integrate physiology and ecology in a more cohesive manner, in order to account for plant distributions in their natural environments. The weakness of this investigation is that the studies have been conducted in a nursery under artificial environmental stresses, and it may be prudent for future work to investigate the species in their natural habitat. Additionally, the role of root associated bacteria and symbiotic associations, such as mycorrhizal fungi should be studied, as they may play a vital role in nutrient and water uptake in resource-limited ecosystems. Although drought caused the most severe stress to the above-ground function of *G. multifolia*, the previous experiments have also shown the below-ground organs to be less able to supply nutrients to the leaves and flowers during the growth phases. These aspects of *G. multifolia*'s physiology indicate that its organs may be more sensitive to stresses than those of *G. villosa*.

According to Tausz et al. (2004) phytochemicals, such as antioxidants may serve as a quantitative indicator of plant stresses such as high light intensities, severe drought, high salt levels, high temperature, low relative humidity, etc.

Antioxidant content-and -capacity during drought and shade

It was also reported by Price et al. (1989) that plants increase their production of their secondary metabolites, such as antioxidative compounds during biotic and abiotic stresses. Other investigations confirmed that the production of polyphenol compounds during these stresses serves as an antioxidant defence system, comprising of enzymes and low molecular antioxidants, to protect plants against the production and damaging effects of reactive oxygen species (ROS) (Tausz et al., 2004). Based on these reports and results from a previous observation study of *G. multifolia* and *G. villosa*, it is hypothesized that *G. multifolia* struggles more to adapt to adverse environmental conditions and consequently will have a higher total polyphenol content with increased antioxidant activity, than *G. villosa*. In comparison to shade stress, this investigation found that both species encountered drought more severely as an environmental stress. The total polyphenols increased significantly under drought stress in the roots of *G. multifolia* when compared to the natural population as well as plant parts of *G. villosa*. Furthermore, the FRAP in *G. multifolia* was also found to be highest in the roots under drought stress when compared to other plant parts and the comparative natural population. Similarly, the highest ORAC was recorded in the roots of *G. multifolia* under drought stress when compared to the other plant parts, the comparative natural population and plant parts of *G. villosa* under both environmental stresses. Results from this study have shown that an increase in the antioxidant profile is highest in the roots of *G. multifolia* under drought stress when compared to *G. villosa*, other plant parts and other growing conditions.

These findings confirm that polyphenolic antioxidants are a powerful quantitative tool for predicting plant performance in a changing environment. These functional predictions have been confirmed by physiological studies involving both whole plant nutrition and photosynthetic gas exchange. The potential weakness of this work is the fact that increasing degrees of drought stress were not investigated as a gradient for plant performance and antioxidant synthesis (Munne-Bosch and Penuelas, 2004). Furthermore, this study also indicated reduced biomass for both species during drought stress and an even more limited flower and consequently fruit production for *G. multifolia* under the same stress. Another weakness of this investigation is that the antioxidant-content and -capacity were not conducted on the flowers and fruit on both species during drought and shade stress and its potential effect on antioxidant-content and -capacity. It has been reported that the flowers and fruit of *Gethyllis* are mostly used for medicinal purposes (Du Plessis and Delpierre, 1973) and therefore these plant parts should be investigated for their antioxidant activity under drought and shade stress in future research projects.

Antioxidant content-and -capacity of the natural populations

It has been hypothesized, based on the pilot studies and the medicinal reports of the genus *Gethyllis*, that certain plant parts of natural populations of both species may have a high total polyphenol content and consequently increased antioxidant activity. In this investigation it was found that, in general, the flowers and fruit of both species showed increased, and in some cases significantly higher antioxidant

activities and total polyphenolic content than the leaves, bulbs and roots. Even though this investigation cannot prove that there is a definite relationship between high polyphenol values, increased antioxidant activity and the medicinal uses of this genus, published reports did indicate mostly the application of the flowers and fruit for medicinal purposes than other plant parts, as mentioned previously.

The total polyphenol content in the fruits of *G. multifolia* and *G. villosa* were found to be in line with other commercial fruits such as raisins, blueberries, raspberries, guavas, marula fruit, strawberries, plums, oranges and red grapes (PR WEB, 2005; Thaipong et al., 2006; Ndhlala et al., 2007). When compared to some commercial vegetables, the polyphenol content of the fruit of both species was higher than that of spinach, broccoli, brussels sprouts, beetroot, and onions (PR WEB, 2005). The ABTS, FRAP and ORAC of the flowers and fruit of both species were significantly higher than those of the other plant parts and in some cases higher than members of the berry range (red raspberry, black raspberry, boysenberry, marion blackberry and evergreen blackberry) (Wang and Lin, 2000) and certain herb species (Zheng and Wang, 2001). The weakness of this study is that only the total polyphenols and the presence of certain flavonoid groups were investigated, but on the other hand, three assays (ABTS, FRAP and ORAC) were used to determine the antioxidant capacity because of their different reaction mechanisms (Prior and Cao, 1999). Furthermore, this study did not indicate which compounds were responsible for the high total polyphenols and antioxidant capacity in certain parts of the two species. For future studies, evaluation of the

ability of these plant parts to inhibit lipid peroxidation using a biological system is advised.

Phytochemical studies and compound extraction

Further phytochemical studies revealed that the chromatographic profiles of the leaves, bulbs and roots of both species were similar, as was found also in a study by Babajide (2010), while higher recoveries were indicated in the roots of *G. multifolia*, hence, a more detailed investigation of the natural product content was thus conducted on this plant part. These detailed studies on the roots of *G. multifolia* led to the isolation and characterization of four flavonoids: 2,3-dihydro-7-hydroxy-2-phenyl-4*H*-1-benzopyran-4-one (**1**), (1-[2.4-dihydroxyphenyl]-3-phenylpropan-1-one) (**2**)**,** 2,3-dihydro-5,7-dihydroxy-2-phenyl-4*H*-1-benzopyran-4-one or pinocembrin (**3**) and 5,7-diacetoxy-2,3-dihydro-2-phenyl-4*H*-1-benzopyran-4-one (**4**). The structure of compound **3** is suggested to be that of a well-known flavanone, pinocembrin, which has been shown to be a flavonoid constituent in several plants, such as litchi (Wang et al., 2011). The weakness of this study is that phytochemistry and natural compound extraction was not conducted on the flowers and fruit of the two species, even though these two plant parts were mostly used by traditional healers and old folk for their medicinal properties.

Antioxidant and phytochemistry outcomes

There seem to be a direct link between the higher (than *G. villosa*) total polyphenol content and antioxidant capacity exhibited by the roots of *G.*

multifolia and the chromatographic profiles of the roots of *G. multifolia* as found in this investigation. In contrast to the fact that flavonoids are generally well-known for their antioxidant properties, as well as reports on high antioxidant properties in plant parts of both *G. multifolia* and *G. villosa*, the literature and the SAR discussion revealed that the isolated compounds in this study (from the roots of *G. multifolia*), in general, exhibit very low antioxidant activity. However, the literature revealed that some of the isolated compounds in this study, exhibit antifungal, antibacterial, antiangiogenic and anticarcinogenic properties *in vitro*, which could be ascribed to the medicinal applications of plant parts of certain species belonging to this genus. This *in vitro* study has further shown that the flowers and fruit of both species may be used as good natural sources of dietary antioxidants, but these results need to be further supported by clinical studies. These results further suggest that under drought stress, *G. multifolia* produces higher levels of polyphenols and flavonols/ flavones than *G. villosa* in the root system, to possibly serve as a protective measure against oxidative stress and the production of ROS. These results could have a significant impact on how traditional healers, pharmaceutical companies and farmers choose conducive environmental conditions for cultivation of the two *Gethyllis* species in order to ensure enhanced polyphenolic content and antimicrobial activity in the relevant plant "parts" that are traditionally used in medicinal practices.

The high antioxidant content, -capacity and flavonoids isolated from plant parts as described in this thesis, do not confirm claims made by traditional healers, but

merely provide us with a better understanding of the chemistry behind the genus *Gethyllis* (Kukumakranka), in specific, *Gethyllis multifolia* and *G. villosa*.

Future directions

The strength of the approach in this thesis is its multi-disciplinary scope, which encompasses normally separate fields of study, and integrates a suite of analytical techniques into a single functional theme, but suggests further investigations on a number of issues. Future research is needed on the fruit and flowers of both species to further elucidate other biological properties of these two endemic plant species, but also to confirm the antioxidant activity *in vivo*. Further studies also need to be conducted on the powerful fragrance and taste of the fruit of *G. multifolia* for possible use in the food and beverages industry. Furthermore, the use of current molecular tools should be further exploited in future work. In this regard, the genes and enzymes involved in antioxidant production should be investigated. In particular, the regulation of the antioxidants by specific gene expressions and post-translational protein modifications, may reveal key aspects of control during environmental and developmental changes. This would be of importance to the pharmaceutical exploitation of *G. multifolia,* in the quest of enhancing its antioxidant capacity.

References

Babajide, O. J., Mabusela, W. T., Green, I. R., Ameer, F., Weitz, F., Iwuoha, E. I. (2010). Phytochemical screening and biological activity studies of five South African indigenous medicinal plants. *Journal of Medicinal Plants Research*, 4: 1924-1932.

Daniels, C.W. (2007). *A comparative study of the propagation and cultivation of Gethyllis multifolia and G. villosa.* Masters thesis, Cape Peninsula University of Technology, Bellville, South Africa: pp. 42-57.

Du Plessis, N., Delpierre, G. (1973). Blommeprag uit eie bodem: Koekemakranka. *Landbouweekblad*, 21 Aug: 37-39.

Hilton-Taylor, C. (1996). *Red data list of southern African plants.* National Biodiversity Institute, Cape Town, South Africa: pp. 57.

IUCN. (1998). *Red list of threatened plants*. IUCN, World Conservation Union, Cambridge, UK, pp. 617.

Munne-Bosch, S., Penuelas, J. (2004). Drought-induced oxidative stress in strawberry tree (*Arbutus unedo* L.) growing in Mediterranean field conditions. *Plant Science*, 166: 1105-1110.

Ndhlala, A.R., Kasiyamhuru, A., Mupure, C., Chitindingo, K., Benhura, M.A., Muchuweti, M. (2007). Phenolic composition *of Flacourtia indica*, *Opuntia megacantha* and *Sclerocarya birrea*. *Food Chemistry*, 103: 82-87.

PR WEB. (2005). Jobelyn, African wonder herb set to rule the world of natural plant antioxidants and phenolics. [Accessed February 29, 2008] http://www.prweb.com/pdfdownload/312415.pdf.

Price, A.H., Atherton, N., Hendry, G.A.F. (1989). Plants under drought stress generated activated oxygen. *Free Radical Research Communications*, 8: 61-66.

Prior, R.L., Cao, G. (1999). In vivo total antioxidant capacity: comparison of different analytical methods. *Free Radical Biology & Medicine*, 27: 1173-1181.

Ruiters, C., McKenzie, B., Raitt, L.M., (1993). Life history studies of the perennial bulbous geophyte species *Haemanthus pubescens* subsp. *pubescens* (Amaryllidaceae) in lowland coastal fynbos, South Africa. *International Journal of Plant Sciences*, 154: 441-449.

Tausz, M., Gonzalez-Rodriguez, M., Wonisch, A., Peters, J., Grill, D., Morales, D., Jiminez, M. (2004). Photostress, photoprotection, and water soluble antioxidants in the canopies of five Canarian laurel forest tree species during a diurnal course in the field. *Flora*, 199: 110-119.

Thaipong, K., Boonprakop, U., Crosby, K., Cisneros-Zevallos, L., Byrne, D.H. (2006). Comparison of ABTS, DPPH, FRAP and ORAC assays for estimating antioxidant activity from guava fruit extracts. *Journal of Food Composition and Analysis*, 19: 669-675.

Valliyodan, B., Nguyen, H.T. (2006). Understanding regulatory networks and engineering for enhanced drought tolerance in plants. *Plant Biology*, 9: 189-195.

Von Maltitz, G.P., Scholes, R.J., Erasmus, B., Letsoalo, A. (2006). Adapting conservation strategies to accommodate impacts of climate change in Southern Africa. *AIACC Working Paper*, 35: 2-3.

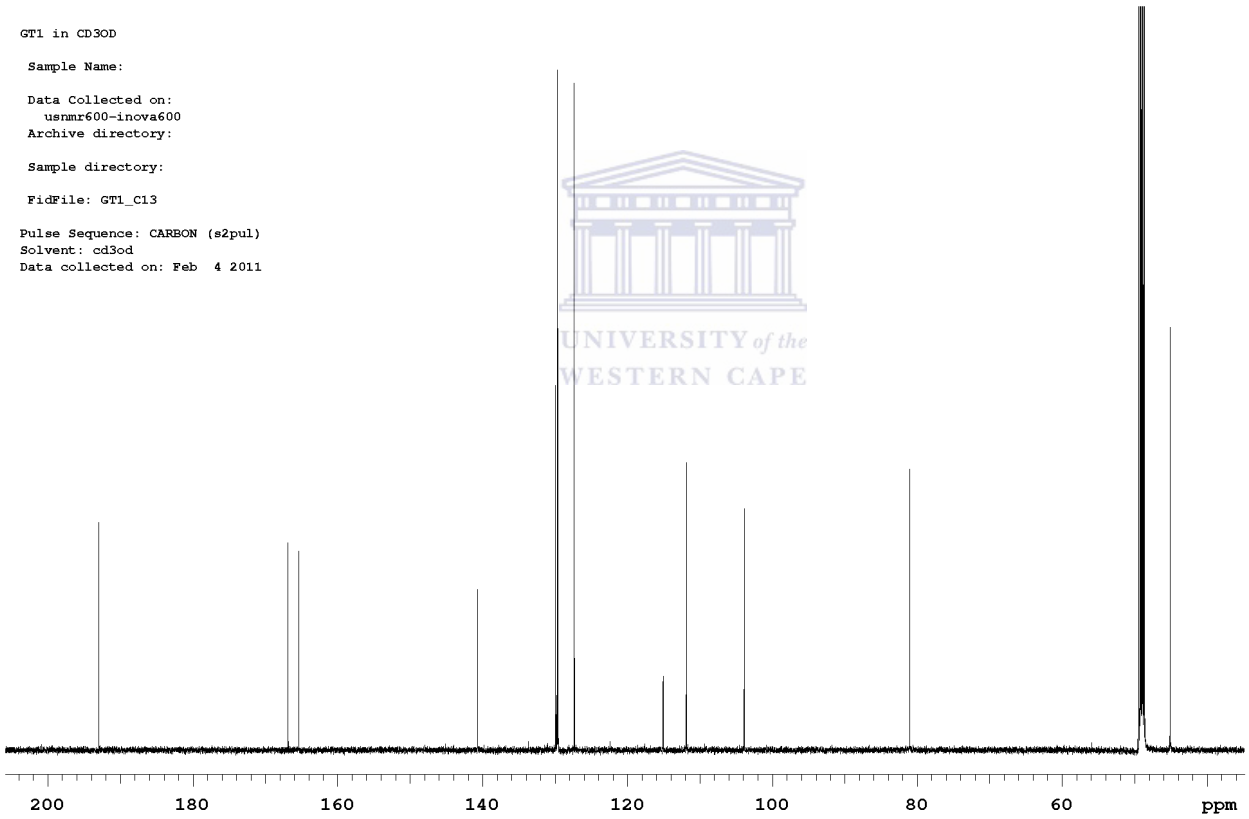
Wang, Q., Xu, W., Dai, S., Song, Y. (2011). Development and application of ice bank precooling equipment used in litchi producing area. New Technology of Agricultural Engineering (ICAE), International Conference, 27-29 May: 1116-1120.

Wang, S.Y., Lin, H. (2000). Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. *Journal of Agricultural and Food Chemistry*, 48: 140-146.

Zheng, W., Wang, S.Y. (2001). Antioxidant activity and phenolic compounds in selected herbs. *Journal of Agricultural and Food Chemistry*, 49: 5165-5170.

ADDENDUM 1:
NMR DATA FOR COMPOUND 1

C13 Plots for Compound 1



GT1 in CD3OD

exp2 CARBON

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nmrsys/data/Data20~		temp	25.0
11/February2011/A_~		gain	not used
Valentine/GT1/GT1_~		spin	not used
	C13.fid	hst	0.008
ACQUISITION		pw90	14.400
sw	41536.9	alfa	10.000
at	0.789	FLAGS	
np	65536	il	n
fb	not used	in	n
bs	16	dp	y
d1	1.000	hs	nn
nt	40000	PROCESSING	
ct	40000	lb	0.50
TRANSMITTER		lsfid	-2
tn	C13	fn	not used
sfrq	150.884	DISPLAY	
tof	4175.5	sp	5252.7
tpwr	53	wp	25802.1
pw	7.200	rfl	9472.8
DECOUPLER		rfp	7392.4
dn	H1	rp	75.2
dof	0	lp	80.2
dm	yyy	PLOT	
decwave	w	wc	250
dpwr	47	sc	0
dmf	13800	vs	4760
		th	3
		ai	cdc ph

GT1 in CD3OD

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Sample directory:

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Solvent: cd3od
Data collected on: Feb 4 2011

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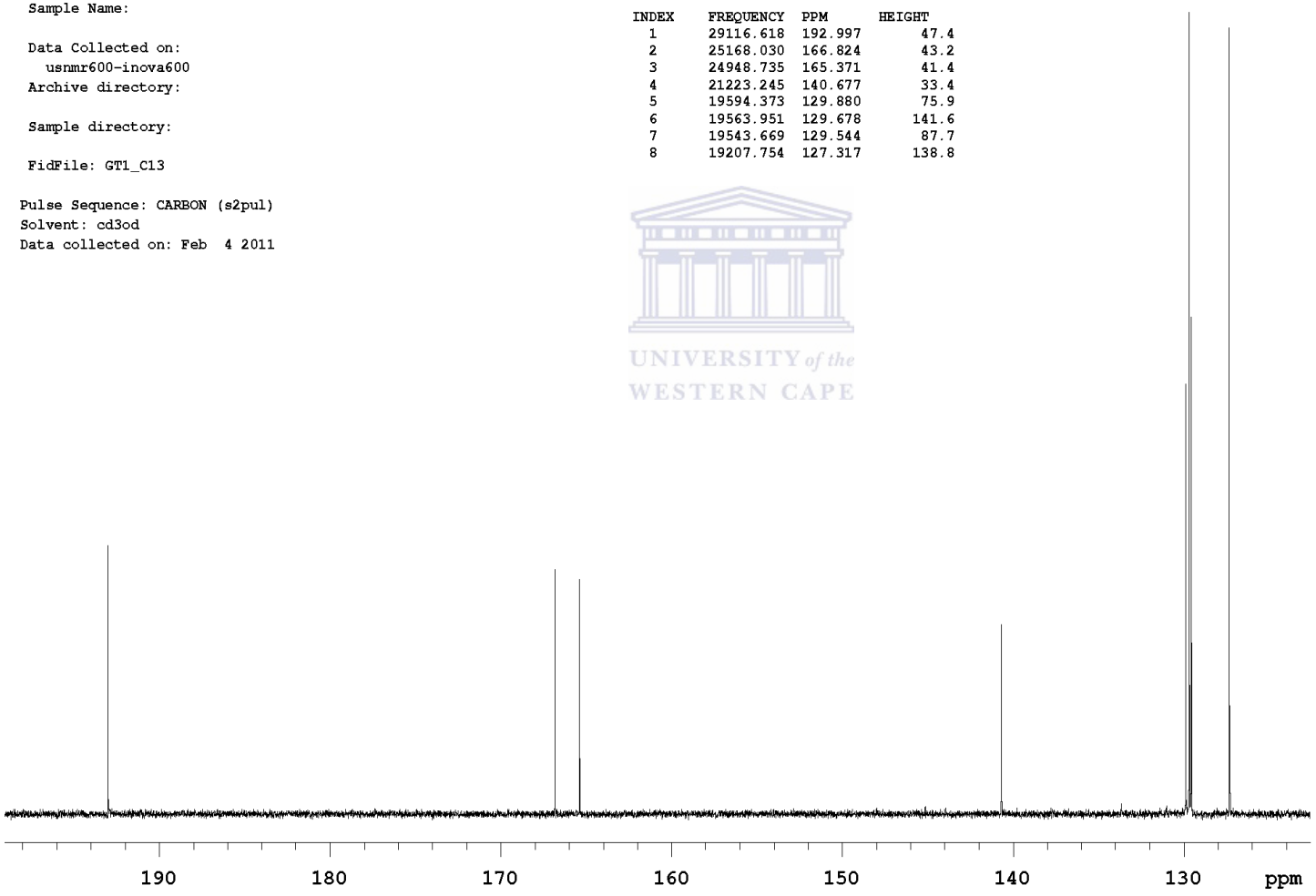
3	24948.735	165.371	41.4
4	21223.245	140.677	33.4
5	19594.373	129.880	75.9
6	19563.951	129.678	141.6
7	19543.669	129.544	87.7
8	19207.754	127.317	138.8



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Solvent: cd3od
Data collected on: Feb 4 2011

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5	7455.793	49.420	255.1
6	7434.243	49.277	684.8
7	7413.962	49.143	1232.3
8	7392.412	49.000	1655.9
9	7370.863	48.857	1575.5
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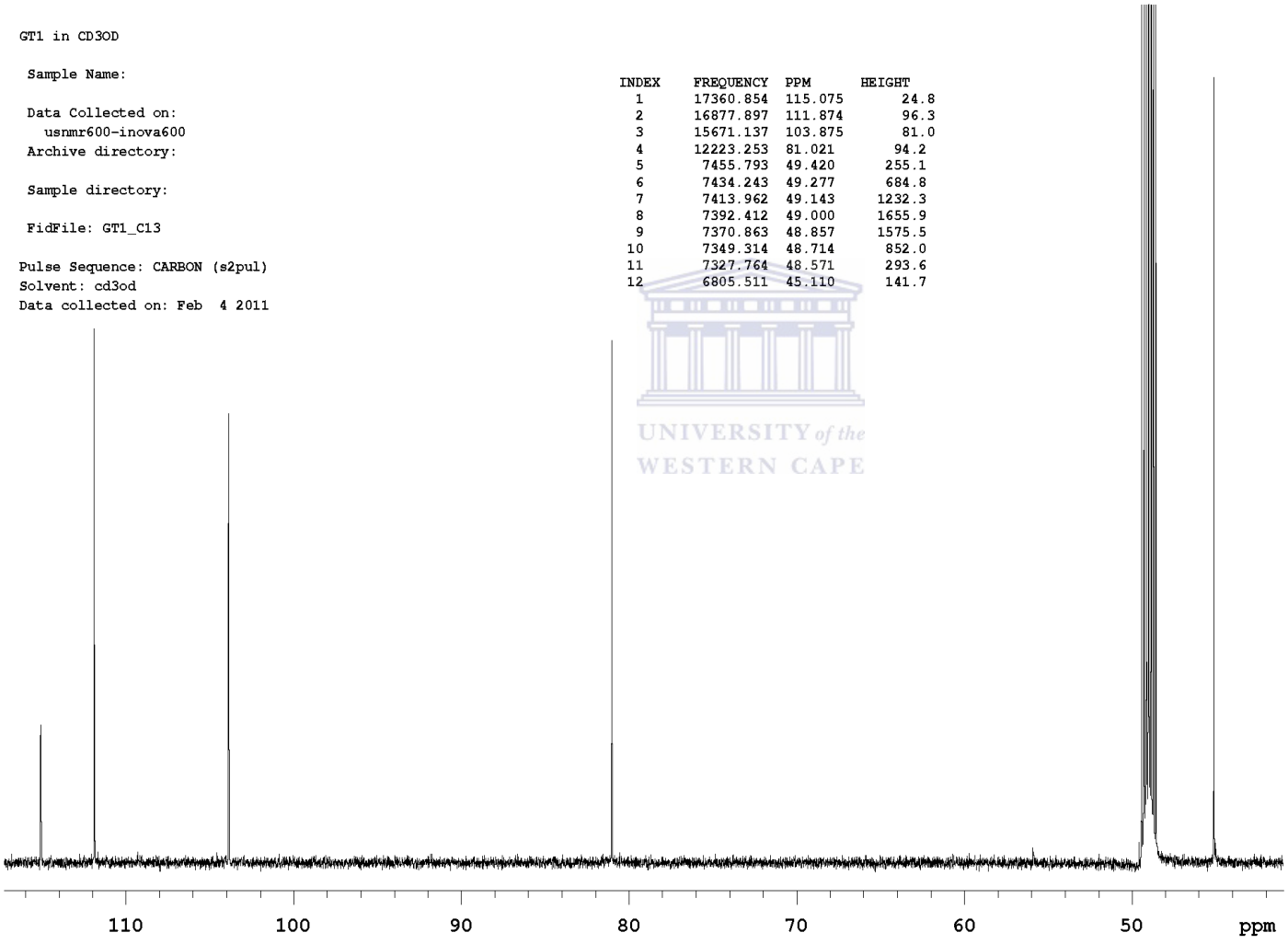
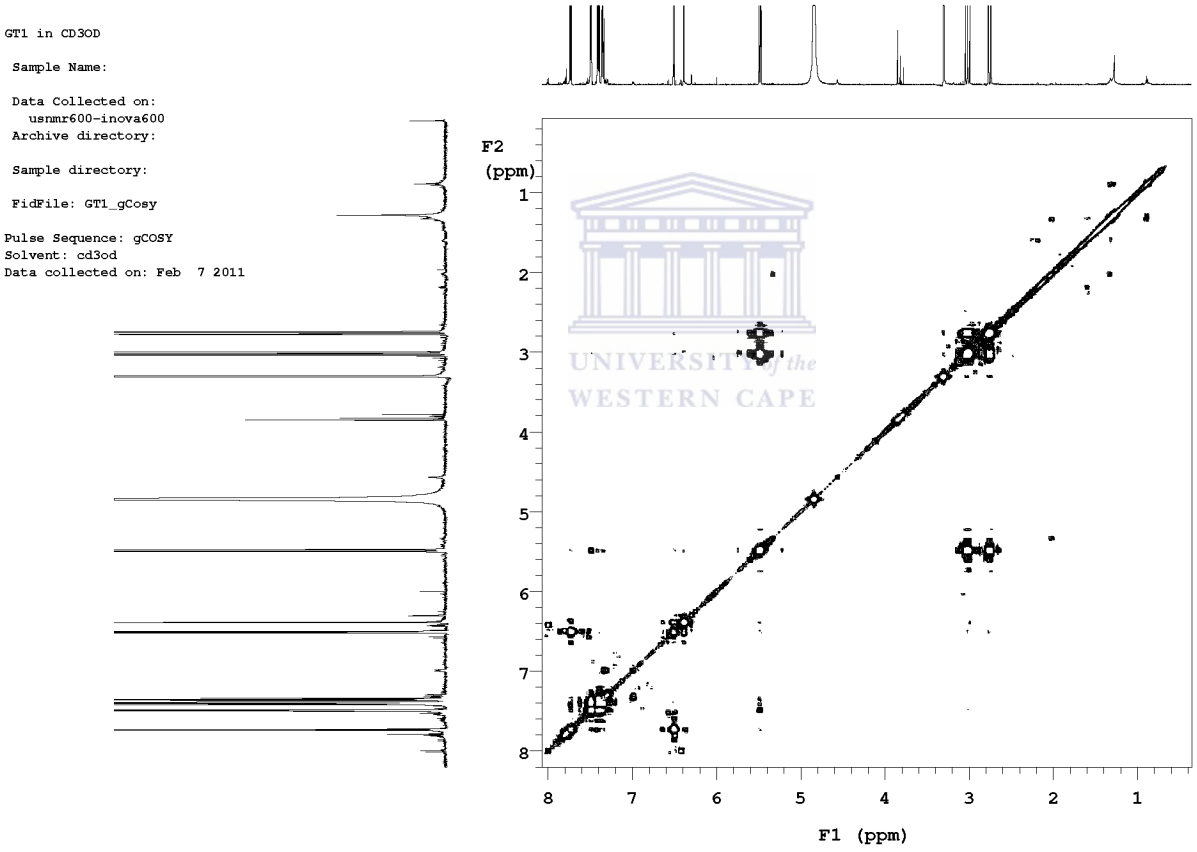


gCOSY Plots for Compound 1



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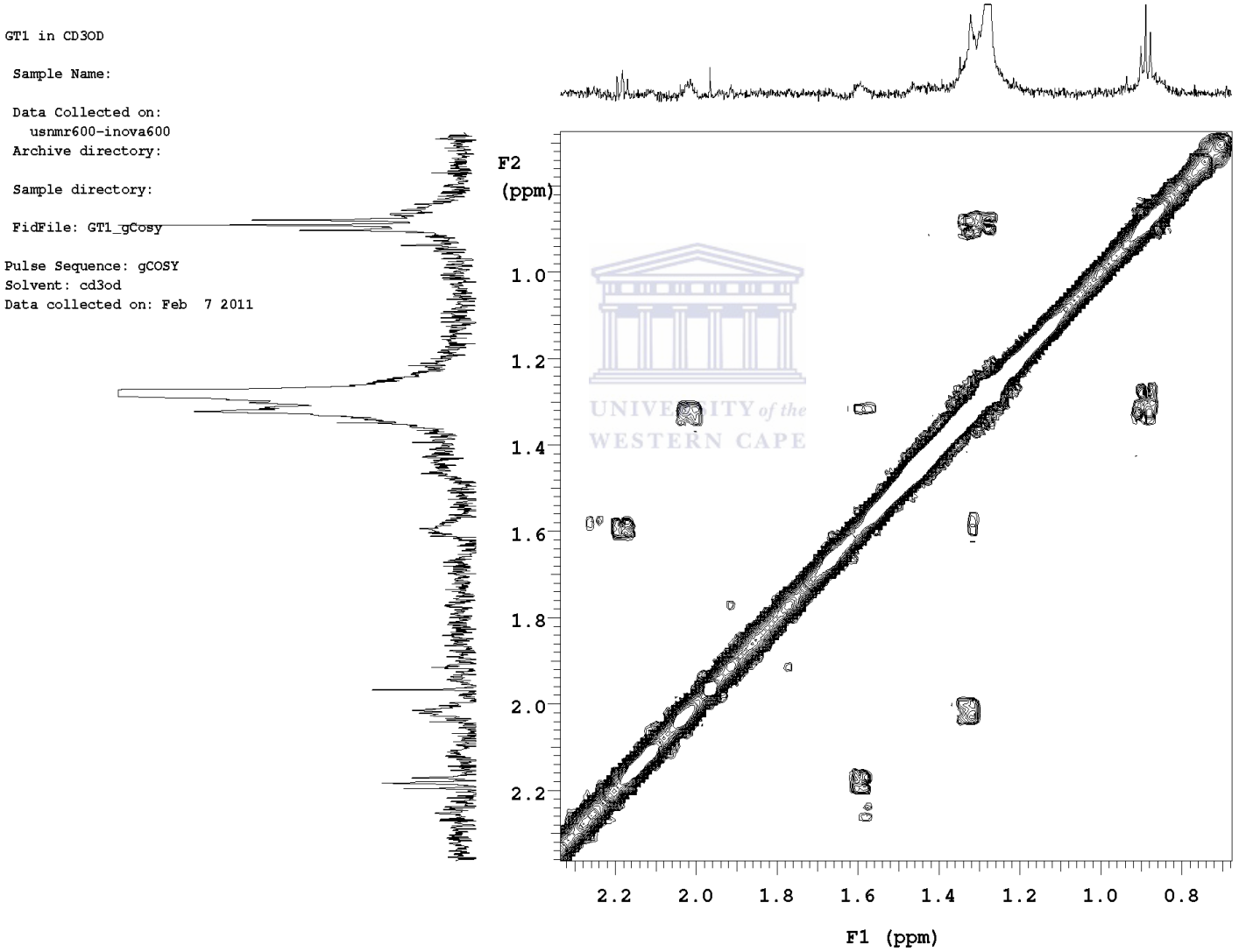

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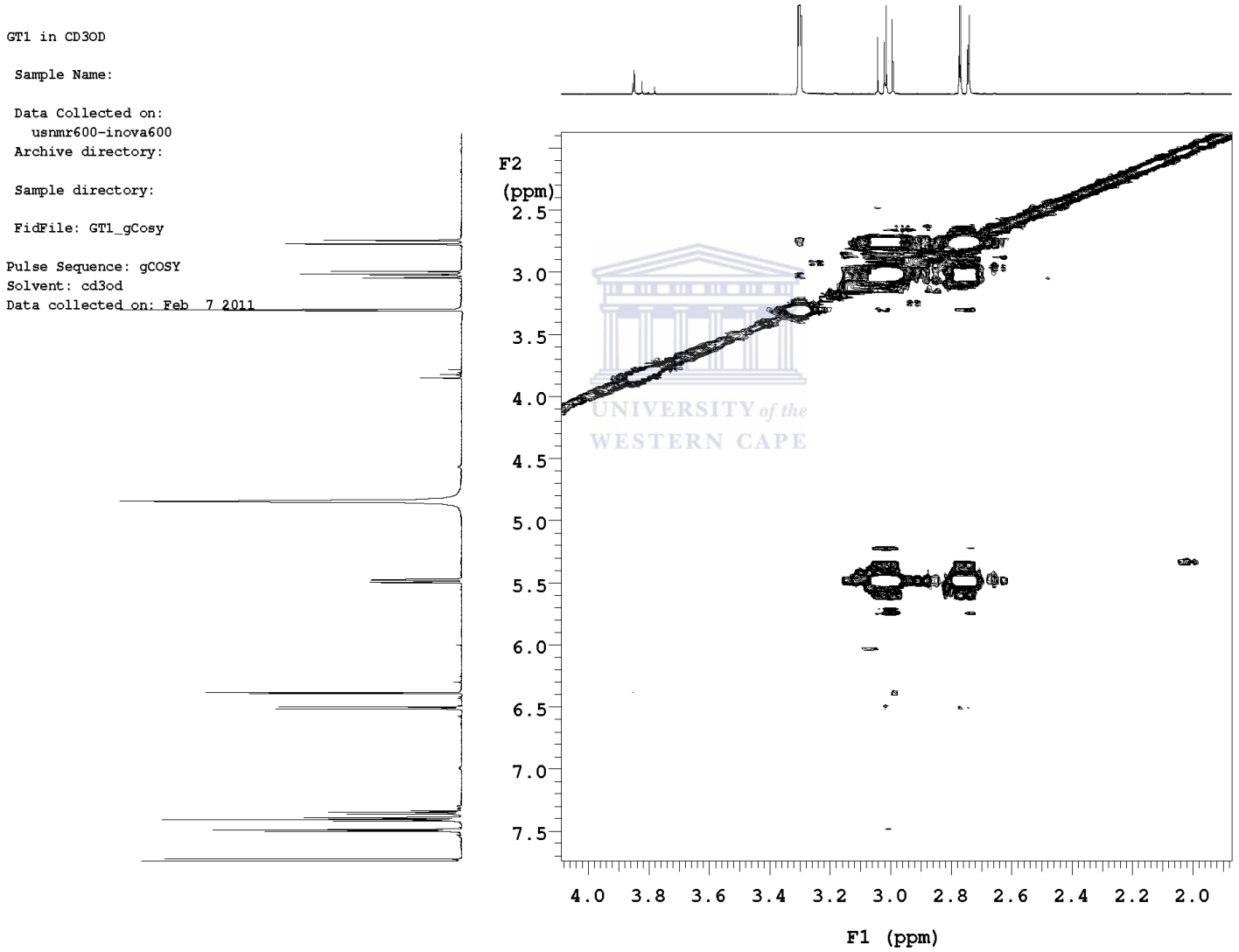

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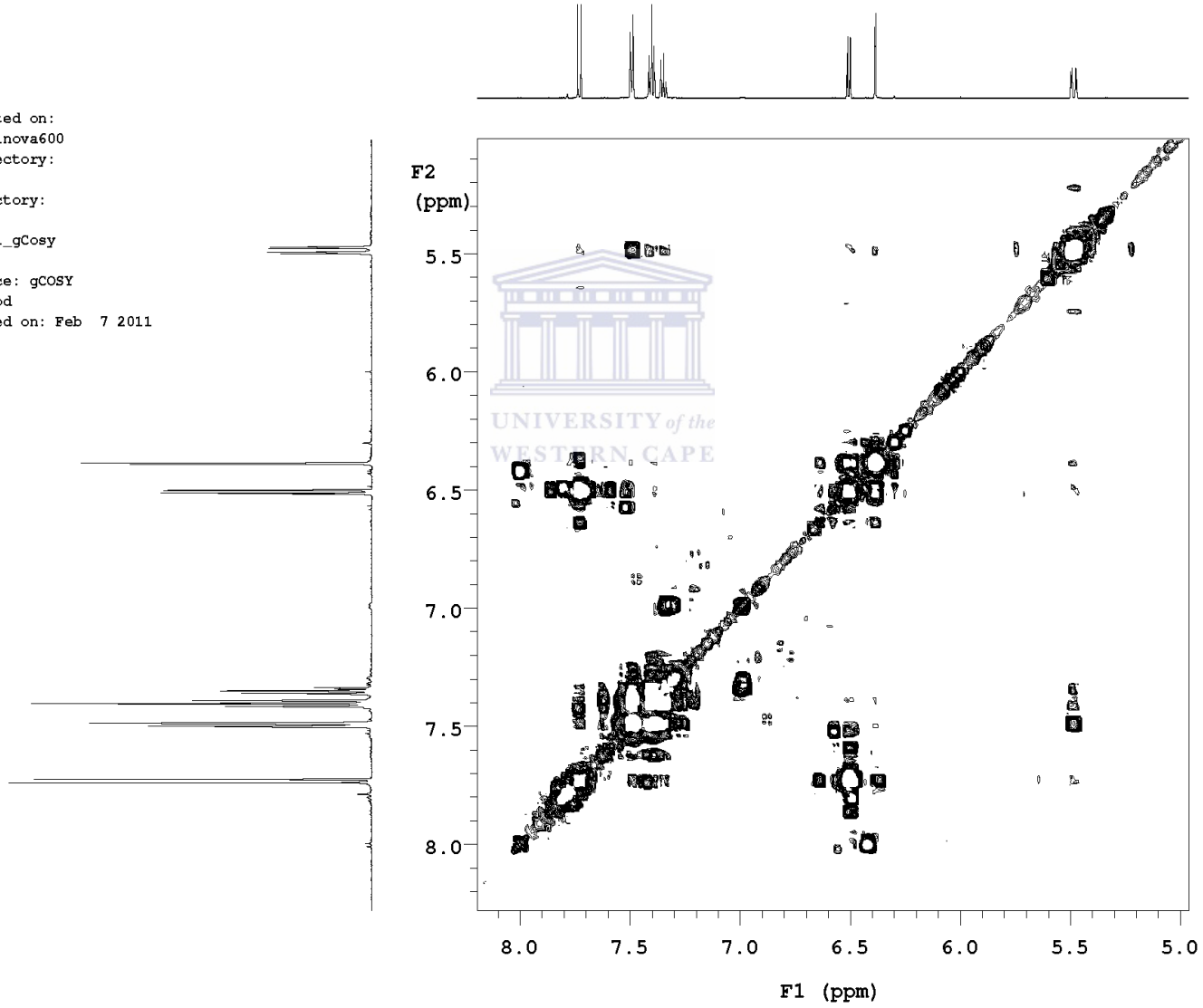
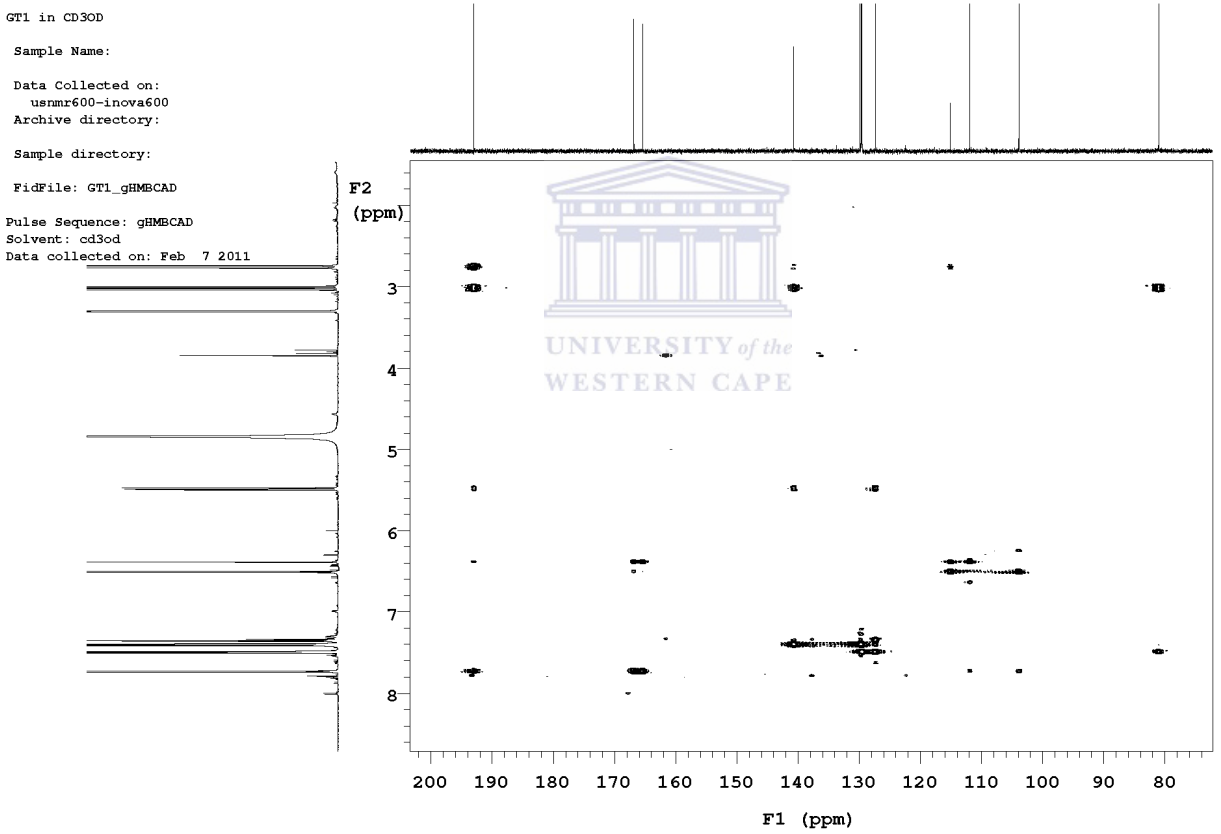
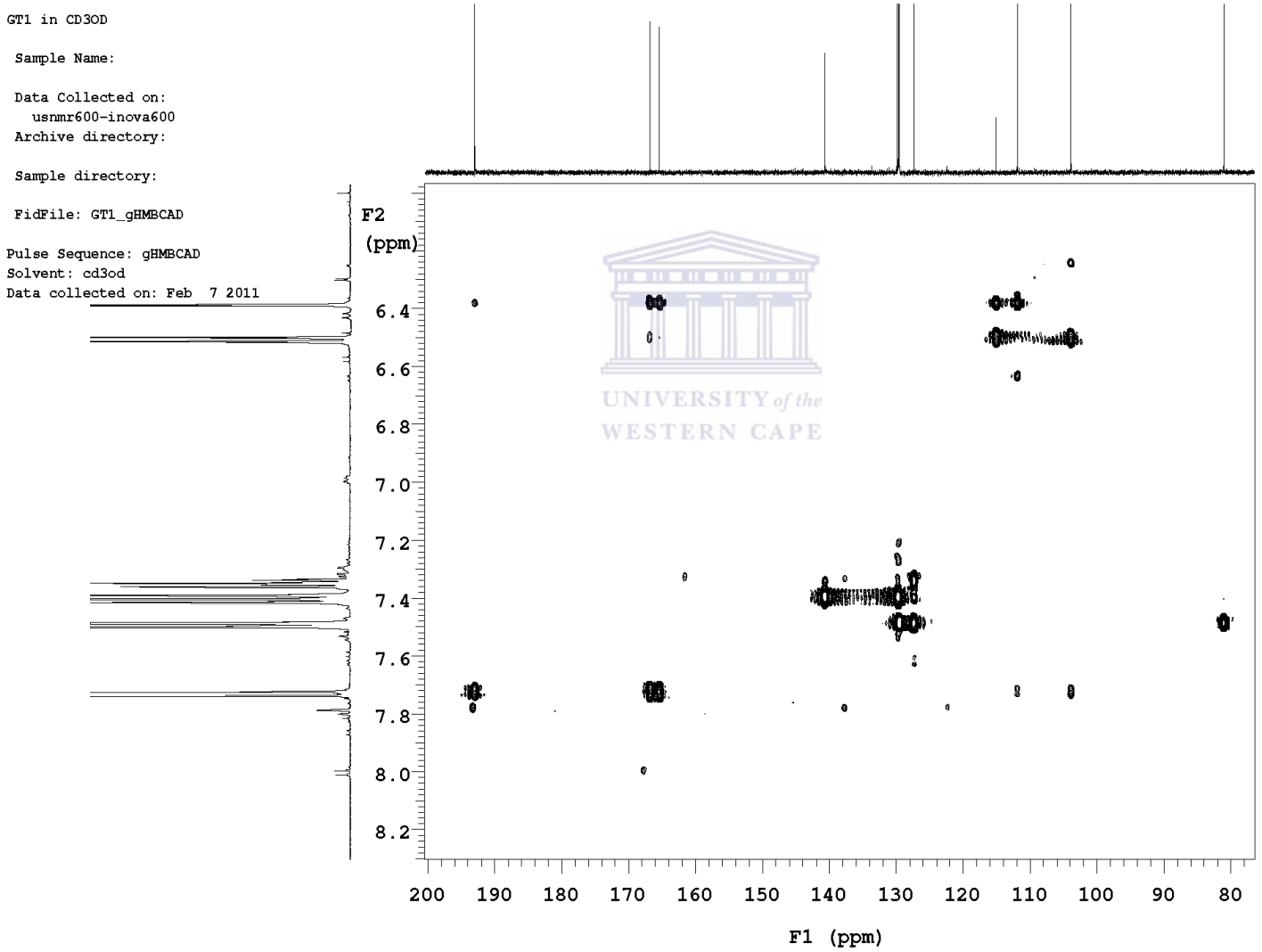
Data collected on: Feb 7 2011

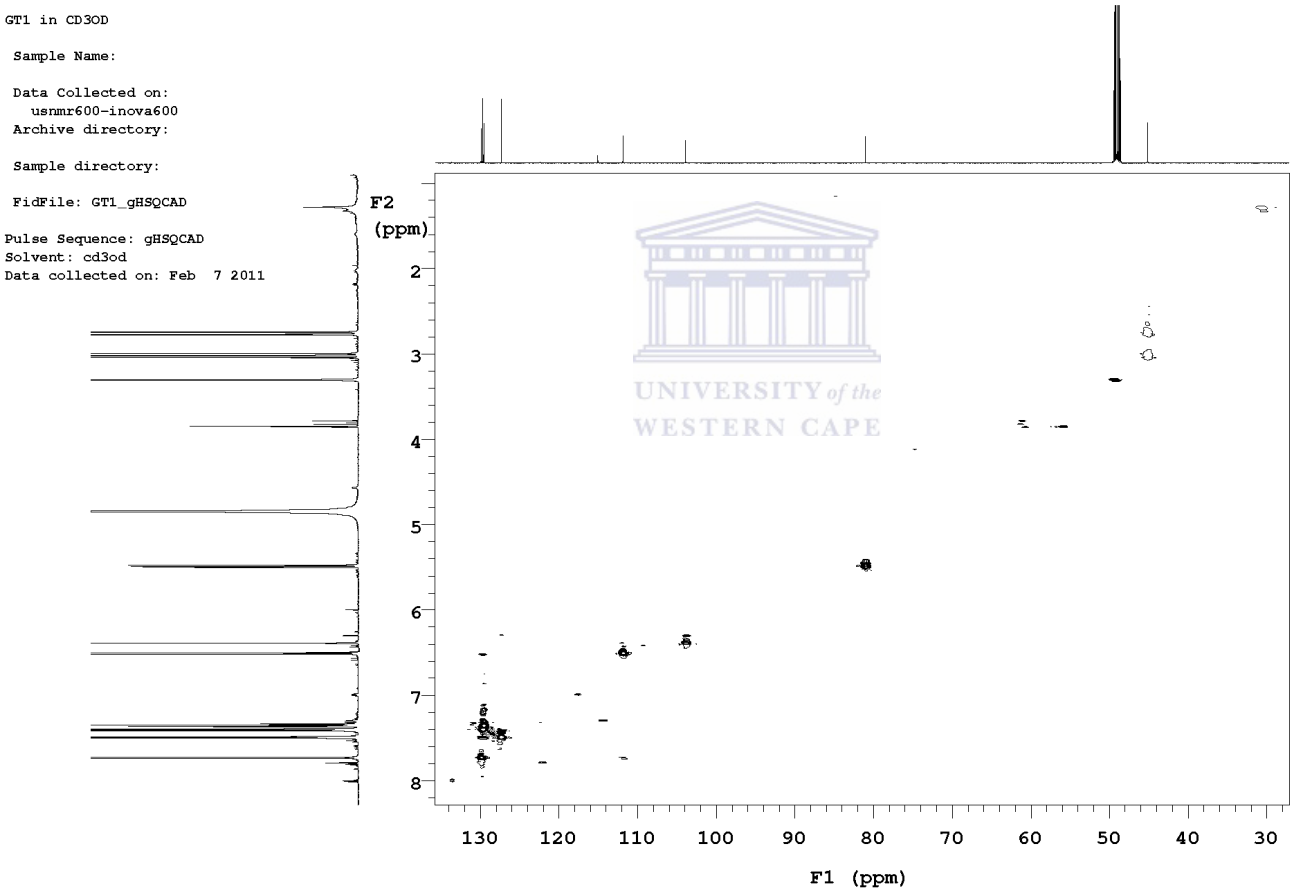


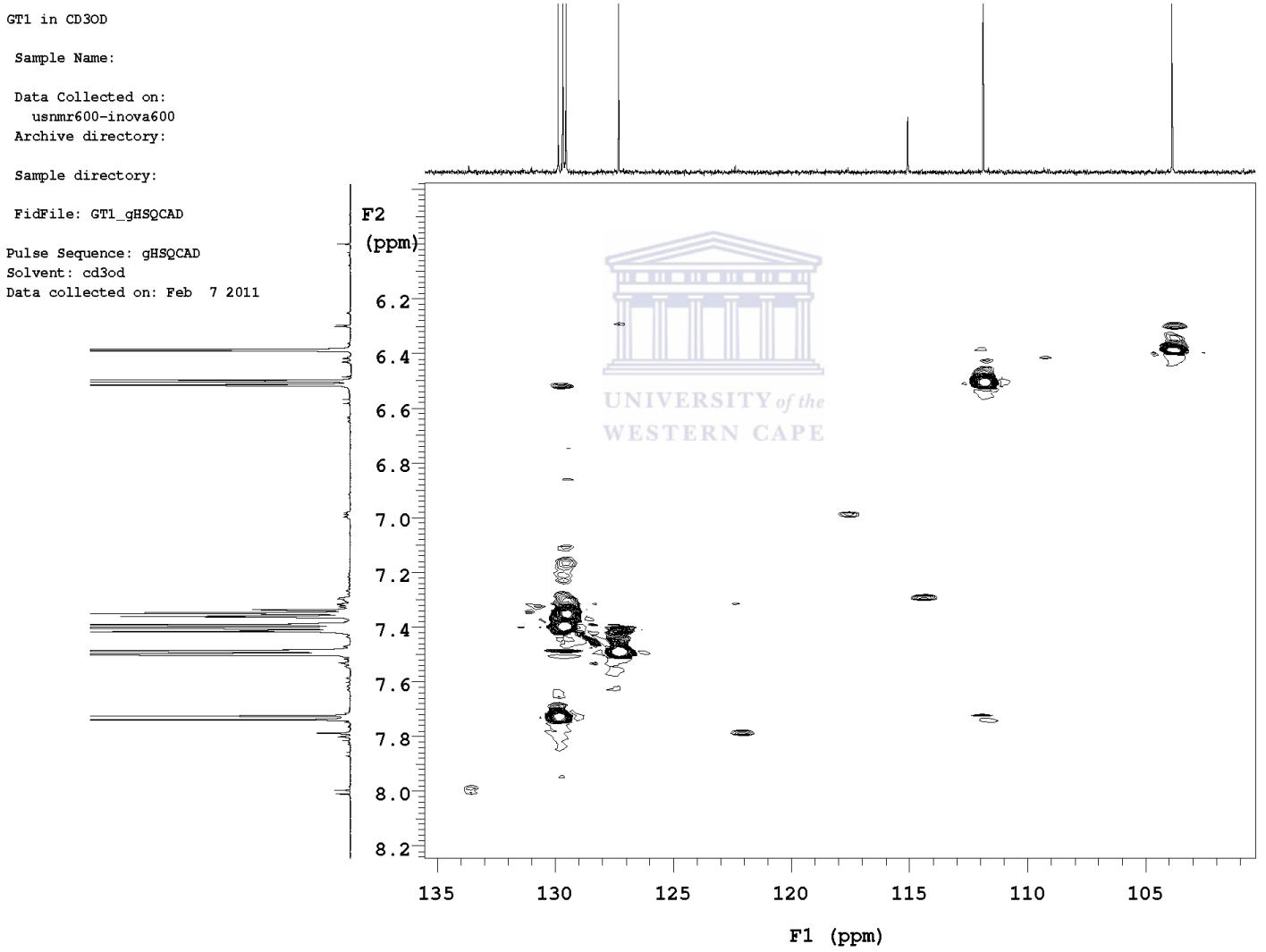
gHMBCAD Plots for Compound 1



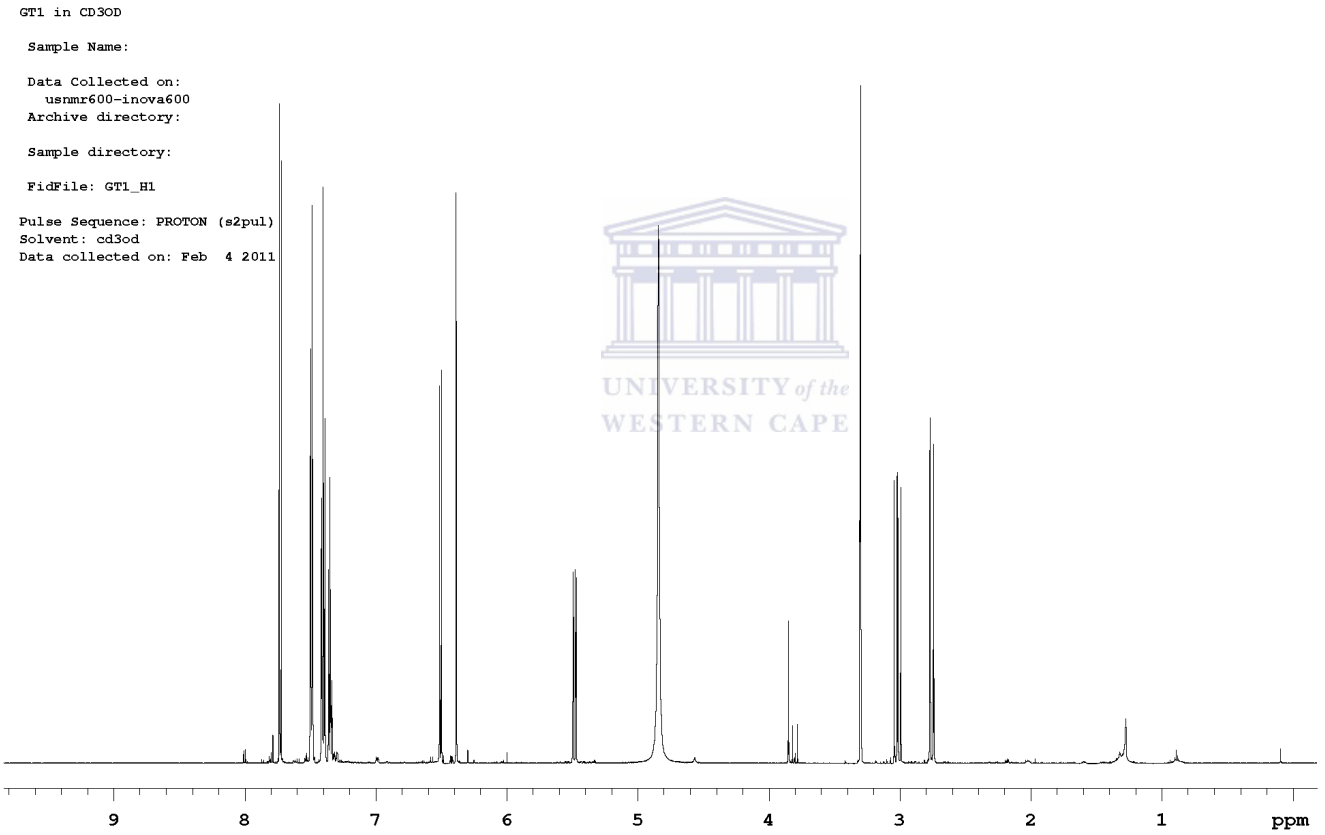


gHSQCAD Plots for Compound 1





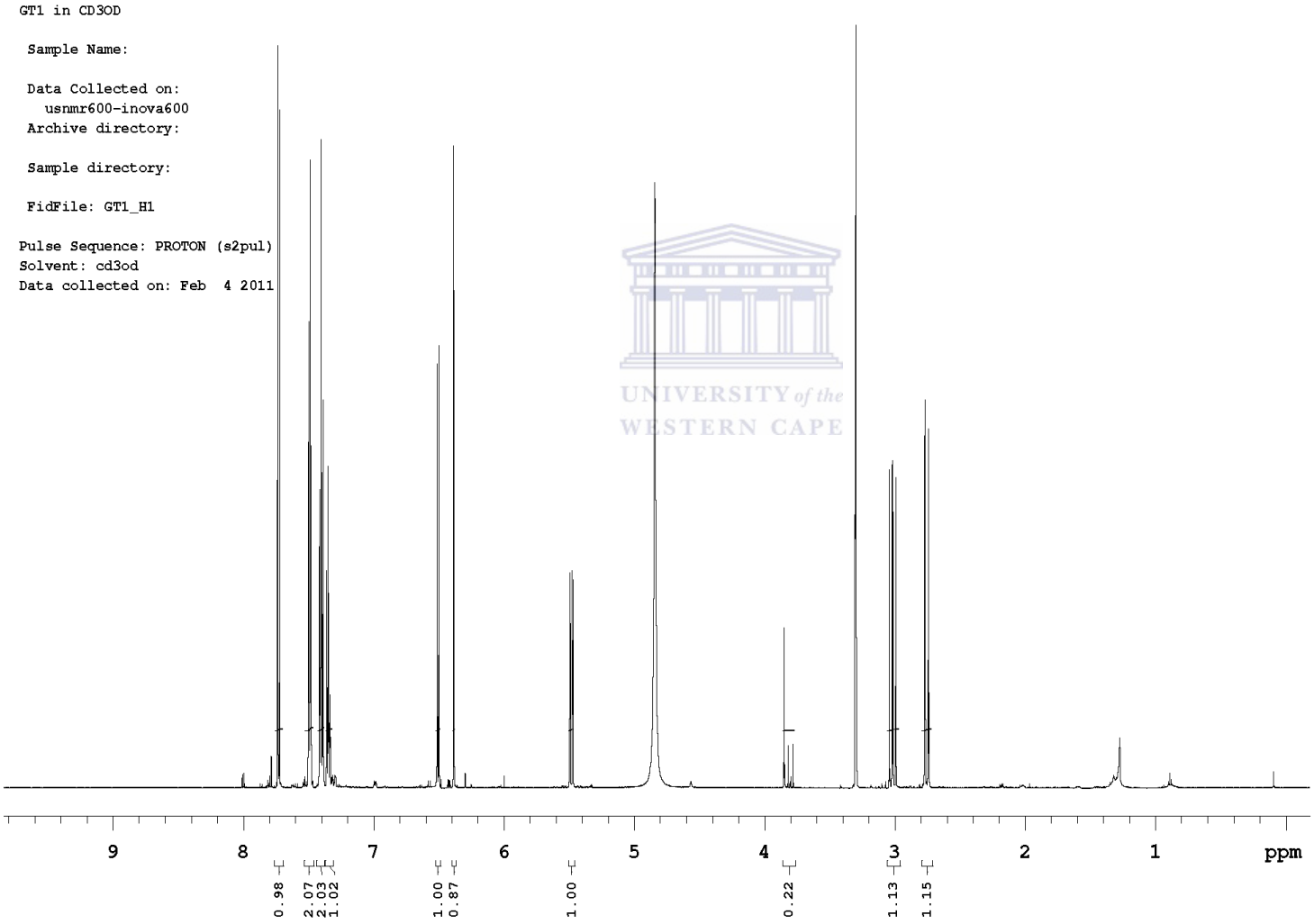
H1 Plots for Compound 1



GT1 in CD3OD

exp4 PROTON

SAMPLE		SPECIAL	
date	Feb 4 2011	temp	25.0
solvent	cd3od	gain	not used
file	/home/vnmr1/v~	spin	not used
nmrsys/data/Data20~		hst	0.008
11/February2011/A_~		pw90	9.200
Valentine/GT1/GT1_~		alfa	6.600
	H1.fid	FLAGS	
ACQUISITION		il	n
sw	9611.9	in	n
at	1.705	dp	y
np	32768	hs	nn
fb	not used	PROCESSING	
bs	32	fn	not used
ss	4	DISPLAY	
d1	1.000	sp	-110.9
nt	32	wp	6013.3
ct	32	rfl	3186.8
TRANSMITTER		rfp	1979.9
tn	H1	rp	125.5
sfrq	599.986	lp	4.3
tof	599.9	PLOT	
tpwr	54	wc	250
pw	4.600	sc	0
DECOUPLER		vs	216
dn	C13	th	3
dof	0	ai	cdc ph
dm	nnn		
dmm	c		
dpwr	40		
dmf	35088		



GT1 in CD3OD

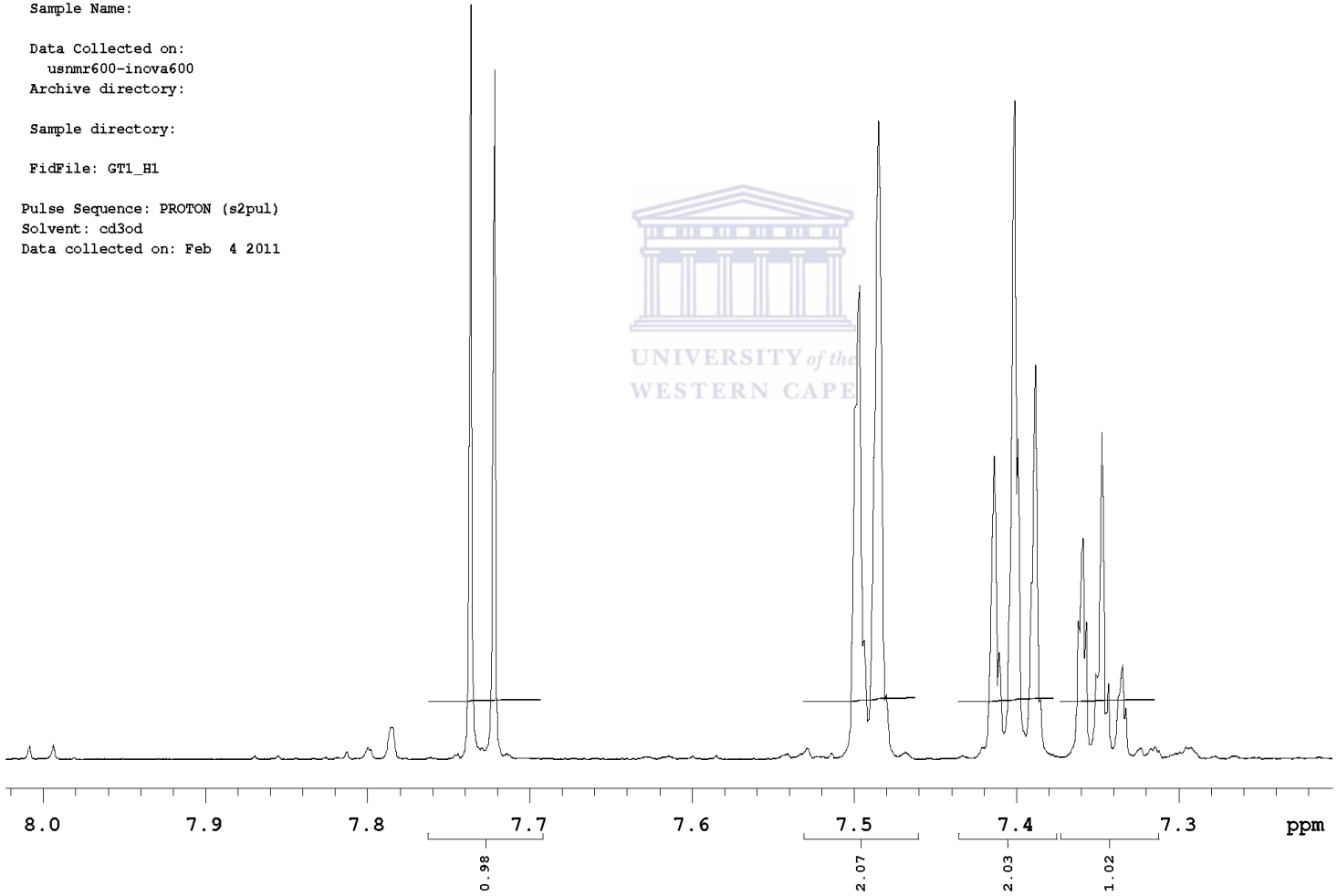
Sample Name:

Data Collected on:
usnmr600-inova600
Archive directory:

Sample directory:

FidFile: GT1_H1

Pulse Sequence: PROTON (s2pul)
Solvent: cd3od
Data collected on: Feb 4 2011



GT1 in CD3OD

Sample Name:

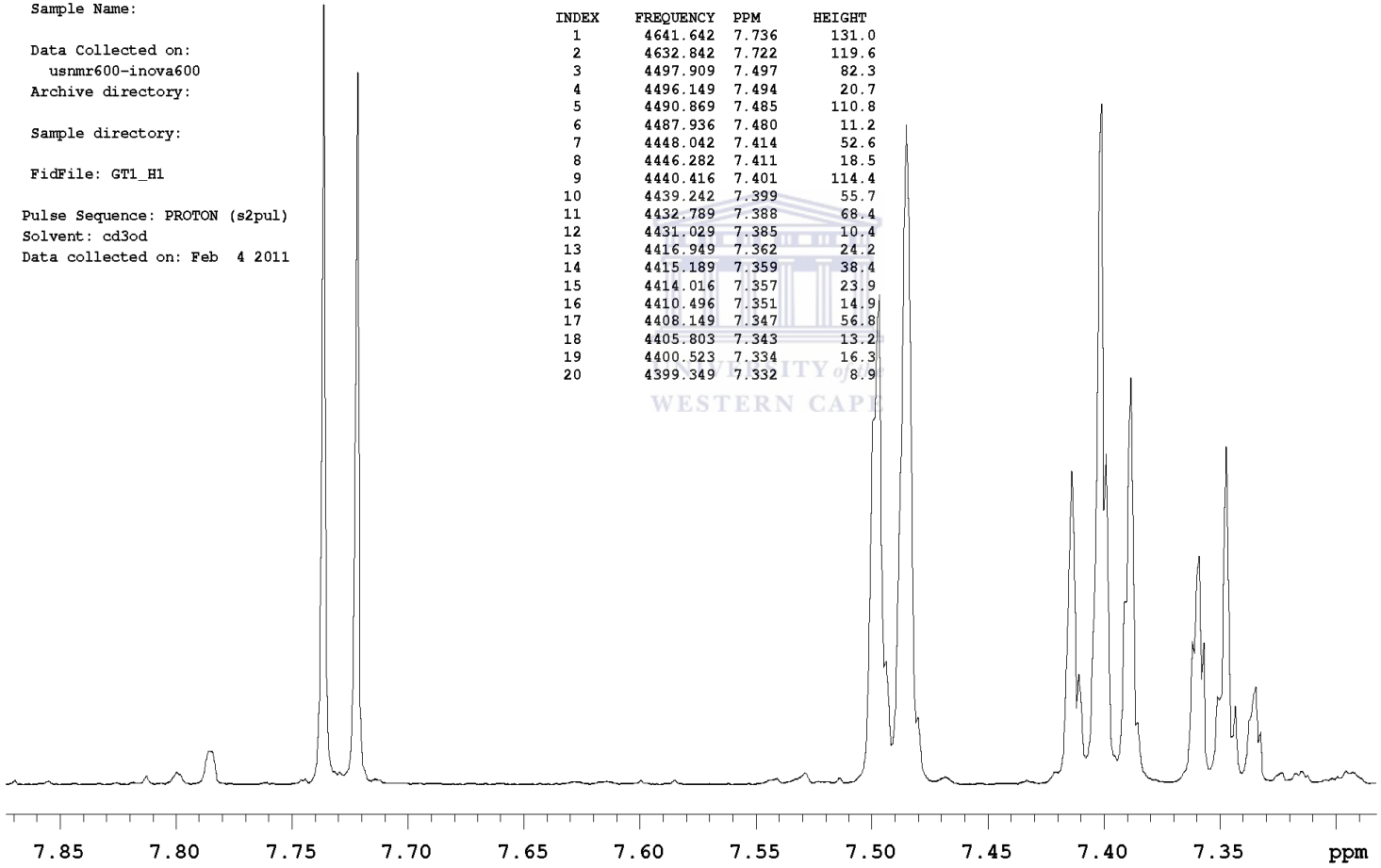
Data Collected on:
usnmr600-inova600
Archive directory:

Sample directory:

FidFile: GT1_H1

Pulse Sequence: PROTON (s2pul)
Solvent: cd3od
Data collected on: Feb 4 2011

INDEX	FREQUENCY	PPM	HEIGHT
1	4641.642	7.736	131.0
2	4632.842	7.722	119.6
3	4497.909	7.497	82.3
4	4496.149	7.494	20.7
5	4490.869	7.485	110.8
6	4487.936	7.480	11.2
7	4448.042	7.414	52.6
8	4446.282	7.411	18.5
9	4440.416	7.401	114.4
10	4439.242	7.399	55.7
11	4432.789	7.388	68.4
12	4431.029	7.385	10.4
13	4416.949	7.362	24.2
14	4415.189	7.359	38.4
15	4414.016	7.357	23.9
16	4410.496	7.351	14.9
17	4408.149	7.347	56.8
18	4405.803	7.343	13.2
19	4400.523	7.334	16.3
20	4399.349	7.332	8.9



GT1 in CD3OD

Sample Name:

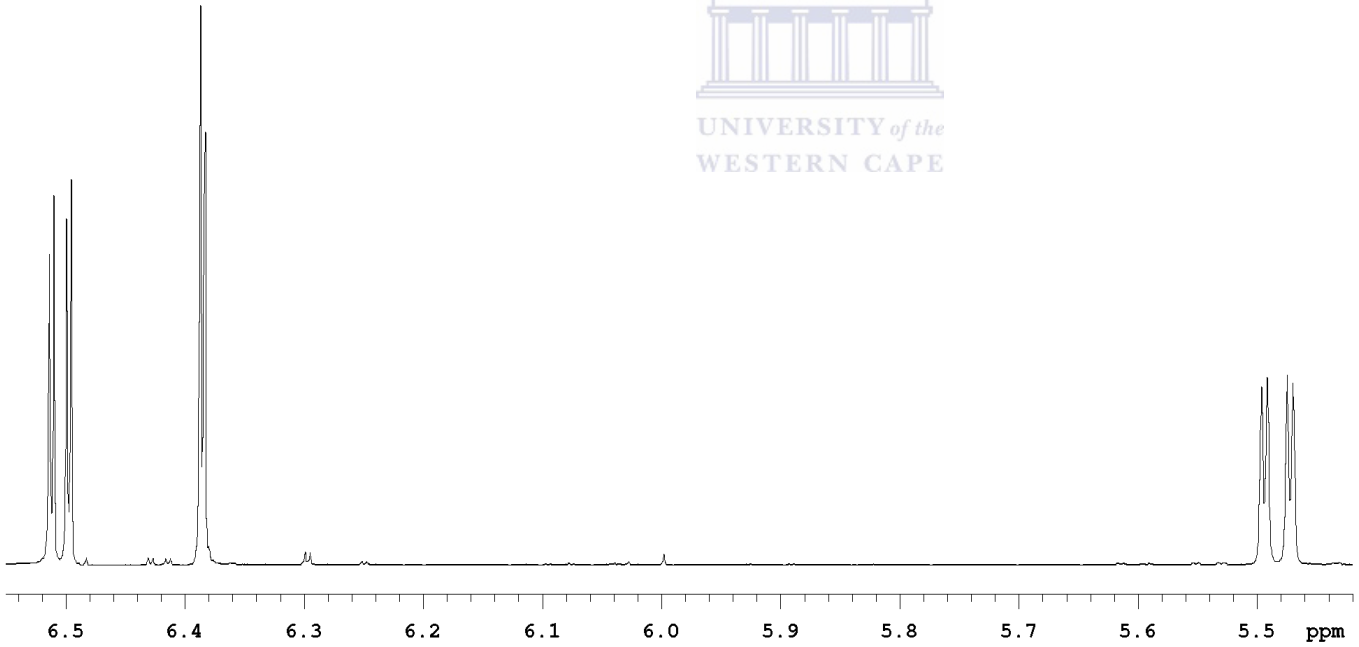
Data Collected on:
usnmr600-inova600
Archive directory:

Sample directory:

FidFile: GT1_H1

Pulse Sequence: PROTON (s2pul)
Solvent: cd3od
Data collected on: Feb 4 2011

INDEX	FREQUENCY	PPM	HEIGHT
1	3908.311	6.514	53.2
2	3905.964	6.510	63.2
3	3899.511	6.499	59.2
4	3897.164	6.495	65.9
5	3832.044	6.387	95.7
6	3829.698	6.383	74.2
7	3297.592	5.496	30.4
8	3294.659	5.491	32.0
9	3284.686	5.475	32.4
10	3281.752	5.470	31.1



GT1 in CD3OD

Sample Name:

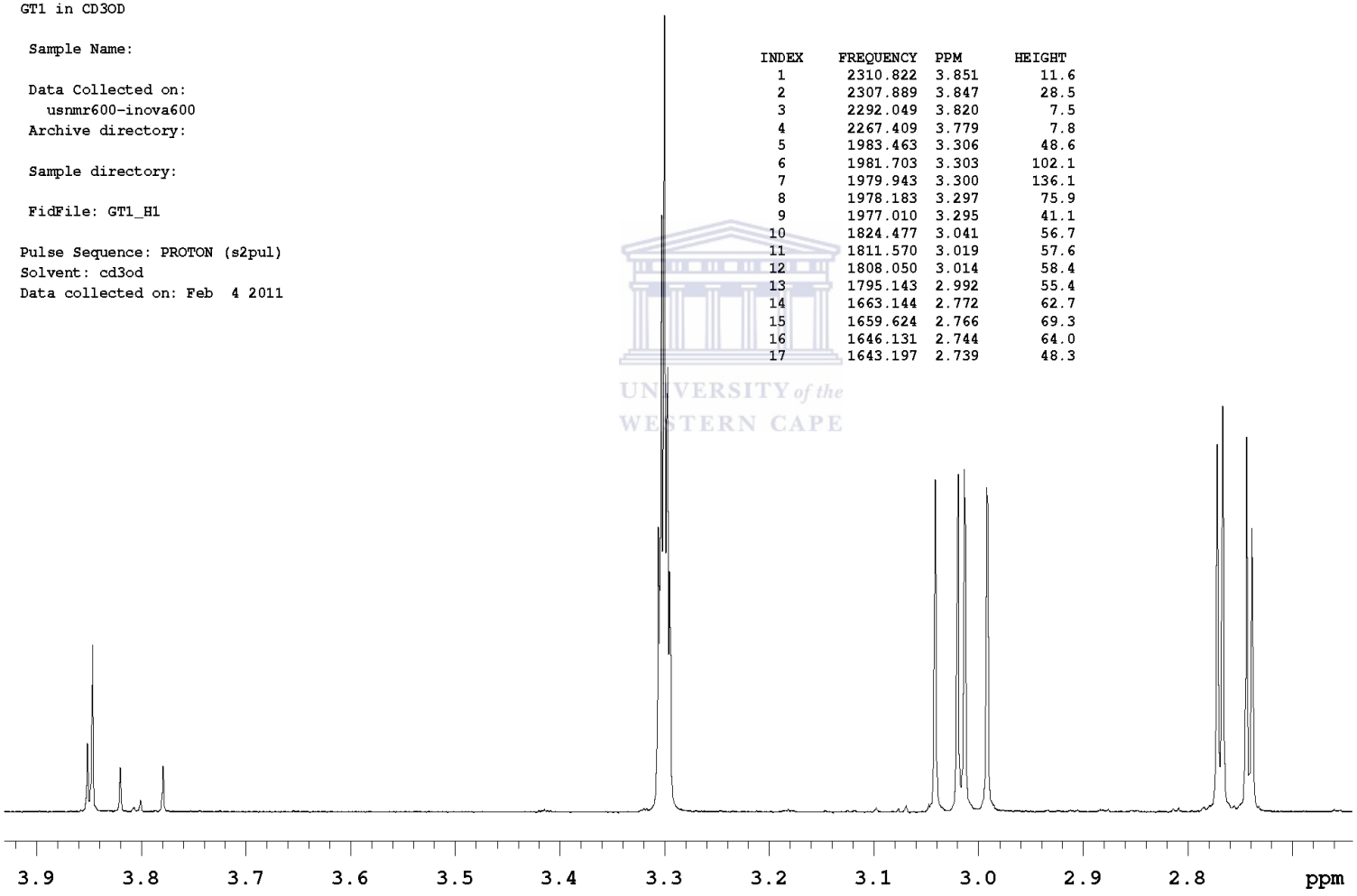
Data Collected on:
usnmr600-inova600
Archive directory:

Sample directory:

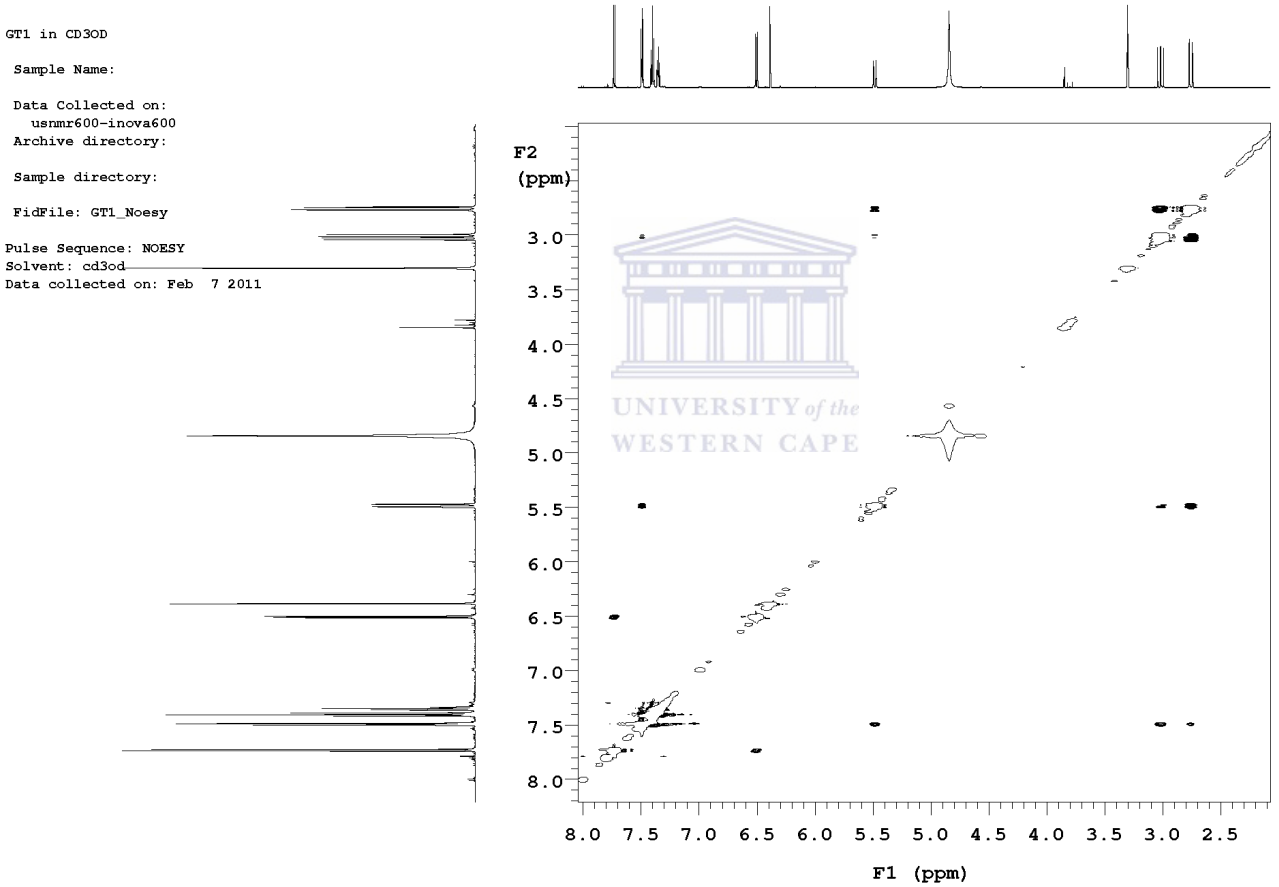
FidFile: GT1_H1

Pulse Sequence: PROTON (s2pul)
Solvent: cd3od
Data collected on: Feb 4 2011

INDEX	FREQUENCY	PPM	HEIGHT
1	2310.822	3.851	11.6
2	2307.889	3.847	28.5
3	2292.049	3.820	7.5
4	2267.409	3.779	7.8
5	1983.463	3.306	48.6
6	1981.703	3.303	102.1
7	1979.943	3.300	136.1
8	1978.183	3.297	75.9
9	1977.010	3.295	41.1
10	1824.477	3.041	56.7
11	1811.570	3.019	57.6
12	1808.050	3.014	58.4
13	1795.143	2.992	55.4
14	1663.144	2.772	62.7
15	1659.624	2.766	69.3
16	1646.131	2.744	64.0
17	1643.197	2.739	48.3



Noesy Plots for Compound 1



GT1 in CD3OD

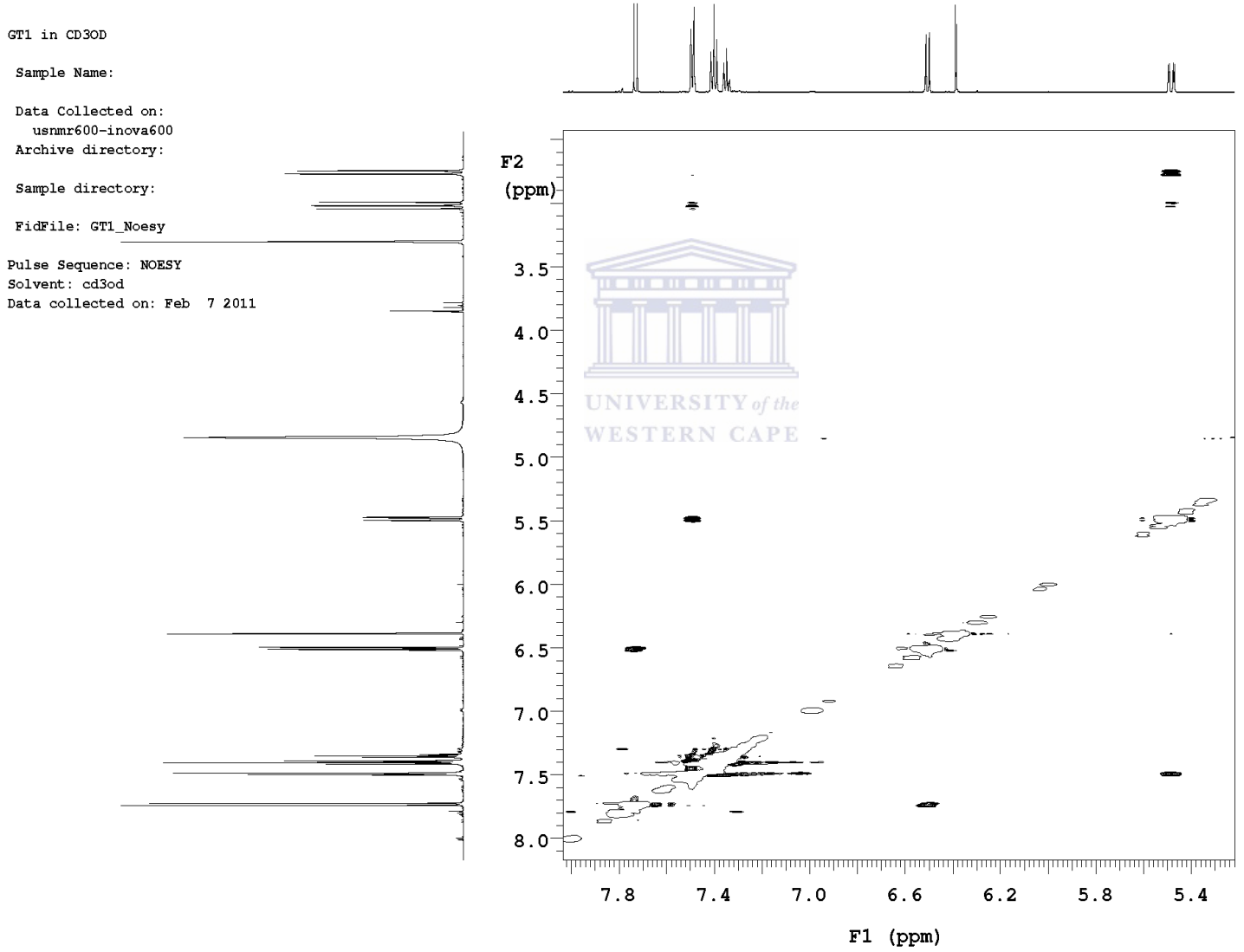
Sample Name:

Data Collected on:
usnmr600-inova600
Archive directory:

Sample directory:

FidFile: GT1_Noesy

Pulse Sequence: NOESY
Solvent: cd3od
Data collected on: Feb 7 2011



GT1 in CD3OD

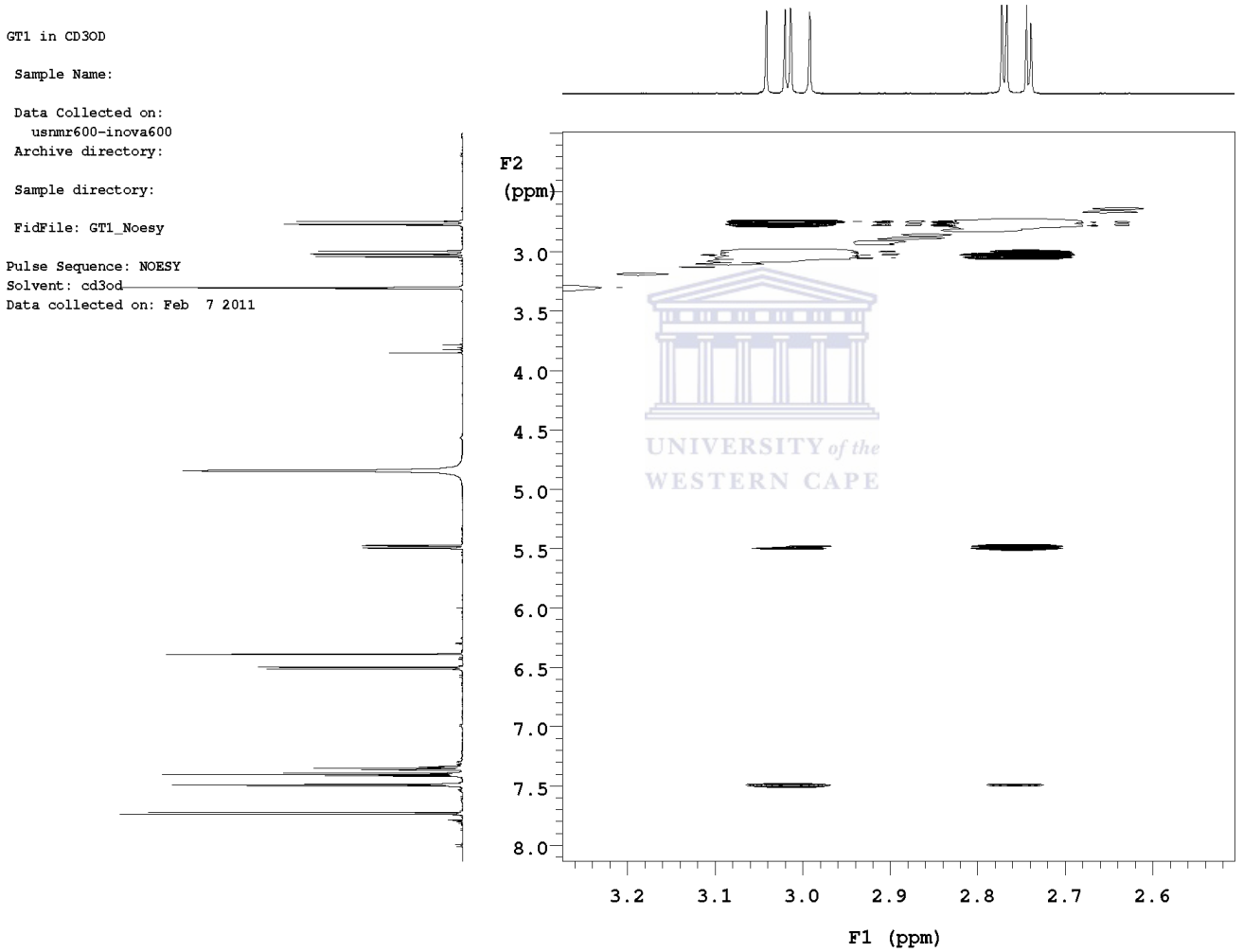
Sample Name:

Data Collected on:
usnmr600-inova600
Archive directory:

Sample directory:

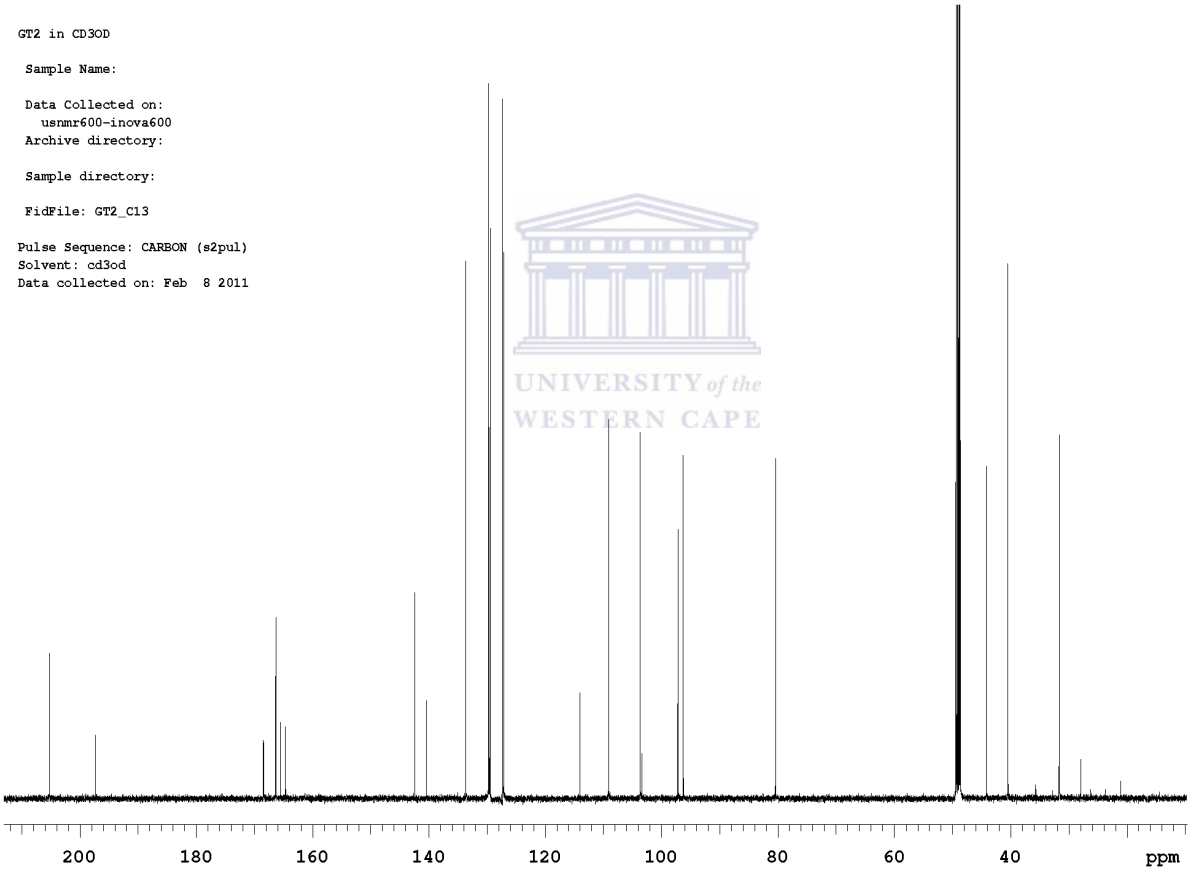
FidFile: GT1_Noesy

Pulse Sequence: NOESY
Solvent: cd3od
Data collected on: Feb 7 2011



ADDENDUM 2:
NMR DATA FOR COMPOUND 2

C13 Plots for Compound 2



GT2 in CD3OD

exp2 CARBON

SAMPLE		PRESATURATION	
date	Feb 8 2011	satmode	n
solvent	cd3od	wet	n
file	/home/vnmr1/v~	SPECIAL	
	nmrsys/data/Data20~	temp	25.0
	11/February2011/A_~	gain	not used
	Valentine/GT2/GT2_~	spin	not used
	C13.fid	hst	0.008
ACQUISITION		pw90	15.400
sw	41536.9	alfa	10.000
at	0.789	FLAGS	
np	65536	il	n
fb	not used	in	n
bs	64	dp	y
d1	1.000	hs	nn
nt	12000	PROCESSING	
ct	12000	lb	0.50
TRANSMITTER		lsfid	-2
tn	C13	fn	not used
sfrq	150.884	DISPLAY	
tof	4175.5	sp	1464.8
tpwr	56	wp	30674.8
pw	7.700	rfl	2294.9
DECOUPLER		rfp	0
dn	H1	rp	83.9
dof	0	lp	70.2
dm	yyy	PLOT	
decwave	w	wc	250
dpwr	36	sc	0
dmf	14400	vs	2393
		th	26
		ai cdc ph	

GT2 in CD3OD

Sample Name:

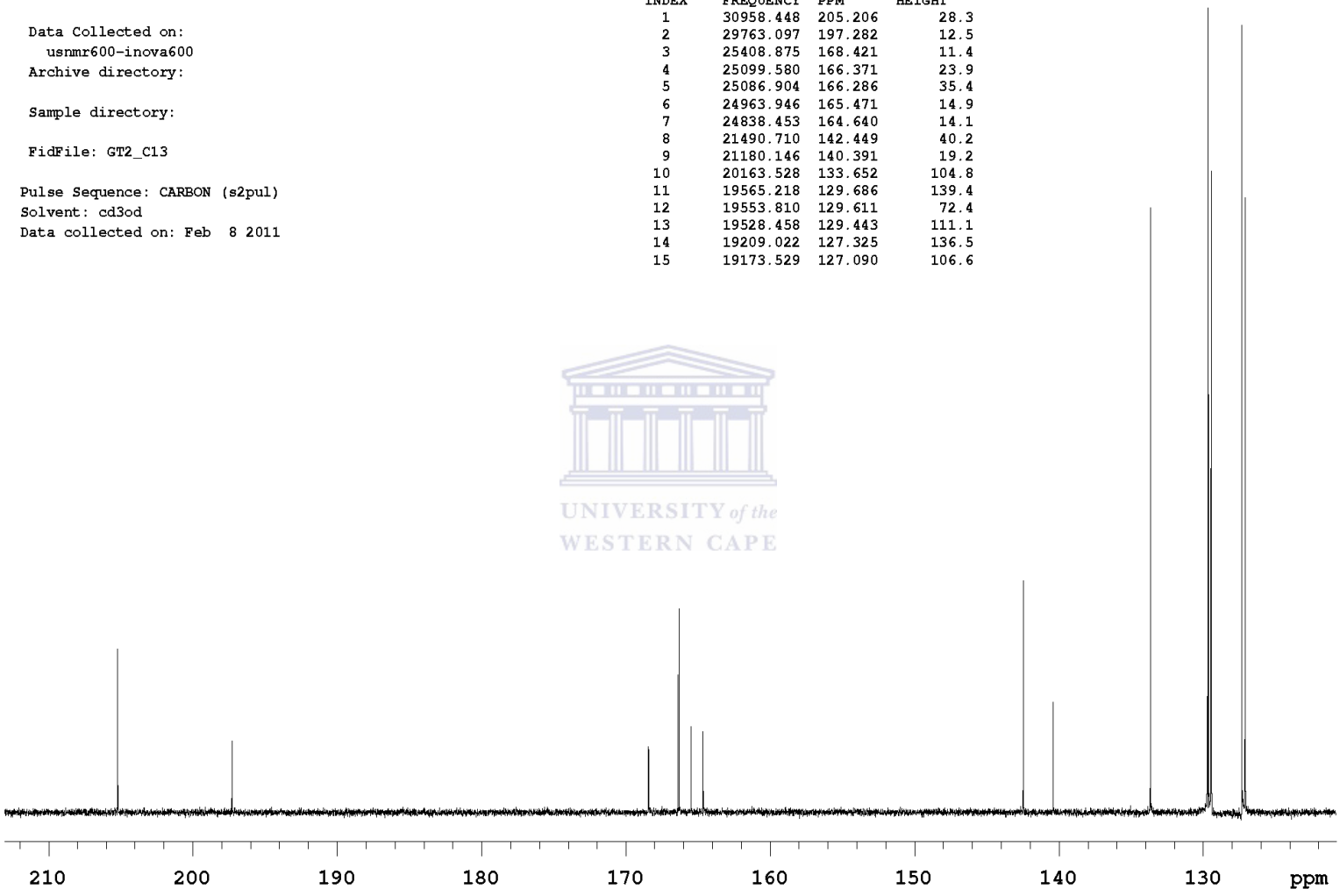
Data Collected on:
usnmr600-inova600
Archive directory:

Sample directory:

FidFile: GT2_C13

Pulse Sequence: CARBON (s2pul)
Solvent: cd3od
Data collected on: Feb 8 2011

INDEX	FREQUENCY	PPM	HEIGHT
1	30958.448	205.206	28.3
2	29763.097	197.282	12.5
3	25408.875	168.421	11.4
4	25099.580	166.371	23.9
5	25086.904	166.286	35.4
6	24963.946	165.471	14.9
7	24838.453	164.640	14.1
8	21490.710	142.449	40.2
9	21180.146	140.391	19.2
10	20163.528	133.652	104.8
11	19565.218	129.686	139.4
12	19553.810	129.611	72.4
13	19528.458	129.443	111.1
14	19209.022	127.325	136.5
15	19173.529	127.090	106.6



GT2 in CD3OD

Sample Name:

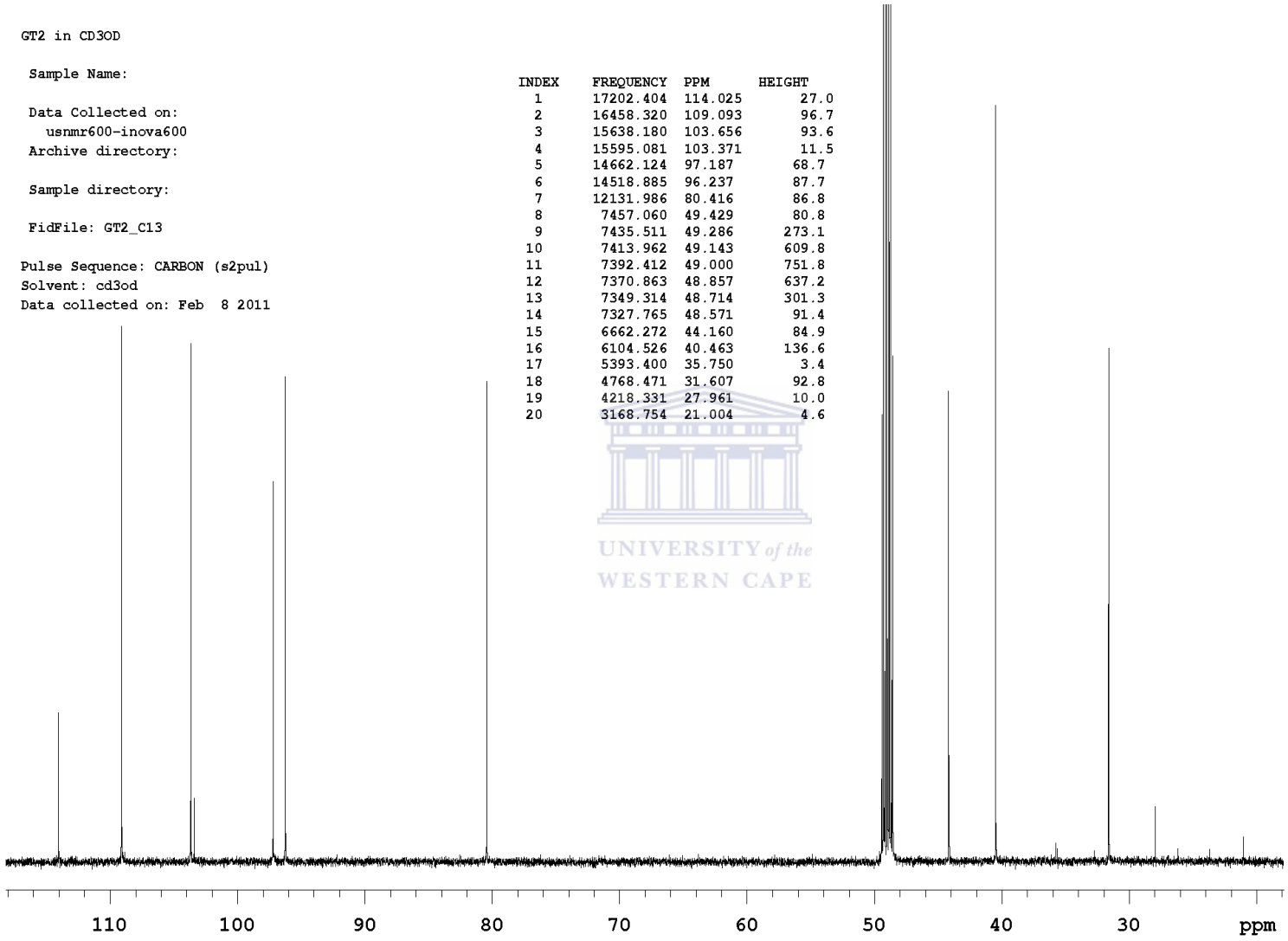
Data Collected on:
usnmr600-inova600
Archive directory:

Sample directory:

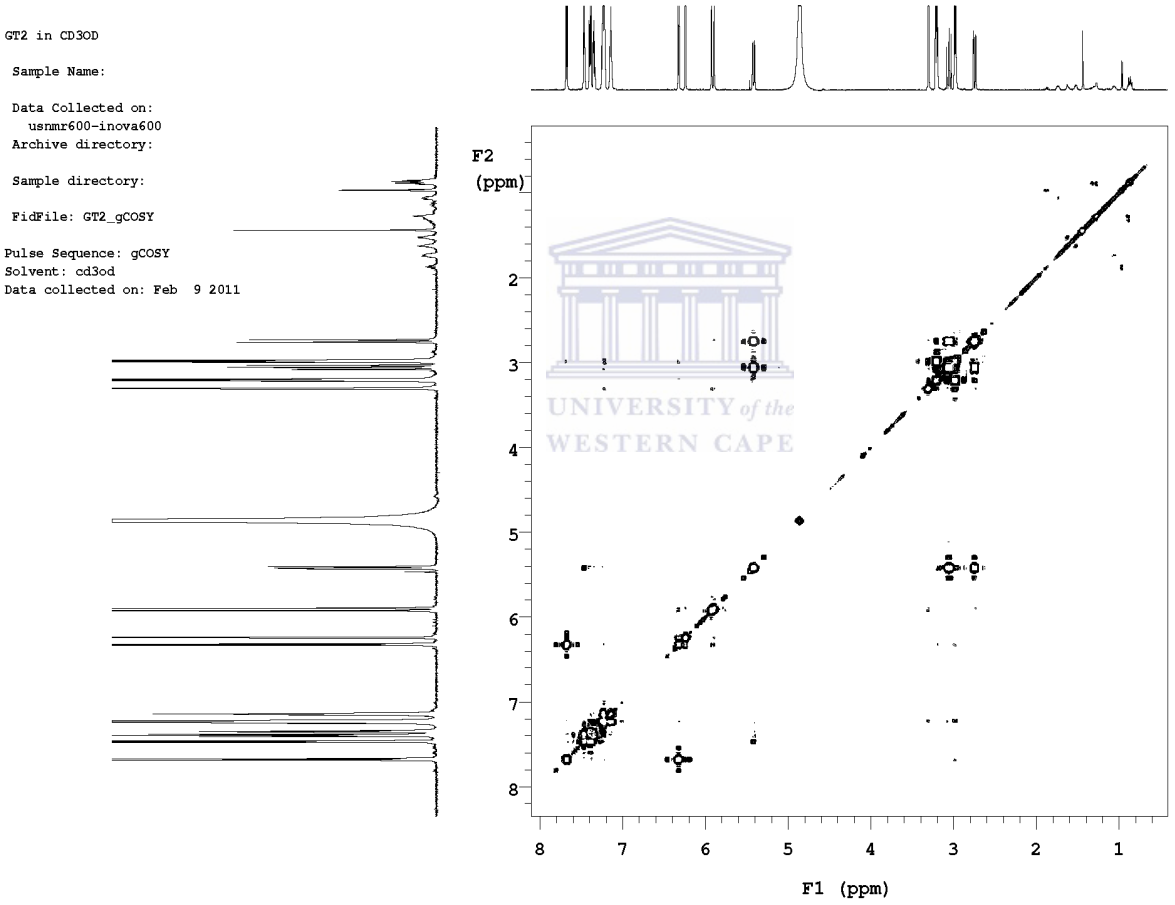
FidFile: GT2_C13

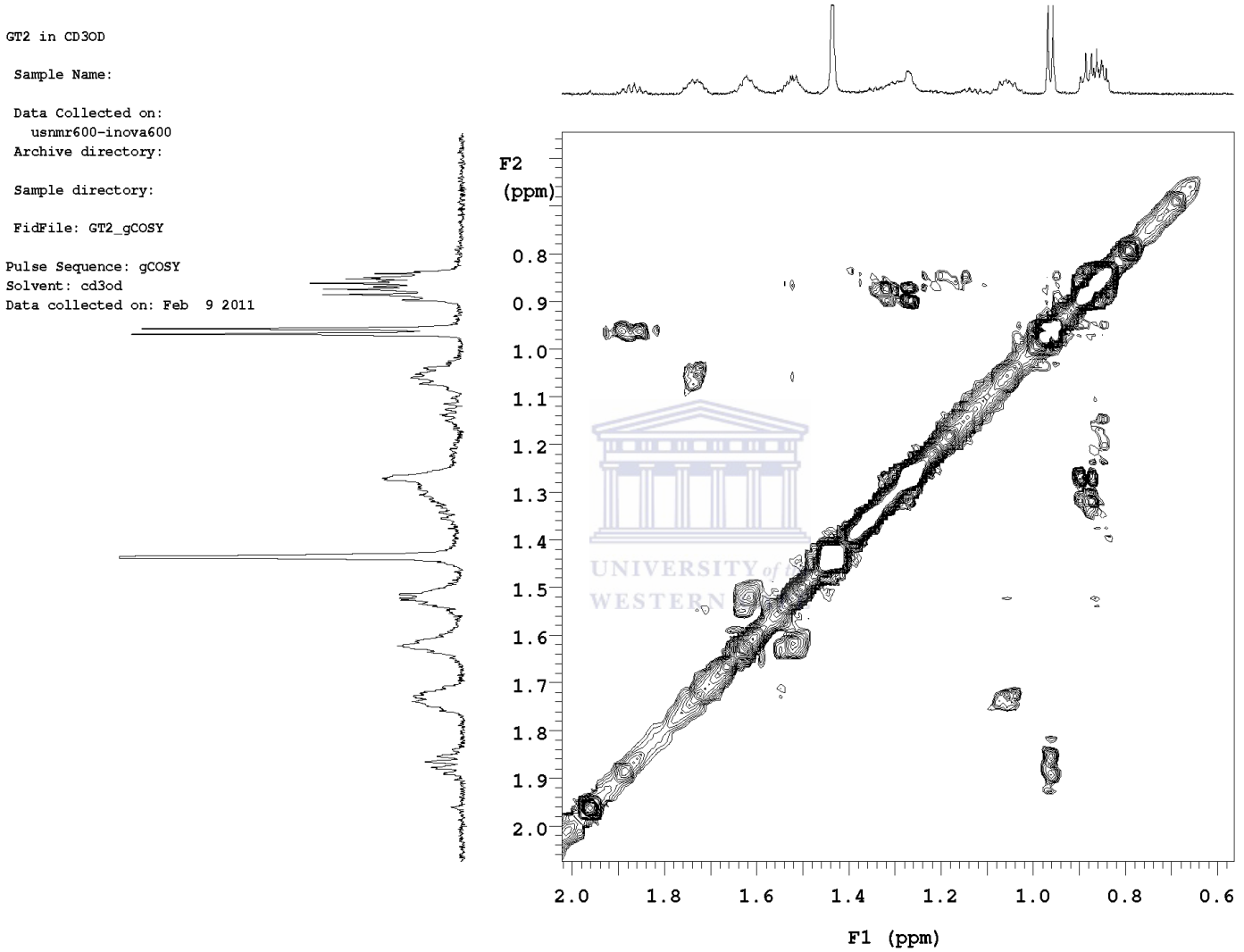
Pulse Sequence: CARBON (s2pul)
Solvent: cd3od
Data collected on: Feb 8 2011

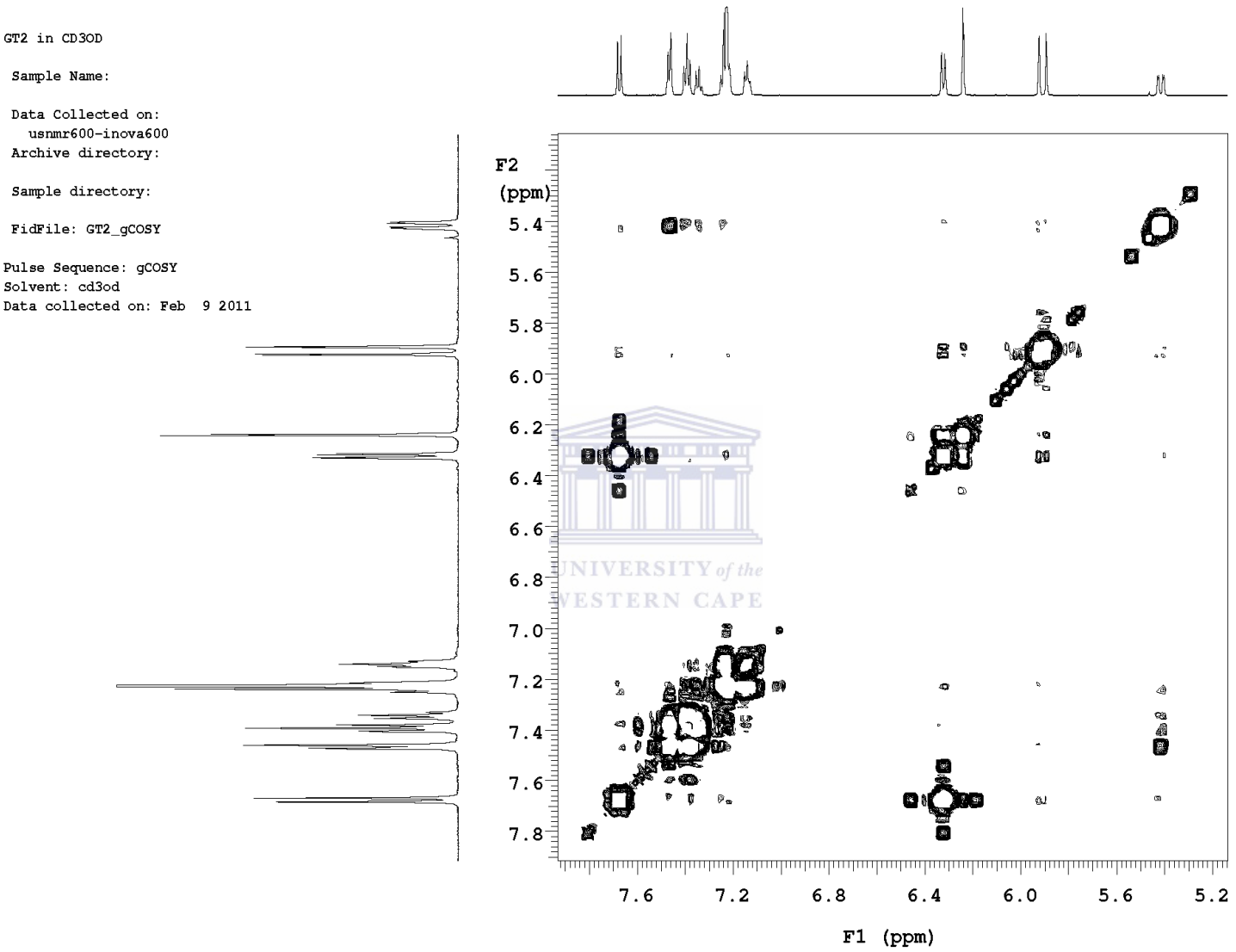
INDEX	FREQUENCY	PPM	HEIGHT
1	17202.404	114.025	27.0
2	16458.320	109.093	96.7
3	15638.180	103.656	93.6
4	15595.081	103.371	11.5
5	14662.124	97.187	68.7
6	14518.885	96.237	87.7
7	12131.986	80.416	86.8
8	7457.060	49.429	80.8
9	7435.511	49.286	273.1
10	7413.962	49.143	609.8
11	7392.412	49.000	751.8
12	7370.863	48.857	637.2
13	7349.314	48.714	301.3
14	7327.765	48.571	91.4
15	6662.272	44.160	84.9
16	6104.526	40.463	136.6
17	5393.400	35.750	3.4
18	4768.471	31.607	92.8
19	4218.331	27.961	10.0
20	3168.754	21.004	4.6

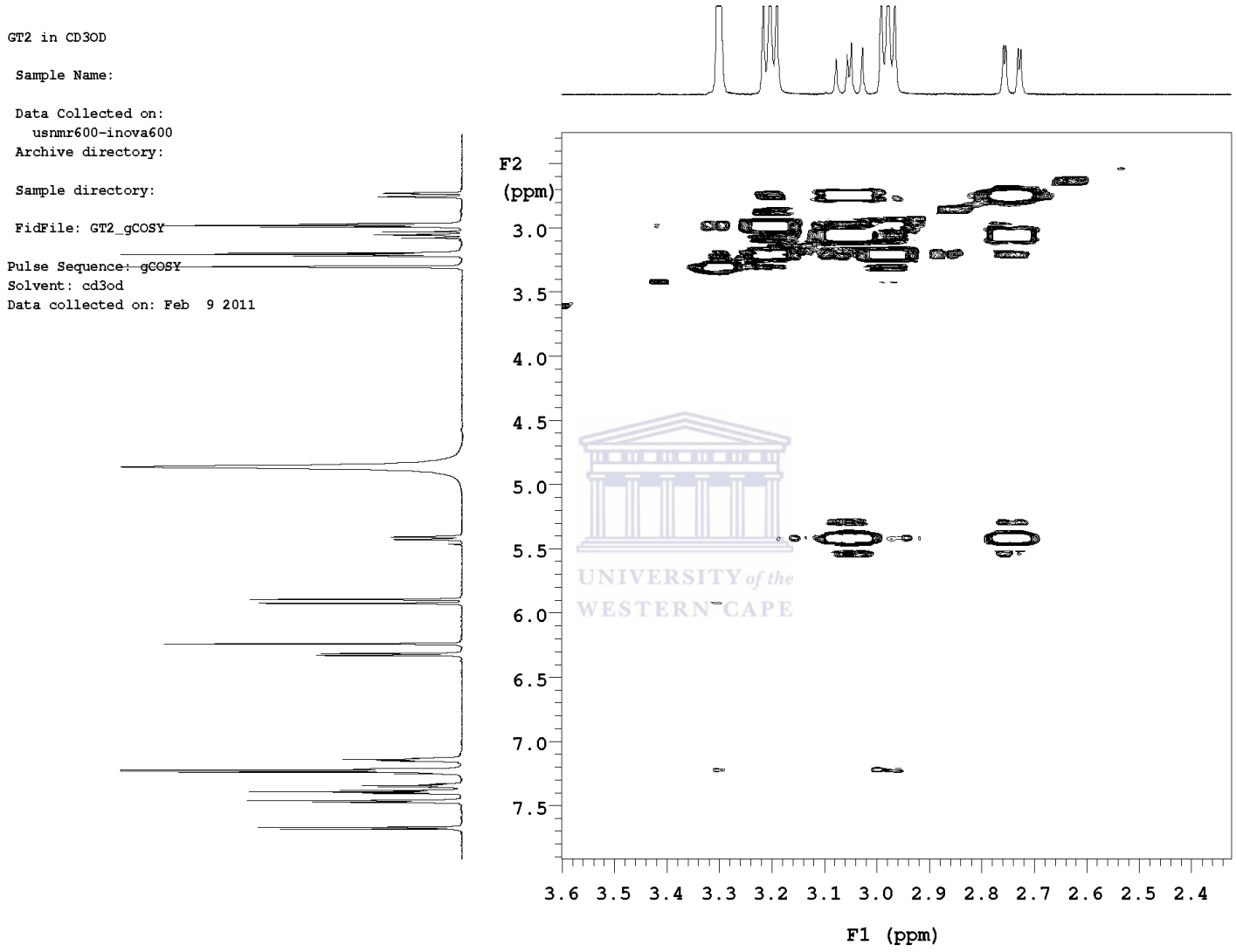


gCOSY Plots for Compound 2

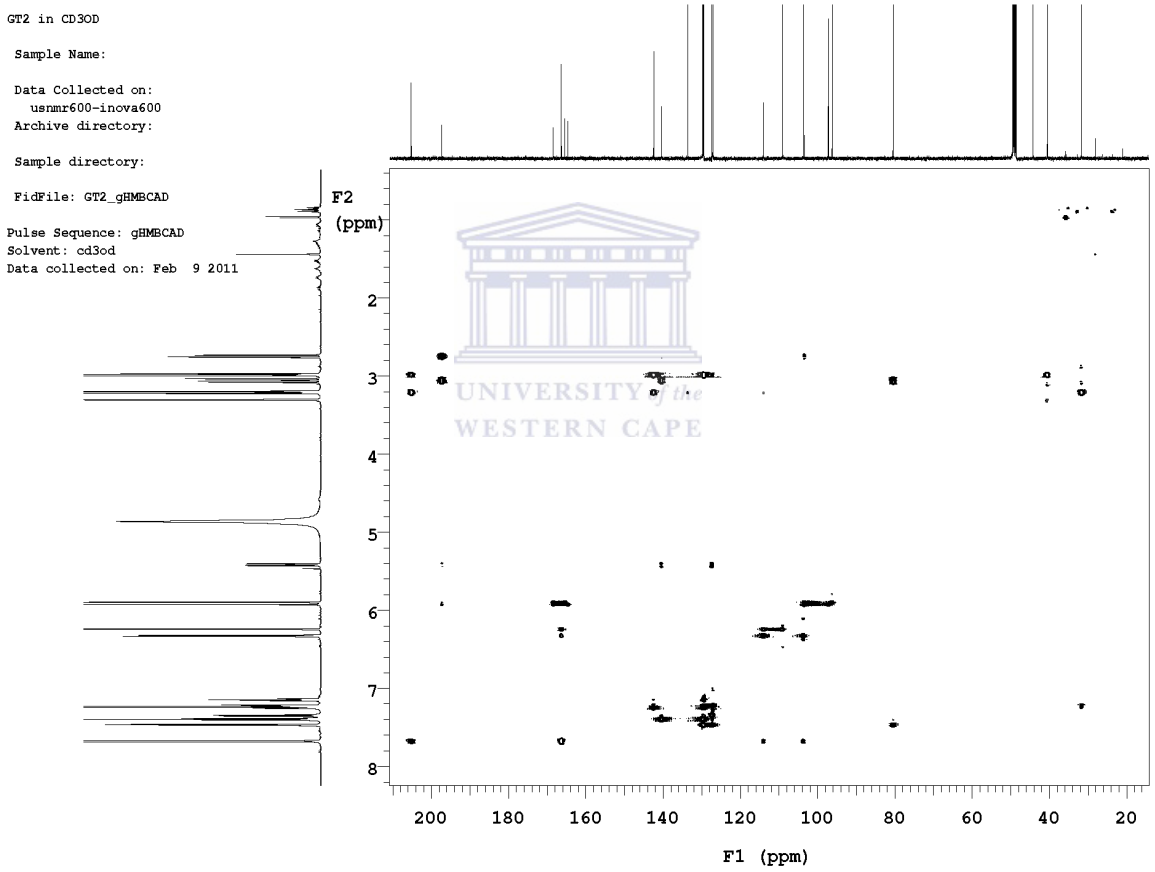


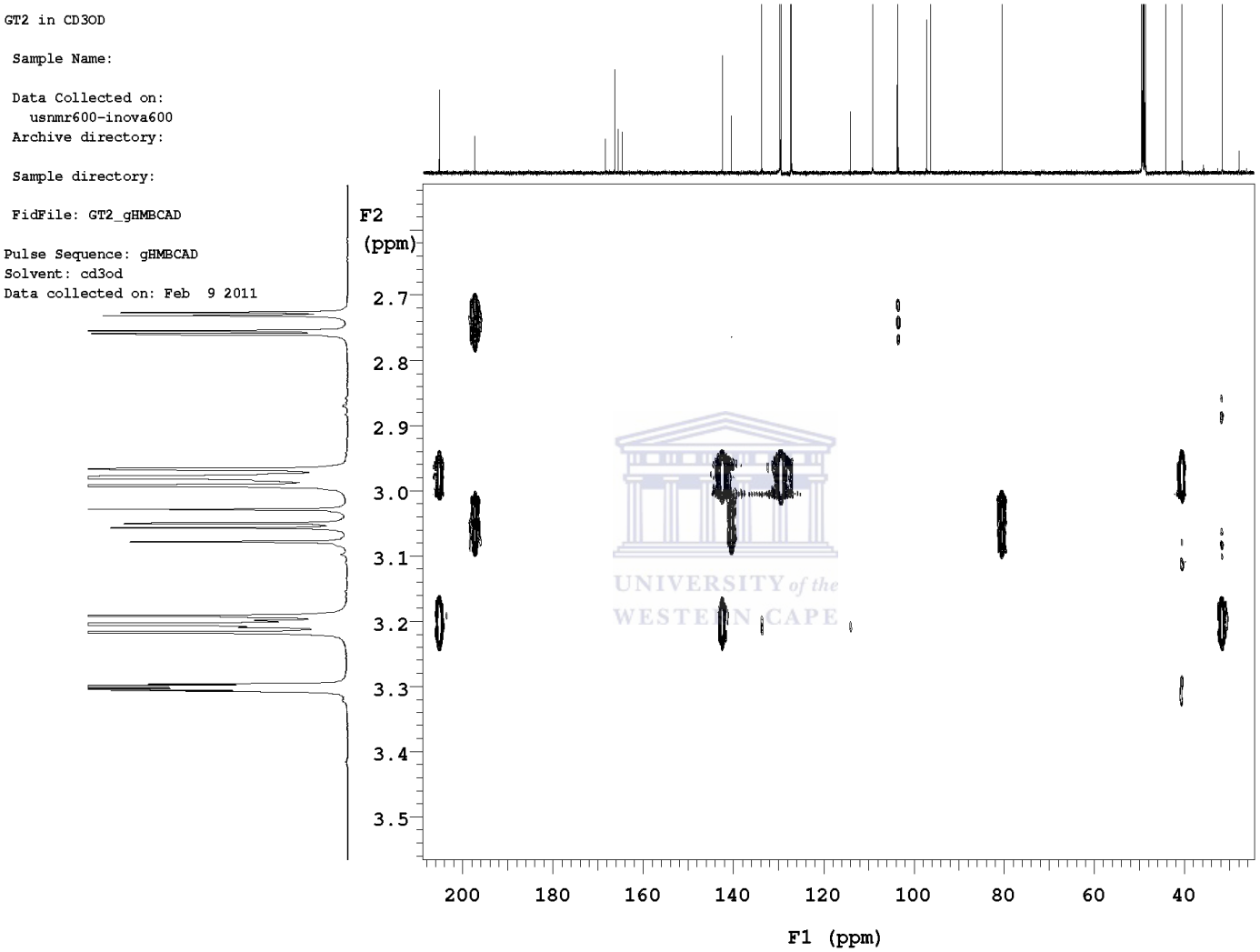

GT2 in CD3OD
Sample Name:
Data Collected on:
usnmr600-inova600
Archive directory:
Sample directory:
FidFile: GT2_gCOSY
Pulse Sequence: gCOSY
Solvent: cd3od
Data collected on: Feb 9 2011
F2
(ppm)
0.8
0.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
2.0
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
F1 (ppm)

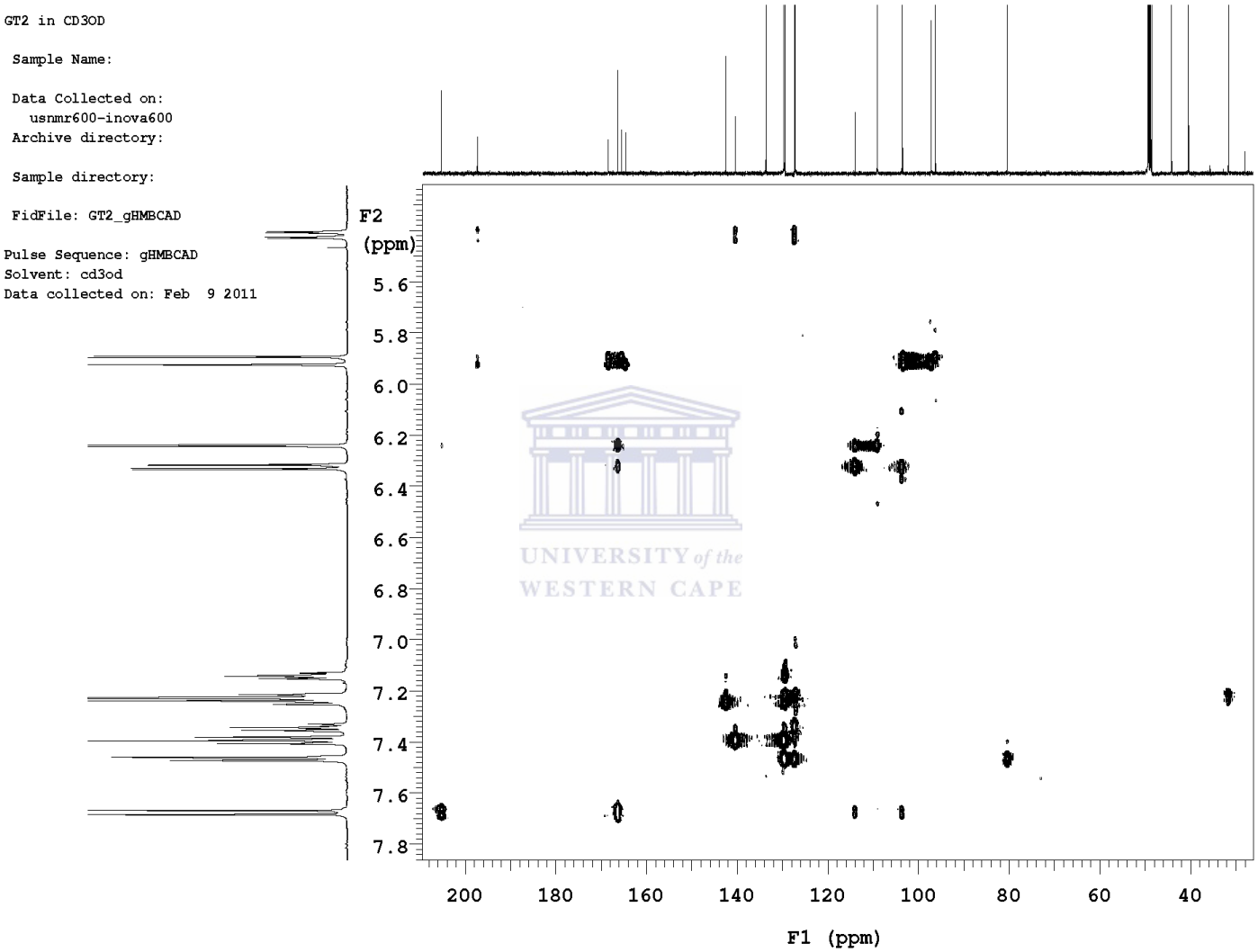




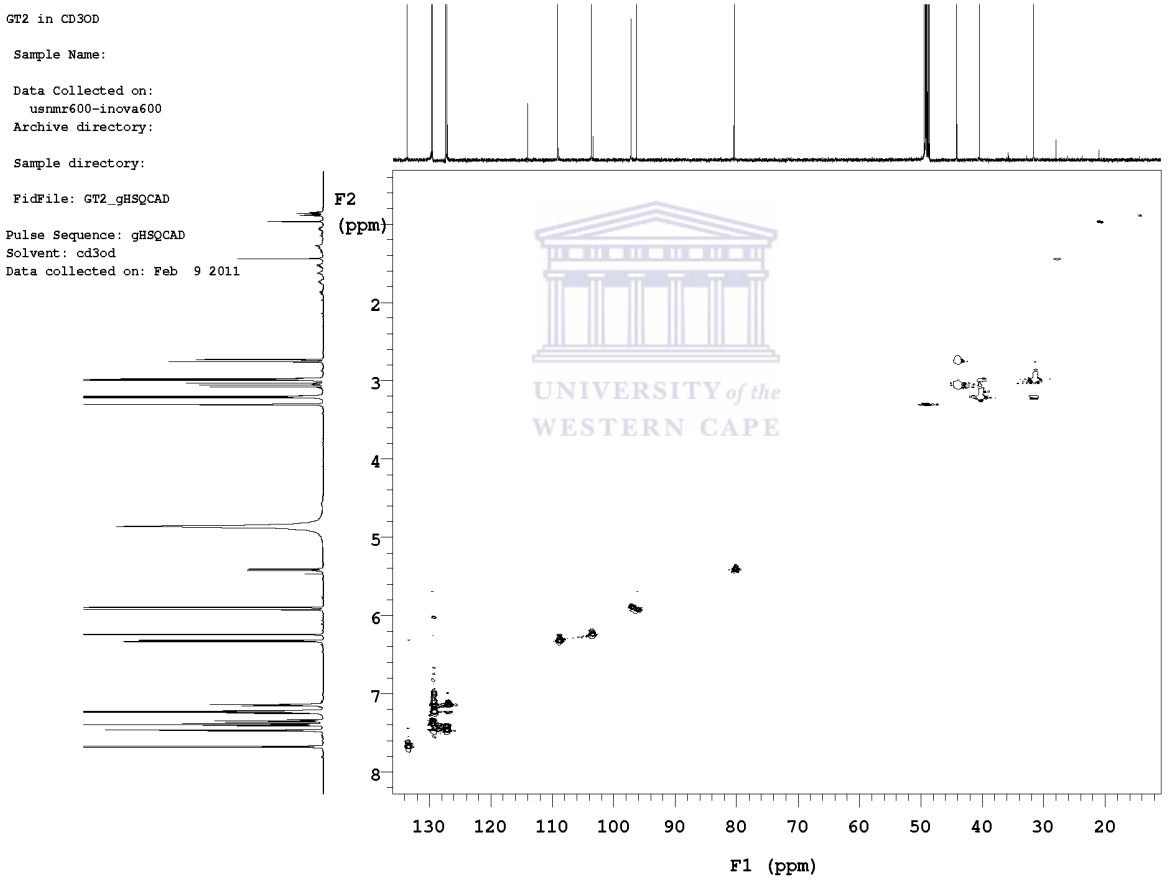
gHMBCAD Plots for Compound 2

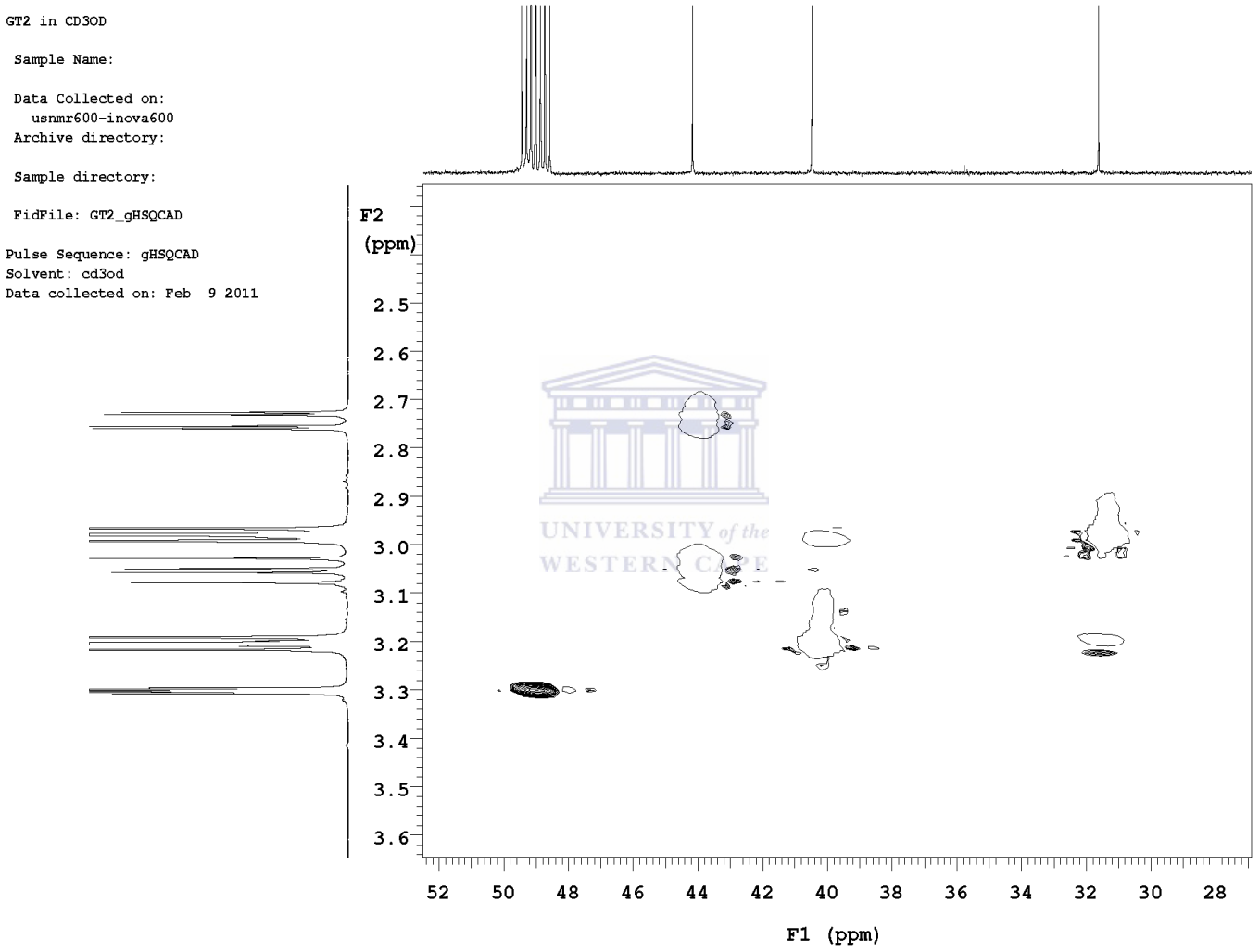


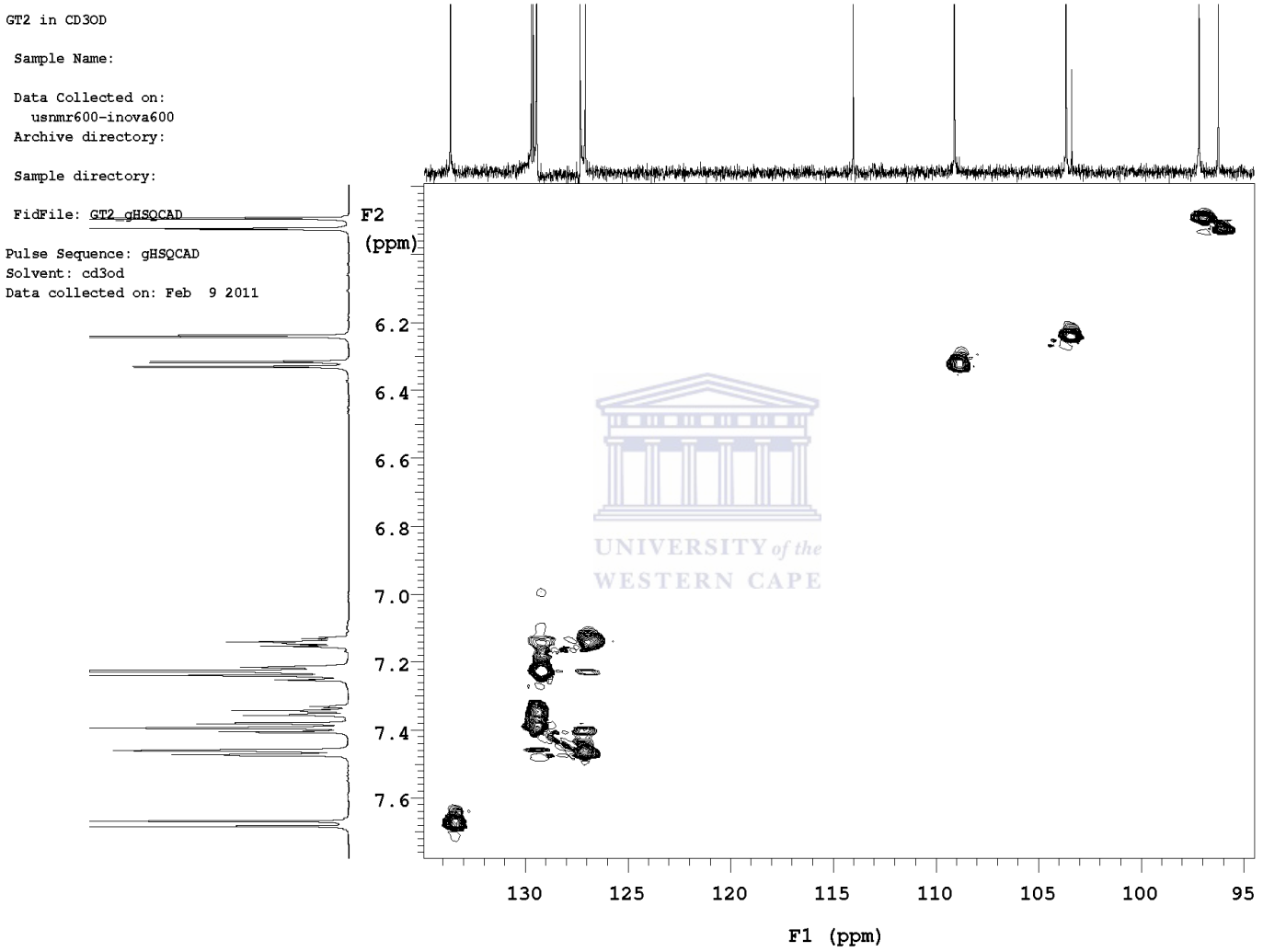




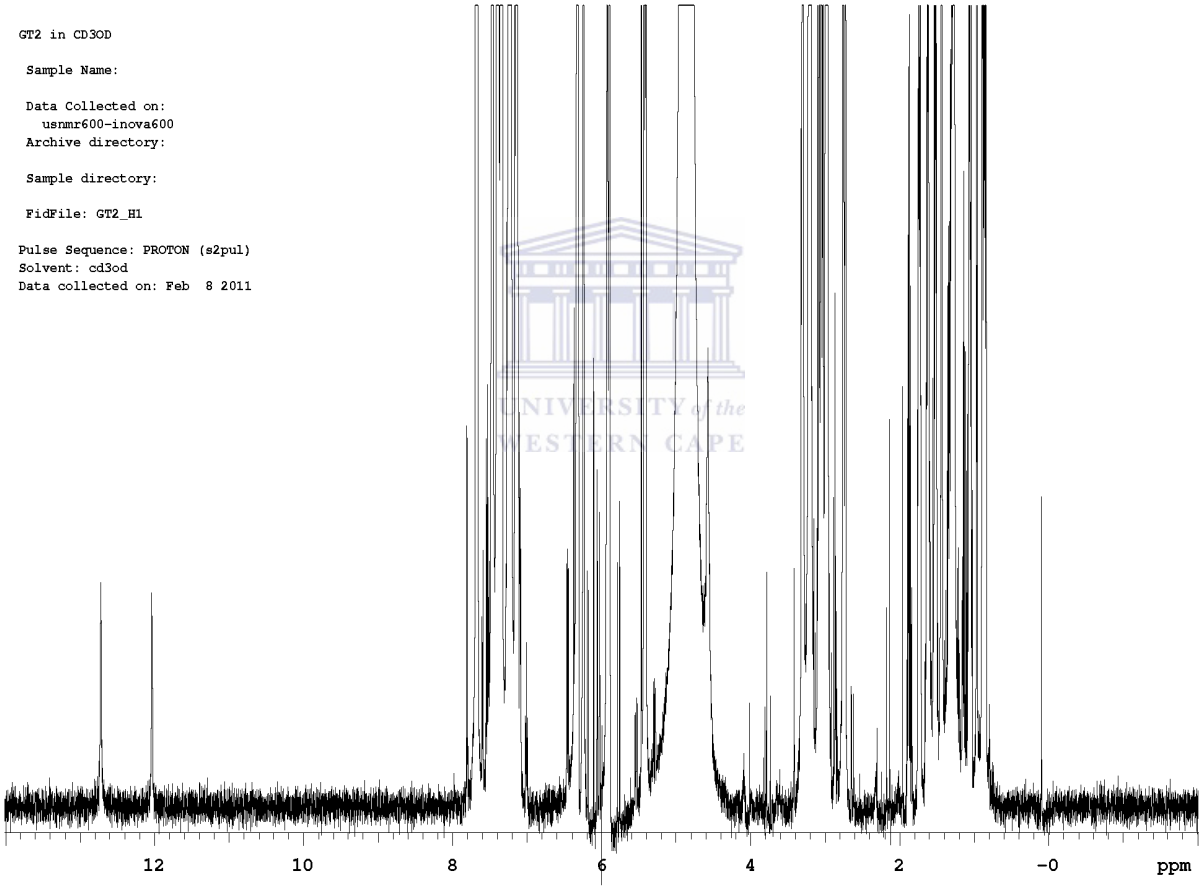
gHSQCAD Plots for Compound 2

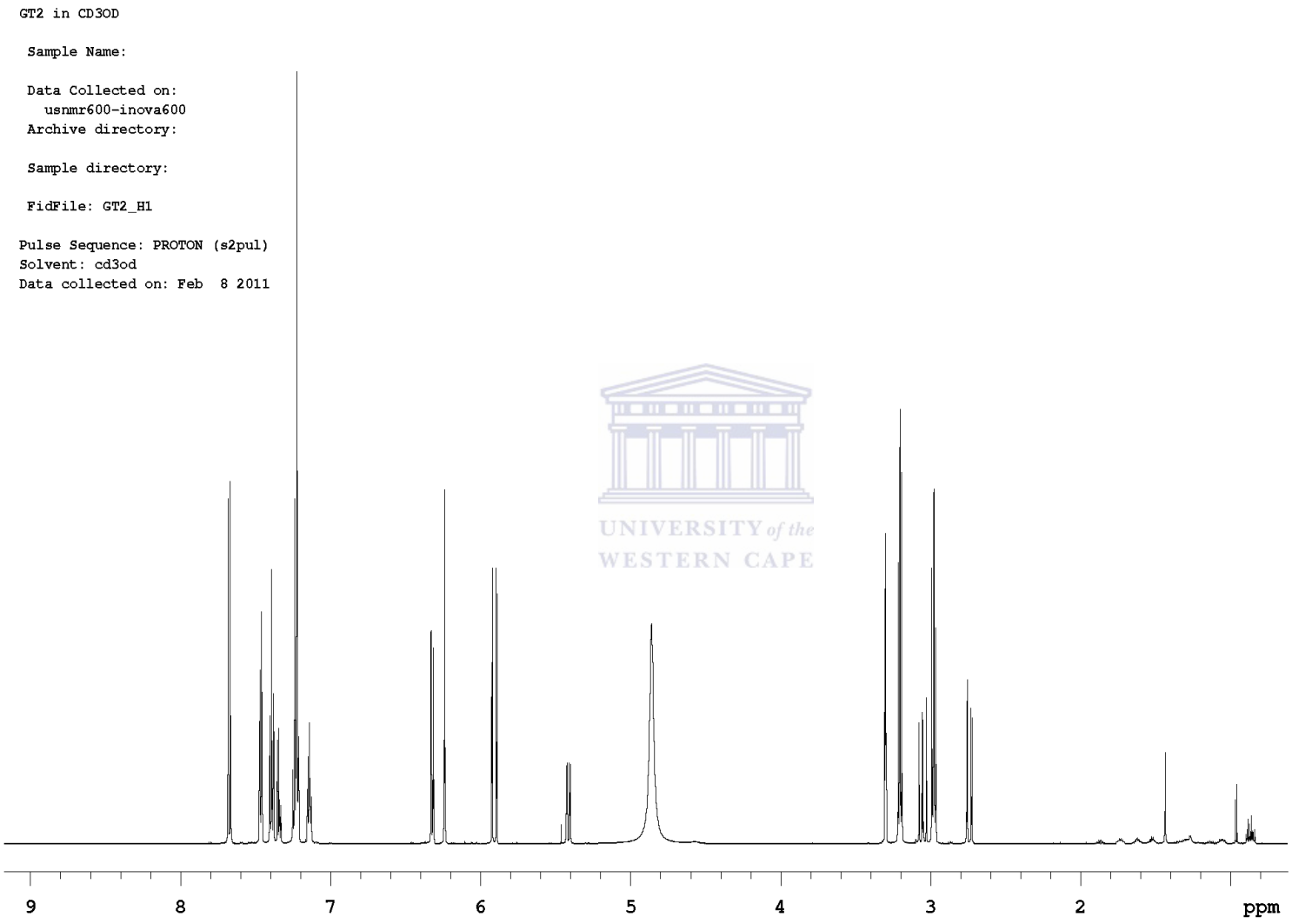






H1 Plots for Compound 2

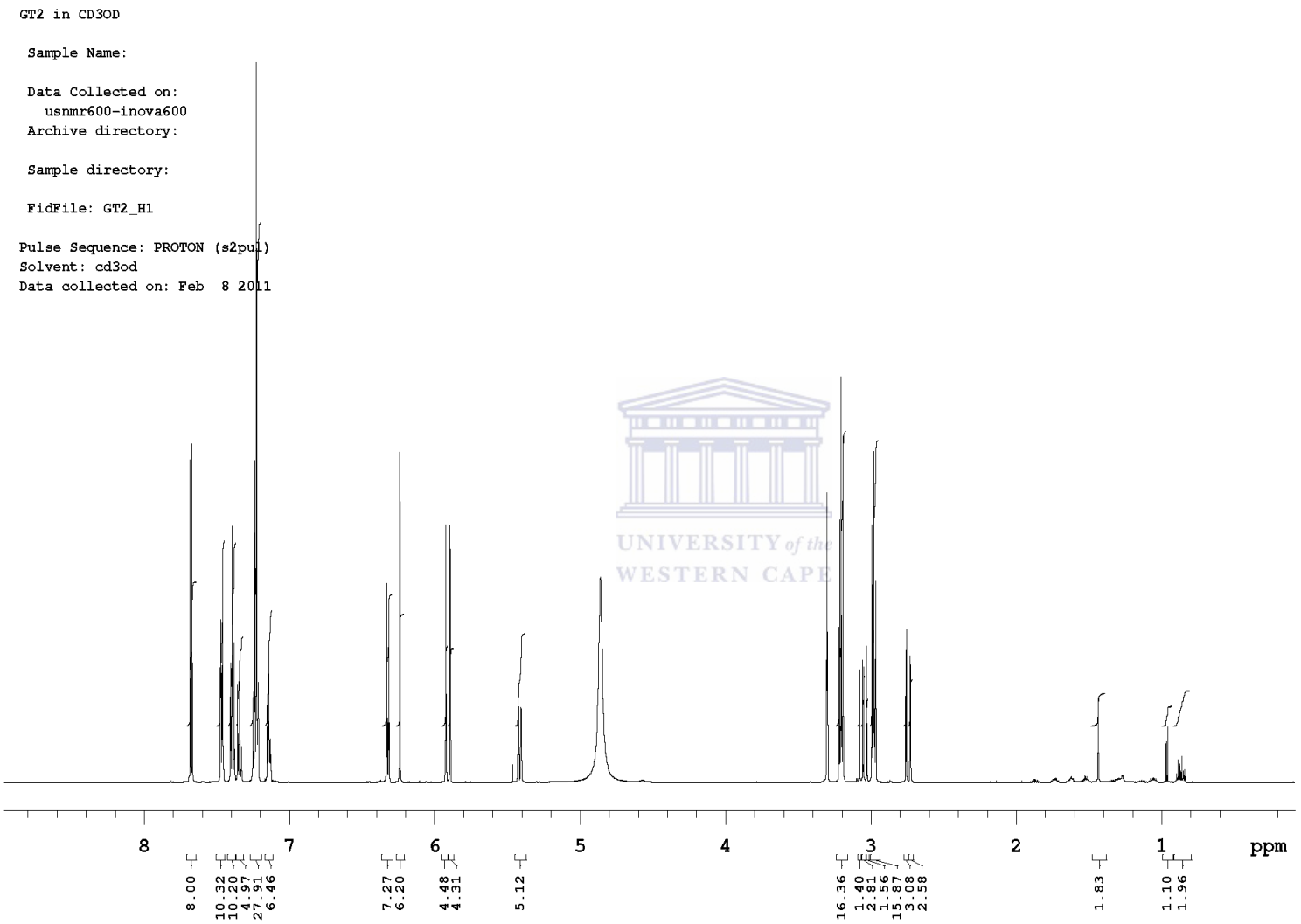


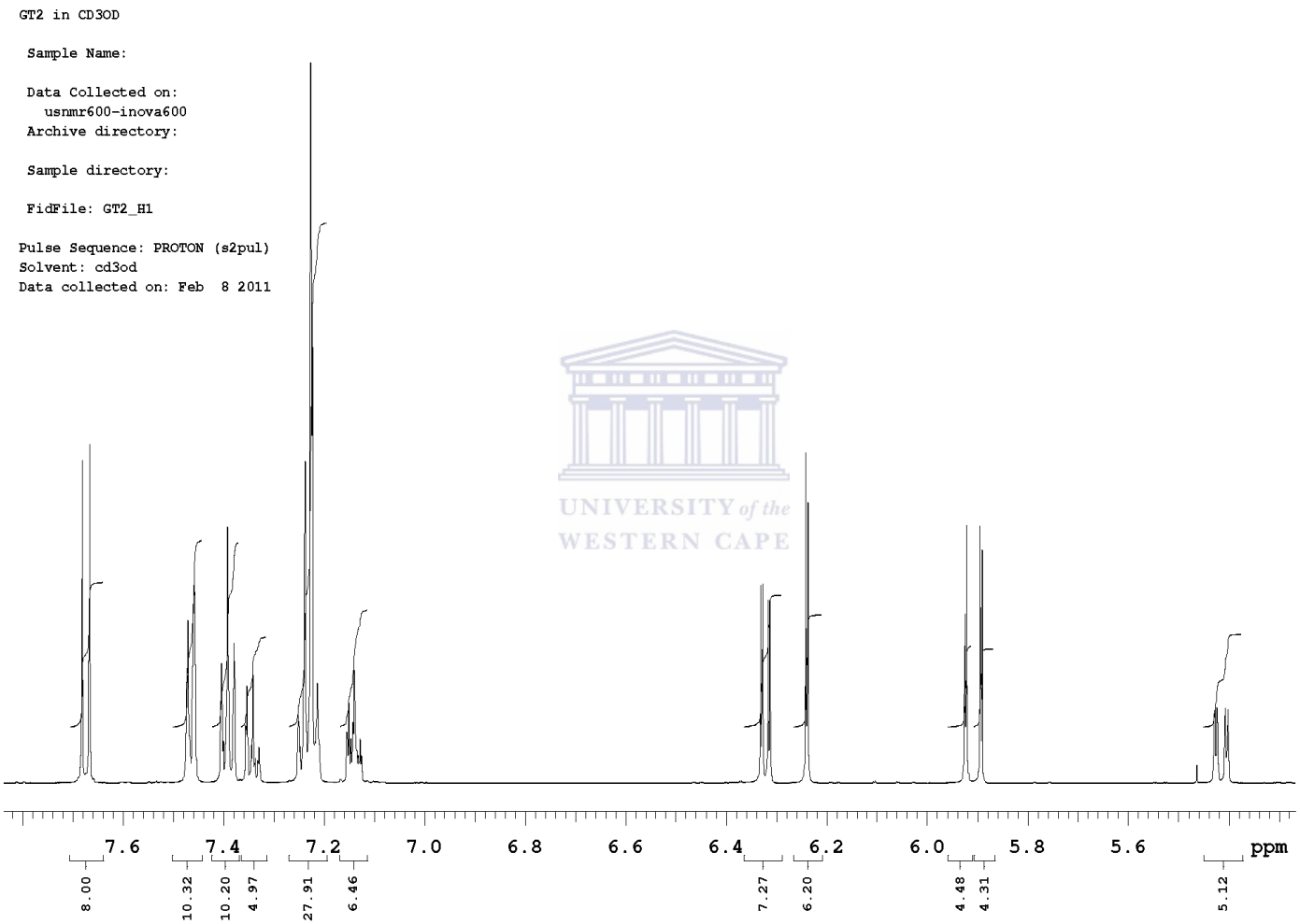


GT2 in CD3OD

exp4 PROTON

SAMPLE		SPECIAL	
date	Feb 8 2011	temp	25.0
solvent	cd3od	gain	not used
file	/home/vnmr1/v~	spin	not used
nmrsys/data/Data20~		hst	0.008
11/February2011/A_~		pw90	9.200
Valentine/GT2/GT2_~		alfa	6.600
	H1.fid	FLAGS	
ACQUISITION		il	n
sw	9611.9	in	n
at	1.705	dp	y
np	32768	hs	nn
fb	not used	PROCESSING	
bs	32	fn	not used
ss	4	DISPLAY	
d1	1.000	sp	371.9
nt	64	wp	5132.7
ct	64	rfl	3186.8
TRANSMITTER		rfp	1979.9
tn	H1	rp	129.2
sfrq	599.986	lp	4.2
tof	599.9	PLOT	
tpwr	54	wc	250
pw	4.600	sc	0
DECOUPLER		vs	156
dn	C13	th	21
dof	0	ai	cdc ph
dm	nnn		
dmm	c		
dpwr	40		
dmf	35088		





GT2 in CD3OD

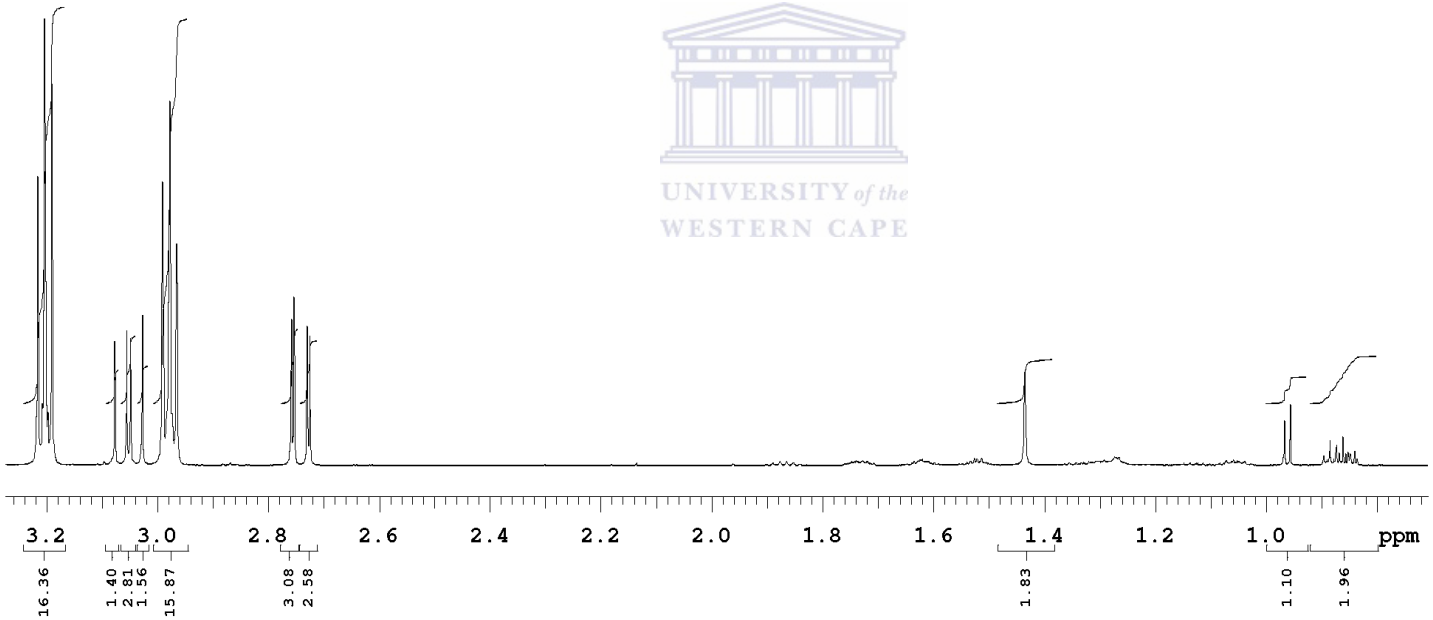
Sample Name:

Data Collected on:
usnmr600-inova600
Archive directory:

Sample directory:

FidFile: GT2_H1

Pulse Sequence: PROTON (s2pul)
Solvent: cd3od
Data collected on: Feb 8 2011



GT2 in CD3OD

Sample Name:

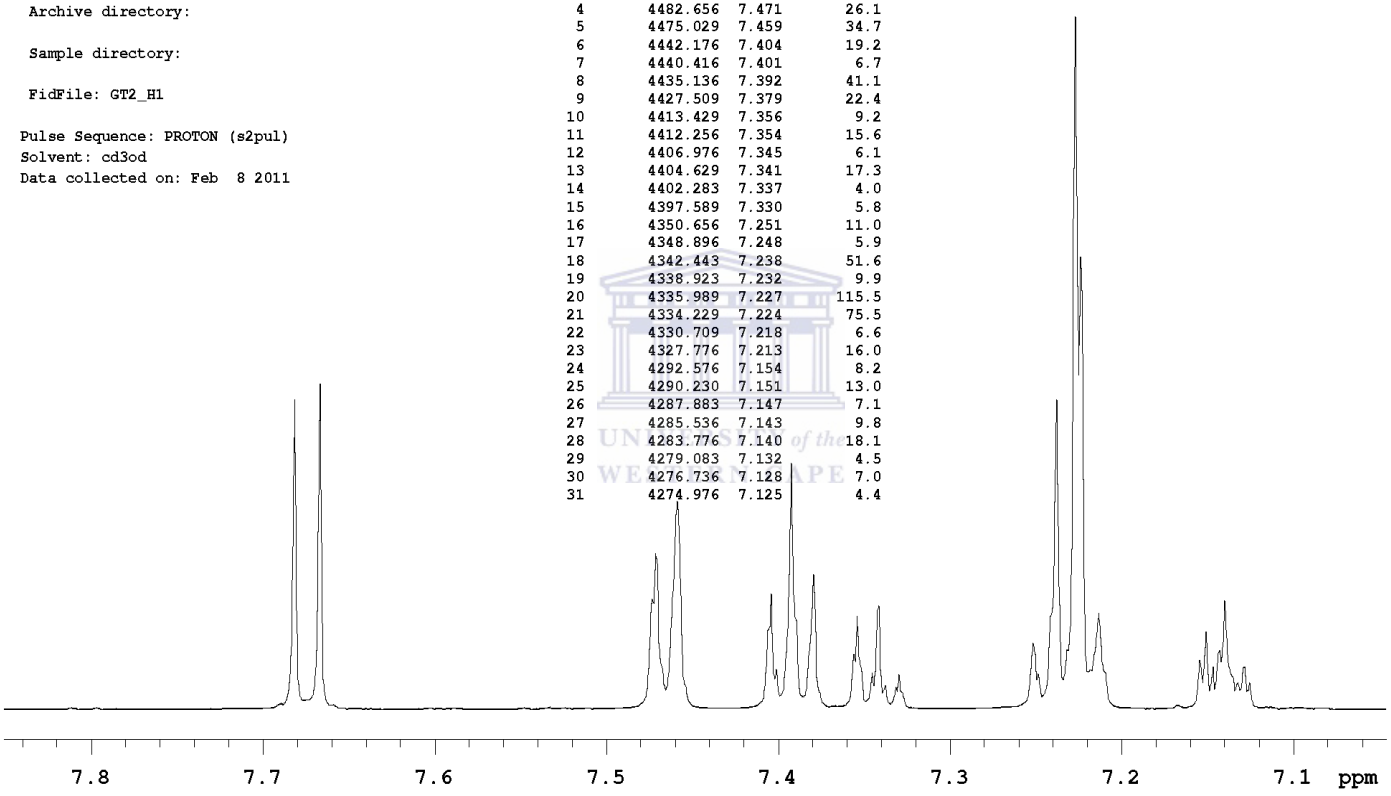
Data Collected on:
usnmr600-inova600
Archive directory:

Sample directory:

FidFile: GT2_H1

Pulse Sequence: PROTON (s2pul)
Solvent: cd3od
Data collected on: Feb 8 2011

INDEX	FREQUENCY	PPM	HEIGHT	INDEX	FREQUENCY	PPM	HEIGHT
1	4608.789	7.682	51.7				
2	4599.989	7.667	54.3				
3	4483.829	7.473	18.4				
4	4482.656	7.471	26.1				
5	4475.029	7.459	34.7				
6	4442.176	7.404	19.2				
7	4440.416	7.401	6.7				
8	4435.136	7.392	41.1				
9	4427.509	7.379	22.4				
10	4413.429	7.356	9.2				
11	4412.256	7.354	15.6				
12	4406.976	7.345	6.1				
13	4404.629	7.341	17.3				
14	4402.283	7.337	4.0				
15	4397.589	7.330	5.8				
16	4350.656	7.251	11.0				
17	4348.896	7.248	5.9				
18	4342.443	7.238	51.6				
19	4338.923	7.232	9.9				
20	4335.989	7.227	115.5				
21	4334.229	7.224	75.5				
22	4330.709	7.218	6.6				
23	4327.776	7.213	16.0				
24	4292.576	7.154	8.2				
25	4290.230	7.151	13.0				
26	4287.883	7.147	7.1				
27	4285.536	7.143	9.8				
28	4283.776	7.140	18.1				
29	4279.083	7.132	4.5				
30	4276.736	7.128	7.0				
31	4274.976	7.125	4.4				



GT2 in CD3OD

Sample Name:

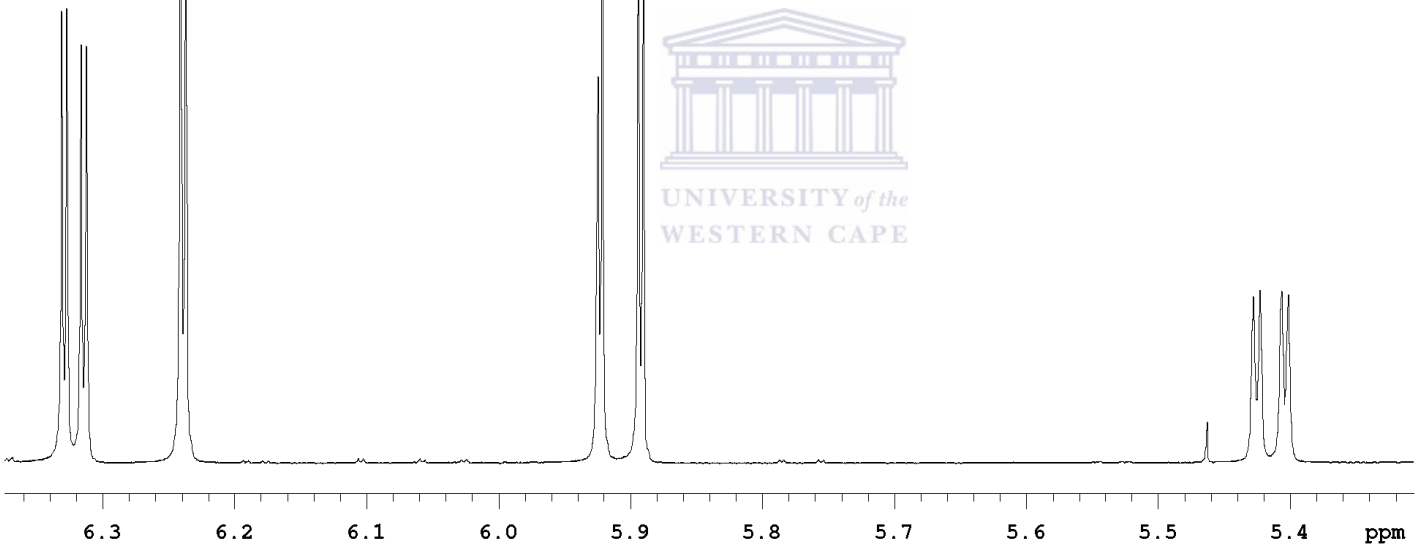
Data Collected on:
usnmr600-inova600
Archive directory:

Sample directory:

FidFile: GT2_H1

Pulse Sequence: PROTON (s2pul)
Solvent: cd3od
Data collected on: Feb 8 2011

INDEX	FREQUENCY	PPM	HEIGHT
1	3798.604	6.331	73.9
2	3796.258	6.327	74.4
3	3789.804	6.317	68.5
4	3787.458	6.313	68.1
5	3744.631	6.241	123.5
6	3742.284	6.237	105.0
7	3554.552	5.924	63.2
8	3552.792	5.921	96.2
9	3536.365	5.894	96.1
10	3534.018	5.890	87.2
11	3277.646	5.463	6.6
12	3256.526	5.428	27.3
13	3253.593	5.423	28.3
14	3243.619	5.406	28.2
15	3240.686	5.401	27.6



GT2 in CD3OD

Sample Name:

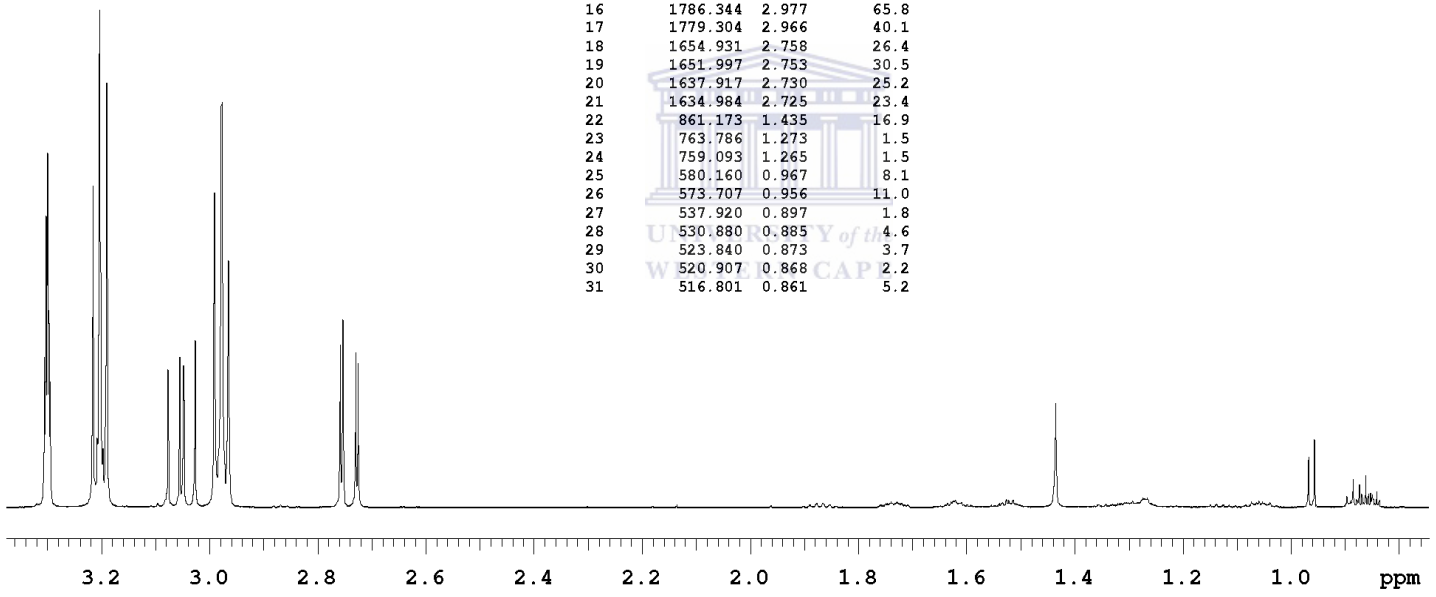
Data Collected on:
usnmr600-inova600
Archive directory:

Sample directory:

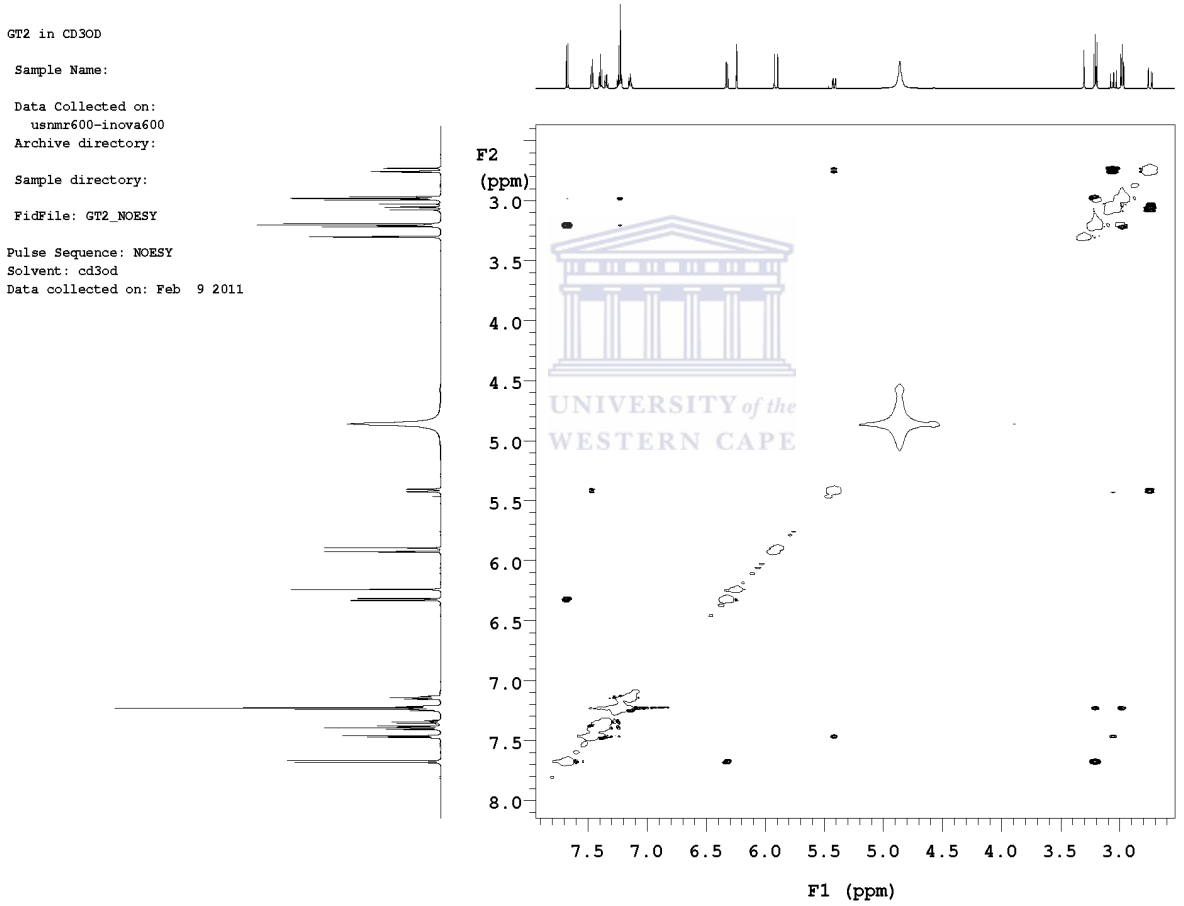
FidFile: GT2_H1

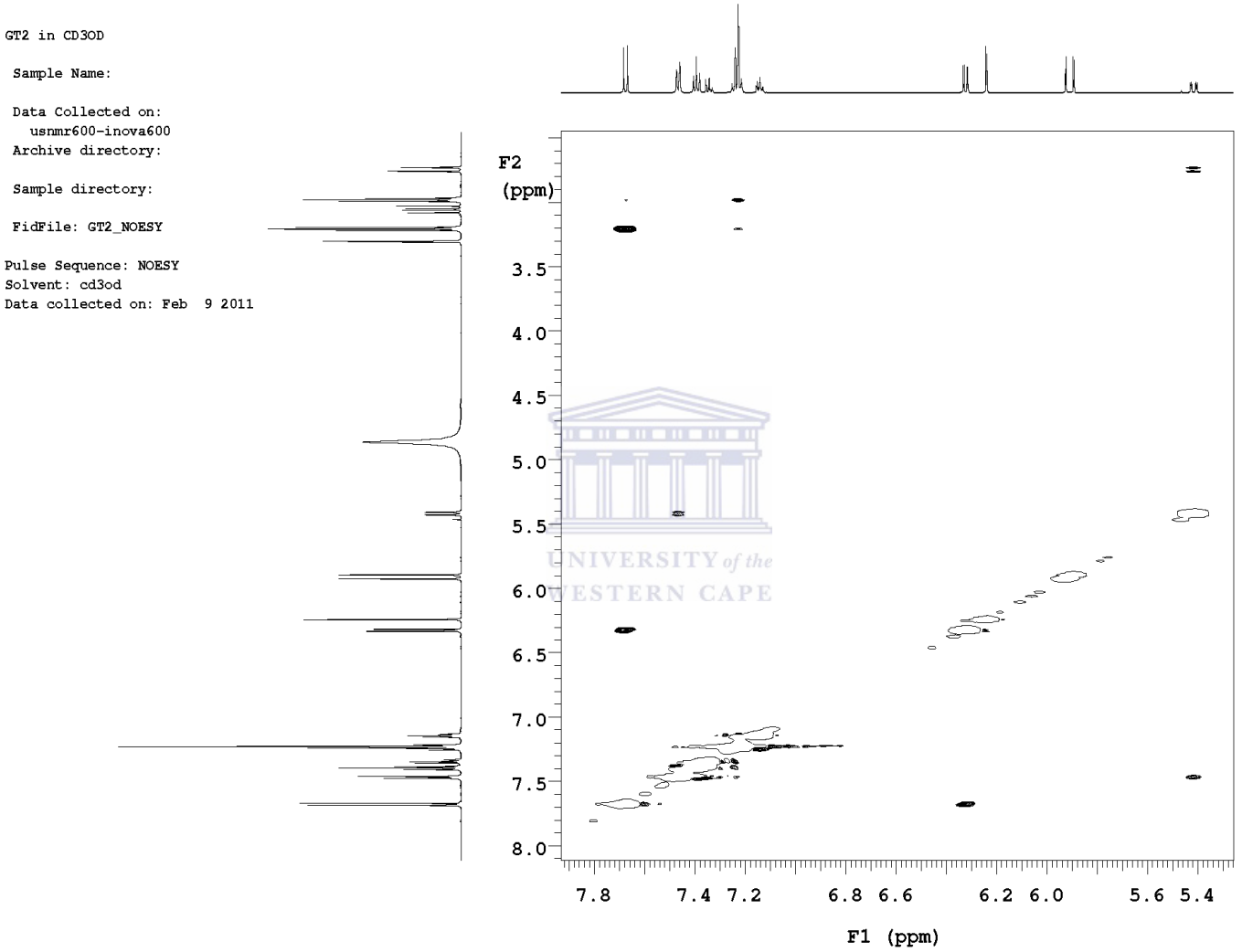
Pulse Sequence: PROTON (s2pul)
Solvent: cd3od
Data collected on: Feb 8 2011

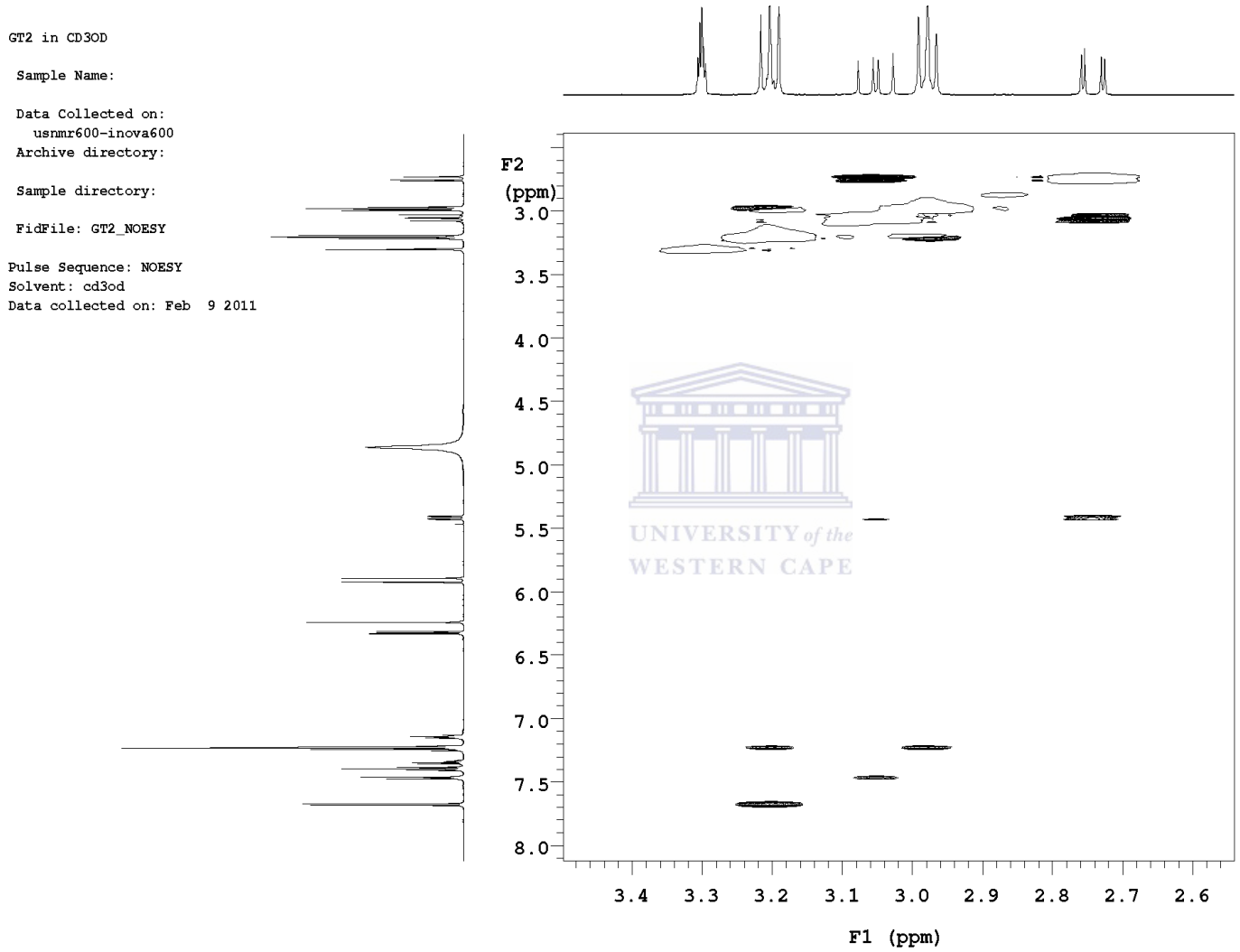
INDEX	FREQUENCY	PPM	HEIGHT	INDEX	FREQUENCY	PPM	HEIGHT
1	1983.463	3.306	24.4	32	513.867	0.856	2.2
2	1981.703	3.303	47.2	33	511.521	0.853	2.4
3	1979.943	3.300	57.5	34	509.174	0.849	2.1
4	1978.770	3.298	32.1	35	504.481	0.841	2.6
5	1977.010	3.295	20.5				
6	1929.490	3.216	52.1				
7	1924.796	3.208	11.2				
8	1921.863	3.203	80.7				
9	1918.343	3.197	9.6				
10	1914.236	3.190	68.9				
11	1846.183	3.077	22.4				
12	1833.277	3.056	24.4				
13	1829.170	3.049	23.0				
14	1816.263	3.027	27.1				
15	1794.557	2.991	51.1				
16	1786.344	2.977	65.8				
17	1779.304	2.966	40.1				
18	1654.931	2.758	26.4				
19	1651.997	2.753	30.5				
20	1637.917	2.730	25.2				
21	1634.984	2.725	23.4				
22	861.173	1.435	16.9				
23	763.786	1.273	1.5				
24	759.093	1.265	1.5				
25	580.160	0.967	8.1				
26	573.707	0.956	11.0				
27	537.920	0.897	1.8				
28	530.880	0.885	4.6				
29	523.840	0.873	3.7				
30	520.907	0.868	2.2				
31	516.801	0.861	5.2				



Noesy Plots for Compound 2

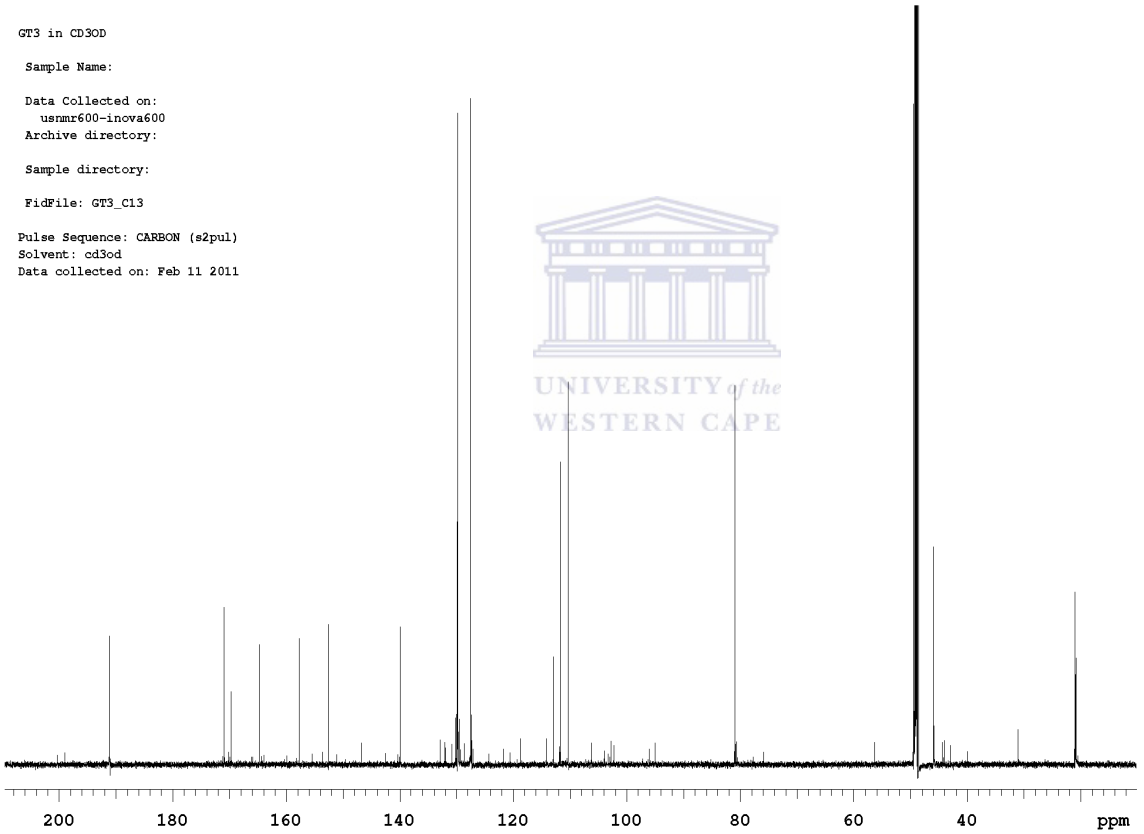






ADDENDUM 3:
NMR DATA FOR COMPOUND 3

C13 Plots for Compound 3



GT3 in CD3OD

exp2 CARBON

SAMPLE		PRESATURATION	
date	Feb 11 2011	satmode	n
solvent	cd3od	wet	n
file	/home/vnmr1/v~	SPECIAL	
nmrsys/data/Data20~		temp	25.0
11/February2011/A_~		gain	not used
Valentine/GT3/GT3_~		spin	not used
	C13.fid	hst	0.008
ACQUISITION		pw90	15.400
sw	41536.9	alfa	10.000
at	0.789	FLAGS	
np	65536	il	n
fb	not used	in	n
bs	16	dp	y
d1	1.000	hs	nn
nt	40000	PROCESSING	
ct	40000	lb	0.50
TRANSMITTER		lsfid	-2
tn	C13	fn	not used
sfrq	150.884	DISPLAY	
tof	4175.5	sp	1539.9
tpwr	56	wp	30072.6
pw	7.700	rfl	9472.8
DECOUPLER		rfp	7392.4
dn	H1	rp	84.2
dof	0	lp	85.2
dm	yyy	PLOT	
decwave	w	wc	250
dpwr	36	sc	0
dmf	14400	vs	4028
		th	14
		ai cdc ph	

GT3 in CD3OD

Sample Name:

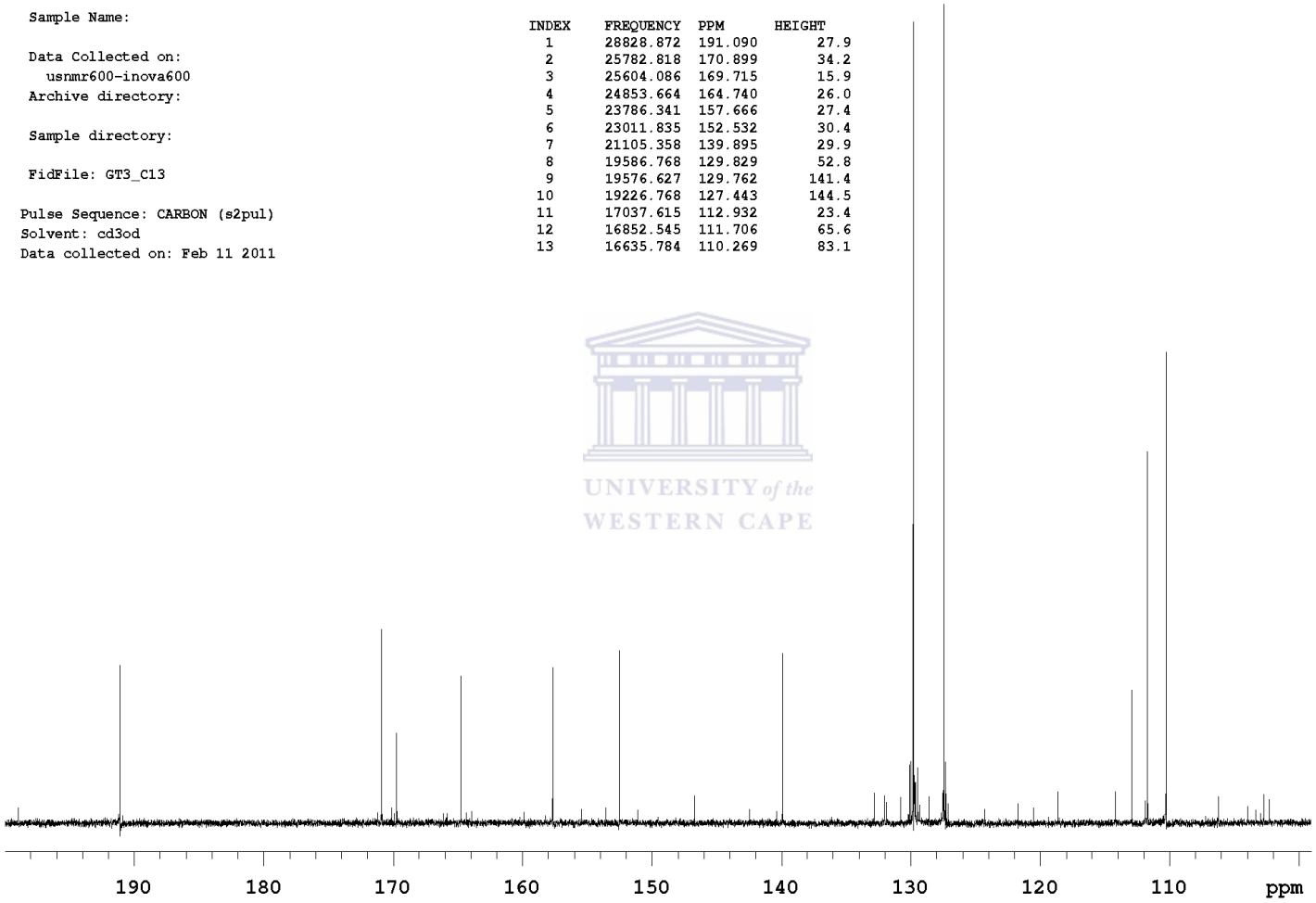
Data Collected on:
usnmr600-inova600
Archive directory:

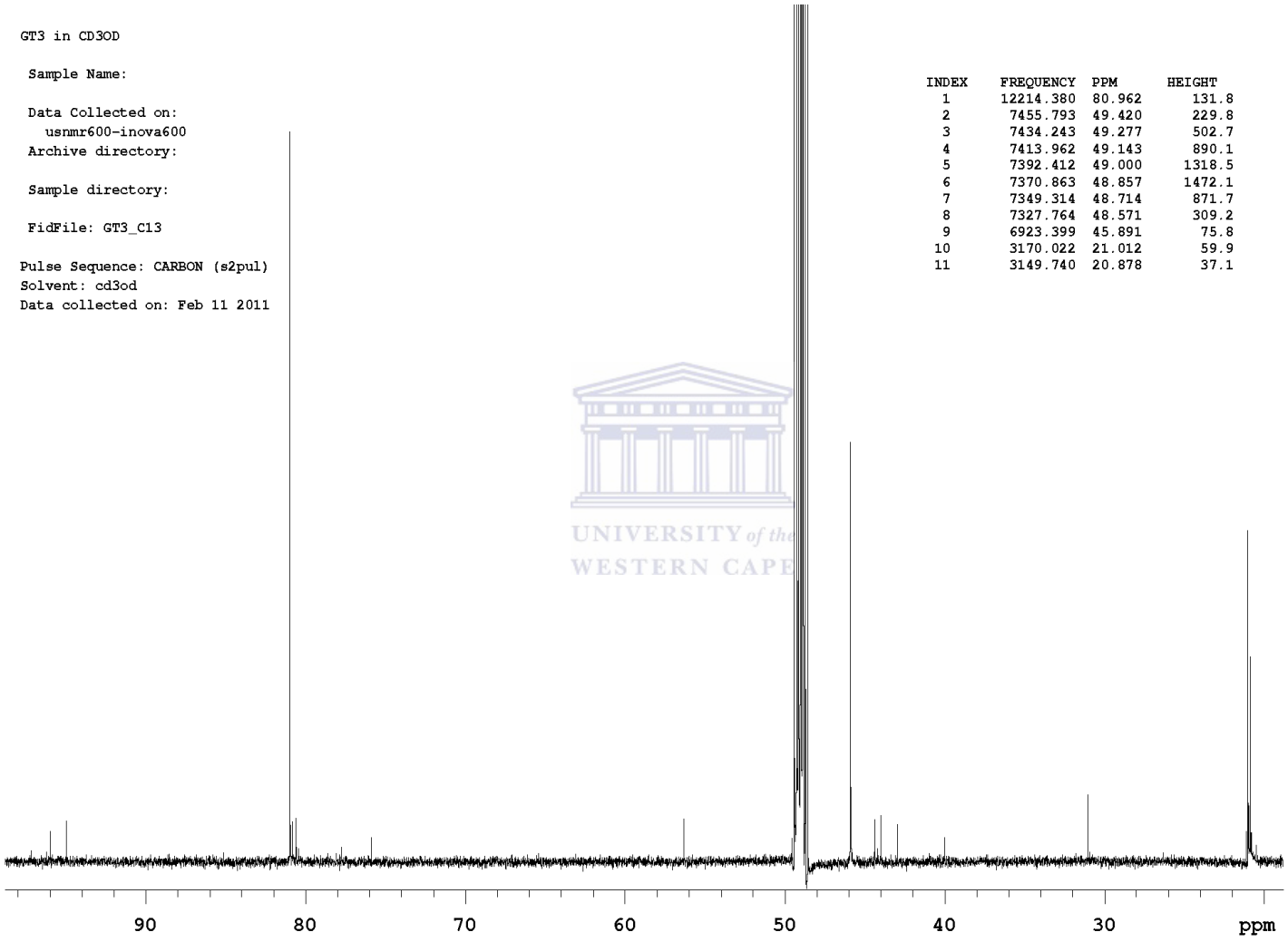
Sample directory:

FidFile: GT3_C13

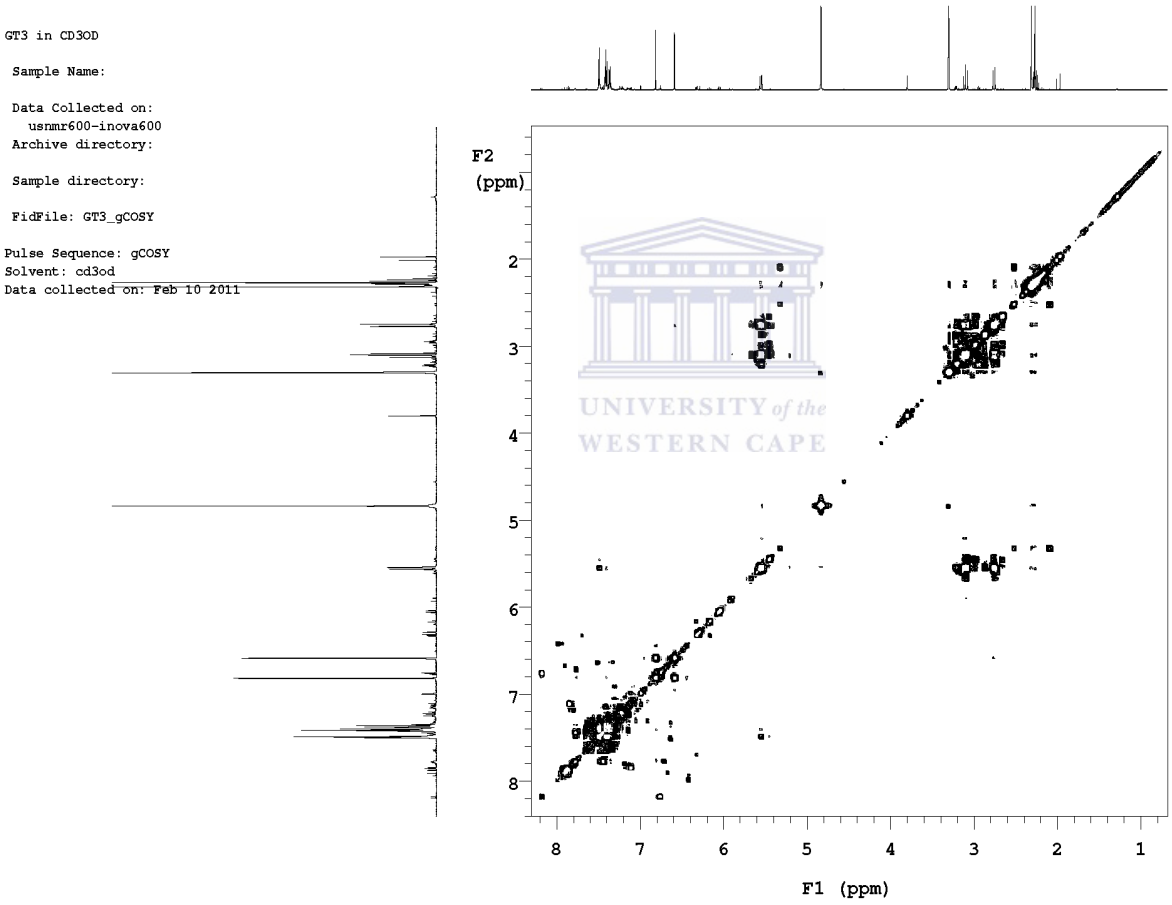
Pulse Sequence: CARBON (s2pul)
Solvent: cd3od
Data collected on: Feb 11 2011

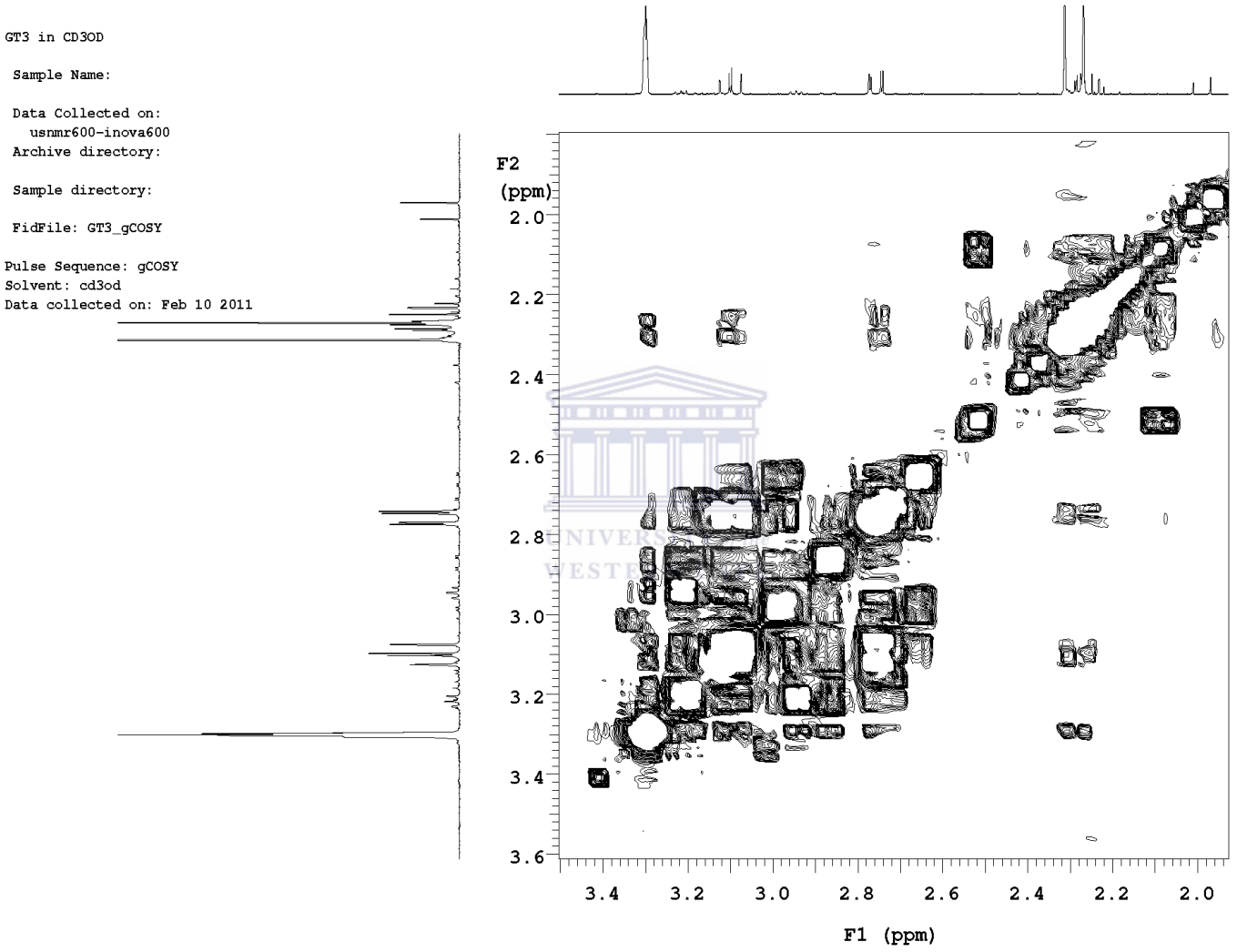
INDEX	FREQUENCY	PPM	HEIGHT
1	28828.872	191.090	27.9
2	25782.818	170.899	34.2
3	25604.086	169.715	15.9
4	24853.664	164.740	26.0
5	23786.341	157.666	27.4
6	23011.835	152.532	30.4
7	21105.358	139.895	29.9
8	19586.768	129.829	52.8
9	19576.627	129.762	141.4
10	19226.768	127.443	144.5
11	17037.615	112.932	23.4
12	16852.545	111.706	65.6
13	16635.784	110.269	83.1

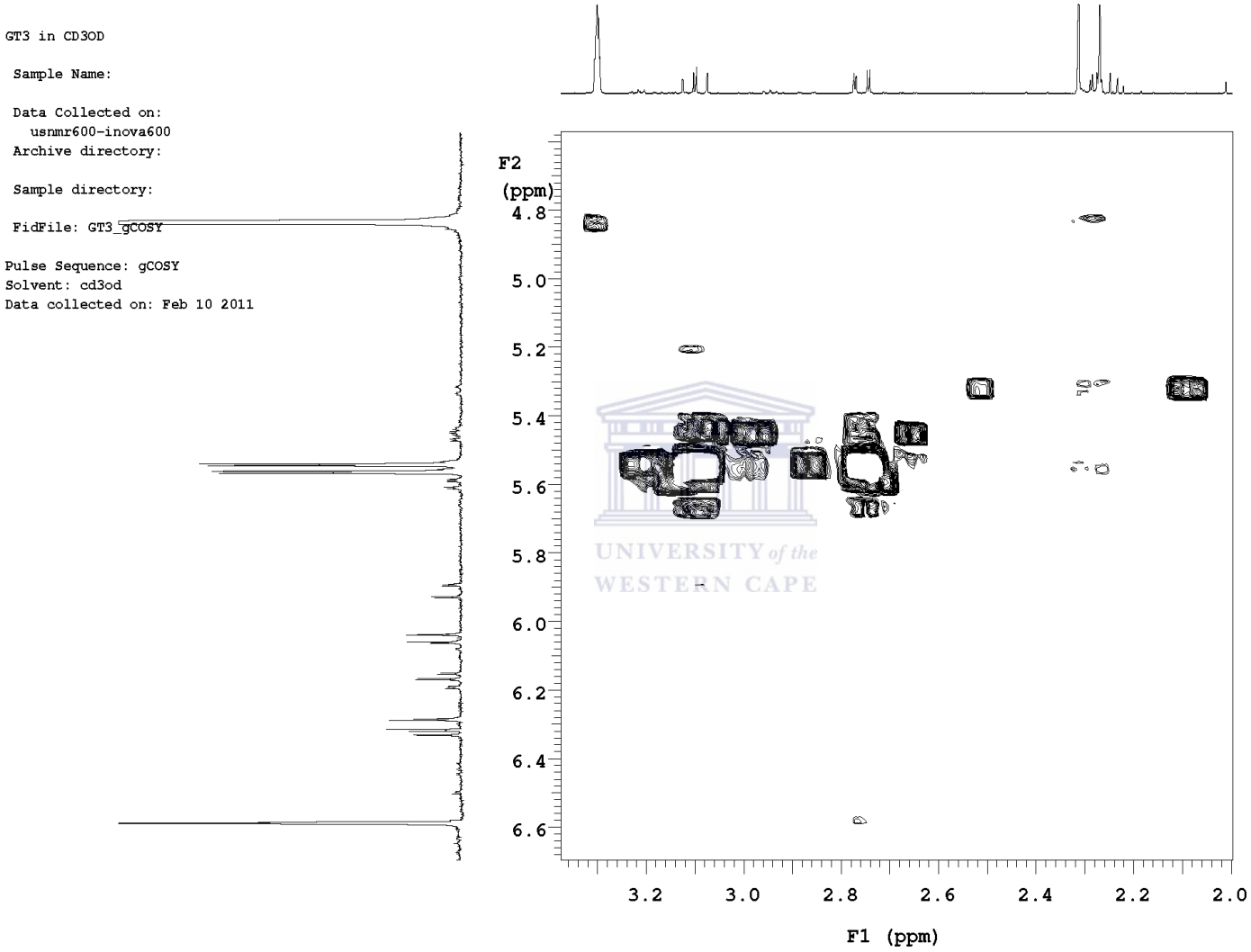


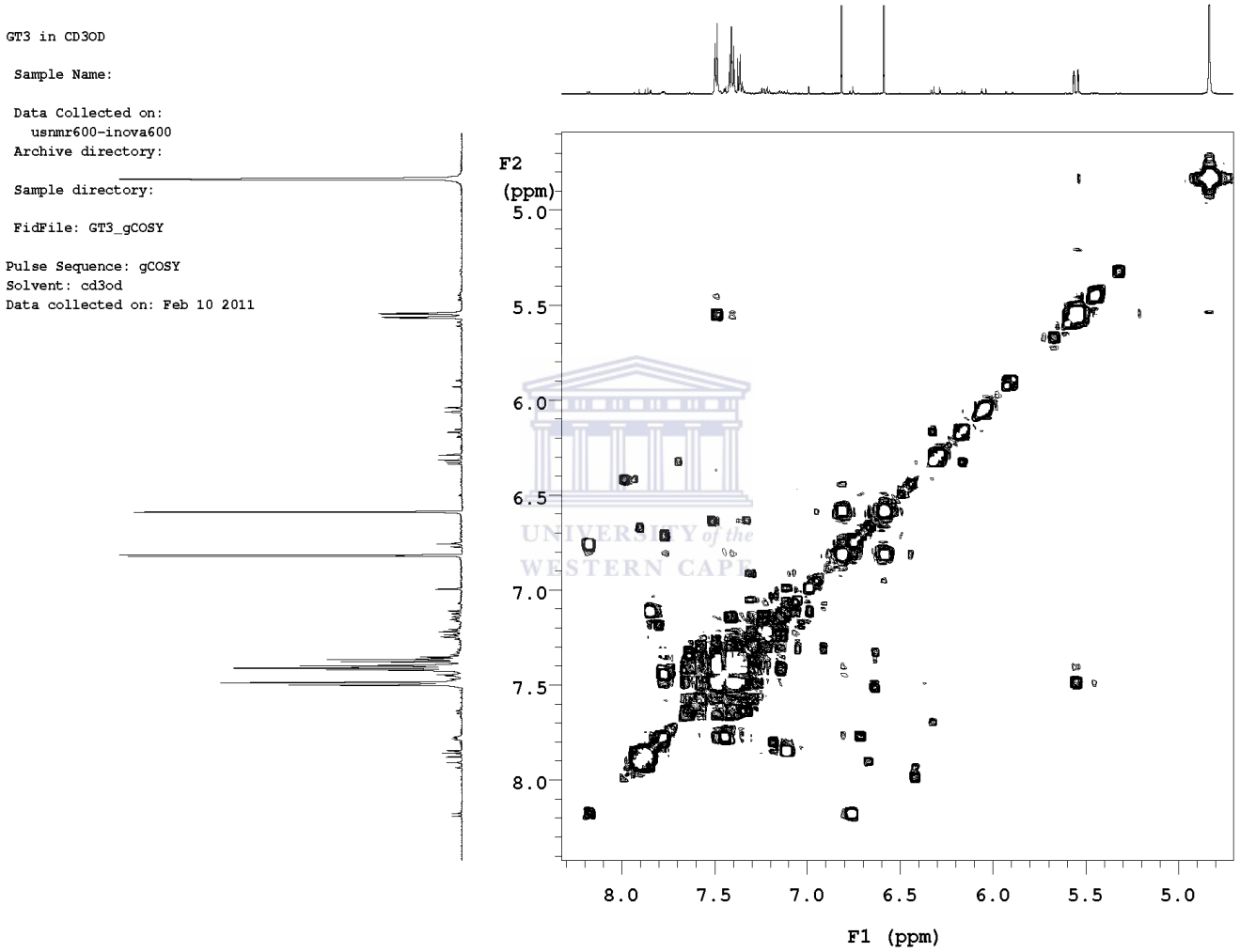


gCOSY Plots for Compound 3

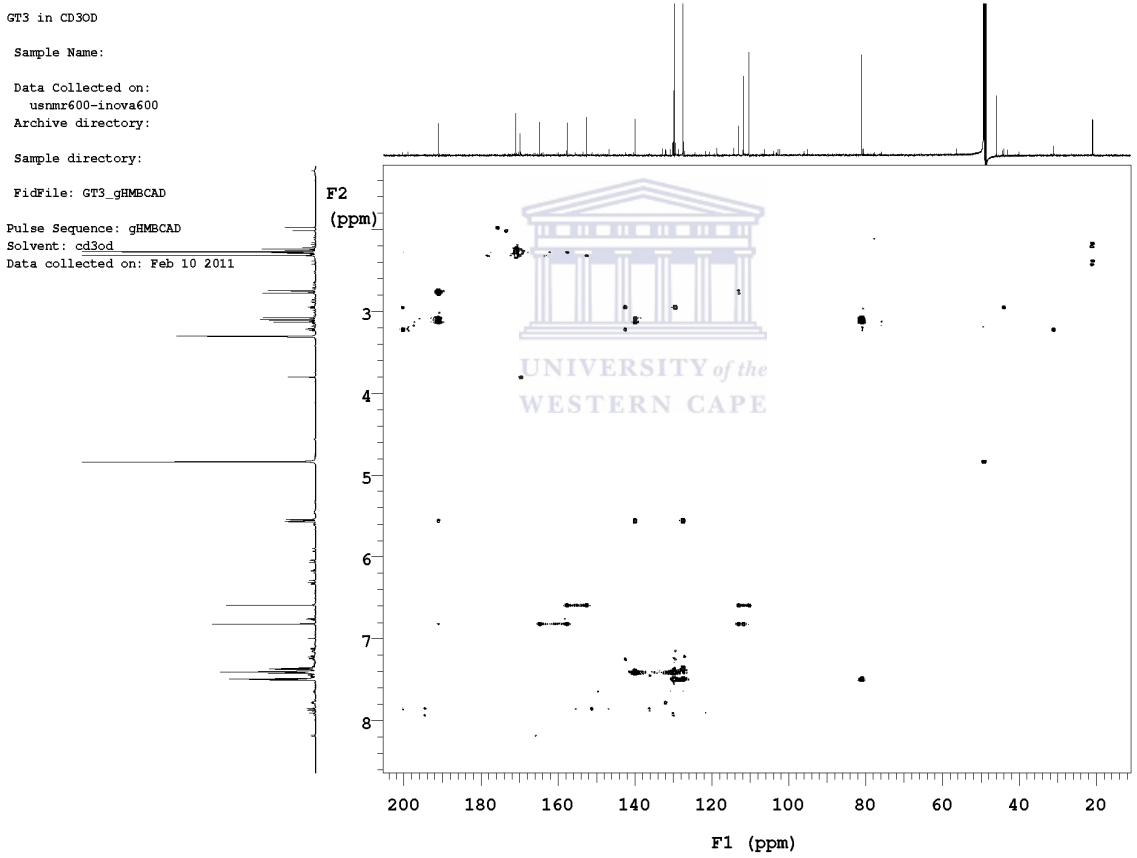


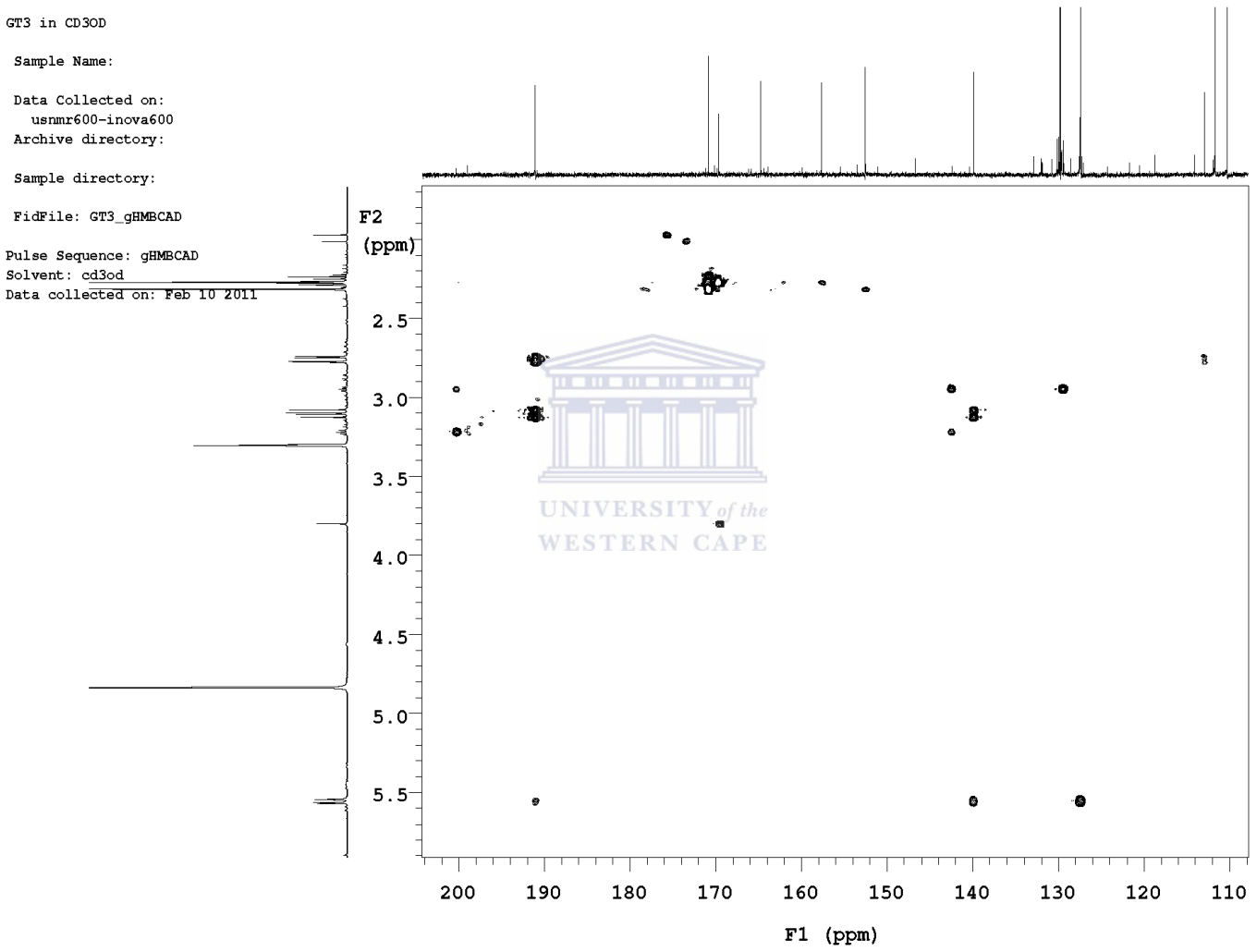

GT3 in CD3OD
Sample Name:
Data Collected on:
usnmr600-inova600
Archive directory:
Sample directory:
FidFile: GT3_gCOSY
Pulse Sequence: gCOSY
Solvent: cd3od
Data collected on: Feb 10 2011
F2
(ppm)
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
F1 (ppm)

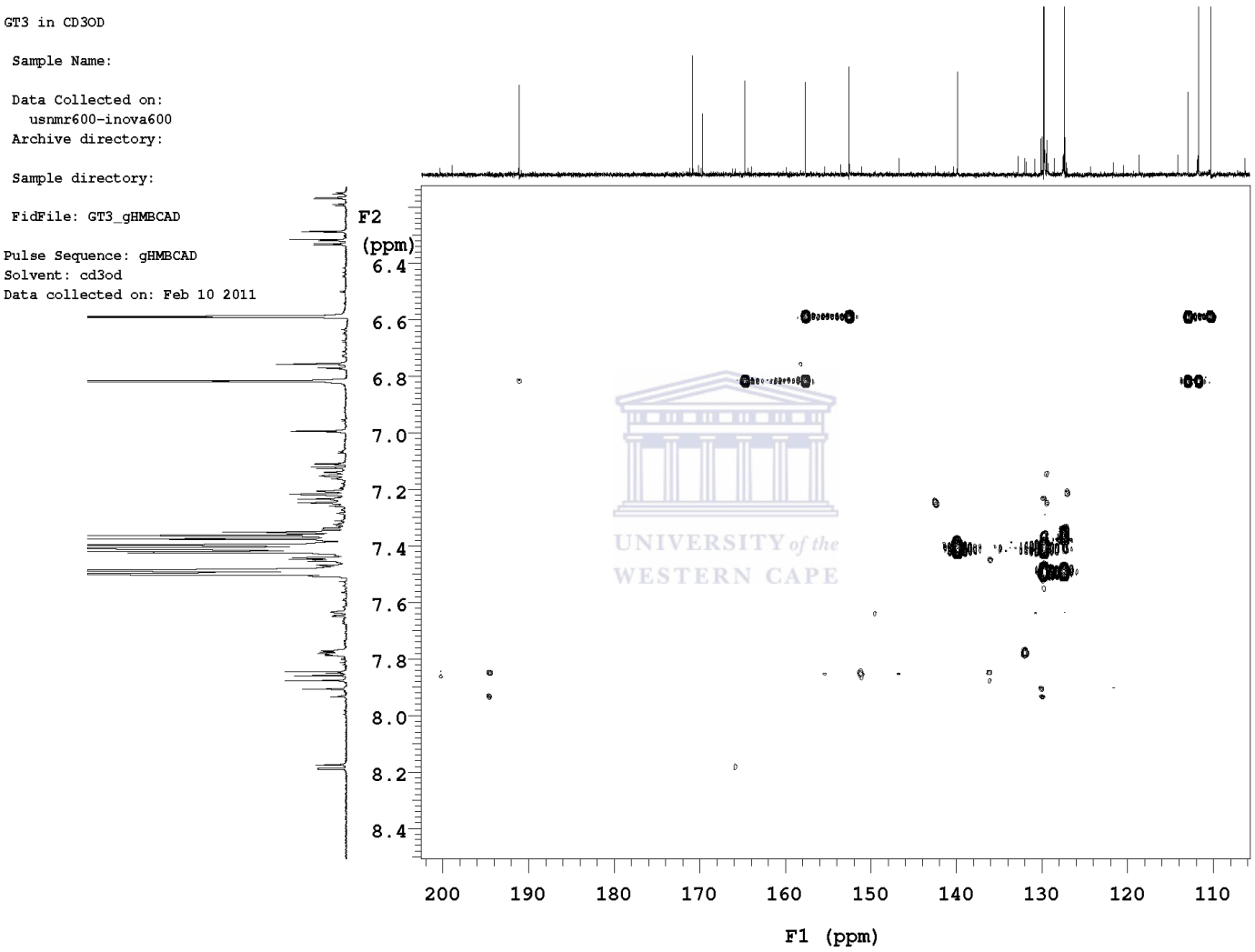




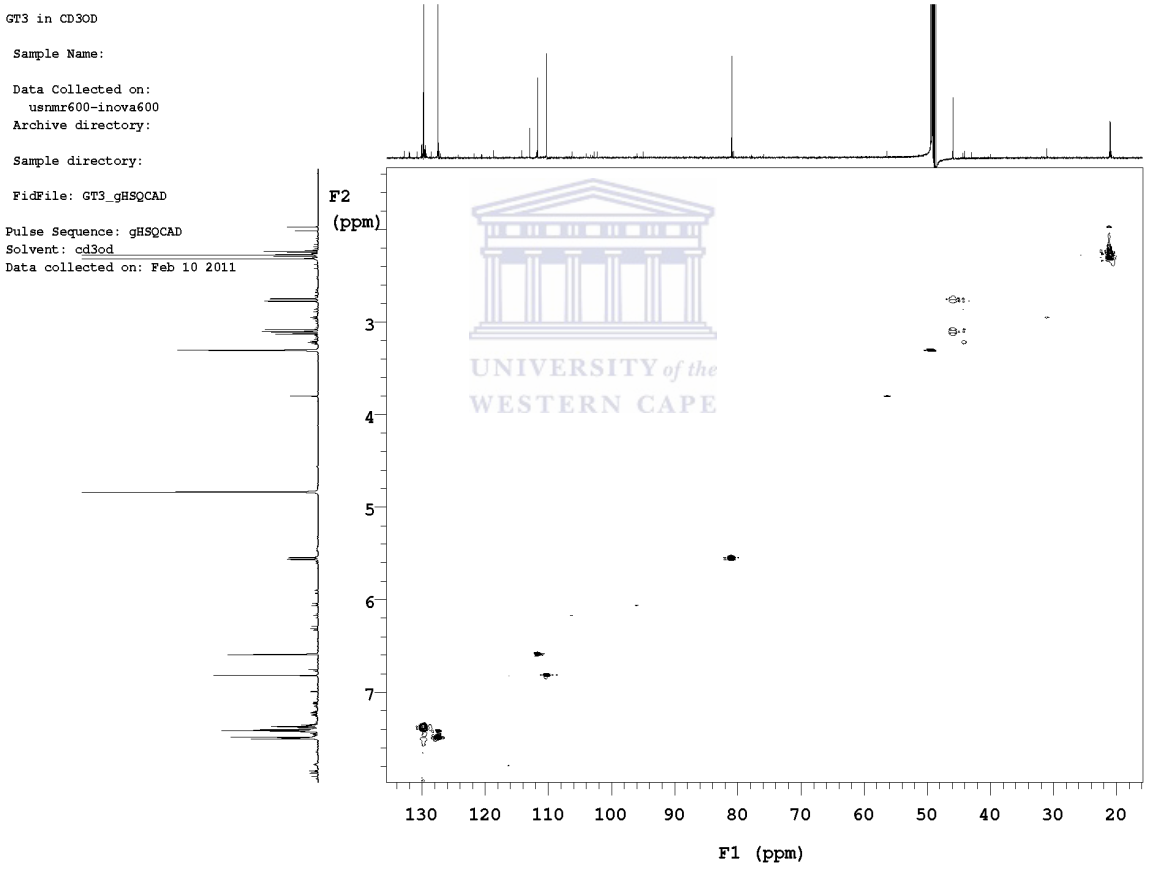
gHMBCAD Plots for Compound 3



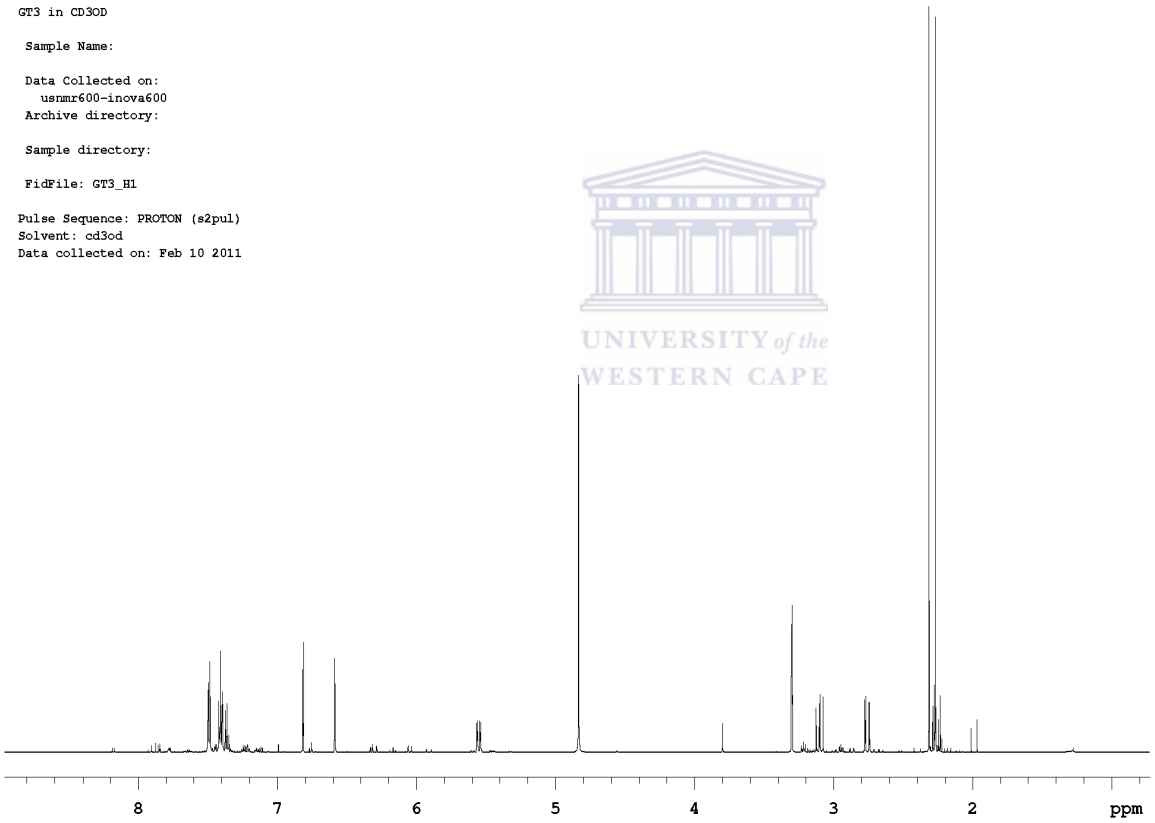




gHSQCAD Plot for Compound 3



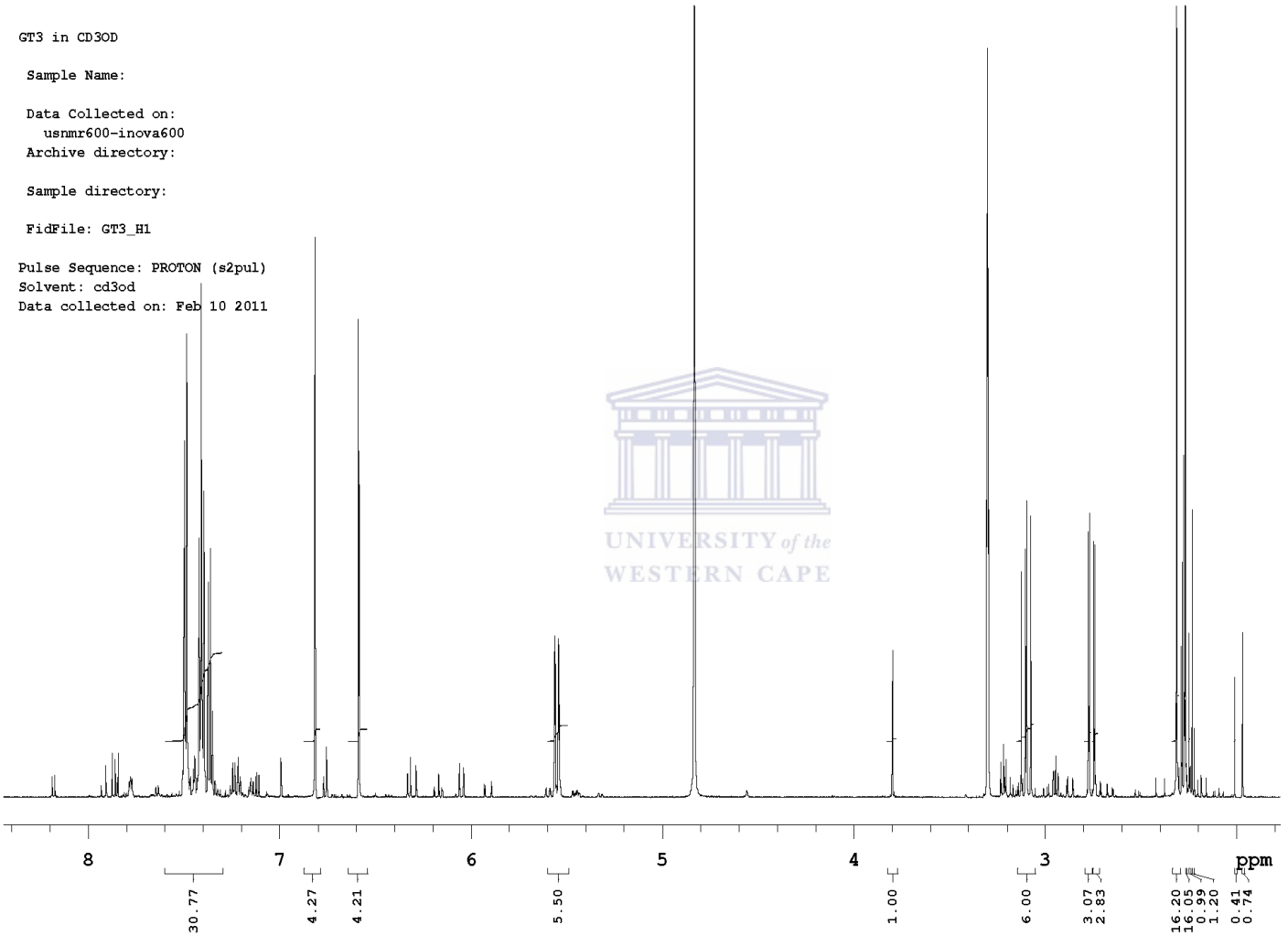
H1 Plots for Compound 3

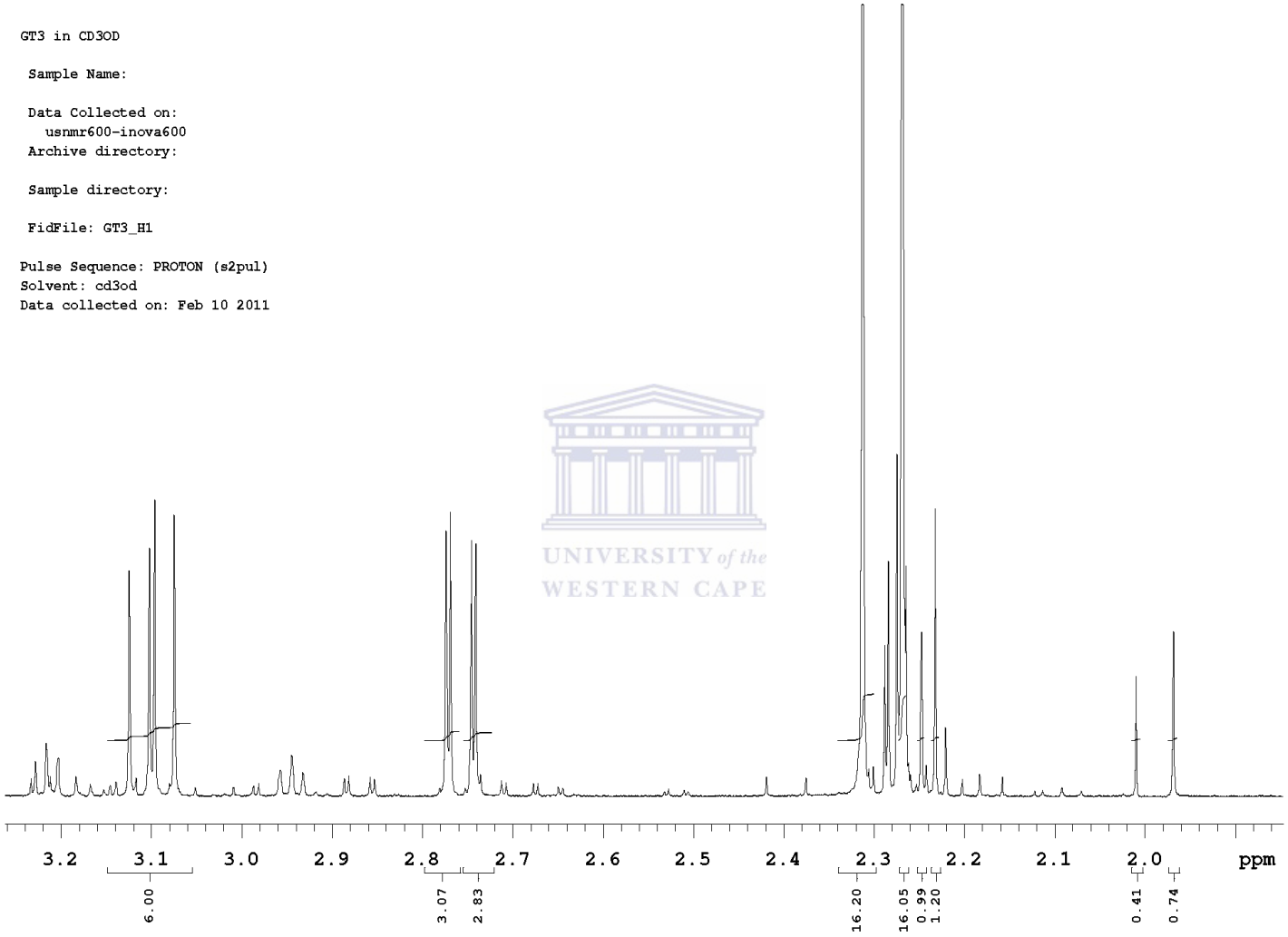


GT3 in CD3OD

exp4 PROTON

SAMPLE		SPECIAL	
date	Feb 10 2011	temp	25.0
solvent	cd3od	gain	not used
file	/home/vnmr1/v~	spin	not used
nmrsys/data/Data20~		hst	0.008
11/February2011/A_~		pw90	9.200
Valentine/GT3/GT3_~		alfa	6.600
	H1.fid	FLAGS	
ACQUISITION		il	n
sw	9611.9	in	n
at	1.705	dp	y
np	32768	hs	nn
fb	not used	PROCESSING	
bs	32	fn	not used
ss	4	DISPLAY	
d1	1.000	sp	436.4
nt	16	wp	4939.7
ct	16	rfl	3186.8
TRANSMITTER		rfp	1979.9
tn	H1	rp	122.4
sfrq	599.986	lp	8.0
tof	600.0	PLOT	
tpwr	54	wc	250
pw	4.600	sc	0
DECOUPLER		vs	63
dn	C13	th	46
dof	0	ai	cdc ph
dm	nnn		
dmm	c		
dpwr	40		
dmf	35088		





GT3 in CD3OD

Sample Name:

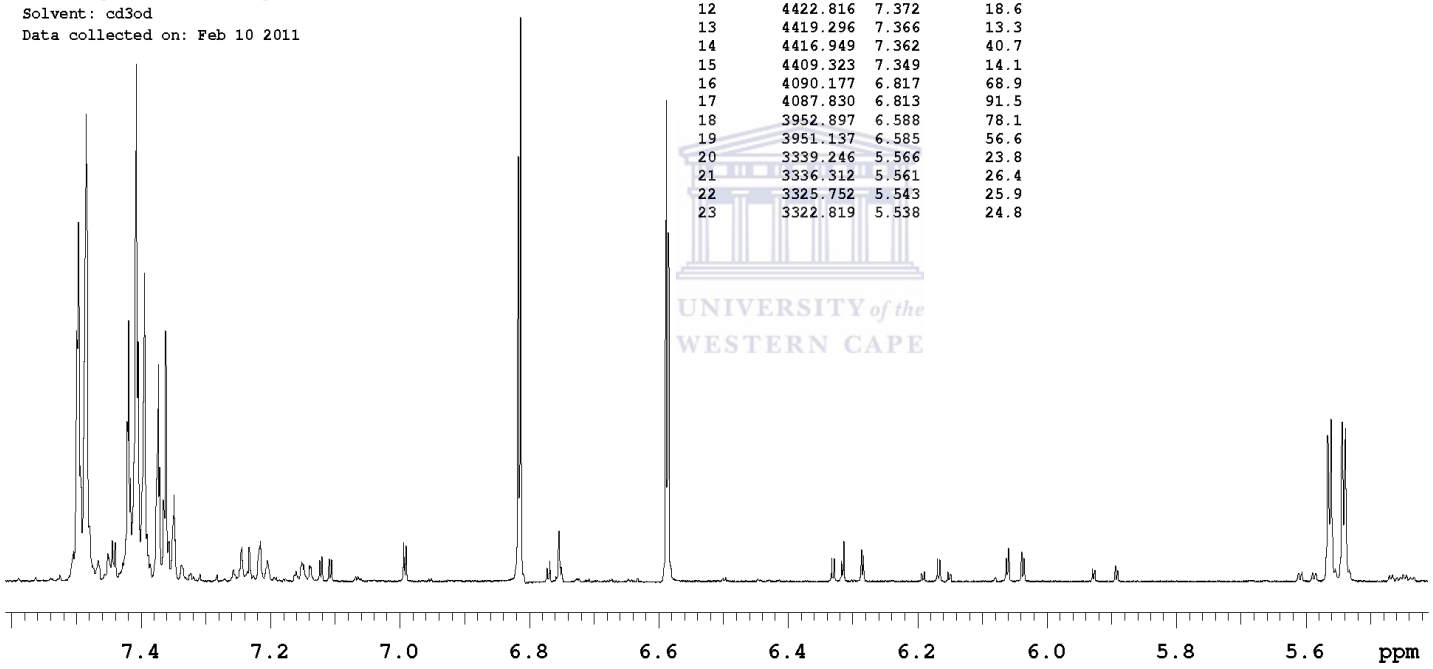
Data Collected on:
usnmr600-inova600
Archive directory:

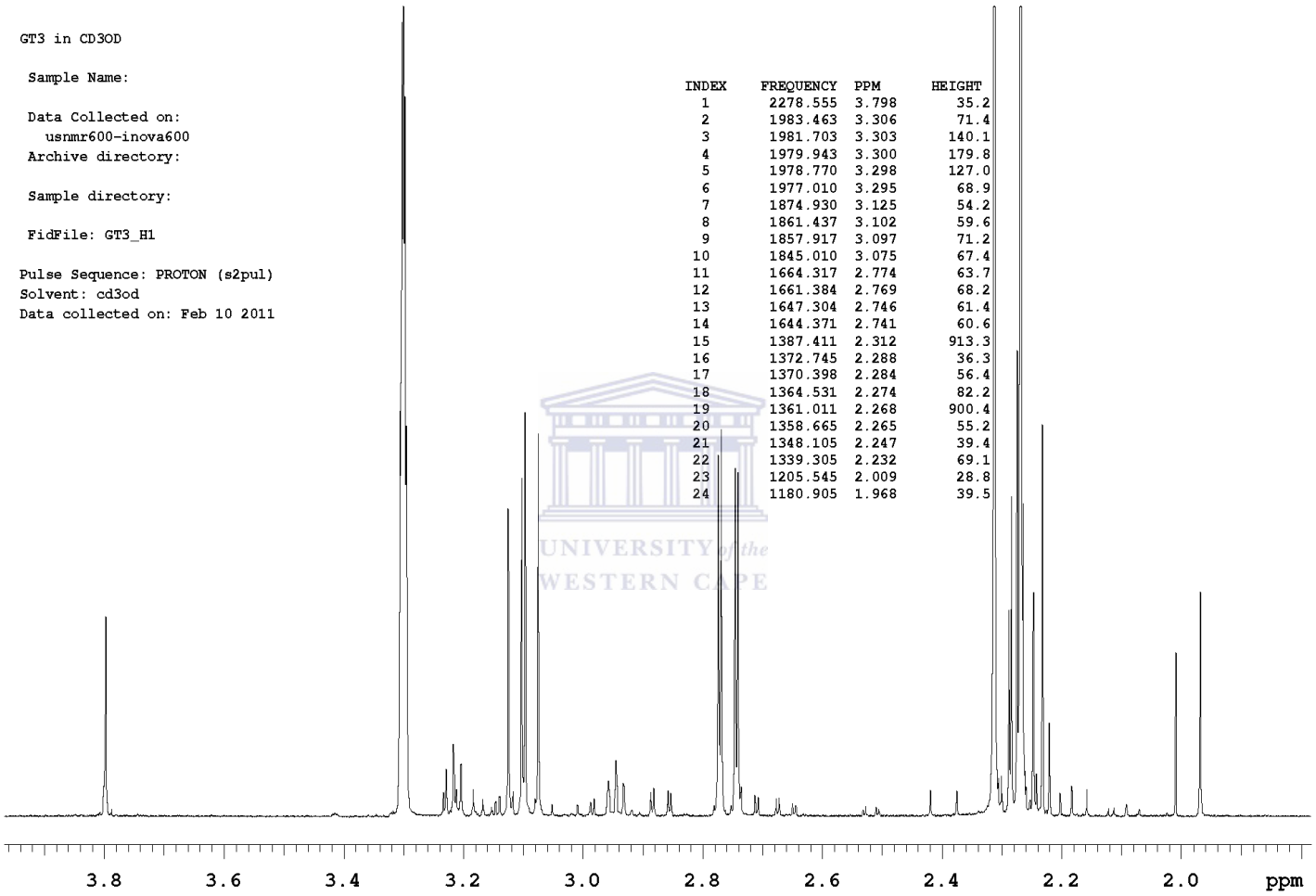
Sample directory:

FidFile: GT3_H1

Pulse Sequence: PROTON (s2pul)
Solvent: cd3od
Data collected on: Feb 10 2011

INDEX	FREQUENCY	PPM	HEIGHT
1	4499.669	7.500	40.7
2	4497.909	7.497	58.3
3	4496.149	7.494	18.7
4	4490.869	7.485	75.8
5	4452.736	7.421	26.0
6	4451.562	7.419	42.3
7	4449.802	7.417	16.8
8	4444.522	7.408	84.0
9	4442.762	7.405	38.9
10	4436.896	7.395	50.0
11	4423.989	7.374	35.1
12	4422.816	7.372	18.6
13	4419.296	7.366	13.3
14	4416.949	7.362	40.7
15	4409.323	7.349	14.1
16	4090.177	6.817	68.9
17	4087.830	6.813	91.5
18	3952.897	6.588	78.1
19	3951.137	6.585	56.6
20	3339.246	5.566	23.8
21	3336.312	5.561	26.4
22	3325.752	5.543	25.9
23	3322.819	5.538	24.8





Noesy Plots for Compound 3