Identification of anti-HIV compounds in *Helichrysum* species (Asteraceae) by means of NMR-based metabolomic guided fractionation

by

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Abstract

The plant kingdom contributes significantly to the natural products that are used for the treatment of a large number of ailments and disease across the globe. Included in these species is the *Helichrysum* genus (Asteraceae), which comprises of more then 600 species across Africa of which 244 species are found in South Africa. *Helichrysum* species are used in many cases for the treatment of coughs, colds, fever, infection, headaches, menstrual pain and are also very popular for wound dressing due to their potential antibacterial properties. The most common *Helichrysum* species used in traditional medicine and for several medicinal purposes are *H. cymosum*, *H.*

odoratissimum, *H. petiolare* and *H. nudifolium*. Previously published research has shown that several of the *Helichrysum* species do have antimicrobial activity with the most relevant to this study being the discovery of antiviral activity of *H. aureonitens* against herpes simplex virus type 1 (HSV-1) as well as the reports of anti-HIV (human immunodeficiency virus) activity of several *Helichrysum* species. With this knowledge, a more in-depth study was initiated to identify the possible active constituents in South African *Helichrysum* species against HIV. Due to the need to speed up drug discovery especially against epidemic diseases like HIV, this study investigated a new tool (nuclear magnetic resonance (NMR) – based metabolomics) to speed up drug discovery form natural products especially when anti-viral constituents are investigated.

In this study very promising anti-HIV results were obtained from several aqueous extracts (1:1 methanol/water) using a full virus model i.e. *Helichrysum populifolium* (IC_{50} 12 µg/ml), *H. appendiculatum* (IC_{50} 17 µg/ml), *H. cymosum* ssp. *clavum* (IC_{50} 19 µg/ml), *H. oxyphyllum* (IC_{50} 19 µg/ml) and *H. cymosum* ssp. *cymosum* (IC_{50} 21 µg/ml). With the use of NMR-based metabolomics and multivariate data analysis (MVA) the specific characteristic that differentiated the active extracts from the non-active extracts was identified by making use of Orthogonal Projections to Latent Structures – Discriminant Analysis (OPLS-DA). This characteristic was then used as a “blue print” or “fingerprint” to guide the process of fractionation and purification. *H. populifolium* showed the highest anti-HIV activity and thus was selected as the candidate extract for further analysis. After a very quick and simple chromatographic fractionation process, seven fractions were compared against the activity profile by making use of their NMR profiles, which then visually indicated which of the fractions had the highest similarity. Fraction 6 had the most similar “fingerprint”. The compounds of this active fraction were then identified with the use of liquid chromatography – ion trap – time of flight (LC-IT-TOF) for quick identification. The analysis revealed the presence of five chlorogenic type compounds, 3,4-dicaffeoyl quinic acid (DCQA), 3,5-DCQA, 4,5-DCQA, 1,3,5- tricaffeoyl quinic acid (TCQA) and 5-malonyl-1,3,4-TCQA of which several are well known to have anti-HIV

activity ranging from 0.85μM to 12μM. We were thus able to show with this study the possibility of using NMR-based metabolomics guided fractionation to guide the process of fractionation and identification from an active characteristic profile to the active constituents within the active *H. populifolium* extract.

Keywords: *Helichrysum*, Asteraceae, HIV, *Helichrysum populifolium*, metabolomics, caffeoyl quinic acid

Declaration

I declare that the thesis, which I hereby submit for the degree Philosophiae Doctor: Option Medicinal Plant Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: …….. DATE: ……..

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LIST OF ABBREVIATIONS

- HIV – human immunodeficiency virus
- AIDS – acquired immunodeficiency syndrome
- ARV – antiretroviral
- NCE – new chemical entity
- ADME – absorption, distribution, metabolism and excretion
- HAART – highly active antiretroviral therapy
- AZT – azidothymidine
- NMR – nuclear magnetic resonance
- UPLC – ultra high performance liquid chromatography
- LC-MS – liquid chromatography – mass spectrometry
- TOF – time-of-flight
- GC-MS – gas chromatography – mass spectrometry
- EI – electron impact
- KZN – KwaZulu-Natal
- EC – Eastern Cape
- FS – Free State
- WC – Western Cape
- NW – North West
- NC – Northern Cape
- TB – tuberculosis
- STD – sexually transmitted disease
- DCM – dichloromethane
- M/W – methanol/water
- HEK293T – human embryonic kidney 293 cells
- DMEM – Dulbecco's Modified Eagle's Medium
- FBS – fetal bovine serum
- IC_{50} – inhibitory concentration
- LC_{50} – lethal concentration
- OPNG – ortho-nitrophenyl-galactopyranoside
- OPN – ortho-nitrophenyl
- DNA - deoxyribonucleic acid
- DMSO - dimethyl sulfoxide
- TMS – tetramethylsilane

- TSP – trimethyl silane propionic acid sodium salt
- ppm – parts per million
- PCA – principal component analysis
- OPLS-DA – orthogonal projections to latent structures – discriminant analysis
- MVA – multivariate data analysis
- DOE – design of experiment
- HTS – high-throughput screening
- R&D – research and development
- TLC – thin-layer chromatography
- MeOH – methanol
- LC-IT-TOF – liquid chromatography – ion trap – time of flight mass spectrometry
- F6 – fraction 6
- DCQA – dicaffeoylquinic acid
- TCQA – tricaffeoylquinic acid
- M_r – molecular weight
- UV – ultra violet light
- HBV – hepatitis B virus

Chapter 1

Introduction

Chapter 1

Introduction

1.1. Background

1.1.1. Burden of disease: HIV and other Infectious disease

Infectious diseases are contributing immensely to the burden of disease in society. It is very important to take the global burden of disease into consideration when conducting research that is directed to address some of the issues surrounding this problem. In the latest "Global burden of disease study" (Lozano et al. 2012) the strain that infectious diseases place on society was tremendous with approximately 9.5 million people succumbing to infectious diseases in 2010. Even though this was a decrease of 12.7% from 1990 it is still a very serious problem to take note of. The trends in the 21st century that will contribute significantly to the large number of infectious diseases are the high mobility and interconnectedness of the world assisting in the spread of disease around the globe. This has been seen in the last few years since diseases spread much easier geographically then ever before (Heymann 2007). Combined with the infectious diseases spreading faster is also the factor of the quicker appearance of new emerging disease. In the period 1997 to 2007 approximately 40 new diseases had appeared that were not known before. The study also reported that in a five year period (2002 – 2007) 1 100 epidemic events occurred world wide (Heymann 2007).

The burden of infectious diseases can roughly be subdivided into the major infectious diseases as can be seen in Table 1.1. The increase in deaths due to AIDS is immense with an increase of 390.4 % in the last 20 years. Neglected diseases and as well as hepatitis have also shown to be on the increase at 9.2 and 46.4% respectively. Even though the other diseases and the total deaths by infectious diseases are showing

a decrease it is nothing to be excited about when nearly 10 million people still die due to infectious diseases. When it comes to children the picture is also horrific (Fig. 1.1). Infectious diseases in 2010 were responsible for more than 50% of all deaths in children between the ages of 1 and 4, which totalled nearly 1 million deaths (Lozano et al. 2012). In sub-Saharan Africa it is noteworthy that out of the top five most deadliest causes of premature deaths, four are due to infectious diseases of which AIDS is the biggest contributor and lower respiratory disease also contributing significantly.

Table 1.1: Summary of serious infectious diseases (adapted from Lozano et al. 2012).

	Deaths (x 1000)		
Disease	**1990**	**2010**	**Δ %**
AIDS	298.8	1 465.4	390.4
Tuberculosis	1 471.5	1 196	-18.7
Diarrhea and Lower Respiratory infections *e.g. Cholera, Rotavirus, Influenza, Meningitis, Measles, Tetanus etc.*	7 772.1	5 276.9	-32 .1
Neglected diseases *e.g. Malaria, Leishmaniasis, Dengue etc.*	1 210.6	1 321.8	9.2
Hepatitis	210.2	307.7	46.4
Total deaths due to infections	**10 963.2**	**9 567.8**	**-12.7**

When the top 10 causes of death in 2008, 2010 and 2011 are taken into consideration, AIDS is always a major contributor with death rates of 1.78 million in 2007 (Organization 2008), 1.47 million in 2010 (Lozano et al. 2012) and 1.70 million in 2011 (WHO 2012). It is further also estimated that 34 million people are living with HIV, which roughly makes out 0.8% of all adults in the world. In the study of Lozano et al. (2012) the change of the effect that AIDS had in 1990 compared to 2010 was once again made very evident as it was indicated that AIDS ranked at 35 of the deadliest diseases in 1990 and was the disease that saw the largest gain up to the 6th place in the 20 year period until 2010.

The new infections rates from 2001 to 2011 give a better and more positive outlook with new HIV infections decreasing from a high in 2001 at 2.4 million to 1.8 million in 2011, but with 1.8 million new infections it is still a long way to have this disease under control (Lozano et al. 2012).

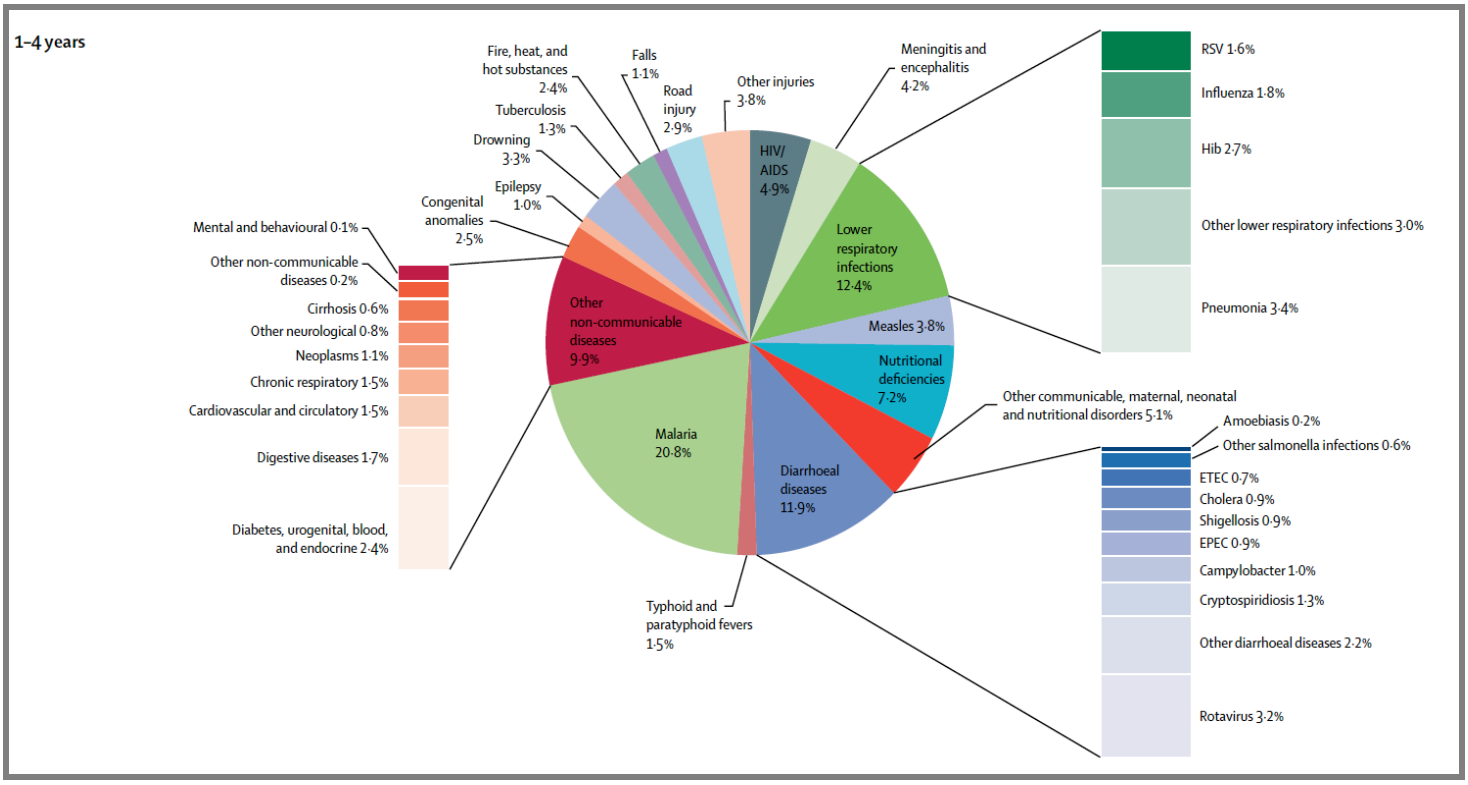

Fig. 1.1. Chart of global child deaths in 2010 by cause (adapted from Lozano et al. (2012).

Sub-Saharan Africa is the region with the most HIV infections, this being indicated by the fact that 1 out of every 20 adults is infected with the virus (prevalence of 5%) and with more then 90% of all children newly infected with the virus, residing in sub-Saharan Africa.

Due to this large prevalence in sub-Saharan Africa, there has been a very large effort to get as many people as possible on antiretroviral (ARV) medication, which has resulted in 8 million people using ARV's at the end of 2011. The use of ARV's has already contributed significantly to the level of "life years" added to low- and middle-

income countries. Since 1995 more then 14 million of these “life years” have been added of which 9 million were in sub-Saharan Africa. Together with these “life years” added, the use of ARV’s has shown to increase the prospects of employment which was supported by an example from KwaZulu-Natal, South Africa where it was revealed that infected patients using ARV’s were in a better position to apply and obtain employment. This thus shows the positive future that is possible with the current ARV treatment (WHO 2012).

It is thus evident that infectious diseases are still very prominent in society and that they will continue to play an immense role in the future. There are many medical interventions taking place across the world, with a large amount of different kinds of resources being widely distributed. Monitoring and evaluation systems are increasingly being employed to be able to keep track of infectious diseases worldwide and control the spread and occurrence of it more effectively.

Research and development on new chemical entities is a vital component of the arsenal that the research community needs to keep focusing on, as new drugs are very important to keep the spread of infections at bay. It is thus in this light that research in drug discovery form natural products needs to be highlighted as a source of new chemical entities for the future fight against infections. The focus of this study will be on the contribution that medicinal plants can make to this fight in the future.

1.1.2. Medicinal Plants

1.1.2.1. Overview of medicinal plants as therapeutics

The use of natural products for medicine has a long history that has delivered many new molecules to the medical field. Even with the huge swing in the last 25 years from natural to synthetic drug discovery and with a huge dip in funding experienced for natural product research in the period spanning 1984 to 2003 the numbers of patents

for natural product-derived, small-molecules have remained quite stable and thus natural products are still an undiminishing source of new pharmaceuticals (Koehn and Carter 2005). In the well-known study of Newmann and Cragg (2007) a huge emphasis was placed on the role of natural products in previous and current market drugs with new chemical entities discovered from natural resources contributing more then 25% when taking into account only natural and natural derived new chemical entities (NCE). It was also mentioned in this study that a specific group of NCE should not be disregarded, this group of molecules were the synthetic NCE that had a natural product-inspired pharmacophore and thus could also be seen as originating from a natural product. Natural products therefore do have an important role to play in the future of drug discovery.

The use of plants as medicine has been a practice even before 3 000 B.C. and is still being used up until today. Raskin and Ripoll (2004) emphasised in their study that since 3000 B.C. till today, medicinal plants have been documented by different nations throughout history. Some of the highlights mentioned by them were the Chinese emperor's book on medicinal plant use in approximately 2700 B.C., the documentation of Indian Ayurvedic herbal medicine, the Greek text of popular herbal medicine of 78 A.D., the well documented use of medicinal medicine by the European monasteries from 500 to 1200 A.D., the isolation of morphine by Wilhelm Serturner in 1803 A.D. and the discovery of Taxol in 1971. This extended history of the use of medicinal plants is a good witness to the fact that plants do possess the necessary potential to produce large amounts of NCE to fight the onslaught of disease in the world.

The large migration away from natural products and with that the move away from botanicals, was mostly due to six very important factors (Koehn and Carter 2005; Raskin and Ripoll 2004):

1) It was very difficult to interface high-throughput screening with the complex nature of botanical extracts.
2) The reproducibility of plant extract composition and with that the same chemical composition was very difficult to attain as these could change easily due to

numerous reasons e.g. environmental conditions, harvesting methods, geographical distribution etc.

3) With the use of high-throughput screening two factors started playing a big role
   i) The increase in the number of new targets that were available for screening on a regular basis prompting shorter drug discovery timelines and
   ii) The high number of good quality extracts needed for screening increased cost significantly.
4) With the isolation of compounds from complex plant extracts it is sometimes too difficult to attain a pure compound or significant amounts of active compounds.
5) Due to samples being of natural origin they need to be collected/cultivated and this brings in a factor of uncertainty as re-supplies can not necessarily be guaranteed.
6) The geopolitical factor that was introduced in 1992 by the signing of the Convention of Biological Diversity, which brought great uncertainty regarding the collection possibility of biological resources of production supply as well as the intrinsic costs associated with compulsory benefit sharing agreement legalities.

Most of these factors made big pharma reconsider their position regarding drug discovery from natural/botanical sources, and most of them chose the synthetic route. It is noteworthy to mention that from 2000 there has been an increase after a decade of slight decline in small-molecule natural-product patents granted (Koehn and Carter 2005).

In a study conducted by Jäger et al. (1996) they indicated that medicinal plants have played an important role in the primary healthcare system in South Africa. In the South African health care system between 70 – 80 % of the rural population consult traditional healers for the treatment of most ailments on a regular basis. This is due to the high cost of western medicine, traditional believes and also the lack of access to

modern health facilities. This threat has been seen in many developing countries in Africa, South America and Asia.

Traditional medicine or herbal medicine are not used in many instances on their own, but are frequently used in combination with western medicine as complimentary medicine and are used less frequently as alternatives to western medicine. This has broadened the user-base and has opened the possibility for research on traditional and herbal medicine, which can potentially offer a rich, and largely unexplored source of NCE for the pharmaceutical industry (Corson and Crews 2007; Iwu and Wootton 2002).

The study of traditional medicine or phytochemistry incorporates three main fields of research i.e. botany, chemistry and pharmacology. Phytochemistry (chemistry of plants) has been very well studied and has been developed in very close association with organic chemistry and with that has enhanced our knowledge of organic chemistry. The same cannot be said of pharmacology that has been associated with traditional medicine because many studies were not followed up with proper mode of action studies or any studies to understand the ADME (absorption, distribution, metabolism and excretion) of the biological active extracts or compounds. The absence of substantial pharmacological studies has thus led to many compounds with significant potential to be lost due to lack of this information (Iwu and Wootton 2002).

An advantage that medicinal plant extracts has over some single bullet – single target drugs, is that plant extracts potentially have a large number of biological active compounds in a single dose/treatment which could affect multiple pharmacological targets, thus increasing the clinical efficacy which is normally not attainable with single compound-based drugs. The use of complete extracts or combination of extracts (herbal mixtures) in many instances has shown to produce a synergistic effect (Williamson 2001). Synergistic effects can increase bioavailability of the main ingredient in a herbal remedy by assisting in transport or solubility, it could also assist in detoxifying certain by-products generated during treatment thus reducing side effects. Secondary compounds other than the major bio-active compound could also have a

different biological target, which could result in access being gained by the major compound to the site of interest (Schmidt et al. 2007; Williamson 2001). Having the potential of an effective multi-targeted medication together with the immense problem of prohibitive costs of health care in developing countries, show the urgent need for more research to find alternative drugs (Elanchezhiyan et al. 1993).

Plants synthesise a very large number of secondary metabolites (metabolites on which a plant does not depend for growth). In a resent study by Ripoll and Raskin (2004) they surveyed different databases and publications with regards to secondary metabolites and it was noteworthy that more than 200 000 secondary metabolites have been characterised in the 250 000 plant species on earth. This is believed to be only a small portion of all the secondary metabolites that exist in the plant kingdom on earth. Many of these secondary metabolites serve as biochemical intermediates or catabolites, internal signal stimuli, communication and defence mechanisms, but many if not most functions of these secondary metabolites still need to be discovered. The three major groups of secondary metabolites are alkaloids (nitrogen-containing), terpenoids and phenolics. Alkaloids have contributed the most to the NCE of the modern pharmacopeia with examples like quinine, vinblastine/vincristine, atropine, etc. Terpenoids contributed the very well known compounds digoxin, paclitaxel (Taxol) and artemisinin. Phenolics are known for their contribution of aspirin and podophyllotoxin to modern medicine (Cragg 1998; Damayanthi and Lown 1998; Dewick 2001; Raskin et al. 2002; Schmidt et al. 2007).

1.1.2.2. Medicinal plants as anti-HIV therapeutics

The human immunodeficiency virus (causative agent of AIDS) is a member of the lentivirus subfamily of the retroviruses. HIV can be subcategorised into HIV-1 and HIV-2 of which HIV-1 is the most pathogenic. With the extensive use of highly active antiretroviral therapy (HAART) that was introduced in 1996 the HIV virus was transformed from a rapid lethal infection to a chronic condition that can be managed for many years (Cos et al. 2008). Therapeutics against the virus are categorised into 10

different groups that correlate to the different life-stages of the virus (Fig. 1.2). The life stages can be summaried as follows: 1) virus cell adsorption, 2) virus-cell fusion, 3) uncoating, 4) reverse transcription, 5) integration, 6) DNA replication, 7) transcription, 8) translation, 9) budding and 10) maturation (Pomerantz and Horn 2003). Thus new medicinal therapeutics are categorised in these groups based on the mode of action that it displays.

Plant-derived compounds with activity against the HIV are quite elaborate and span all three groups of secondary metabolites. The study of Cos et al. (2008) was a very elaborate and well-documented summary of the use of plant-derived compounds against HIV. This study showed the potential of medicinal plants as a source of NCE in the fight against this very deadly virus. The diversity of the compounds that showed activity against the virus featured several alkaloids, some carbohydrates, coumarins, as well as phenolics and flavonoids, contributing a significant number of compounds with anti-HIV activity. In similar fashion, the study of Gambari and Lampronti (2006) also showed examples of a larger number of plants that have contributed to new NCE against HIV. They also highlighted that the potential of plant-derived compounds have the potential to exceed that of current drugs on the market like AZT (Ayisi and Nyadedzor 2003). These were very encouraging studies for phytochemists world-wide as it very clearly showed that phytochemical compounds as therapeutics against HIV is still alive and also very important to keep on supplying new molecules and new scaffolds in the fight against HIV and social-economical important viruses. Another study which reviewed some medicinal plants with activity against HIV found that the activity associated with medicinal plant extracts was very specific and linked to only a few plants (Gambari and Lampronti 2006). The specificity and diversity of molecules and mode of action that was very clearly shown in the study of Cos et al. (2008), asked the question on how to reduce the time of analysis from medicinal plant extract to pure compounds with the highest possible hit rate of compounds active against HIV. This study will attempt to address this specific question.

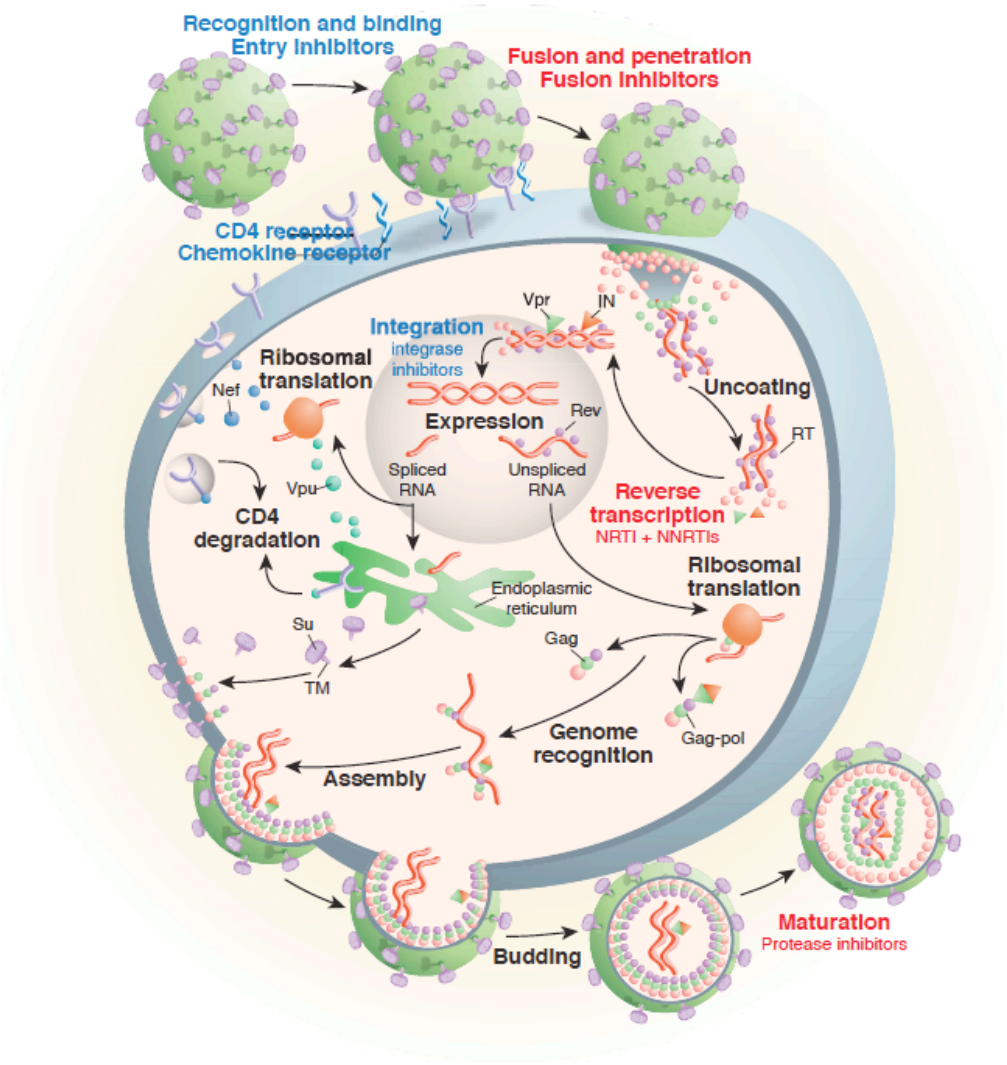

Fig. 1.2. An illustrative diagram of the HIV-1 life cycle. Current targets being inhibited by FDA approved therapeutics are indicated. The targets in blue could be inhibited by therapeutics that will likely be available in the near future (adapted from Pomerantz and Horn 2003).

The biological assays that are used to test for activity against HIV are very time consuming and very expensive at all levels of research. This thus excludes proper bioassay guided fractionation practises, as these will not make it economical for many research groups. Usual practise suggests that testing should be carried out on a specific target e.g. HIV reverse transcriptase, HIV integrase, etc. The numerous targets

available for testing of HIV then makes the testing very specific and introduces the risk of missing potential molecules that could have been active against other targets.

It is thus very important that a method is incorporated that analyse the medicinal plant extract holistically (as many compounds in the extract as possible) and at the same time can determine the effects of the medicinal plant extract on the complete, living HI virus. Analysis of this type will take the chemical properties of the medicinal plant extract into account from the beginning together with the effect of the extract against the HI virus. The advantage of such an approach will be that a faster link between the active ingredients within the extract and the mode of action of the HI virus could possibly be made. In this study NMR-based metabolomics was used as a holistic analysis method to search for anti-HIV actives in 64 *Helichrysum* plant extracts.

1.1.3. Metabolomics

Metabolomics is the study of all the metabolites in a sample with the aim to identify and quantify as many of them as possible. It has been mentioned several times before that the “holy grail” of plant metabolomics is to be able to gain a complete overview of the entire metabolome of a plant within a single snapshot analysis (or small series of analyses) – which is currently impossible due to the vast biochemical complexity of plant metabolites, but at the rate that new technologies are being developed and improved, we could in the future be pleasantly surprised (Hall 2006).

To understand metabolomics it is imperative that one understands all the different aspects of metabolite analysis in order to be able to differentiate metabolomics from the other methods. The following list provides an overview of all the relevant terms that are associated with metabolite analysis (Dettmer et al. 2007; Holmes et al. 2008):

- Metabolites: small molecules that play a role in general metabolism and that are needed for maintenance, growth and normal function of the organism/cell.
- Metabolome: collective term for all the metabolites in an organism.

- Metabolomics: identification and quantification of all the metabolites in an organism/cell/sample.
- Metabonomics: a holistic measurement approach of a dynamic metabolic response of a living system (body fluids) to biological stimuli or genetic manipulation (mostly used as a non-plant term).
- Metabolic profiling: quantitative analysis of a set of targeted metabolites of a specific class or in a selected biochemical pathway.
- Metabolic fingerprinting: an unbiased analysis in search of a "metabolite fingerprint" or pattern in response to any of several foreign influences e.g. disease, environmental changes, etc. to be able to identify adulterances or discriminating metabolites.
- Metabolic foot-printing: an analyses of the resulting extracellular environment due to metabolite extraction or uptake of cells/organism e.g. cell culture.

For any metabolomics analysis different techniques can be employed to be able to analyse samples and understanding the chemical complexity, metabolic heterogeneity and dynamic range of plant samples. For any metabolomics platform the important criteria to consider are, reproducibility, ease of quantification and identification, the time needed for analysis and sample preparation and the number of metabolites that can be detected (Verpoorte et al. 2007). The main techniques used in metabolomics that cover all these criteria will briefly be discussed to highlight the different aspects each of the techniques brings to the field of metabolomics as emphasised and summarised by Hall (2006).

1.1.3.1. Ultra high performance liquid chromatography (UPLC) and Liquid Chromatography – Mass Spectrometry (LC-MS)

High performance liquid chromatography (HPLC) or LC is relatively easy to perform and can be fully automated to give sensitive, selective and high-resolution data. For the identification of metabolites after proper separation, MS is added to give an extensive

overview of a sample (Tistaert et al. 2011). LC-MS has the capability of analysing large groups of secondary metabolites. The advances made and those that are still being made in chromatography technologies (UPLC – reduced analysis time up to a factor of eight with higher resolution and without the loss of data), as well as the advances made in column technology, have made it possible to significantly improve separation and analysis of complex plant samples. The use of modern LC techniques with especially high sensitive and accurate MS systems like time-of-flight (TOF) have been the examples of how complex metabolite mixtures of plants can be comprehensively analysed. Being able to also use both negative and positive modes in MS has significantly broadened the range of metabolites that can be analysed and also the time of analysis as separate analysis for the different modes have now been made redundant (Hall 2006).

With all the positive aspects that LC-MS brings to metabolomics, the biggest negative aspect that has been an active topic of research for many years is the lack of comprehensive libraries for LC-MS data, but this is receiving considerable attention in recent years (Hall 2006).

1.1.3.2. Gas Chromatography – Mass Spectrometry (GC-MS)

GC is very well known and used as a tool for comprehensive characterization and identification of volatile compounds and in combination with MS is a very powerful technique for the analysis of complex plant metabolite mixtures (Tistaert et al. 2011). The use of GC-MS has become very popular and has been seen to be the most popular global analysis method in this field. This is mostly due to its robustness both as separating technique and the use of the highly effective electron impact (EI) spectrometry technique. Putting these together with the excellent deconvolution and metabolite identification software makes it possible for complete metabolite identification and quantification. So it is understandable that GC-MS has developed into a technique that is widely used as a basic and general metabolomics tool (Kopka 2006). GC-MS has been primarily known to be applicable for the analysis of volatile metabolites

(temperatures of up to 300°C), but through chemical derivatisation non-volatile and polar metabolites can be analysed. After derivatisation, plant samples can then be analysed by GC-MS to obtain detailed metabolic information on specific metabolite content of the plants sample (Hall 2006).

Research groups are in many cases motivated to use GC-MS as a metabolomic tool due to the relative low cost of the GC-MS facility when compared to the expensive LC-MS and even more so the NMR and LC-NMR. Secondly the superior chromatographic resolution, versatility and reproducibility as well as the accurate fingerprinting capabilities of the EI MS all add to the decisions to acquire a GC-MS as metabolomic tool. The most significant drawback that is experienced with the GC-MS as a tool for metabolomics is the requirement in many instances for chemical derivatisation prior to analysis (Kopka 2006) which extends analysis time and the possibility of operator error. Another very important aspect in GC-MS analysis is that the compounds that are to be tested, should be stable at high temperatures, which adds to the undesirable aspects of GC-MS (Schripsema 2010). GC-MS analysis is limited to the analysis of mostly molecules that are in a small range of polarity and molecular weight, thus limiting the analysis of complex structures like glycosides (Verpoorte et al. 2008).

1.1.3.3. Nuclear Magnetic Resonance (NMR)

The use of NMR as a tool in metabolomics has been used extensively with huge success in many different kind of projects. In the phytochemistry review of Verpoorte et al. (2008) the advantages that NMR has over chromatography and MS-based methods was clearly indicated. It provides very rapid and exhaustive analysis of the bio-molecular composition of a crude plant extract. Sample preparation associated with NMR analysis is very simple, thus reducing operator-handling error. The modern NMR equipment also have been improved for more sensitivity which then enables it to serve better as an universal detector in which the signals detected give undeviating structural information of the molecules in the plant extract. The NMR spectrum is a product of the physical character of a compound/extract, which means that if the exact same

procedure and the same NMR solvent in one experiment is used, the second experiment will give a duplication of the previous results. This major advantage that NMR metabolomics has provides data that will last a life-time and which will be available for data-mining for a long time to come (Verpoorte et al. 2008).

NMR based metabolomics in plant analysis has in many instances played second fiddle to the MS-based methods as it has much poorer sensitivity. One of the negative aspects of NMR based metabolomics is that the number of compounds detected in a single run are limited to a few dozen at most, whereas with MS based methods the numbers are much higher. The use of NMR analysis has been the favour of choice for the analysis of mostly the major metabolites in a sample and this thus resulted in many other important compounds not being detected due to the low NMR detection threshold. To counter this limitation modern instrumentation and instrumentation design have been focussed to increase sensitivity (e.g. cryo-probes, field strengths, etc.), this together with improved databases (currently under development) will probably prove to be a big turning point for NMR based metabolomics (Hall 2006; Schripsema 2010; Verpoorte et al. 2007; Verpoorte et al. 2008).

Even though the sensitivity of the NMR analysis is very low, the added advantage like structural information, reproducibility and quantification aspects that accompanies NMR analysis, will possibly lead to NMR based metabolomics to become more popular in future for most metabolomic applications. It was thus on this basis that NMR based metabolomics was used in this study to search for anti-HIV constituents in the complex 64 *Helichrysum* extracts.

1.2. Objectives

- To determine if the selected *Helichrysum* species have anti-human immunodeficiency virus (HIV) activities.

- To determine the differences in metabolite profile of 30 *Helichrysum* species and one infraspecific species.

- To investigate the relationship between the metabolite profiles of 64 selected *Helichrysum* plant extracts and their anti-HIV activity using nuclear magnetic resonance (NMR) - based metabolomics and identifying co-occurrence patterns and distinct differences between the different *Helichrysum* species in relation to their activity.

- To use a metabolomic contribution plot profile to guide the purification and identification of the active compounds from the most active *Helichrysum* extract.

1.2.1. *Hypothesis*

NMR-based metabolomic guided fractionation can be used to identify and isolate active constituents from complex *Helichrysum* plant extracts and speed up drug discovery.

1.3. Structure of thesis

This study comprised of an investigation to find an easier and quicker way to correlate the bio-activity of a medicinal plant extract to the constituents within the extract responsible for the activity. Due to HIV bioassays being very time-consuming and expensive, analysing large quantities of plant samples through this bioassay is not feasible for most drug discovery research laboratories. Faced with this problem, it was necessary to find a way to reduce the time and resources spend on the bioassay.

With the use of NMR-based metabolomics and multivariate data analysis software it was possible to analyse 30 *Helichrysum* species and one infraspecific species in search of bio-active constituents in the active plant extracts. NMR data of the *Helichrysum* spp. were analysed in conjunction with bio-activity results to obtain a NMR contribution

profile correlating the specific chemical character of the active constituents responsible for the activity. Using the NMR contribution profile it was possible to narrow down and focus the purification and isolation process, which then led to four molecules which were known for their anti-HIV properties. Though this is the beginning of the road, this study indicated that metabolomics might be used to speed up the process of identifying, purifying and isolating bio-active constituents.

Chapter 1: The introductory chapter consists of the general background of natural products and also medicinal plants, as a source of therapeutics. Metabolomics is also defined and a brief view in the analytical capabilities used in metabolomics is given.

Chapter 2: This chapter entails a detailed discussion of all 30 *Helichrysum* species and one infraspecific species. The chapter covers aspects of geographical distribution, morphology, traditional use and previous phytochemical discoveries.

Chapter 3: This chapter covers the screening of 64 *Helichrysum* spp. extracts for their bio-activity against the human immunodeficiency virus (HIV). The chapter covers the background of the virus, the DeCIPhR bioassay and the results of the NMR-based metabolomic comparison study between all the different extracts.

Chapter 4: In this chapter the use of NMR-based metabolomics as a method to guide the fractionation process is described. With the use of the contribution plot from chapter 3, it was possible to be guided through the purification process and to ultimately identify five compounds of which four where known to have potent anti-HIV activity. As far as we know all compounds have been isolated for the first time from *H. populifolium.*

Chapter 5: This chapter summarises the whole study and gives a general discussion and conclusions of the study.

Chapter 6: Acknowledgements are made in this chapter.

Chapter 7: The Appendix includes all the additional NMR used throughout this study.

1.4. References

Ayisi, N. K. & Nyadedzor, C. (2003). Comparative in vitro effects of AZT and extracts of *Ocimum gratissimum*, *Ficus polita*, *Clausena anisata*, *Alchornea cordifolia*, and *Elaeophorbia drupifera* against HIV-1 and HIV-2 infections. Antiviral Research 58, 25-33

Corson, T. W. & Crews, C. M. (2007). Molecular understanding and modern application of traditional medicines: triumphs and trials. Cell 130, 769-74 doi: 10.1016/j.cell.2007.08.021

Cos, P., Maes, L., Vlietinck, A. & Pieters, L. (2008). Plant-derived leading compounds for chemotherapy of human immunodeficiency virus (HIV) infection - an update (1998 - 2007). Planta Medica 74, 1323-37 doi:10.1055/s-2008-1081314

Cragg, G. M. (1998). Paclitaxel (Taxol): a success story with valuable lessons for natural product drug discovery and development. Medicinal Research Reviews 18, 315-31

Damayanthi, Y. & Lown, J. W. (1998). Podophyllotoxins: current status and recent developments. Current Medicinal Chemistry 5, 205-52

Dettmer, K., Aronov, P. A. & Hammock, B. D. (2007). Mass spectrometry-based metabolomics. Mass Spectrometry Reviews 26, 51-78 doi: 10.1002/mas.20108

Dewick, P. M. (2001). The mevalonate and deoxyxylulose phosphate pathways: terpenoids and steroids medicinal natural products. John Wiley & Sons, Ltd. pp. 167-289

Elanchezhiyan, M., Rajarajan, S., Rajendran, P., Subramanian, S. & Thyagarajan, S. P. (1993). Antiviral properties of the seed extract of an Indian medicinal plant,

Pongamia pinnata, Linn., against herpes simplex viruses: *in-vitro* studies on Vero cells. Journal of Medical Microbiology 38, 262-4

Gambari, R. & Lampronti, I. (2006). Inhibition of immunodeficiency type-1 virus (HIV-1) life cycle by medicinal plant extracts and plant-derived compounds. In: Mahmud, T. H. K., A. Arjumand (eds) Advances in Phytomedicine. Volume 2. Elsevier. pp. 299-311

Hall, R. D. (2006). Plant metabolomics: from holistic hope, to hype, to hot topic. New Phytologist 169, 453-68 doi: 10.1111/j.1469-8137.2005.01632.x

Heymann, D. L. (2007). The World Health Report 2007: Global Public Health Security in the 21st Century, WHO.

Holmes, E., Wilson, I. D. & Nicholson, J. K. (2008). Metabolic phenotyping in health and disease. Cell 134, 714-717

Iwu, M. M. & Wootton, J. (2002). Ethnomedicine and drug discovery, Elsevier Science.

Jäger, A. K., Hutchings, A. & van Staden, J. (1996). Screening of Zulu medicinal plants for prostaglandin-synthesis inhibitors. Journal of Ethnopharmacology 52, 95-100 doi: http://dx.doi.org/10.1016/0378-8741(96)01395-5

Koehn, F. E. & Carter, G. T. (2005). The evolving role of natural products in drug discovery. Nature Reviews Drug Discovery 4, 206-20 doi: 10.1038/nrd1657

Kopka, J. (2006). Current challenges and developments in GC–MS based metabolite profiling technology. Journal of Biotechnology 124, 312-322 doi: http://dx.doi.org/10.1016/j.jbiotec.2005.12.012

Lozano, R., Naghavi, M., Foreman, K., Lim, S., Shibuya, K., Aboyans, V., Abraham, J., Adair, T., Aggarwal, R., Ahn, S. Y., AlMazroa, M. A., Alvarado, M., Anderson, H.

R., Anderson, L. M., Andrews, K. G., Atkinson, C., Baddour, L. M., Barker-Collo, S., Bartels, D. H., et al. (2012). Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. The Lancet 380, 2095-2128

Pomerantz, R. J. & Horn, D. L. (2003). Twenty years of therapy for HIV-1 infection. Nature Medicine 9, 867-73 doi: 10.1038/nm0703-867

Raskin, I., Ribnicky, D. M., Komarnytsky, S., Ilic, N., Poulev, A., Borisjuk, N., Brinker, A., Moreno, D. A., Ripoll, C., Yakoby, N., O'Neal, J. M., Cornwell, T., Pastor, I. & Fridlender, B. (2002). Plants and human health in the twenty-first century. Trends in Biotechnology 20, 522-31

Raskin, I. & Ripoll, C. (2004). Can an apple a day keep the doctor away? Current Pharmaceutical Design 10, 3419-29

Schmidt, B. M., Ribnicky, D. M., Lipsky, P. E. & Raskin, I. (2007). Revisiting the ancient concept of botanical therapeutics. Nature Chemical Biology 3, 360-366

Schripsema, J. (2010). Application of NMR in plant metabolomics: techniques, problems and prospects. Phytochemical Analysis 21, 14-21 doi: 10.1002/pca.1185

Tistaert, C., Dejaegher, B. & Heyden, Y. V. (2011). Chromatographic separation techniques and data handling methods for herbal fingerprints: A review. Analytica Chimica Acta 690, 148-161 doi:http://dx.doi.org/10.1016/j.aca.2011.02.023

Verpoorte, R., Choi, Y. H. & Kim, H. K. (2007). NMR-based metabolomics at work in phytochemistry. Phytochemistry Reviews 6, 3-14 doi: 10.1007/s11101-006-9031-3

Verpoorte, R., Choi, Y. H., Mustafa, N. R. & Kim, H. K. (2008). Metabolomics: back to basics. Phytochemistry Reviews 7, 525-537 doi:10.1007/s11101-008-9091-7

WHO (2008). The top 10 causes of death. Fact sheet No. 310. In: Update October 2008. http://www.who.int/entity/mediacentre/factsheets/fs310_2008.pdf Accessed 12 April 2013 2013

WHO (2010). Global report: UNAIDS report on the global AIDS epidemic 2010. Geneva. WHO.

WHO (2012). 2012 UNAIDS Report on the Global AIDS Epidemic. Geneva. WHO.

Williamson, E. M. (2001). Synergy and other interactions in phytomedicines. Phytomedicine 8, 401-9 doi:10.1078/0944-7113-00060

Chapter 2

Helichrysum species selected

Chapter 2

Helichrysum species selected

2.1. Introduction

This study investigated 30 species and one infraspecific species in the *Helichrysum* genus (Table 2.1). This genus is part of the Asteraceae family (Inuleae tribe and Gnaphaliinae sub-tribe), which is known to be the largest family of flowering plants. It has over 1600 genera and consists of more then 23 000 species of which many are well known for their importance as medicinal, ornamental and/or economical uses (Wang et al. 2004). Of these 23 000, 300 are known to be used for their medicinal use in China of which *Artemisia annua* is probably the most renowned plant species for its active ingredient, artemisinin, a very potent antimalarial drug (Pigott et al. 2012). The *Helichrysum* genus is known to be a prolific producer of secondary metabolites (Appendino et al. 2007; Jakupovic et al. 1986).

The *Helichrysum* genus is a large genus with between 500 - 600 species residing on the African continent including the island of Madagascar. Of these, 240 - 250 species occur in southern Africa of which approximately 70 - 80 species are found in the collection sites of this study (Fig. 2.2.), Ukhahlamba Drakensberg Mountains in KwaZulu-Natal, south-eastern KZN coastline and in the Eastern Cape (EC) region of South Africa. Due to the massive morphological diversity the genus was subdivided into 30 morphological groups based on the shape and the size of the flower heads (Hilliard 1983; Pooley 1998; Pooley 2003).

As stated earlier, the *Helichrysum* genus, and specifically the southern African *Helichrysum* genus, has proven to be an enormous source of novel and interesting secondary metabolites as can be seen in the in-depth phytochemical analyses of Bohlmann et al. in the 70's and 80's (Bohlmann and Abraham 1979a; Bohlmann and

Abraham 1979b; Bohlmann and Abraham 1979c; Bohlmann et al. 1980a; Bohlmann and Zdero 1979; Bohlmann et al. 1980b; Bohlmann et al. 1978b; Bohlmann et al. 1979a). The review study done by Lourens et al. (2008) also indicated the extensive traditional use of 73 species of the genus. *Helichrysum* species occur as herbs and shrubs and the leaves and flowers usually have a pleasant odour of which curry is a most prominent scent in many of the species (Appendino et al. 2007; Pooley 2003).

Table **2.1:** *Helichrysum* species selected for extraction

Selected plants	Abbreviation	Author			PRU* voucher no.
H. acutatum	HACU	DC.			117098
H. allioides	HALL	Less.			117113
H. anomalum	HANO	Less.			117112
H. appendiculatum	HAPP	(L.f.) Less.			117101
H. aureonitens	HAU-1	Sch. Bip.			117111
H. cephaloideum	HCEP	DC.			117127
H. chionosphaerum	HCHI	DC.			117097
H. confertum	HCON	N.E. Br.			96720
H. cymosum	HCCY	(L.) D.Don	ssp.	*cymosum*	117120
H. cymosum	HCCL	Hilliard	ssp.	*clavum*	117142
H. difficile	HDIF	Hilliard			117122
H. drakensbergense	HDRA	Killick			117121
H. herbaceum	HHER	(Andrews) Sweet			117099
H. melanacme	HMEL	DC.			117107
H. miconiifolium	HMIC	DC.			117102
H. natalitium	HNAT	DC.			117669
H. nudifolium	HNUN	(L.) Less.	var.	*nudifolium*	117104
H. odoratissimum	HODO	(L.) Sweet			117106
H. oreophilum	HOR-1	Klatt			117096
H. oxyphyllum	HOXY	DC.			117670
H. pallidum	HPAL	DC.			117108
H. panduratum	HPAN	O. Hoffm			117662
H. pannosum	HPANA	DC.			117144
H. pilosellum	HPIL	(L.f.) Less.			117110
H. pilosellum†	HNUP	(L.f.) Less.			96717
H. populifolium	HPOP	DC.			117138
H. rugulosum	HRUG	Less.			117114
H. splendidum	HSPL-1	(Thunb.) Less.			117124
H. subluteum	HSUB	Burtt Davy			117123
H. sutherlandii	HSUT	Harv.			117115
H. umbraculigerum	HUMB	Less.			117100
H. vernum	HVER	Hilliard			117116

* H.G.W.J. Schweikerdt Herbarium of the University of Pretoria

† Earlier classification was uncertain, but after further analysis this species was reclassified as *H. pilosellum* (HNUP).

In this metabolomic study, 30 *Helichrysum* species and one infraspecific species were collected mostly in the Ukhahlamba Drakensberg Mountains in KwaZulu-Natal (Fig. 2.2.), south-eastern KZN coastline and some being collected in the EC region of South Africa. In the search for anti-human immunodeficiency virus (anti-HIV) activity, non-polar and polar extracts of these species were made and using the new approach, NMR-based metabolomics guided fractionation; the extracts were tested against the full HI virus. The selected species are described below with regard to geographical distribution, morphology and traditional use and previous phytochemical discoveries, where available. The excellent review of Lourens et al. (2008) of *Helichrysum* species in South Africa was extensively used for the description of the species in the current study.

After collection of the plants a preliminary identification was made for one of the *H. pilosellum* plants due to uncertainty regarding its morphology. This plant was only later correctly reclassified as *H. pilosellum* (HNUP), this resulted in a duplication of this species throughout the study. As these two plants of the same species were collected in two different geographical sites, the results of the chemical variation could still be interesting and was thus not removed.

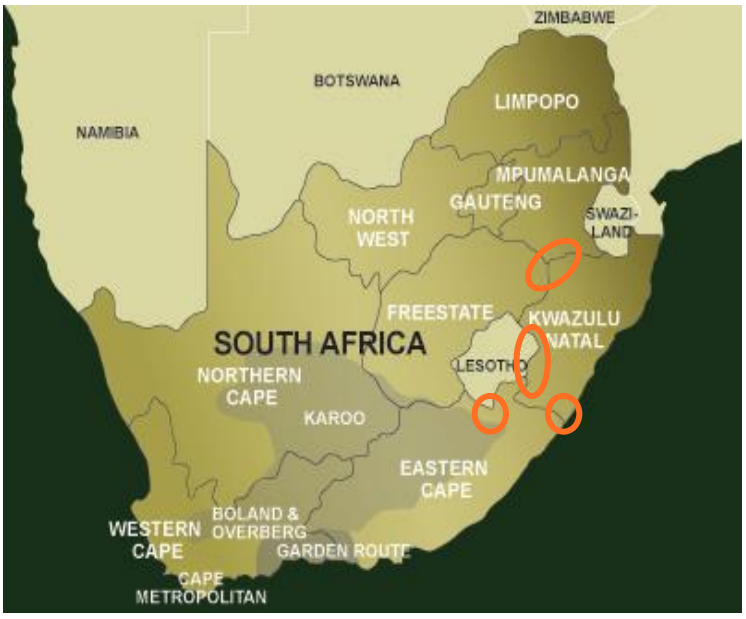

Fig. 2.1. Map with this study's plant collection sites.

Fig. 2.2. Plant collection in the Ukhahlamba Drakensberg Mountains in KwaZulu-Natal.

2.1.1. *Helichrysum acutatum* DC. (HACU)

This silvery grey perennial herb is woody and has woolly hairs on flowering stems and leaf margins. The name *acutatum* is a reflection of the sharply pointed bracts that this species has. It can be found in Mpumalanga, southern KZN northern Limpopo and Swaziland in rocky grasslands up to 2000m above sea level. Flowering of *H. acutatum* occurs from September to January (Hilliard 1983; Pooley 2003).

H. acutatum cannot be linked to any specific use but it is used widley and is sold commercially for the use in traditional medicine. This plant is known to have produced isolated compounds like terpenoids, flavonoid derivatives and the compounds pinocembrin and pinocembrin chalcone, which have been shown to have activity against *Staphyloccocus* bacteria (Bohlmann and Abraham 1979a). Isolated compounds are illustrated in Figure 2.3.

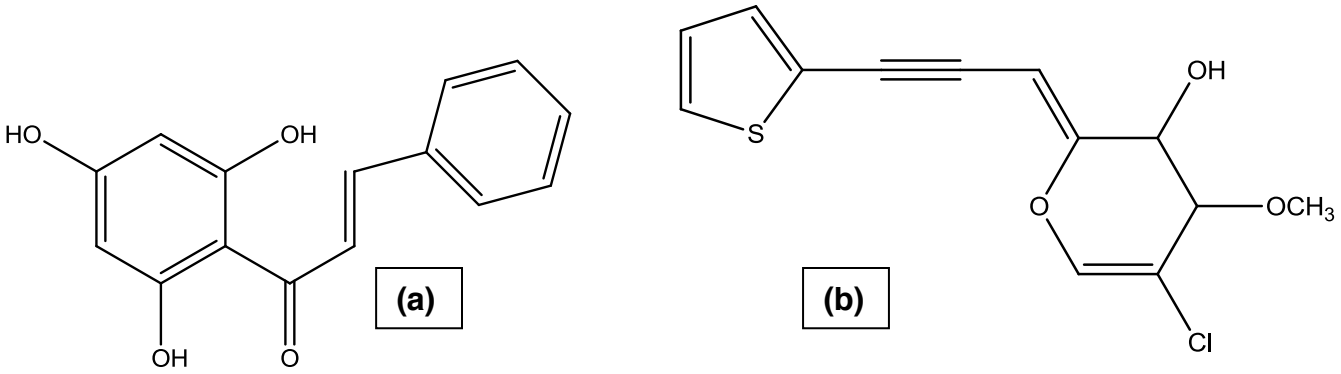

Fig. 2.3. A (a) 2,4,6-trihydroxychalcone and a (b) chloronated acetylenic thiophene isolated form *H. acutatum* (Bohlmann and Abraham 1979a).

2.1.2. *H. alliodes* Less. (HALL)

This plant is also a perennial herb that is woody with thin, long leaves and solitary flowering stems. Leaf surfaces are very woolly with the lower surface having more hairs than the upper surface. The plant is found in colonies in Mpumalanga and KZN, down the coast all the way through to the central EC, and sometimes also in the eastern parts of the Free State (FS), usually in open grasslands up to 1250m above sea level. Flowering occurs from August to December (Hilliard 1983; Pooley 2003).

No biological activity has been reported for this species, but there has been the isolation of an acetylenic compound shown below in Figure 2.4.

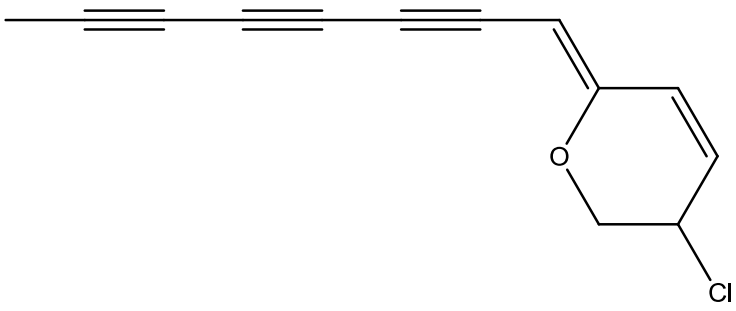

Fig. 2.4. A chloroenolether polyine isolated form *H. alliodes* (Rauter et al. 2005).

2.1.3. *H. anomalum* DC. (HANO)

This species is a dwarf shrub that is found in the eastern parts of the Western Cape (WC), the EC, southern KZN and Swaziland. It usually grows in rocky areas or rough grassland and flowering occurs between September and May. Leaves are reduced and grey woolly hairs cover the entire surface area of the leaves (Hilliard 1983).

This species is known to contain phloroglucinol derivatives as well as a ∝ humulene compound in its aerial parts, there are no reports of any biological activity from this species (Jakupovic et al. 1989).

2.1.4. *H. appendiculatum* (L.F.) Less (HAPP-1)

H. appendiculatum is a thick, woody perennial herb with woolly hairs on the upper and lower surfaces of the leaves. The plant is distinguished from other species by its dull involucral bracts. The flower-heads form compact clusters and have a branched inflorescence. Flowering of *H. appendiculatum* (Fig. 2.5) takes place from December to February. The plant has a wide distribution and can be found in Limpopo, Mpumalanga, Lesotho, Swaziland, KZN, FS, and the Eastern and WC (Hilliard 1983; Pooley 1998).

In traditional medicine the leaves of this species are eaten raw to treat chest problems or infections of the respiratory tract. The whole plant is used to treat smallpox and parasitic worms, while the roots are used to treat colds and coughs. Roots are also ground and burnt and smeared on the body to reduce swelling. Leaves are used as wound dressings by rubbing ground leaves onto areas of the wound or cramps. The leaves are also used as a tea drunken for several ailments (Arnold et al. 2002; Watt and Breyer-Brandwijk 1962). In a metabolomic study done by Heyman (2009), *H. appendiculatum* 50% methanolic extract showed significant anti-HSV (herpes simplex virus) activity at 50 ug/ml (cytopathic effect (CPE)).

Fig. 2.5. *H. appendiculatum* in Cathedral Peak area in the Drakensberg Mountains.

Nine different oils have also been isolated from this species which are: 8α-hydroxy-α-gurjunene, 8α-acetoxy-α-gurjunene, 14-gnaphaladien-8-ol, 1-methyl-4-(1,5,9-trimethyl-4,8-decadienyl)-1,3-cyclohexadiene, helinudichromene quinone, helinudifolin (Fig. 2.6a), helinudiquinone (6-Me ether), helinudiquinone (Fig. 2.6b) and isocomen-5-one (Jakupovic et al. 1986).

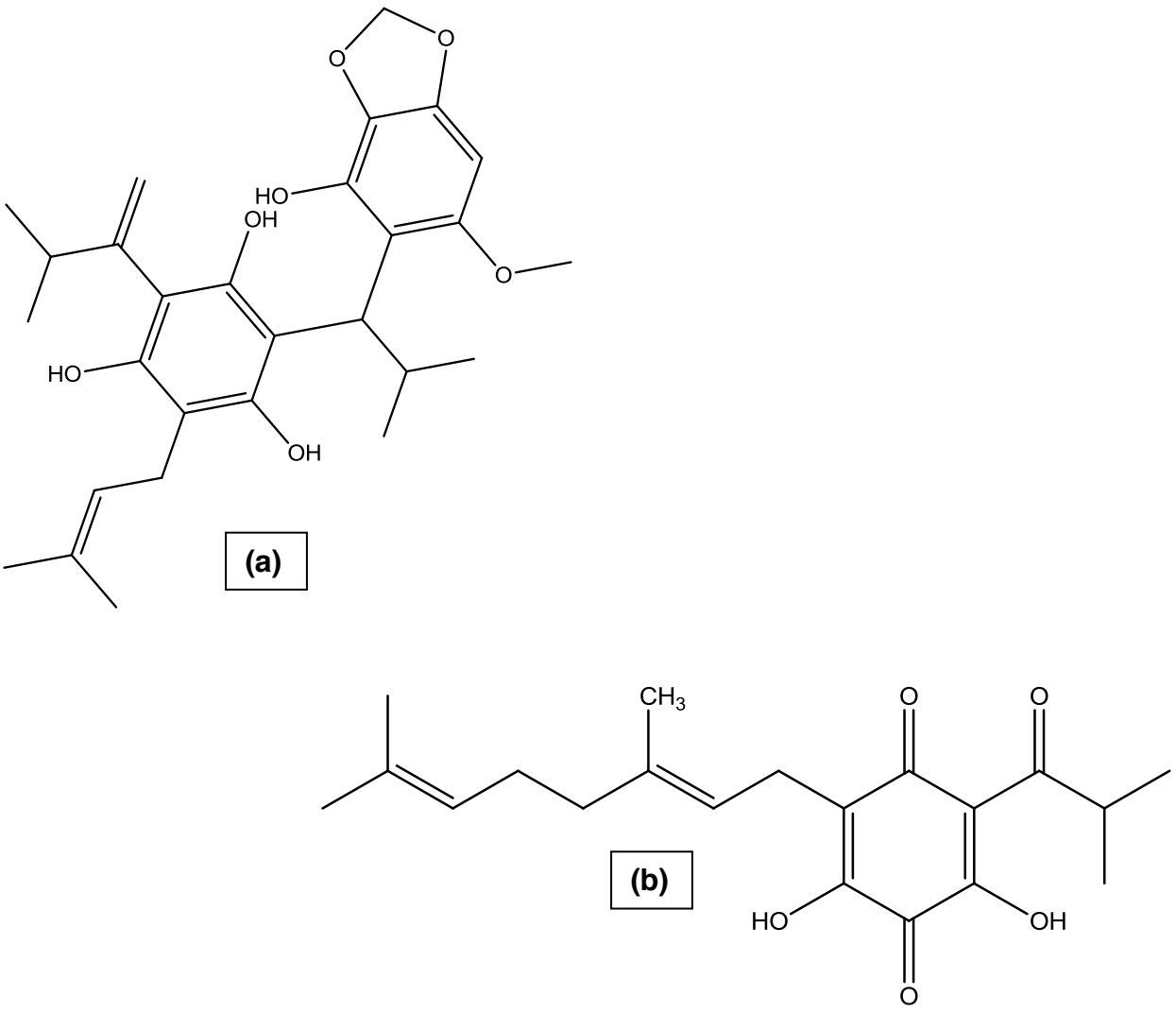

Fig. 2.6. a) Helinudifolin and b) helinudiquinone etc.

2.1.5. *H. aureonitens* Sch. Bip. (HAU-1)

This golden everlasting, as it is also known, is found in Limpopo, the North West Province (NW), Gauteng, Mpumalanga, FS, KZN and northern parts of the EC. Flowering takes place from September to February and woolly hairs are present on both surfaces of leaves. Involucral bracts are present and may range from brown to yellow. These plants prefer damp grassland, has a grey colour and are very widespread (Hilliard 1983; Pooley 1998), but care should be taken not to confuse it with *H. psilolepis,* which looks very similar.

In traditional medicine this plant's leaves and stems are burnt as an insence to induce trances. Leaves and stems are also used to control urination in children, to treat skin infections like herpes simplex and to treat microbial infections since it has antibacterial and antifungal activities (Afolayan and Meyer 1997; Hutchings et al. 1996; Mathekga and Meyer 1998; Mathekga 2001; Meyer et al. 1996; Pooley 1998; Pooley 2003). Terpenes have mostly been isolated from *H. aureonitens*, but also aninteresting tetrahydrofuran derivative shown in Fig. 2.7a (Bohlmann and Ziesche 1979). There has also been the isolation of the antimicrobial and antiviral compound known as galangin from this species, shown in Fig. 2.7b below (Afolayan and Meyer 1997). In other studies galangin was reported to be active against resistant *Staphylococcus aureus* strains as well as having anti-fungal activity, thus supporting the use of *H. aureonitens* extracts for skin infections (Mathekga 2001).

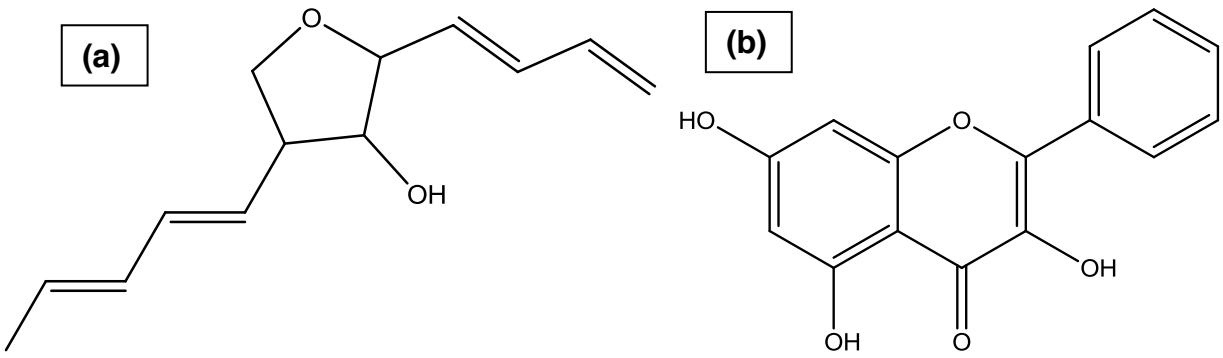

Fig. 2.7. The chemical structure of a a) tetrahydrofuran derivative and b) galangin isolated from *H. aureonitens* (Afolayan and Meyer 1997; Bohlmann and Ziesche 1979).

2.1.6. *H. cephaloideum* DC. (HCEP)

This woody perennial herb has loosely scattered grey hair on the upper and lower surfaces of leaves. Its inflorescence is compact and roundish and can reach 30mm in width which are normally webbed together at the base with wool (Pooley 2003). Its flowering season is from November to May. It is usually found in grasslands in Limpopo, NW, KZN, Mpumalanga, the EC, Swaziland and Lesotho (Hilliard 1983).

This plant is known to be very poisonous to sheep (Watt and Breyer-Brandwijk 1962). It contains several flavonols, a few terpene compounds and numerous phloroglucinol derivatives (Fig. 2.8), isolated from its aerial parts and roots.

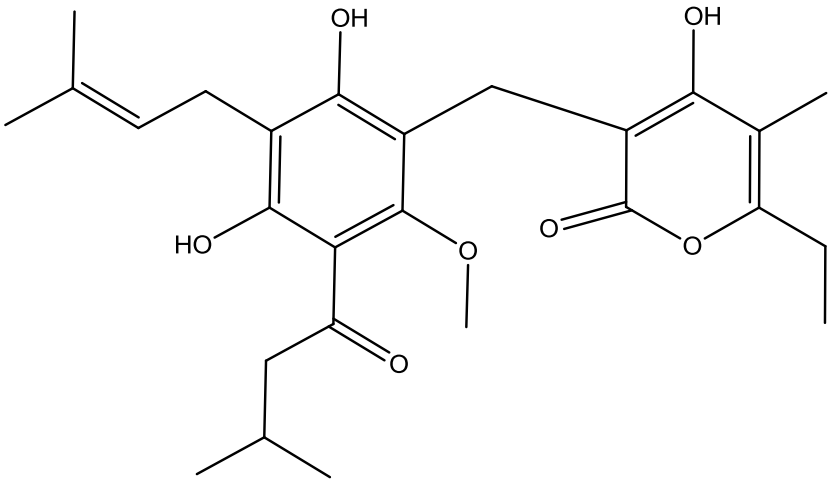

Fig. 2.8. A phloroglucinol deriviative with a pyrone moeity isolated form *H. cephaloideum* (Hänsel et al. 1980).

2.1.7. *H. chionosphaerum* DC. (HCHI)

H. chionosphaerum also known as the dwarf everlasting, is a shrub that grows horizontally, covering the ground surface, containing many stems and branches that have white or silver woolly hairs giving it a silky appearance. These plants occur in stony grassland areas in Limpopo, Mpumalanga, KZN, Swaziland, Lesotho and the northern parts of the EC (Hilliard 1983; Pooley 1998).

In this species mostly diterpene and other terpenes have been isolated (Fig. 2.9) in the studies of Bohlmann et al. (1980a) and Jakupovic et al. (1989).

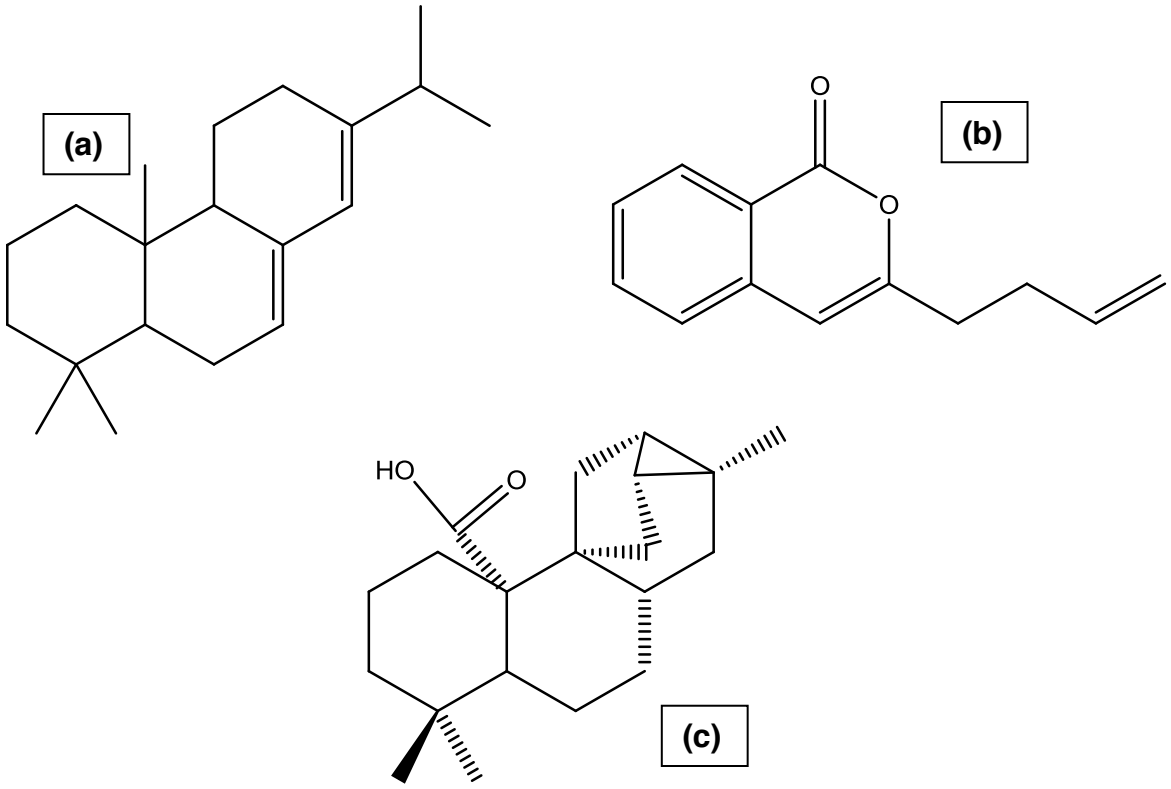

Fig. 2.9. A a) abieta-7,13-diene, b) artemedin and c) helifulvanic acid isolated from *H. chionosphaerum* (Bohlmann et al. 1980a).

2.1.8. *H. confertum* N.E. Br. (HCON)

This species is a dwarf shrub that is well branched and has leaves that appear grey with woolly hairs only on the upper surfaces. Flowering occurs from July to September and these plants can be found only close to the Drakensberg Mountains in KZN and near Lesotho close to the border of the EC and usually on cliff faces at altitudes of between 1800 and 3000m (Hilliard 1983; Pooley 1998).

In this species only a few diterpene compounds in its roots have been isolated (Bohlmann et al. 1978b).

2.1.9. *H. cymosum* sp. *calvum* Hilliard (HCCL)

This subspecies differs from *H. cymosum sp. cymosum* in that it has narrower leaves and the branches are more densely clustered with its flower-heads being smaller than that of *H. cymosum* sp. *cymosum*. Its glabrous ovaries usually distinguish it, and it occurs more specifically in the EC and in KZN. Flowering coincides with its subspecies cousin starting in September until April (Fig. 2.10). In contrast with *H. cymosum* sp. *cymosum,* it only grows in areas that are higher than 1200m above sea level up to a maximum of approximately 3170m (Hilliard 1983; Pooley 1998).

Fig. 2.10. A beautiful flowering shrub of *H. cymosum* sp. *clavum*.

This species is known to contain several flavonoid derivative compounds, and a few terpene compounds (Bohlmann et al. 1979b).

2.1.10. *H. cymosum* sp. *cymosum* D. Don. (HCCY)

The main species is a shrub that usually grows up to 1m tall standing erect with grey-white hairs on the upper surfaces of the leaves. Leaves become reduced in size closer to the top of the shrub and flowering occurs between September and April. These plants can be found in the WC and EC and all along the coast up to KZN. It usually grows in clusters and often in moist places with higher humidity (Hilliard 1983).

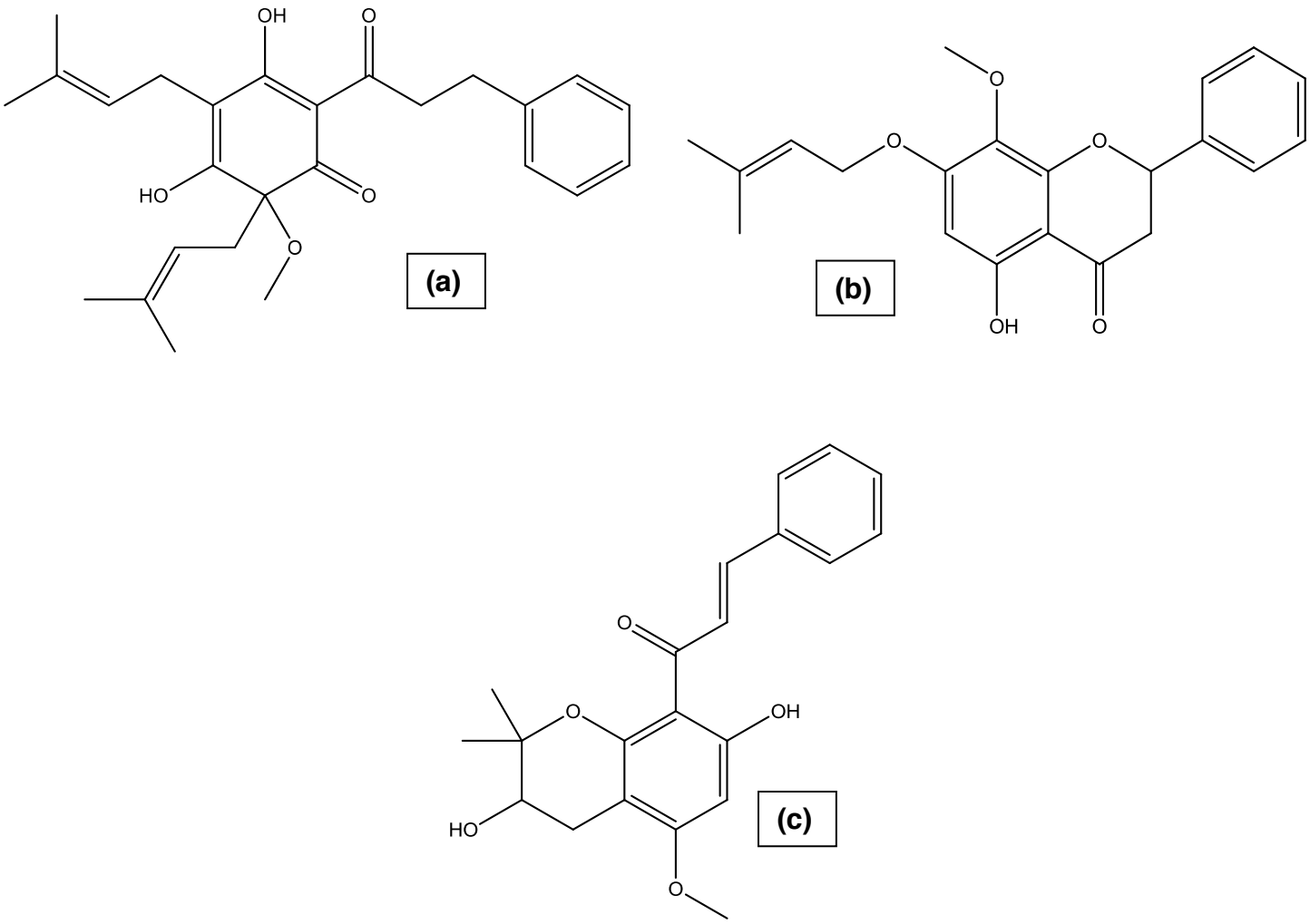

Fig. 2.11. (a) Helihumulone, (b) 5-hydroxy-8-methoxy-7-prenyloxyflavanone and (c) a helichromanochalcone isolated from *H. cymosum* sp. *cymosum* (Jakupovic et al. 1989; van Vuuren et al. 2006).

This species is known for the isolation of helihumulone (Fig. 2.11a), an oily phloroglucinol derviative with substantial antimicrobial activity. Other interesting compounds isolated from this species are 5-hydroxy-8-methoxy-7-prenyloxyflavanone (Fig. 2.11b), and a flavone derivative, helichromanochalcone (Fig. 2.11c) which was isolated from the roots (Jakupovic et al. 1989; van Vuuren et al. 2006). This plant is used in traditional medicine for inducing trances, while roots are used to induce vomiting and as a purgative or laxative. The leaves of *H. cymosum* sp. *cymosum* are used traditionally in the form of tea to treat coughs or colds or in the form of vapour from a vapour bath to treat headaches (Bhat and Jacobs 1995).

2.1.11. *H. difficile* Hilliard (HDIF)

H. difficile occurs mostly in Mpumalanga, Gauteng, Swaziland, NW and northern KZN and flowers between March and April. Leaves are smaller in size with white hairs on the lower surfaces (Hilliard 1983).

No traditional use, biological activity or phytochemical analysis has yet been reported from this species.

2.1.12. *H. drakensbergense* Killick (HDRA)

This is a bushy shrub more or less 40cm tall with grey hairs growing on branches to give a woolly appearance. Leaves are more prevalent in the lower half of the plant with woolly hairs on upper and lower surfaces. This species is endemic to the Drakensberg Mountains and only occurs in a small area in these mountains. It is usually seen on grassy slopes high above sea level in large colonies close to streams (Hilliard 1983).

This species is known to contain pyrones, a few diterpenes as well as other terpenes and other biologically active compounds in its roots and aerial parts (Bohlmann and Suwita 1979b) of which hydroxyacetophenone (Fig. 2.12) was shown to have significant antibacterial activity (Melliou and Chinou 2005).

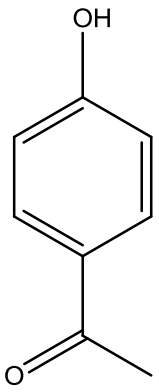

Fig. 2.12. Hydroxyacetophenone isolated from *H. drakensbergense* (Bohlmann and Suwita 1979b).

2.1.13. *H. herbaceum* (Andr.) Sweet (HHER)

This plant has long flowering stems that are woolly in nature with radical leaves with coarse white hairs on the lower surfaces and a cobweb-like nature on upper surfaces. It has been named the monkey-tail everlasting due to its appearance (Fig. 2.13). It is more herb-like rather than woody, like many other species. This plant has been found to grow at very high locations above sea level, usually in bare and disturbed grassland areas. *H. herbaceum* can occur in Limpopo, KZN, FS, the EC and Lesotho and rarely in the WC. Flowering season starts in October and end around April (Hilliard 1983).

Fig. 2.13. *H. herbaceum*, illustrating the cone shaped flowering buds.

H. herbaceum is mainly used to invoke goodwill of the ancestors and is sold heavily in the informal market (Pooley 2003). Some flavones have been isolated from this species with alpinetin (Fig. 2.14) being the best known flavanone isolated. This species is also known to contain some phloroglucinol compounds as well as a number of terpenes in its aerial parts as was reported by Bohlmann et al. (1979a).

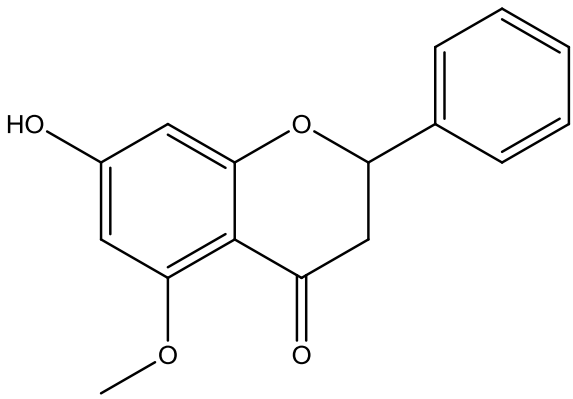

Fig. 2.14. Alpinetin isolated from *H. herbaceum* (Bohlmann et al. 1979a).

2.1.14. *H. melanacme* DC. (HMEL)

This species, like *H. herbaceum* has hairs on the lower surfaces and a cobweb-like nature on upper surfaces of leaves and usually contains numerous flowers (Fig. 2.15) occurring from December through to April. This plant can be found in KZN, FS, Lesotho and Western Swaziland usually on grassy mountain slopes (Hilliard 1983).

Fig. 2.15. *H. melanacme* in full bloom at the foot hill of Mikes Pass in the Catherdral Peak area.

This species is known to be used traditionally in the form of a tea to treat coughs, fevers, headaches, colds and chest pains (Arnold et al. 2002). Some anti-viral and anti-TB activity have been reported with two chalcones (Fig. 2.16) being isolated by Lall et al. (2006).

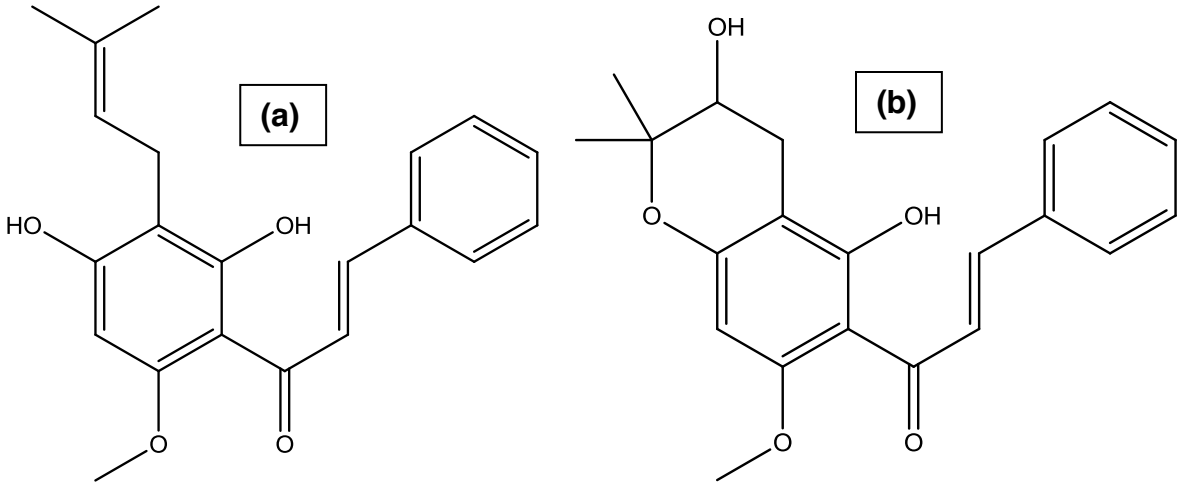

Fig. 2.16. Two chalcones, a) 2,′4′,6′-trihydroxy-3′-prenylchalcone and b) 4′,6′,5″-trihydroxy-6″,6″-dimethyldihydropyrano-[2″,3″-2′,3′] chalcone isolated from *H. melanacme.* (Lall et al. 2006).

2.1.15. *H. miconiifolium* DC. (HMIC)

This is a woody perennial herb with long flowering stems usually occurring individually and is covered in woolly hairs. Leaves are radical or cauline with long petioles present with hairs on the bottom surfaces but smooth upper surfaces. This species grows in grasslands in Limpopo, NW, southern Lesotho, Swaziland, KZN and the EC and flowering starts in November and continues through to February (Hilliard 1983).

This plant is used traditionally in the Xhosa culture by grinding and boiling the leaves and using it to wash the body to take away pain after circumcision. The roots are powdered and used against intestinal parasites and against ticks in poultry (Arnold et al. 2002). This species is known to contain a few diterpene compounds in its roots (Bohlmann et al. 1980b).

2.1.16. *H. natalitium* DC. (HNAT)

This plant has erect stems that can grow to approximately a meter tall with thin grey-white hairs occurring on branches. Branches are covered with many leaves that

decrease in size upward, forming inflorescence bracts with the possibility of hairs occurring on both surfaces. This species is known to grow in the central and southern Drakensberg mountains where it can grow at locations high above sea level near moist streamlines or in damp and rough grasslands, with flowering starting in January and continuing until April (Hilliard 1983).

This is also a species used extensively as incense when burnt to invoke goodwill with the ancestors (Pooley 2003). It is known to contain several phloroglucinols (Fig. 2.17 a and b), a few terpene compounds and other biologically active compounds in its aerial parts and roots (Bohlmann and Zdero 1979).

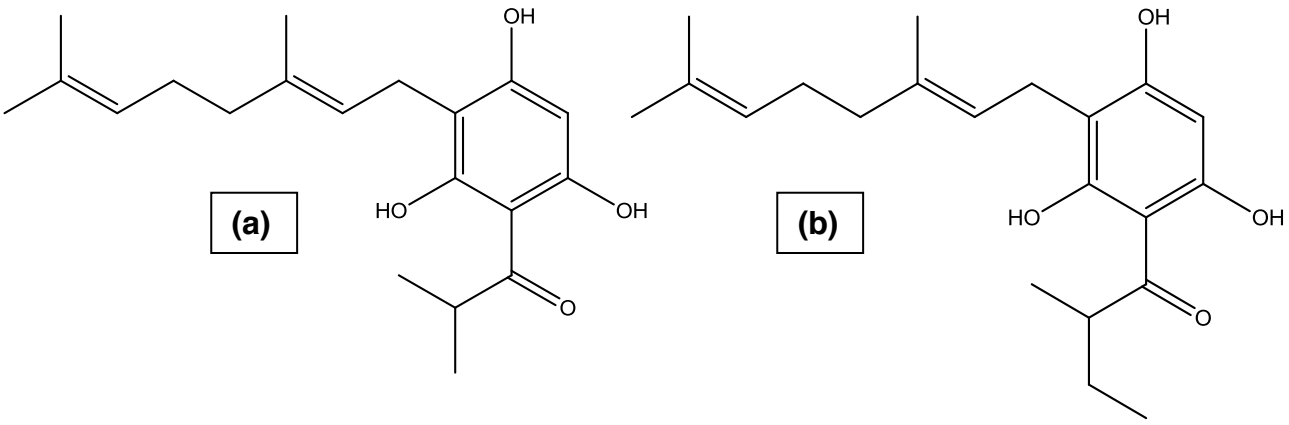

Fig. 2.17. Two phloroglucinol derivatives isolated from *H.* natalitium. A a) 2-Geranyl-4-(2-methylpropanoyl)phloroglucinol and another acylphloroglucinol b) 2-Geranyl-4-(2-methylbutanoyl)phloroglucinol (Bohlmann and Zdero 1979).

2.1.17. *H. nudifolium* var. *nudifolium* (L.) Less (HNUN)

There are many different variants of this perennial herb also called the Hottentot's tea. It has solitary flowering stems that can reach heights of 1.5m with the inflorescence being contracted and head-like. Leaves are often spread flat on the ground and can be thinly or thickly woolly with white hairs occurring on all surfaces of the leaves. This species

can grow up to 2500m above sea level and is found throughout Africa and all around South Africa except in the NC, with flowers being formed from November to March (Hilliard 1983; Pooley 2003).

The traditional use of this species is very elaborate, it is used to invoke goodwill with the ancestors, used to treat colds (Zulu medicine), used as wound dressing, effective as a treatment fo colic in children, to treat rectal prolapse, etc.. The species has also been invesitgated for its antibacterial and antifungal activity. A comprehensive overview of all the uses and biological activity can be obtained from the review of Lourens et al. (2008). A number of compounds have been isolated from this species, of which a number of them being phloroglucinols as was also isolated from *H. appendiculatum* (Fig. 2.4) (Jakupovic et al. 1989), as well as diterpenes and terpenes (Bohlmann et al. 1980b; Bohlmann et al. 1978b).

2.1.18. *H. odoratissimum* (L.) Sweet (HODO)

This species is known for its aromatic presence, very sweet smelling and bushy nature with many stems rooting at the base of the plant, which are covered in thin greyish-white hairs which cover the leaves on the upper as well as the lower surfaces. It grows in large clumps, in damp grasslands and near the foot of cliffs up to an altitude of 2400m. *H. odoratissimum* has two distinct flowering seasons with the south western parts of South African, WC and EC, experiencing it flowering from August to December whereas in the summer rainfall areas; KZN, Mpumalanga, Limpopo, Lesotho and Swaziland flowering takes place from January to (Hilliard 1983; Pooley 2003).

This plant's leaves are used traditionally against wounds and burns; to induce vomiting (by eating the leaves in ash form); against coughs, heartburn, headaches, convulsions, fevers, pain, female sterility, colic and conjunctivitis (inflammation of the eyelids). The whole plant is sometimes burnt and the smoke is inhaled to treat insomnia or as a protective cleanser. The roots are used to treat coughs and colds and is also used as a laxative. It is generally seen as an essential ingredient for herbalists when

trying to invoke a trance (Pooley 2003). Investigations into its antibacterial, antifungal as well as anti-TB activity have been investigated (Lourens et al. 2008).

This species is one of the most commonly used *Helichrysum* species in traditional medicine and it has been studied several times phytochemically. It was shown to contain several chalcone and flavanol compounds, some phloroglucinol compounds, pyrones as well as some diterpene compounds (Hänsel et al. 1980; Lourens et al. 2008; Rauter et al. 2005; Van Puyvelde et al. 1989).

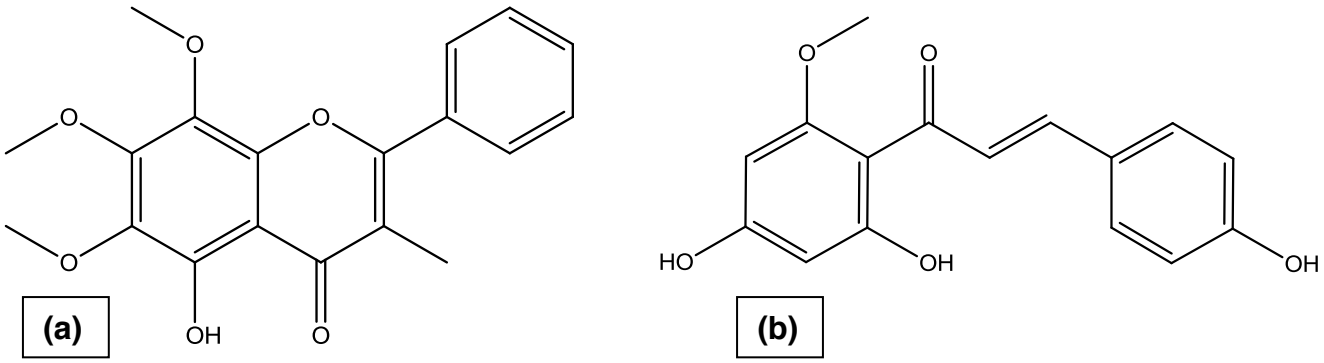

Fig. 2.18. Two of the compounds isolated from *H. odoratissimum*, a) 3,5-Dihydroxy-6,7,8-trimethoxyflavone and b) helichrysetin.

2.1.19. *H. oreophilium* Klatt (HOR-1)

This woody herb has rhizomes and an erect flowering stem with a silky-grey appearance on the stem. Leaves are reduced in size towards the upper parts of the plant with grey hairs occurring on all leaf surfaces especially on the main nerve on lower surfaces. This species can be found in areas were overgrazing or erosion is prominent. It can be found in Lesotho, Swaziland, Limpopo, Mpumalanga, western parts of KZN and south-eastern side of the FS. Flowering can be observed between October and December (Hilliard 1983; Pooley 2003).

This species is used in traditional medicine as a preventative measure for different illnesses. It was also tested for it anti-bacterial and anti-fungal activity

(Mathekga and Meyer 1998). Flavanone, chalcone, phloroglucinol, diterpenes and terpene compounds have been isolated from its aerial parts (Bohlmann et al. 1980b; Jakupovic et al. 1986).

2.1.20. *H. oxyphyllum* DC. (HOXY)

H. oxyphyllum has thin flowering stalks which are covered with thin woolly hairs. Leaves have extended petioles with the lower surfaces being white-felted because of hairs while upper surfaces have three or sometimes five veins. It occurs from Limpopo through Mpumalanga to Swaziland and KZN and all the way down the coast in the Eastern and WC, and it flowers from August through to January (Hilliard 1983).

The traditional use is mostly confined to being used in a magical sence as a protective charm (Arnold et al. 2002). Bohlmann et al. (1980b) conducted a phytochemical analysis on this species together with other *Helichrysum* species. They isolated a few flavonoid dervitatives as well as some diterpenes and terpenes.

2.1.21. *H. pallidum* DC. (HPAL)

This species is very similar to *H. oxyphyllum* and *H. nudifolium* but has shorter petioles, and its distribution differs in that it can also be found in Lesotho and sometimes even in the south-eastern parts of the FS. It does not occur in the WC but can be found in the north-eastern parts of the EC. Flowering also starts in August but may continue until February with flower-heads being creamy to pale yellow (Hilliard 1983; Pooley 1998).

H. pallidum is also very popular for the use in magical rituals, where it is used as a protective charm against illness (Arnold et al. 2002). There were some diterpenes and terpenes isolated from this species in the extensive phytochemical study of Bohlmann et al. (1980b).

2.1.22. *H. panduratum* O. Hoffm. (HPAN)

H. panduratum obtained its name because of its panduriform leaves which are sessile. It is a soft woody sub-shrub and grows in large, tangled clumbs in mixed grassland near to the forest margins. *H. panduratum* can be found from the EC all the way up to Kenya. Flowering starts in December and carries on to January and the flowers are honey-scented. This species only occur in KZN and the EC (Hilliard 1983; Pooley 1998).

This plant is used in traditional medicine to treat convulsions and malaria in children and is also frequently used as a herbal tea (Pooley 1998). Antiviral activity has been investigated for *H. panduratum* and it was found that it is active against the herpex simplex virus (HSV) at 50 ug/ml (CPE), similar to *H. appendiculatum* (Heyman 2009). There is only one known compound isolated from this species, a thioderivative known as helipandurin, as shown in Figure 2.19 (Bohlmann and Abraham 1979c).

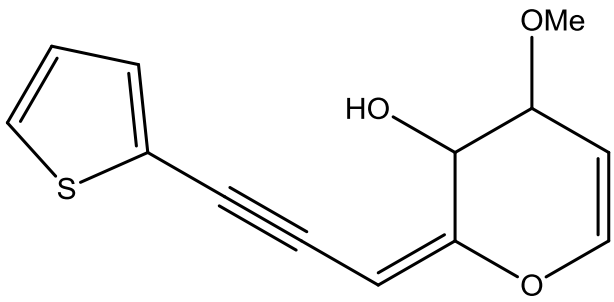

Fig. 2.19. Chemical structure of helipandurin which is similar to the acetylenic thiopene isolated from *H. acutatum* with the exception of the chlorine substitute (Bohlmann and Abraham 1979c).

2.1.23. *H. pannosum* DC. (HPANN)

This perennial herb can grow up to 1m tall and its leaves are reduced in size on the upper parts of the plant. Leaves have grey-white hairs on upper and lower surfaces.

Flowering of *H. pannosum* usually starts in April and last up to June. This species can be found in grassland often on hill slopes in a small area of coastal KZN (Hilliard 1983).

No traditional use, biological activity or phytochemical analysis has been reported on this species.

2.1.24. *H. pilosellum* (L.f.) Less. (HPIL & HNUP)

This species can only grow to a height of approximately 450mm and its leaves usually only occur close to the base of the plant. The upper surfaces of leaves are densely covered with hairs with long, coarse, multicellular hairs covering the surface while the lower surface is also somewhat covered with strong veins being present on the lower surface. It is common in open grassland up to 2400m and widely spread in Limpopo, Lesotho, Swaziland, Mpumalanga, KZN, FS and the EC, and flowering occurs from August to December (Hilliard 1983; Pooley 2003).

H. pilosellum is traditionally used as an antispetic or as treatment for stomach ailments (Arnold et al. 2002). The only compounds that have been isolated from it were different derivatives of ent-kaurene molecules as shown below (Fig. 2.20.).

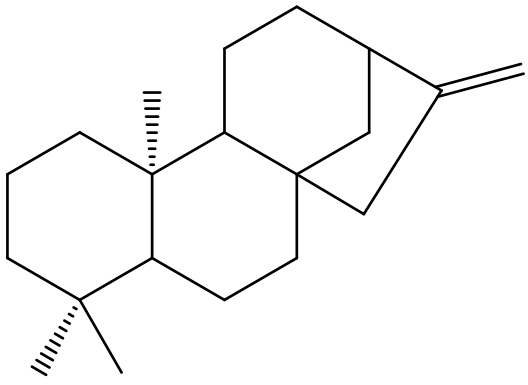

Fig. 2.20. A ent-kaurene compound isolated from *H. pilosellum* (Jakupovic et al. 1986).

2.1.25. *H. populifolium* D.C. (HPOP)

This species is very distinct from other *Helichrysum* species as it grows up to 2m tall contrary to other species being relatively small shrubs or even creepers. It has a leaf blade that is broadly ovate, sub-rotund, acute and obtuse and the petioles are not winged. The leaves are congested in clusters that are arranged in large spreading corymbose panicles. The involucral bracts are minutely radiating, white or silvery. *H. populifolium* has large poplar-like leaves that also make it very distinctive from other species. It grows in large outcropping masses of rock or along the cliffs above gorges usually not higher than c. 800m above sea level and is mostly confined to the Natal Group Sandstone. Flowering usually takes place between February and May (Hilliard 1983; Pooley 1998).

There is no traditional use known for *H. populifolium* and only one study previously reported the isolation of a compound (Fig. 2.21) (Bohlmann et al. 1980b). It has only really been used for its attractive foliage in the garden (Pooley 1998). The study by Heyman (2009) revealed that *H. populifolium* has significant CPE activity against both the HS virus (50 ug/ml) and HI virus (200 ug/ml HIV-reverse transcriptase activity).

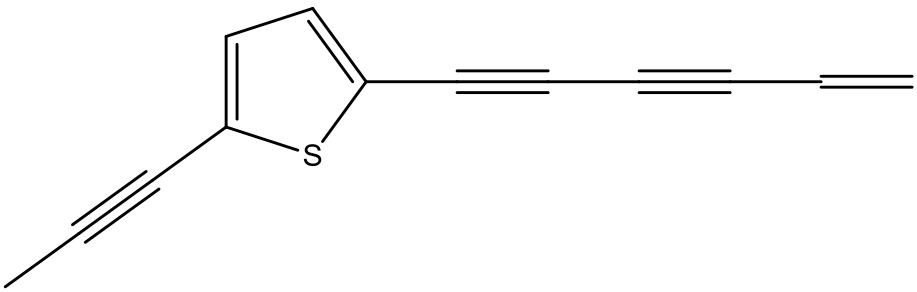

Fig. 2.21. Isolated compound from *H. populifolium* (Bohlmann et al. 1980b).

2.1.26. *H. rugulosum* Less. (HRUG-1)

This species is a perennial herb that has tufted flowers with flowering starting in December and continuing through to March. It has small sessile leaves that diminish in size towards the top. Leaves have a cobweb-appearance on the upper surfaces while lower surfaces are covered in white, stringy hairs. This plant is widely distributed in South Africa, usually in stony areas, roadsides, overgrazed areas or sandy grassland, and only doesn't occur in the Northern Cape (NC). It is sparingly found in the WC (Hilliard 1983).

This plant is used in traditional medicine as an ingredient of an enema to treat colic. It is also sometimes used to treat colds. Matekga and Meyer (1998) tested *H. rugulosum* for its antibacterial and it was active at IC_{50} of 100 ug/ml against most Gram positive bacteria. Chemically two very similar flavones have been isolated from this species with the only difference being the replacement of a alcohol group at position 5 with a methoxy group (Fig. 2.22.). Several other flavonone and dihydrochalcone compounds have also been isolated (Bohlmann and Misra 1984).

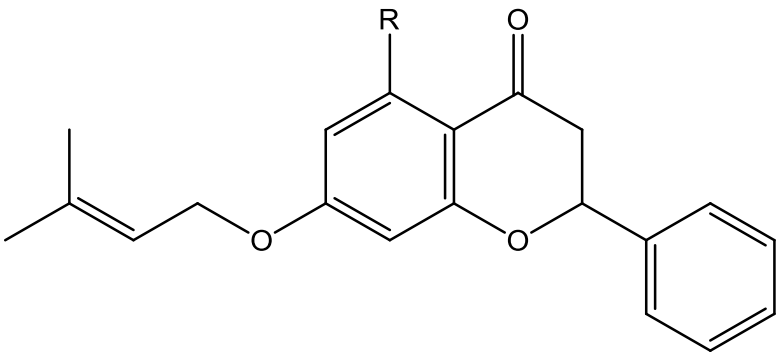

Fig. 2.22. 5-hydroxy-7-prenyloxyflavanone (R = OH) and 5-methoxy-7-prenyloxyflavanone (R = Me) both isolated from *H. rugulosum* (Bohlmann and Misra 1984)

2.1.27. *H. splendidium* Less. (HSPL-1)

H. splendidium is a shrub that can grow to a height of approximately 1.5m with grey-hair covered branches that may contain leaf scars. Upper and lower surfaces of leaves may be thickly or thinly covered with grey-white hairs. This species is also a very aromatic and has a scent very similar to lavender. It is also known as the Cape Gold everlasting, occurs in most South African provinces except for the FS and the NC and infrequently in the WC. Flowering occurs between October and January/February (Hilliard 1983; Pooley 1998).

This plant is used in traditional medicine to treat rheumatism and against pimples. It is known to contain a few dihydrochalcone compounds, a few flavonols, numerous terpenes of which a very unique terpene in the *Helichrysum* genus was isolated as is depicted in Fig. 2.23 (Bohlmann and Suwita 1979a; Jakupovic et al. 1989).

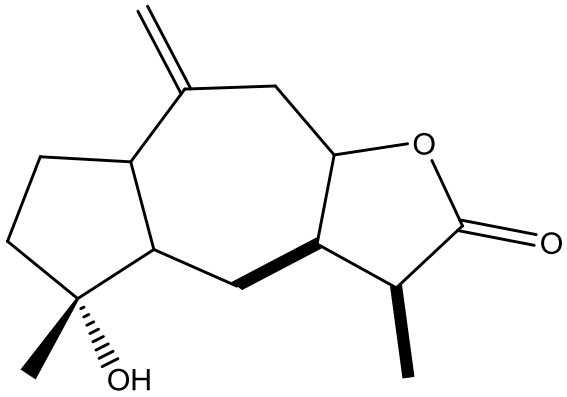

Fig. 2.23. A sesquiterpene of the guaianolide type isolated form *H. splendidium* (Bohlmann and Suwita 1979a).

2.1.28. *H. subluteum* Burtt Davy (HSUB)

This plant is covered throughout its surface with greyish-white hairs, with the hairs on the adaxial surface being longer than those on the abaxial surface. This plant may flower from September to December but flowering mainly occurs in November. This

plant can be found in Limpopo, Mpumalanga, Lesotho, Swaziland and KZN (Hilliard 1983).

No traditional use, biological activity or phytochemical analysis has been reported on this species.

2.1.29. *H. sutherlandii* Harv. (HSUT)

This plant is a shrub that only grows to a height of approximately 0.4m and does not grow completely erect. Plants usually grow in clumps that are suspended from Cave Sandstone cliffs, rocky outcrops and cliffs close to streams. The adaxial surfaces of the leaves are silver-grey and woolly while the abaxial surfaces are white felted with prominent veins. Flowers are yellow with long stalks, up to a meter long; while flower heads are barrel-shaped. Plants are found especially in the Drakensberg area as well as in Gauteng, the FS and KZN. Flowering takes place from mid-February to July with most flowers being produced in April (Hilliard 1983).

The whole plant is burned, powdered and then applied into the cuts or wounds of a patient (Arnold et al. 2002; Pooley 1998; Pooley 2003; Watt and Breyer-Brandwijk 1962). Phytochemical analyis revealed mainly chalcone derivatives with some diterpenes isolated from the roots of this species (Bohlmann et al. 1978a; Bohlmann et al. 1980b)

2.1.30. *H. umbraculigerum* Less. (HUMB)

This species has very long flower stalks with shocking yellow flowers that are white underneath and almost form an umbrella shape, thus being commonly known as the woolly umbrella *Helichrysum*. It is an erect herb that grows fast to reach a height of approximately 1m. Leaf forms may vary according to distribution, but young plants are often woolly and plants become woody with aging. Leaves are green on the adaxial surface and silver-grey on the abaxial surface with small hairs also present on the

abaxial surface. Flowering takes place from late January to April and flowers retain their colour for long periods of time. The plant generally requires a summer rainfall period and is found in the EC and other areas of South Africa as well as in Zimbabwe's highlands, grasslands and forest edges up to an altitude of 2500m (Hilliard 1983; Pooley 2003).

No traditional use is known for this species, but it is known to be heavily grazed by livestock (Pooley 1998). In a study of Bohlmann and Hoffmann (1979), they isolated, very suprisingly, cannabinoid-type (Fig. 2.24) compounds from *H. umbraculigerum*. These cannabinoids-type compounds are very similar to those produced in *Cannabis sativa*, thus *H. umbraculigerum* is only one of two plants that have been reported to produce compounds like these (Appendino et al. 2011).

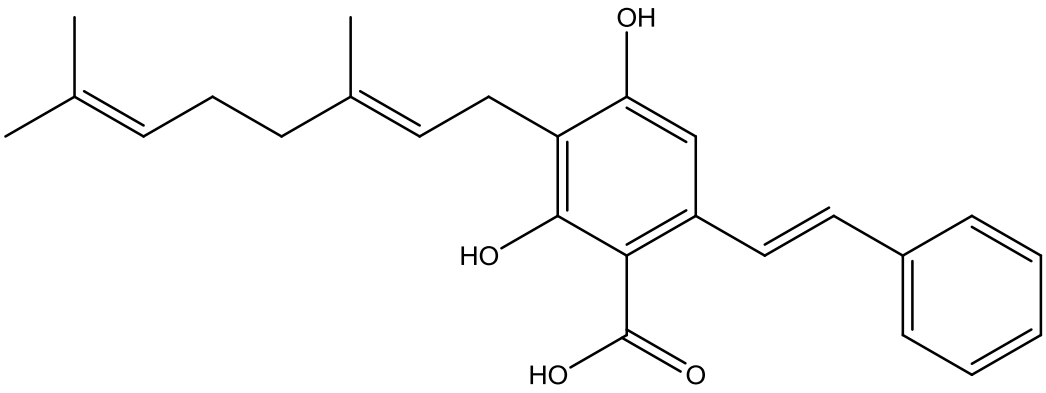

Fig. 2.24. Cannibigerol isolated form *H. umbraculigerum* (Bohlmann and Hoffmann 1979).

2.1.31. *H. vernum* Hilliard (HVER)

This plant arises from a thick, woody underground stem which branches to create a mat-forming ground cover (Fig. 2.25). Leaves are thick and leathery and often have a prominent mid-vein. This species occurs mainly on the stony grassy slopes of the foothills of the Drakensberg in KZN and Lesotho. Flowering takes place from September to December (Hilliard 1983).

Fig. 2.25. Flowering *H. vernum* in the Drakensberg mountains.

There is no known traditional use of this species and only a few diterpenes and terpenes (Fig. 2.26) have been isolated from it (Bohlmann et al. 1980b).

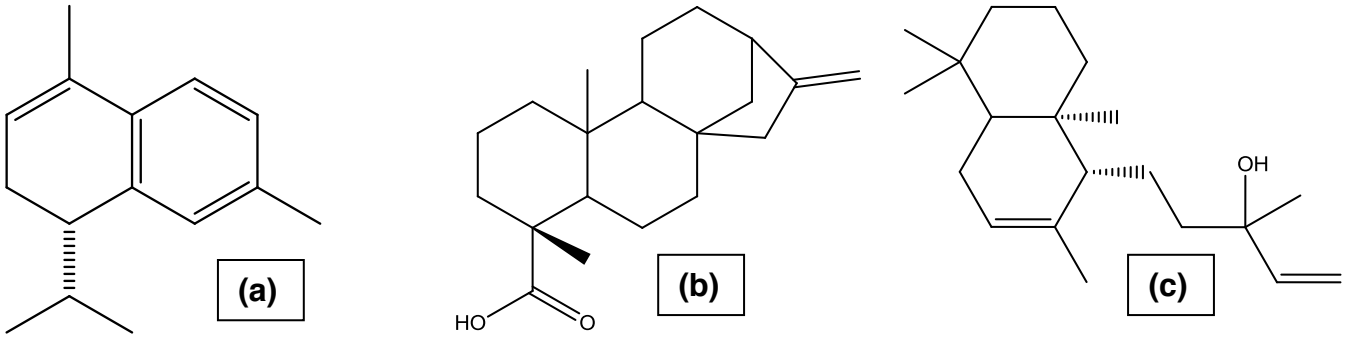

Fig. 2.26. a) 1,3,5,9-cadinatetraene, b) kaurenolide and c) 7,14-labdadien-13-ol isolated from *H. vernum* (Bohlmann et al. 1980b).

Fig. 2.27. All good things need to come to an end.

2.2. References

Afolayan, A. J. & Meyer, J. J. M. (1997). The antimicrobial activity of 3,5,7-trihydroxyflavone isolated from the shoots of *Helichrysum aureonitens*. Journal of Ethnopharmacology 57, 177-181 doi:http://dx.doi.org/10.1016/S0378-8741(97)00065-2

Appendino, G., Chianese, G. & Taglialatela-Scafati, O. (2011). Cannabinoids: occurrence and medicinal chemistry. Current Medicinal Chemistry 18, 1085-99

Appendino, G., Ottino, M., Marquez, N., Bianchi, F., Giana, A., Ballero, M., Sterner, O., Fiebich, B. L. & Munoz, E. (2007). Arzanol, an anti-inflammatory and anti-HIV-1 phloroglucinol alpha-Pyrone from *Helichrysum italicum* ssp. *microphyllum*. Journal of Natural Products 70, 608-12 doi:10.1021/np060581r

Arnold, T. H. & Killick, D. J. B. (2002). Medicinal and Magical Plants of Southern Africa: An Annotated Checklist, National Botanical Institute.

Bhat, R. B. & Jacobs, T. V. (1995). Traditional herbal medicine in Transkei. Journal of Ethnopharmacology 48, 7-12

Bohlmann, F. & Abraham, W. R. (1979a). Neue diterpene aus *Helichrysum acutatum*. Phytochemistry 18, 1754-1756 doi: http://dx.doi.org/10.1016/0031-9422(79)80208-3

Bohlmann, F. & Abraham, W. R. (1979b). Neue diterpene und weitere inhaltsstoffe aus *Helichrysum calliconum* und *Helichrysum heterolasium*. Phytochemistry 18, 889-891 doi:http://dx.doi.org/10.1016/0031-9422(79)80043-6

Bohlmann, F., Abraham, W.-R. (1979c). Neue, chlorsubstituierte thiophenacetylenverbindungen mit ungewöhnlicher struktur aus *Helichrysum*-arten. Phytochemistry 18, 839-842 doi: http://dx.doi.org/10.1016/0031-9422(79)80024-2

Bohlmann, F., Abraham, W. R. & Sheldrick, W. S. (1980a). Weitere diterpene mit helifulvan-gerüst und andere inhaltsstoffe aus *Helichrysum chionosphaerum*. Phytochemistry 19, 869-871 doi:http://dx.doi.org/10.1016/0031-9422(80)85128-4

Bohlmann, F. & Hoffmann, E. (1979). Cannabigerol-ähnliche verbindungen aus *Helichrysum umbraculigerum*. Phytochemistry 18, 1371-1374 doi: http://dx.doi.org/10.1016/0031-9422(79)83025-3

Bohlmann, F., Mahanta, P. K. & Zdero, C. (1978a). Neue chalkon-derivate aus südafrikanischen *Helichrysum*-arten. Phytochemistry 17, 1935-1937 doi: http://dx.doi.org/10.1016/S0031-9422(00)88737-3

Bohlmann, F. & Misra, L. N. (1984). New prenylflavanones and chalcones from *Helichrysum rugulosum*. Planta Medica 50, 271-2 doi:10.1055/s-2007-969699

Bohlmann, F. & Suwita, A. (1979a). Ein neues guajanolid und ein secoguajanolid aus *Helichrysum splendidum*. Phytochemistry 18, 885-886 doi: http://dx.doi.org/10.1016/0031-9422(79)80041-2

Bohlmann, F. & Suwita, A. (1979b). Weitere phloroglucin-derivate aus *Helichrysum*-arten. Phytochemistry 18, 2046-2049 doi: http://dx.doi.org/10.1016/S0031-9422(00)82740-5

Bohlmann, F. & Zdero, C. (1979). Neue phloroglucin-derivate aus *Helichrysum natalitium* und *Helichrysum bellum*. Phytochemistry 18, 641-644 doi: http://dx.doi.org/10.1016/S0031-9422(00)84276-4

Bohlmann, F., Zdero, C., Abraham, W.-R., Suwita, A. & Grenz, M. (1980b). Neue diterpene und neue dihydrochalkon-derivate sowie weitere inhaltsstoffe aus *Helichrysum*-arten. Phytochemistry 19, 873-879 doi: http://dx.doi.org/10.1016/0031-9422(80)85129-6

Bohlmann, F., Zdero, C., Hoffmann, E., Mahanta, P. K. & Dorner, W. (1978b). Neue diterpene und sesquiterpene aus südafrikanischen *Helichrysum*-arten.

Phytochemistry 17, 1917-1922 doi: http://dx.doi.org/10.1016/S0031-9422(00)88733-6

Bohlmann, F., Zdero, C. & Ziesche, J. (1979a). Neue flavone und phloroglucin-derivate aus *Helichrysum herbaceum* und *Helichrysum chrysargyrum*. Phytochemistry 18, 1375-1378 doi: http://dx.doi.org/10.1016/0031-9422(79)83026-5

Bohlmann, F. & Ziesche, J. (1979). Ein ungewöhnliches tetrahydrofuran-derivat aus *Helichrysum aureonitens*. Phytochemistry 18, 664-665 doi: http://dx.doi.org/10.1016/S0031-9422(00)84287-9

Bohlmann, F., Ziesche, J. & Mahanta, P. K. (1979b). Neue chalkon-derivate und humulon-ähnliche verbindungen aus *Helichrysum*-arten. Phytochemistry 18, 1033-1036 doi: http://dx.doi.org/10.1016/S0031-9422(00)91472-9

Hänsel, R., Cybulski, E.-M., Çubukçu, B., Meriçli, A. H., Bohlmann, F. & Zdero, C. (1980). Neue pyron-derivate aus *Helichrysum*-arten. Phytochemistry 19, 639-644 doi: http://dx.doi.org/10.1016/0031-9422(80)87030-0

Heyman, H. (2009). Metabolomic comparison of selected *Helichrysum* species to predict their antiviral properties. University of Pretoria

Hilliard, O. M. (1983). Flora of southern Africa: Asteraceae (Compositae). Inuleae. Gnaphaliinae (first part), National Botanical Institute.

Hutchings, A., Scott, A. H., Lewis, G. & Cunningham, A. (1996). Zulu medicinal plants: An inventory. University of Natal Press, Pietermaritzburg.

Jakupovic, J., Kuhnke, J., Schuster, A., Metwally, M. A. & Bohlmann, F. (1986). Phloroglucinol derivatives and other constituents from South African *Helichrysum* species. Phytochemistry 25, 1133-1142 doi: http://dx.doi.org/10.1016/S0031-9422(00)81569-1

Jakupovic, J., Zdero, C., Grenz, M., Tsichritzis, F., Lehmann, L., Hashemi-Nejad, S. M. & Bohlmann, F. (1989). Twenty-one acylphloroglucinol derivatives and further

constituents from south african *Helichrysum* species. Phytochemistry 28, 1119-1131 doi: http://dx.doi.org/10.1016/0031-9422(89)80195-5

Lall, N., Hussein, A. A. & Meyer, J. J. M. (2006). Antiviral and antituberculous activity of *Helichrysum melanacme* constituents. Fitoterapia 77, 230-232 doi: http://dx.doi.org/10.1016/j.fitote.2006.01.007

Lourens, A. C. U., Viljoen, A. M. & Van Heerden, F. R. (2008). South African *Helichrysum* species: A review of the traditional uses, biological activity and phytochemistry. Journal of Ethnopharmacology 119, 630-652 doi: http://dx.doi.org/10.1016/j.jep.2008.06.011

Mathekga, A. & Meyer, J. J. M. (1998). Antibacterial activity of South African *Helichrysum* species. South African Journal of Botany 64, 293-295

Mathekga, A. D. M. (2001). Antimicrobial activity of *Helichrysum* species and the isolation of a new phloroglucinol from *Helichrysum caespititium*, University of Pretoria.

Melliou, E. & Chinou, I. (2005). Chemistry and bioactivity of royal jelly from Greece. Journal of Agricultural and Food Chemistry 53, 8987-92 doi:10.1021/jf051550p

Meyer, J. J. M., Afolayan, A. J., Taylor, M. B. & Engelbrecht, L. (1996). Inhibition of herpes simplex virus type 1 by aqueous extracts from shoots of *Helichrysum aureonitens* (Asteraceae). Journal of Ethnopharmacology 52, 41-43 doi: http://dx.doi.org/10.1016/0378-8741(96)01387-6

Pigott, E., Roberts, W., Ovenden, S. B., Rochfort, S. & Bourne, D. (2012). Metabolomic investigations of *Ricinus communis* for cultivar and provenance determination. Metabolomics 8, 634-642 doi:10.1007/s11306-011-0355-7

Pooley, E. (1998). A Field Guide to Wild Flowers: KwaZulu-Natal and the Eastern Region, Natal Flora Publication Trust.

Pooley, E. (2003). Mountain flowers: Field guide to the flora of the Drakensberg and Lesotho. Natal Flora Publication Trust.

Rauter, A. P., Martins, A., Borges, C., Ferreira, J., Justino, J., Bronze, M.-R., Coelho, A. V., Choi, Y. H. & Verpoorte, R. (2005). Liquid chromatography–diode array detection–electrospray ionisation mass spectrometry/nuclear magnetic resonance analyses of the anti-hyperglycemic flavonoid extract of *Genista tenera*: Structure elucidation of a flavonoid-C-glycoside. Journal of Chromatography A 1089, 59-64 doi: http://dx.doi.org/10.1016/j.chroma.2005.06.046

Van Puyvelde, L., De Kimpe, N., Costa, J., Munyjabo, V., Nyirankuliza, S., Hakizamungu, E. & Schamp, N. (1989). Isolation of flavonoids and a chalcone from *Helichrysum odoratissimum* and synthesis of helichrysetin. Journal of Natural Products 52, 629-33

Van Vuuren, S. F., Viljoen, A. M., Van Zyl, R. L., Van Heerden, F. R. & Başer, K. H. C. (2006). The antimicrobial, antimalarial and toxicity profiles of helihumulone, leaf essential oil and extracts of *Helichrysum cymosum* (L.) D. Don subsp. *cymosum*. South African Journal of Botany 72, 287-290 doi: http://dx.doi.org/10.1016/j.sajb.2005.07.007

Wang, Y., Tang, H., Nicholson, J. K., Hylands, P. J., Sampson, J., Whitcombe, I., Stewart, C. G., Caiger, S., Oru, I. & Holmes, E. (2004). Metabolomic strategy for the classification and quality control of phytomedicine: A case study of Chamomile flower (*Matricaria recutita* L.). Planta Medica 70, 250-255 doi:10.1055/s-2004-815543

Watt, J. M. & Breyer-Brandwijk, M. G. (1962). The medicinal and poisonous plants of southern and eastern Africa: being an account of their medicinal and other uses, chemical composition, pharmacological effects and toxicology in man and animal, E. & S. Livingstone.

Chapter 3

Analysis of several South African *Helichrysum* species by means of NMR-based metabolomics in search of anti-HIV constituents

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3.1. Introduction

In the search for natural products as anti-HIV drugs, plant sources are especially a good starting point. This statement is supported by the research done by Newman and Cragg as reported in their review, "Natural Products as Sources of New Drugs over the Last 25 Years" (Newman and Cragg 2007), where emphasis was placed on expanding and not decreasing the search for new chemical entities (NCE) from natural sources. Newman and Cragg also showed very clearly that natural and naturally derived compounds contributed significantly to NCE with more than 27% of all NCE in the period 01/1981 – 06/2006, having a natural origin or were derived from a natural origin. Plant based research thus has huge potential in discovering new leads and scaffolds to treat many diseases over the world.

In contrast to the single ingredient drug use of the modern pharmaceutical industry, primary medicine, especially before the 20th century relied mostly on multicomponent medicine obtained from natural resources. This is a trend that is starting to appear again due to the dramatic decline in NCE being discovered in the last 10 years (Newman and Cragg 2007; Schmidt et al. 2007). Combinatorial treatment has been particularly important in the treatment of certain infectious diseases like HIV, tuberculosis (TB) and malaria (Agrawal et al. 2006; Blomberg and Fourie 2003; Butler 2004; Sams-Dodd 2006; Schellenberg et al. 2006). The use of natural products in combination with existing antibiotics or other treatments have

also shown in many instances to restore their previous activity against resistant forms of the pathogens (Hemalswarya and Doble 2006). The edge that these and other natural products have over synthetic compounds lies in the molecular complexity, the richness in stereochemistry, the ring-system diversity, scaffold variety etc. and also the large number of secondary metabolites found. On the other hand, the use of low cost combinatorial libraries and the ease and speed that these bring to identify bio-actives from these libraries, together with the use of high throughput screening, present massive challenges for natural product drug discovery (Schmidt et al. 2007), unless new ways of speeding up drug discovery from natural products are explored.

In this study the focus is placed on metabolomics as a way to speed up drug discovery in search of bioactive compounds. Plant metabolomics has the goal of analysing a plant sample to obtain a total metabolite composition view (Kim et al. 2006). The metabolite composition can then be compared to a secondary variable, i.e. biological activity, taxonomical data, geographical data, etc. in search of distinct differences or co-occurrence patterns directly associated with the secondary variable. The use of NMR-based plant metabolomics is well summarised in the study "Quality Control of Herbal Material and Phytopharmaceuticals with MS and NMR based Metabolic Fingerprinting" (Van der Kooy et al. 2009). In this study it was shown that NMR-based metabolomics has been used as a tool for classification and identification of adulterants, the identification of biomarkers for quality control purposes, to characterise different cultivars of *Cannabis sativa* on the basis of the metabolite composition etc. These examples highlight the possibility of systematic and phytochemical analyses being possible within plant metabolomics and especially using NMR as the investigating tool. The focus of the current study was therefore to investigate the use of NMR-based metabolomics to identify the link between the biological activity and metabolite composition within the phytochemical diverse *Helichrysum* genus, to speed up drug discovery.

The Asteraceae family has many interesting genera regarding drug discovery of which the most familiar species probably is *Artemisia annua*, producing the anti-malaria drug, artemisinin. In this family the interesting and diverse *Helichrysum*

genus also resides. The *Helichrysum* genus has been used extensively in traditional practices as was indicated by Lourens et al. (2008). Traditional usage include the treatment of coughs, fever, intestinal ailments, gonorrhoea, sore eyes, skin infections, etc. The skin infection treatment is mainly associated with the treatment of wounds that normally are associated with sexual transmitted diseases (STD) often caused by unhygienic practices during circumcision in rural villages (Lourens et al. 2008). The traditional use and the identified chemical diversity of the genus motivated this study of 30 *Helichrysum* species and one infraspecific species, as a first step in search of novel anti-HIV drug leads by means of NMR-based metabolomics.

3.2. Materials and Methods

3.2.1 Plant collection

The *Helichrysum* species selected for this study were collected within the Ukhahlamba Drakensberg Mountains in KwaZulu-Natal as well as the eastern Cape region of South Africa (Permit no: OP 4928/2010). The aerial parts (flower, leaves and stems) of 30 species of *Helichrysum* and the one infraspecific species were collected from their natural habitat during December 2010 and Herbarium specimens were prepared and stored in the HGWJ Schweickerdt Herbarium (PRU), University of Pretoria, South Africa (Table 2.1).

3.2.2. Extract preparation

Dried aerial plant parts of all 30 species and the one infraspecific species (8g - 32g) were extracted with a solution of 300 ml of 50% dichloromethane (DCM) and 300ml of 50% methanol/water (M/W) (1:1). The extracts were homogenised for 5 min with a handheld homogeniser (Heidolph, Germany), sonicated for 20 min in an ultrasonic bath (Elma, Germany), filtered (Whatman no. 3), the two liquid phases were separated with a separating funnel (aqueous from organic) and each concentrated to dryness at reduced pressure at room temperature. The resultant residue was used for both the bioassay and chemical analysis.

3.2.3. Cell culture

The reporter cell line HeLa-SxR5 was used for the anti-HIV bioassay, which stably expresses the CD4 receptor and the CXCR4/CCR5 chemokine receptors. It also contains a HIV-1 long terminal repeat fused upstream of the bacterial reporter gene LacZ, which encodes for β-galactosidase, all of which is stably integrated into the cellular genome. The human embryonic kidney cell line HEK293T (Human Embryonic Kidney 293 cells) was used for transfection experiments with HIV-producing plasmids. Both cell lines were maintained in high-glucose DMEM (Dulbecco's Modified Eagle's Medium) medium supplemented with 10% Fetal Bovine Serum (FBS) and were cultivated at 37°C in a 7% CO_2 atmosphere (Vidal et al. 2012)

3.2.4. HIV Assay and Cytotoxicity

All 64 extracts (32 DCM and 32 M/W) were screened in duplicate for anti-HIV activity using a colorimetric cell-based assay at two concentrations, 25 µg/ml and 2.5 µg/ml (Fig 3.1) (Postive control: Efavirenz (5% DMSO) 300 nM – 1 nM, Negative control: PBS / 5% DMSO - 0% inhibition). The bioassay produces replication-competent HIV, allowing up to four rounds of replication in a time window of 4 days (deCIPhR) as described in Vidal et al. (2012). Cytotoxicity and normal cell growth was microscopically investigated after incubation to get an indication of the toxicity of the plant extracts, especially those that had inhibitory properties against the HI virus. Addition of β-galactosidase to the reaction mixture resulted in a yellow colour change in wells with active virus and infected cells. The wells where the plant extract inhibited the proliferation of the virus had little or no colour change, which was quantified by an ELISA read-out.

From the 64 extracts screened, 22 were selected on the following criteria with regards to the screening results (Table 3.2) to be further bio-assayed to establish the individual inhibitory concentrations (IC_{50}) and cytotoxicity concentrations (LC_{50}) over an extended concentration range (Fig. 3.2):

1.) > 50% inhibition at 25 µg/ml, and > 10% inhibition at 2.5 µg/ml with no toxicity at 25 µg/ml (4 DCM extracts and 6 M/W extracts).

2.) > 50% inhibition at 25 µg/ml and no inhibition at 2.5 µg/ml with limited toxicity at 25 µg/ml (12 DCM extracts).

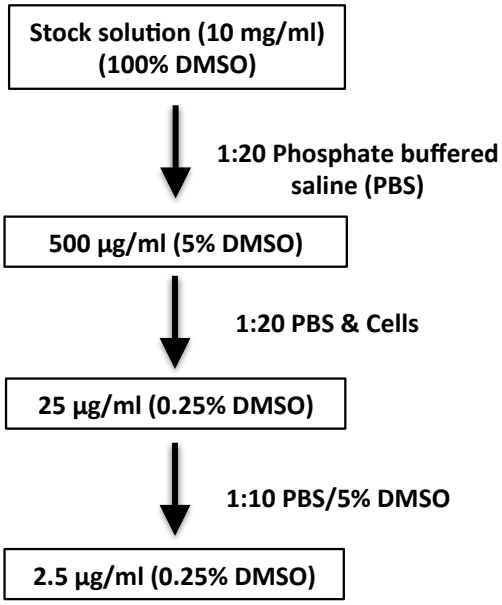

Fig **3.1.** Dilution scheme for the screening of all 64 *Helichrysum* plant extracts (DCM and M/W).

The cell-based assay utilises HEK293T cells that are transfected with the full-length pro-viral plasmid pNL4-3 (CXCR4-tropic) or derivatives thereof, using Lipofectamine2000 (Invitrogen, USA) as lipofectant. On the day of transfection, the transfected cells are dispensed into 96-well plates and incubated for 4 days (37°C) in co-culture with HeLa-SxR5 reporter cells in the presence of the respective test substances. At the end of the incubation period β-galactosidase activity was determined using a colorimetric readout for the ortho-nitrophenyl-galactopyranoside (ONPG) to ortho-nitrophenyl (ONP) conversion (at 405nm). This readout was transformed to “percent viral inhibition” following normalization of the data with positive (full virus production in the absence of inhibitor) and negative controls (full suppression by addition of a characterized HIV-inhibitor, positive drug control Efavirenz at 300nM – 1nM) included in each 96-well plate (Klimkait et al. 1998; Louvel et al. 2008). Where appropriate, the effect of extracts as anti-HIV agents was

modelled as a dose response curve with XLfit (ID Business Solutions, UK) for proper alignment.

For the determination of any cytotoxicity caused by the extract the identical cell culture conditions as used for the anti-HIV activity determination were utilized with the exception that no pro-viral DNA was transfected (mock transfection). After the four days incubation period cytotoxicity was determined by using the AlamarBlue reagent (Invitrogen, USA). In order to eliminate any unspecific signal reductions which could have been caused by either a reduced cellular metabolism (cytostatic) or even cytotoxicity activity, the readouts of both the HIV-assay and the cytotoxicity assay where compared by superimposing the graphs to establish any significant reduction that needed to be taken into account.

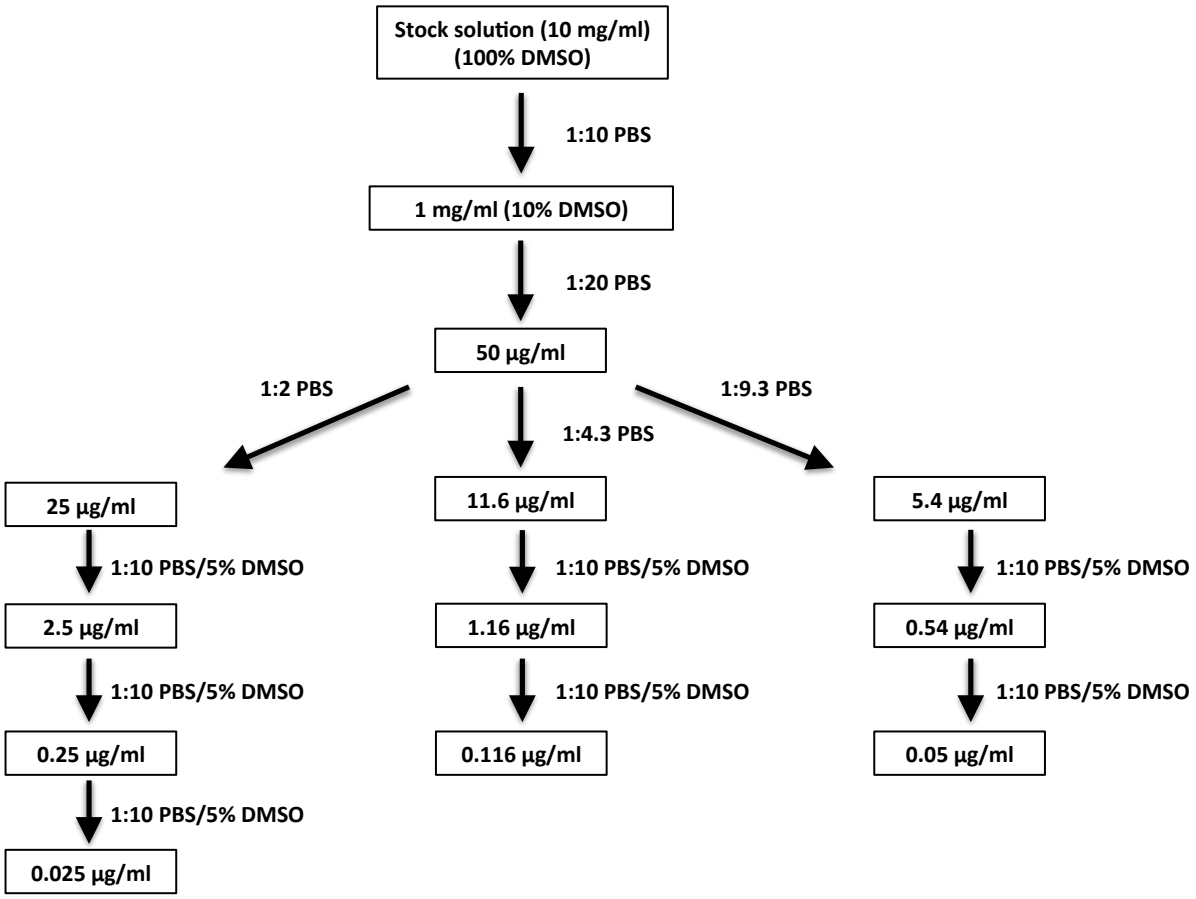

Fig **3.2.** Dilution scheme for the dose response determination of the 22 active *Helichrysum* extracts.

3.2.5. ^{1}H-NMR analysis

NMR analysis was done on a Bruker 600MHz spectrometer (Leiden University, The Netherlands) operating at a proton NMR frequency of 600.13 MHz. The organic fractions were re-dissolved to 15 mg/ml in $CDCl_3$ with 0.1% tetramethylsilane (TMS) as internal standard (0.00 ppm). The M/W fractions were also re-dissolved to 15 mg/ml in a buffered mixture of CD_3OD and KH_2PO4 - D_2O solution, with the pH adjusted to pH 6.0 with NaOD (1M). The internal standard trimethylsilane propionic acid sodium salt (0.1% TSP – 0.00ppm) was used for spectral referencing of the 50% methanolic samples. The samples (600 ul) were transferred to 5 mm NMR tubes and queued for analyses overnight. For each spectrum 32 scans were recorded with a spectral width of 14 ppm. Temperature was kept constant for each run at 25°C and all ^{1}H NMR spectra were referenced to the respective internal standards and manually phased and baseline corrected (Whittaker smoother).

3.2.6. Multivariate data analysis

The ^{1}H NMR spectra were reduced to ASCII files using MestReNova 8.1.1 (Mestrelab Research). Normalisation was done by scaling the spectral intensities to 0.1% of TMS (organic extracts) and 0.1% TSP (50% methanolic extract) and the region of 0.00 – 10.00 ppm was reduced to bins of 0.04 ppm in width. The region ranging from 3.28 – 3.36 ppm (residual MeOH) and 4.60 – 5.00 ppm (residual water) were removed prior to statistical analyses for the methanolic samples. The ASCII files generated were then imported into Microsoft Excel 2010 for secondary variable labelling after which the files were imported into SIMCA-P 13.0.0 (Umetrics, Umeå, Sweden). The data was Pareto scaled before being subjected to PCA and OPLS analysis.

3.3. Results and Discussion

The anti-HIV screening of the 64 extracts (Table 3.1) showed that 22 of the extracts had more than 50% inhibition against the virus at 25 µg/ml with little or no cytotoxicity (microscopically investigated).

Table **3.1:** Anti-HIV activity of selected *Helichrysum* species (% inhibition) using the DeCIPhR method.

	DCM		W/M	
Selected plants	25 µg/ml	2.5 µg/ml	25 µg/ml	2.5 µg/ml
H. acutatum	113*	69	41	-11**
H. allioides	112*	-21**	9	-8**
H. anomalum	95‡	-7**	27	-10**
H. appendiculatum	50	-38**	82	-28**
H. aureonitens	88	0	11	-13**
H. cephaloideum	58	-57**	35	-33**
H. chionosphaerum	104‡	-45**	3	-14**
H. confertum	104*	3	35	2
H. cymosum ssp. *cymosum*	90‡	-32**	75	-15**
H. cymosum ssp. *clavum*	114‡	-33**	70	-18**
H. difficile	16‡	10	21	8
H. drakensbergense	49	-22**	-14**	-30**
H. herbaceum	39	4	-19**	-37**
H. melanacme	84‡	11	-26**	-33**
H. miconiifolium	42	-30**	1	-43**
H. natalitium	117*	-33**	13	-14**
H. nudifolium var. *nudifolium* (1)	87‡	-18**	16	10
H. odoratissimum	116*	-4**	10	-12**
H. oreophilum (1)	112‡	2	23	-4**
H. oxyphyllum	113*	-13**	102	19
H. pallidum	96‡	-19**	8	-1**
H. panduratum	-67**	-23**	3	-34**
H. pannosum	-55**	-21**	25	-11**
H. pilosellum	109‡	-51**	23	3
H. pilosellum†	11	-8**	15	12
H. populifolium	58	0	84	8
H. rugulosum	51	-15**	-31**	-3**
H. splendidum (1)	85‡	-11**	12	-31**
H. subluteum	51	-3**	42	16
H. sutherlandii	-64**	-56**	32	-30**
H. umbraculigerum	109‡	-40**	23	-26**
H. vernum	113*	-19**	23	13

* high cytotoxicity ‡ medium cytotoxicity ** negative values can be explained by a read-out induction due to a gene-activating activity in the respective fractions † Earlier classification was uncertain, but after further analysis this species was reclassified as *H. pilosellum* (HNUP).

DCM – Dichloromethane M/W – Methanol/Water

Positive control: Efavirenz (5% DMSO) 0.5 – 1.0 nM (testing range 300nm – 1nm) / Negative control: PBS/5%DMSO - 0% inhibition

The IC_{50} and LD_{50} of these 22 extracts were determined and the most active extracts were the M/W extracts of *Helichrysum populifolium* (IC_{50} 12 µg/ml), *H. appendiculatum* (IC_{50} 17 µg/ml), *H. cymosum* ssp. *clavum* (IC_{50} 19 µg/ml), *H. oxyphyllum* (IC_{50} 19 µg/ml) and *H. cymosum* ssp. *cymosum* (IC_{50} 21 µg/ml) (Table 3.2). These were identified as the most active M/W extracts on the basis that in both the screening and IC_{50} analysis they showed significant activity and mostly no cytotoxicity. These would then serve as the active sample group for the multivariate data analysis with the purpose of establishing how these differ compared to the non-active samples based on their chemistry.

Table **3.2:** Anti-HIV activity (IC_{50} µg/ml) and cytotoxicity (LD_{50} µg/ml) of the best dichloromethane (DCM) and water/methanol (W/M) *Helichrysum* extracts using the DeCIPhR method.

	IC**50** (LD**50**)	
Selected plants	DCM	W/M
H. acutatum	4 (4) [a]	N/T
H. anomalum	23 (>50) [a]	N/T
H. appendiculatum	N/T	17 (>50)
H. aureonitens	9 (33)	N/T
H. cephaloideum	19 (>50)	N/T
H. chionosphaerum	26 (>50) [a]	N/T
H. cymosum ssp. *cymosum*	14 (>50) [a]	21 (>50)
H. cymosum ssp. *clavum*	10 (>50) [a]	19 (>50)
H. melanacme	10 (50) [a]	N/T
H. nudifolium var. *nudifolium* (1)	48 (>50) [a]	N/T
H. oreophilum (1)	46 (>50) [a]	N/T
H. oxyphyllum	N/T	19 (>50)
H. pallidum	31 (>50) [a]	N/T
H. pilosellum	20 (>50) [a]	N/T
H. populifolium	33 (>50)	12 (>50)
H. splendidum (1)	7 (>50) [a]	N/T
H. umbraculigerum	17 (>50) [a]	N/T
Efavirenz (DMSO)	0.5 – 1.0 nM	

N/T - not tested [a] - observed altered cell morphology due to extract
The activity range of the positive control Efavirenz (EFV) used for each sample set is included and is expressed in nM.

Most of the DCM extract showed significant cytotoxicity (morphological and LD_{50}) thus eliminating them as candidates for further analysis. *H. populifolium* was

identified as the most active extract that had no morphological or cytotoxic effect on the cells and thus was selected as the extract of choice for the purification, identification and isolation (if applicable) after the establishment of a common activity profile.

For the metabolomic analysis all the extracts were analysed under the same conditions to limit operating errors in the data generated. Only the M/W extracts were subjected to metabolomic analysis as the organic fractions showed in several instances cytotoxic tendencies as mentioned above (Table 3.1 and 3.2).

The NMR spectra off all the M/W extracts showed significant incidences of aromatic compounds (6.00 – 10.00 ppm) and also carbohydrate moieties (3.00 – 6.00 ppm) (Fig. 3.3.). Using the processed NMR data to compare the phytochemical composition of all the extracts it was possible to generate a Principal Component Analysis (PCA) (Fig 3.4.). With the use of Distance to Model X (DMod[X]) and Hotelling's T2Range Plots (T2Range) as well as the observations of the PCA scores plot, two extracts (*H. drakensbergense* and *H. herbaceum*) were identified as significant outliers and were excluded from the dataset. The new dataset (Table 3.4.), with all seven principle components (PC) included in the PCA scores plot (Fig 3.5.), did not show any distinct grouping that correlated to the activity of the extracts. Multivariate PCA data analysis is most commonly interpreted by means of R^2 and Q^2 values of the fitted model. R^2 of the fitted model indicates the amount of data explained by the model and gives a general overview of the fitness of the model. The Q^2 on the other hand gives and indication of the predictability of the model. Models that explain the data well and are well fitted usually have R^2 and Q^2 values close to 1 (Gao et al. 2012). In this study the high variability observed in the PCA and the OPLS of the 32 *Helichrysum* samples was possibly due to:

1.) Being collected in different geographical areas,

2.) Being from different morphological groups (Hilliard 1983), or

3.) Due to the fact that they were different species.

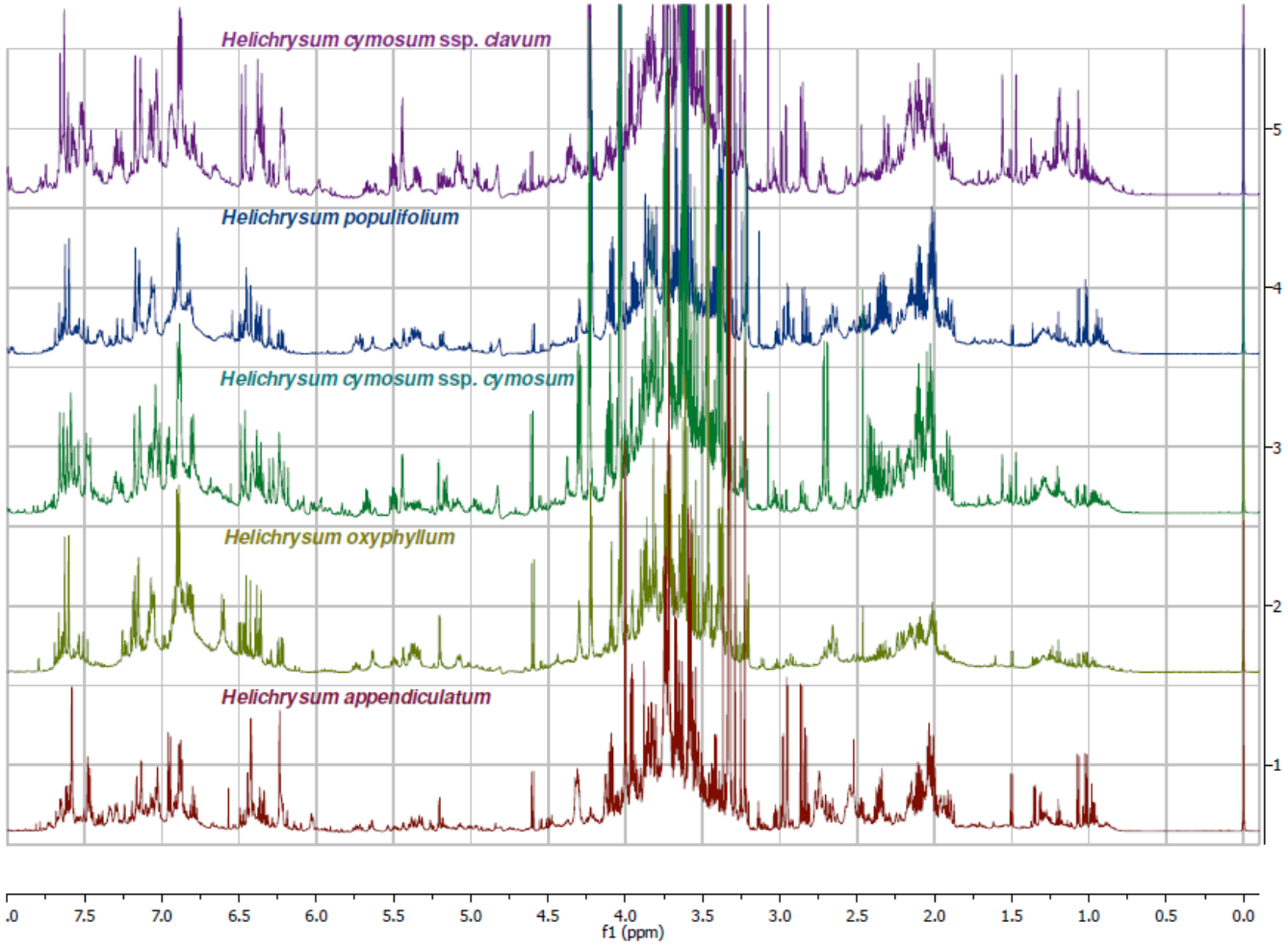

Fig. **3.3.** ^{1}H NMR stacked spectra of the most promising *Helichrysum* plant extracts showing significant similarities.

These factors contributed to R^2 and Q^2 values of 0.39 and 0.2 respectively (Table 3.3). Thus approximately 40% of the data could be explained, with predictability at 20% thus the prediction of the outcome of future samples' would not be very accurate. It was also very difficult to identify what the PCA explained exactly as the differences could have been due to the different factors mentioned above. It was thus very important to include an additional factor, i.e. the anti-HIV activity of the different samples. This would allow for a biased analysis that could identify what the chemical differences are, when the anti-HIV activity is taken into account.

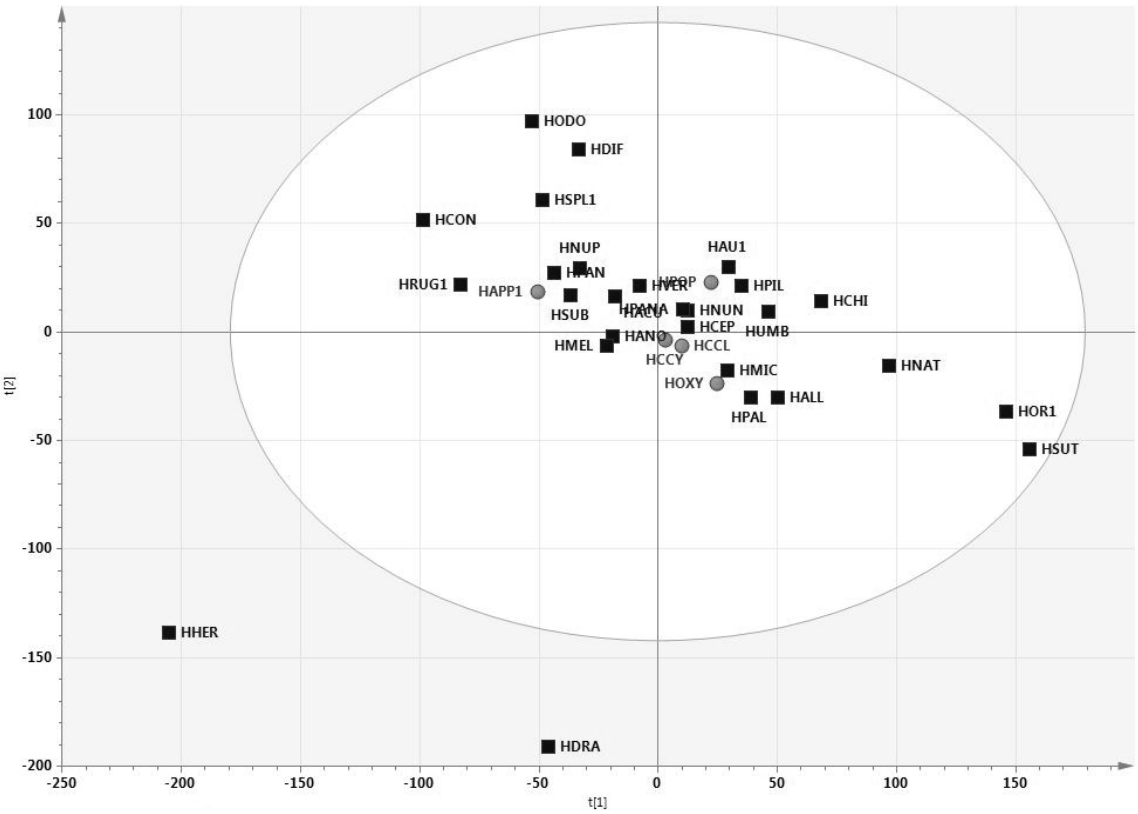

Fig. **3.4.** PCA scores plot which did not show any significant correlation between active (●) and non-active (■) *Helichrysum* extracts.

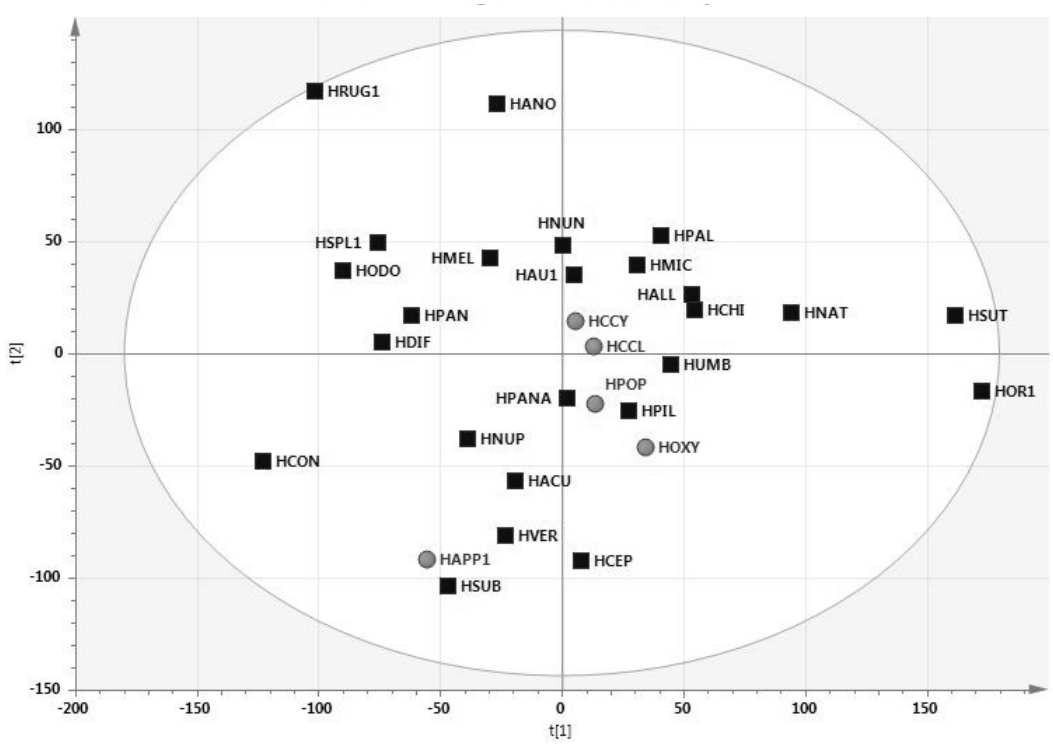

Fig. **3.5.** PCA scores plot which did also not show any significant correlation between active (●) and non-active (■) *Helichrysum* extracts with outliers removed.

Table **3.3:** The Principal Component Analysis (PCA) workset's Principal Component (PC) breakdown.

Component	R2X	R2X(cum)	Eigenvalue	Q2	Q2(cum)	Significance	Iterations
0	Cent.						
1	0.242	0.242	7.25	0.119	0.119	R1	30
2	0.155	0.396	4.64	0.092	0.2	R1	20

This prompted the use of Orthogonal Projections to Latent Structures – Discriminant Analysis (OPLS-DA) where the activity data was included as a secondary variable, which then enabled us to correlate the phytochemical composition to the biological activity. In Fig. 3.6 it can be seen how the active extracts grouped together and are separated from the non-active extracts, indicating that on the basis of activity, there is a phytochemical difference between these two groups.

With OPLS-DA analysis in this study it is important to look into the amount of variation that can be explained between the two groups (active and non-active) of datasets, which is indicated by the R^2Y value (Table 3.4). It is also important to evaluate the amount of variation in X, which can be related to the separation of the two groups, which will be represented by the value R^2X (Eriksson et al. 2008). The OPLS-DA analysis resulted in a significant amount of data being explained in the analysis of the active vs. non-actives, with the R^2Y value being 1.00. The variation in X on the other hand does not explain as much of the variation with the R^2X (cumulative) only being 21%. The predictive component (P1) is also of concern with it only explaining 6% of the variation in X related to the separation of the samples based on the activity. The OPLS-DA models was thus partially able to differentiate between the active samples from the non-active samples based on the phytochemical composition (R^2Y = 1.00, R^2X = 0.21 and R^2 = 0.75) (Table 3.4). The predictability of the model was once again not very significant with Q^2 at only 0.3. This could probably be attributed to the previously mentioned factors influencing the variability of the data, but it should be noted that the narrow (few observations), but wide (large number of variables, buckets) datasets sometimes associated with metabolomics analysis could have contributed to the low predictability. With such a

variable dataset the inclusion of more replicates (biological replicates) could have been beneficial to improve the accuracy of the predication property.

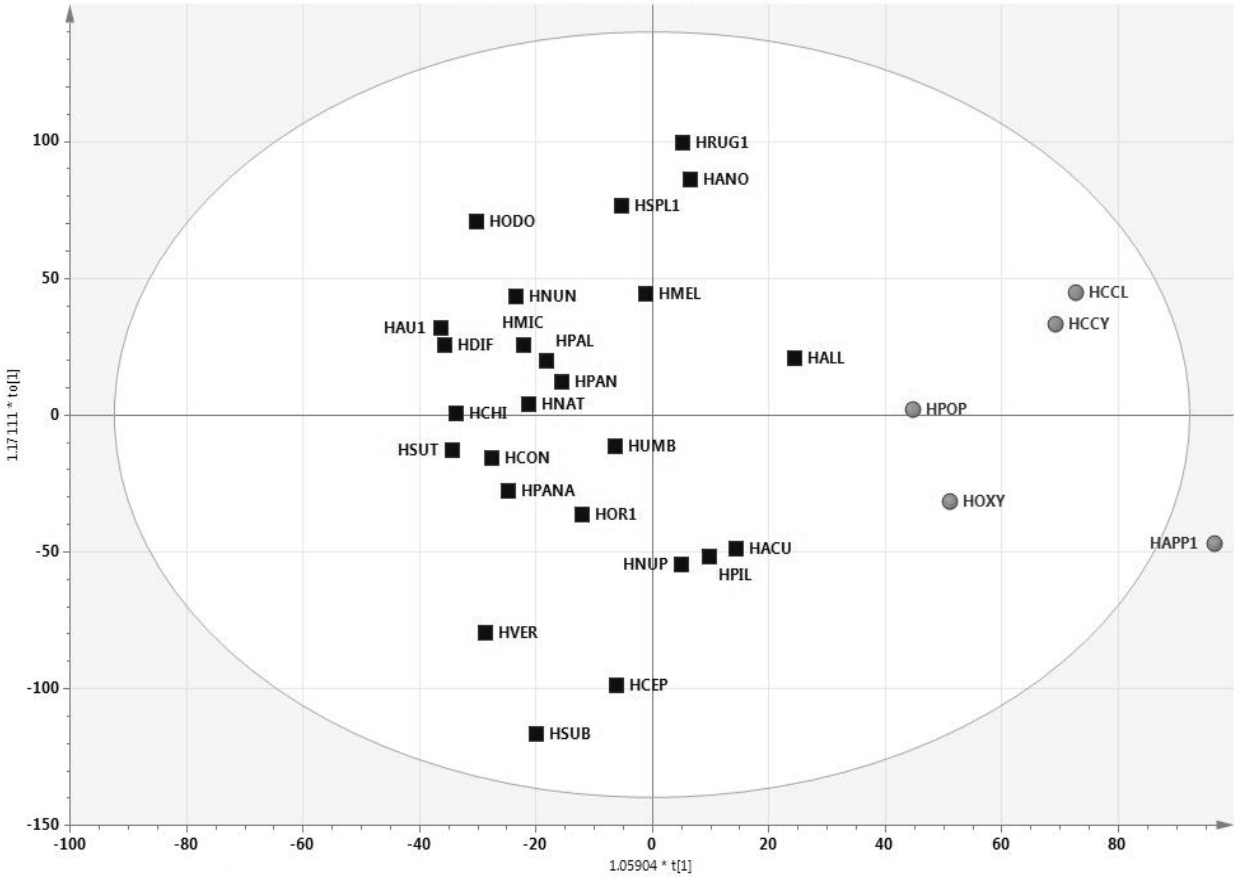

Fig. **3.6.** OPLS scores plot depickting the correlation between active (●) and non-active (■) *Helichrysum* extracts.

With the use of multivariate data analysis it is important to incorporate a form of validation. In this study cross validation (CV)-ANOVA was done to validate the OPLS-DA of the active vs. non-active extracts (Yuliana et al. 2011). The CV-ANOVA validation revealed that the OPLS analysis was significant with $p < 0.055$.

Table **3.4:** The Orthogonal Projections to Latent Structures - Discriminant Analysis (OPLS-DA) workset's Principal Component (PC) breakdown.

Component	R2X	R2X (cum)	Eigenvalue	R2	R2 (cum)	Q2	Q2 (cum)	R2Y	R2Y (cum)	Eigenvalue Y
Model		0.21			0.753		0.3		1	
Predictive		0.0638			0.753		0.3		1	
P1	0.0638	0.0638	1.91	0.753	0.753	0.3	0.3	1	1	2
Orthogonal in X(OPLS)		0.146			0					
O1	0.146	0.146	4.39	0	0					

By making use of a OPLS scores plot, it was possible to generate a contribution plot which takes the loading plots of both the non-active and active groups of *Helichrysum* extracts into consideration and generates a profile that can then be analysed for specific regions of interest, which highlights the phytochemical differences of the active extracts compared to the non-active extracts. In Fig. 3.7 the contribution plot indicates the regions of 2.56 – 3.08 ppm, 5.24 – 6.28 ppm, 6.44 – 7.04 ppm and 7.24 – 8.04 ppm (bars protruding up) as being responsible for activity and that the other chemical shifts (sugars, amino acids, etc.) do not seem to play a role in anti-HIV activity. Furthermore using a S-plot profile to interpret the OPLS better by splitting the uncorrelated variations orthogonally from the predicted variations (Yuliana et al. 2011), it was then clearly shown that the eight buckets (Fig. 3.8) i.e. 2.76, 6.24, 6.44, 6.96, 7.28, 7.32 and 7.48 ppm (2.76 and 6.96 ppm are specific for *H. populifolium*) are very crucial for an extract to be active. These specific regions could be indicative of several compounds (derivatives of each other) having these specific chemical shifts or it could be indicating a combination of compounds working together (synergistically) to achieve the activity.

3.4. Conclusion

The use of traditional bioassay guided fractionation techniques for isolating and identifying NCE has worked very well with the use in antibacterial and antifungal studies, but when it comes to cell based bioassays, bioassay guided fractionation is

no longer ideal. Any technique that could possibly reduce the number of bioassays performed will ultimately benefit the drug discovery process.

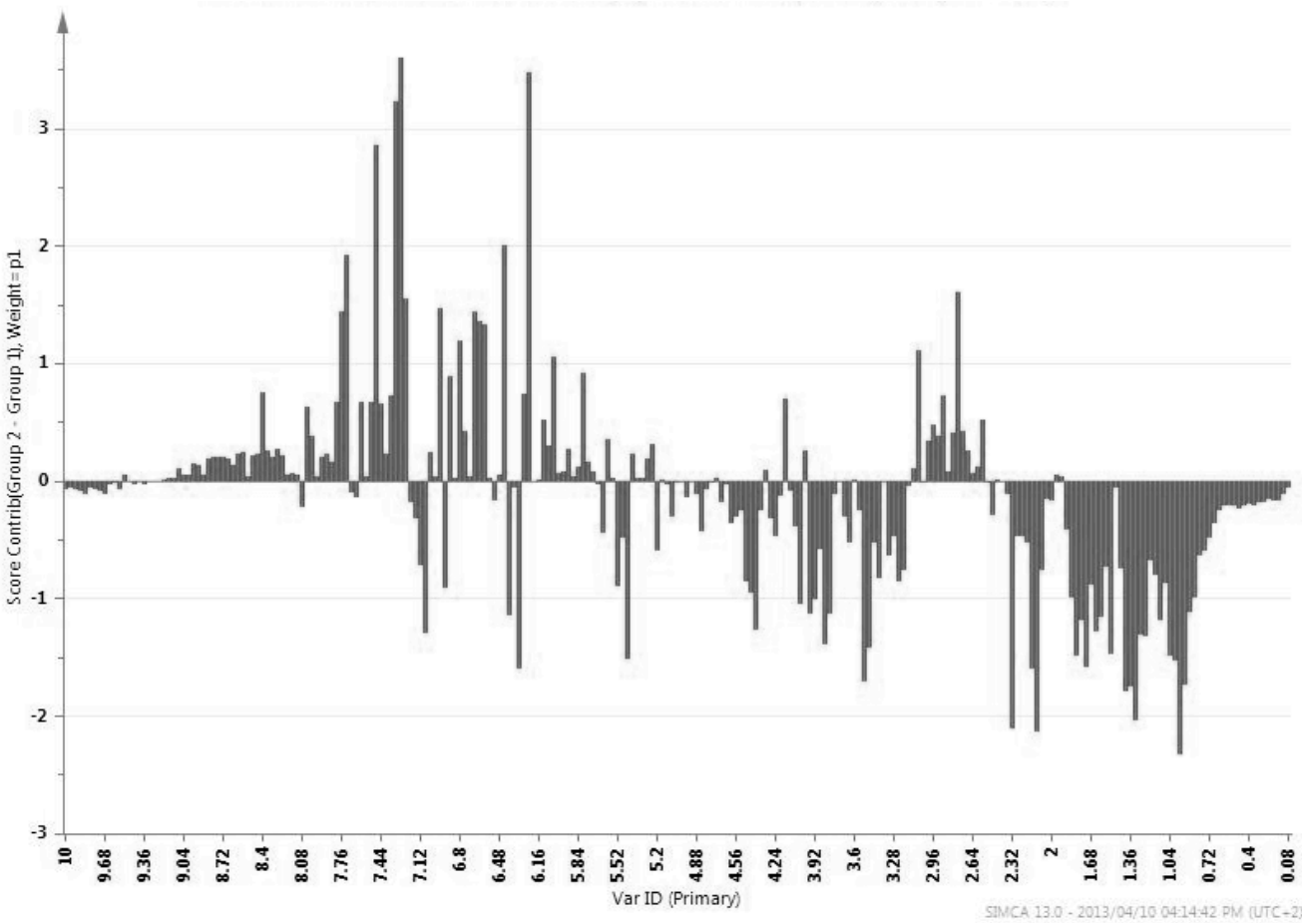

Fig. **3.7.** The contribution plot generated by comparing the active vs. non-active *Helichrysum* extracts. The positive buckets represent the specific regions of the *Helichrysum* NMR spectrum responsible for the activity. The buckets protruding down (negative bars) represent areas that are very prominent in non-active extracts.

By making use of NMR-based metabolomics as a screening technique this study showed an improved method of identifying potential anti-HIV characteristics in the complex plant extracts. With the information gained from a single full virus bioassay screening and the NMR data of the 32 M/W *Helichrysum* extracts, NMR-based metabolomics was able to provide an activity related profile. This profile is the first step in establishing what in the specific active extracts should be focussed on when continuing with purification, identification and finally, if applicable, isolation of

the active constituent(s). Just as important as the active profile are the regions that are identified not to contribute to the activity, as these can be immediately avoid to reduce time wasted on purifying unwanted and most probably non-active constituents.

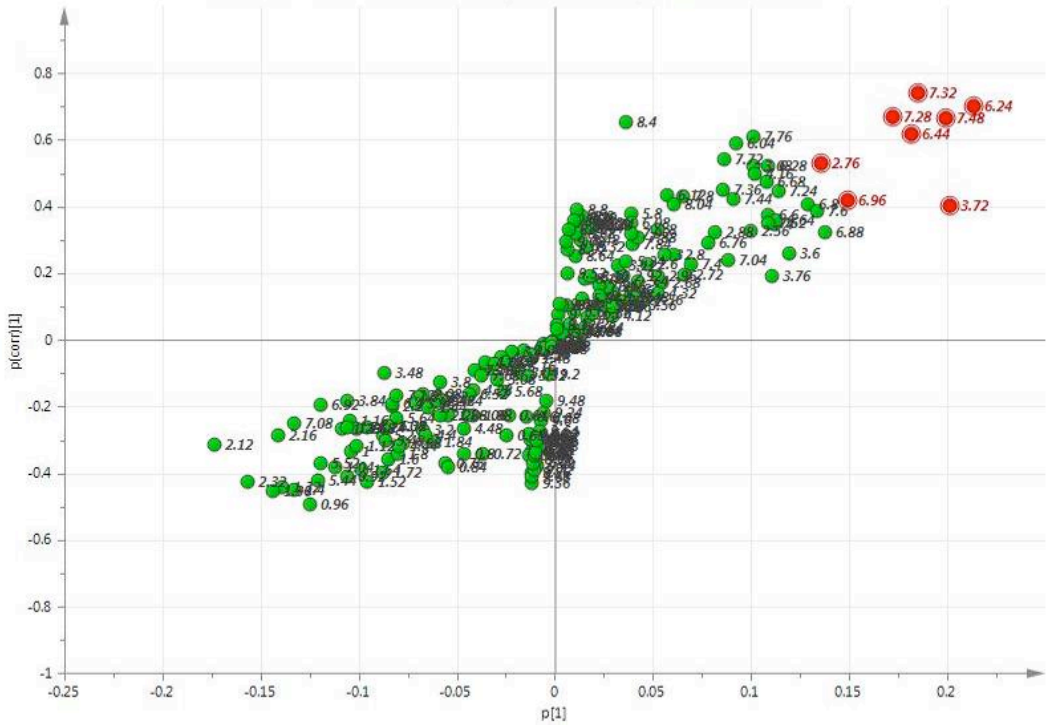

Fig. **3.8.** The loadings S-plot indicating the buckets (red circles) that are most relevant to the activity of the active *Helichrysum* extracts.

When the chemical shifts of interest in the study are studied closer it is interesting to notice that they show patterns that are characteristics to the signals of cinnamoyl-quinic acid type compounds. The typical signals of cinnamoyl units can be observed and on the contribution plot two important groups are present in the region of 6.2 to 6.5 ppm and of 7.5 to 7.7 ppm. These specific chemical shifts could be an indication of the olefinic protons of the trans-cinnamoyl moieties. More downfield regions represent the quinic moiety especially the methylene protons in the 2 to 3 ppm region. Several multiplets in the regions between 3.5 and 6 ppm could be associated with the protons located on the carbons 3, 4 or 5 on the quinic moiety, which are very dependant on their esterification condition. With these observations of the contribution plot it was already possible to focus the search of active constituents

to a specific group or class of compounds (chlorogenic type compounds) of which the chemical properties were known, thus simplifying the purification process.

This screening method thus could potentially accelerate the discovery of NCE with anti-HIV activity, in that it will reduce the number of bioassay runs as well as directing the purification and isolation process in a more focussed manner in search of only the identified anti-HIV characteristic in the plant extract of interest. Having the knowledge of the type of compounds involved can assist in the identification of the proper analytical techniques to be used for the purification step. In this instance, knowing that we are possibly targeting a chlorogenic type compound the use of silica gel chromatography will immediately be avoided as this will cause the loss of these compounds due to their extreme retention to silica resin. It is also possible at this stage of the process to evaluate if the profile is of a known group of compounds and thus establish the necessity to isolate or merely identify the compounds of interest to determine whether these are novel compounds or previously isolated compounds. This will have the advantage of saving time and cost in trying to isolate an already know compound(s).

In this part of the study we were consequently able to identify to a certain extend a pattern that can be linked to the anti-HIV activity of *Helichrysum* methanolic (50%) extracts. With this pattern it will now be possible to continue with purification, identification and isolation of the most active *Helichrysum* extract. Each of the purified fractions could then be compared to this pattern and with that the isolation process will be very narrowly focussed in finding the bioactive compound(s) or synergistically working compounds in the *Helichrysum* extract. It could also be beneficial to use this pattern to compare other *Helichrysum* species (not tested in this study), closely related genera or other chemotypes in search of anti-HIV compounds.

For the purpose of this study there were 32 M/W and 32 DCM extracts, of which the DCM extracts, were not further researched due to their cytotoxicity. The multivariate data analysis (MVA) from the 32 M/W extracts revealed R^2 and Q^2 values that could be argued not to represent a predictable model, as the number of

samples should have been increased to include more biological replicates to improve the predictability. This supports the notion that design of experiment (DOE) should be a more prominent role in the development of research projects to avoid unnecessary repetitions due to low statistical predictability. Increased number of biological replicates would probably increase the predictability as well as the specificity of the activity profile, thus in the case of this study, potentially directing the purification to more specific derivatives of chlorogenic type of compounds, e.g. specific di- or tri-substituted caffeoylquinic acids.

3.5. References

Agrawal, L., Lu, X., Jin, Q. & Alkhatib, G. (2006). Anti-HIV therapy: Current and future directions. Current Pharmaceutical Design 12, 2031-2055 doi:10.2174/138161206777442100

Blomberg, B. & Fourie, B. (2003). Fixed-dose combination drugs for tuberculosis. Drugs 63, 535-553 doi:10.2165/00003495-200363060-00002

Butler, M. S. (2004). The role of natural product chemistry in drug discovery. Journal of Natural Products 67, 2141-2153

Eriksson, L., Trygg, J. & Wold, S. (2008). CV-ANOVA for significance testing of PLS and OPLS® models. Journal of Chemometrics 22, 594-600 doi:10.1002/cem.1187

Gao, L., Xu, X., Nan, H., Song, J., Zhu, Y., Liu, C. & Cheng, S. (2012). Isolation of cinnamic acid derivatives from the root of *Rheum tanguticum* Maxim. ex Balf. and its significance. Journal of Medicinal Plant Research 6, 929-931

HemaIswarya, S. & Doble, M. (2006). Potential synergism of natural products in the treatment of cancer. Phytotherapy Research 20, 239-249 doi:10.1002/ptr.1841

Hilliard, O. M. (1983). Flora of southern Africa: Asteraceae (Compositae). Inuleae. Gnaphaliinae (first part), National Botanical Institute.

Kim, H. K., Choi, Y. H. & Verpoorte, R. (2006). Metabolomic analysis of *Catharanthus roseus* using NMR and principal component analysis. In: Saito, K., Dixon, R. & Willmitzer, L. (eds) Plant Metabolomics. Biotechnology in agriculture and forestry, Vol 57. Springer Berlin Heidelberg. pp. 261-276

Klimkait, T., Stauffer, F., Lupo, E. & Sonderegger-Rubli, C. (1998). Dissecting the mode of action of various HIV-inhibitor classes in a stable cellular system. Archives of Virology 143, 2109-31

Lourens, A. C. U., Viljoen, A. M. & Van Heerden, F. R. (2008). South African *Helichrysum* species: A review of the traditional uses, biological activity and phytochemistry. Journal of Ethnopharmacology 119, 630-652 doi: http://dx.doi.org/10.1016/j.jep.2008.06.011

Louvel, S., Battegay, M., Vernazza, P., Bregenzer, T., Klimkait, T. & Hamy, F. (2008). Detection of drug-resistant HIV minorities in clinical specimens and therapy failure. HIV Medicine 9, 133-141

Newman, D. J. & Cragg, G. M. (2007). Natural products as sources of new drugs over the last 25 years. Journal of Natural Products 70, 461-477 doi:10.1021/np068054v

Sams-Dodd, F. (2006). Drug discovery: selecting the optimal approach. Drug Discovery Today 11, 465-472

Schellenberg, D., Abdulla, S. & Roper, C. (2006). Current Issues for anti-malarial drugs to control *P. falciparum* malaria. Current Molecular Medicine 6, 253-260

Schmidt, B. M., Ribnicky, D. M., Lipsky, P. E. & Raskin, I. (2007). Revisiting the ancient concept of botanical therapeutics. Nature Chemical Biology 3, 360-366

Van der Kooy, F., Maltese, F., Choi, Y. H., Kim, H. K. & Verpoorte, R. (2009). Quality control of herbal material and phytopharmaceuticals with MS and NMR based metabolic fingerprinting. Planta Medica 75, 763-775

Vidal, V., Potterat, O., Louvel, S., Hamy, F., Mojarrab, M., Sanglier, J. J., Klimkait, T. & Hamburger, M. (2012). Library-based discovery and characterization of daphnane diterpenes as potent and selective HIV inhibitors in *Daphne gnidium*. Journal of Natural Products 75, 414-9 doi:10.1021/np200855d

Yuliana, N. D., Khatib, A., Verpoorte, R. & Choi, Y. H. (2011). Comprehensive extraction method integrated with NMR metabolomics: A new bioactivity screening method for plants, adenosine A1 receptor binding compounds in *Orthosiphon stamineus* Benth. Analytical Chemistry 83, 6902-6906 doi:10.1021/ac201458n

Chapter 4

NMR-based metabolomic guided fractionation and identification of anti-HIV active compounds

Chapter 4

NMR-based metabolomic guided fractionation and identification of anti-HIV active compounds

4.1. Introduction

The search of new chemical entities (NCE) from natural products was a very active field and also probably the main field of drug discovery until the late 1970's. Since the 1980's drug discovery had a huge turning point with the major pharmaceutical companies moving away from natural products as a source of NCE and focusing their time, energy and funds on high-throughput screening (HTS) of synthetic combinatorial chemistry libraries in search of NCE, which was intended for the mass production of novel drugs and the resultant high revenue windfalls (Koehn and Carter 2005). It is thus ironic that in 2001, 20 years after the introduction of combinatorial chemistry, NCE discovery had hit a 20 years low with only 37 NCE being discovered and the FDA only receiving 16 new drug applications in the same year (Newman et al. 2003). It is thus these unrealized expectations of the combinatorial chemistry research and development (R&D) strategy as well as new emerging trends in drug discovery that are renewing the interest in natural products as source of NCE (Koehn and Carter 2005).

The potential of natural products as a source for NCE was discussed in several studies reviewing the history of natural products as well as the future potential of natural products in drug discovery (Butler 2004; Koehn and Carter 2005; Newman and Cragg 2007; Newman et al. 2003). The result of their studies showed that natural products played a significant role in the development of the pharmaceutical industry and on top of that still delivered significant amounts of NCE in the last three decades. Of the 1184 NCE discovered or synthesised between 1981

and 2006 more than 34% either originated from natural products or where natural derived products compared to the 37% of purely synthetical NCE (Newman and Cragg 2007).

Plant based compounds contribute significantly to the NCE discovered and still play a very important role in supplying leads as well as new precursors for organic synthesis. In a study conducted by Raskin et al. (2002), the contribution of plants was emphasised with estimated world-wide sales in 2002 of alkaloids, terpenes and steroids, glycosides and other medicinal compounds reaching more than US$ 30 billion. At the beginning of the 21st century 27 of the 252 drugs considered by the World Health Organisation as essential and fundamental were exclusively from flowering plants (Rates 2001). Taking into consideration that there are approximately 250 000 living plants species on earth with a much greater diversity of bio-active compounds than any chemical library, most researchers argue that plants are playing and will still play a large role as a source for molecular diversity and novel chemo-types in drug discovery research. It is thus very clear that natural products, especially plants, are a very significant source of small molecules in the search of new leads; it will thus be important and crucial for biologists, organic chemists and clinicians to work together and make use of the rich source of potential/possible leads available in nature.

As plants are poised to, once again, become significant contributors in drug discovery due to the advantages discussed above, it is very important to make the drug discovery research relevant and worthwhile to the industry (Koehn and Carter 2005; Raskin et al. 2002). To stay relevant it is very important that drug discovery from natural products stays competitive and for this to happen new technologies need to be developed and applied to improve screening (rapid screening), hit identification, isolation of hits, characterisation of hits and improved hit-to-lead development of bio-active NCE. There is thus the need to develop multiplex approaches capable of detecting beneficial correlations within plants and other natural products on a multi-targeted approach to be able to discover a multiplet of analogues against specific conditions or pathogens. Such an approach is being investigated in this study with the use of metabolomics as a holistic approach to systems biology.

Traditionally the use of bioassay-guided fractionation was used to direct the isolation and purification process of the extract(s) of interest. Bioassay-guided fractionation integrates the process of separation of constituents in an active plant extract using various analytical methods with the results obtained from a biological assay(s). The process is initiated with testing of an extract(s) of which the active or most active extract is then used for crude separation/purification. Crude fractions are then tested after which active fractions are used for further fractionations and purification. Purified fractions are tested again and the process of bio-activity testing and fractionation continues until a pure compound(s) is obtained. Inactive extracts, fractions or compounds are set aside or discarded and active ones are used for further analysis (Jamil et al. 2012). Bioassay-guided fractionation of plant extracts has been very successful in many plant species as well as the use of it in combination with high-throughput screening, but there are several limitations to such a fractions process. To be successful such a method needs to quick, relative inexpensive and easy to perform to be able to integrate it sufficiently. This has thus lead to fractionation and isolation of constituents being target specific and the loss of the holistic approach. This approach reduces the possibility of finding a NCE with different targets in the same pathogen (Jamil et al. 2012; Mbah et al. 2012; Phillipson 2001).

This study investigates the potential of NMR-based metabolomics to link the bio-activity of a plant extract(s) against an intact pathogen (e.g. HIV virus) to the active constituents, faster and easier. This was done by determining if NMR-based metabolomics can guide the process of fractionation to the identification of the bio-active constituent(s) in *Helichrysum populifolium* by tracing / following the NMR-based profile generated in chapter 3.

4.2. Materials and Methods

4.2.1. Plant selection and collection

Helichrysum populifolium was selected for this study, because it was the most active of the M/W extracts against HIV (IC_{50} 12 µg/ml). In a previous study on *Helichrysum* species, Heyman (2009) also showed that *H. populifolium* had the highest activity against HIV. The aerial parts (flower, leaves and stems) of *H. populifolium* were collected from the wild (Permit no: OP 1926/2012) in southern KwaZulu-Natal in the Umtamvuna Nature Reserve, Port Edward, South Africa at the beginning of 2011 and a herbarium specimen (PRU no. 117138) was stored in the HGWJ Schweickerdt Herbarium, University of Pretoria, South Africa.

4.2.2. Extract preparation

Air-dried plant material (30g) was pulverized and then placed in a SpeedExtractor E916 (Buchi, Switzerland), with 40 ml pressure vessels. Six extraction cycles (heat-up, hold and discharge) with methanol/water (1:1) were used (Table 4.1) to extract the plant material effectively. The extracts were concentrated to dryness using a centrifugal evaporator (EZ 2 Plus, GeneVac, UK). The extraction yielded 6.652 g of *H. populifolium* 50% methanolic extract (22 % yield), which was then used for NMR analysis and fractionation.

Table **4.1:** Extraction parameters used to extract *H. populifolium*.

Cycle no:	Heat-up (50°C)	Hold (100 bar)	Discharge (N_2 gas)
1	1 min	10 min	5 min
2	1 min	10 min	5 min
3	1 min	9 min	5 min
4	1 min	9 min	5 min
5	1 min	6 min	5 min
6	1 min	6 min	5 min

4.2.3. Purification and Identification

Sephadex LH-20 (Sigma, USA) was used for the fractionation of the plant extract. The Sephadex column (dimensions: 25mm (id) x 360 mm (height)) was loaded with 800 – 1800 mg (1500 mg being optimum) of the 50% methanolic extract re-dissolved in methanol/water (1:1). The applied extract was then eluted with water containing increasing proportions of ethanol (0%, 20%, 50% and 100%). For each run 50 – 55 fractions were collected which were then combined after TLC analysis (thin-layered chromatography) on their similar chemistry, resulting in seven final fractions. The yields of each fraction after five runs were as follows: F1 – 756.4 mg, F2 – 1134.6 mg, F3 – 510.6 mg, F4 – 733.7 mg, F5 – 113.5 mg, F6 451.1 mg and F7 – 75.6 mg.

4.2.4. ^{1}H-NMR analysis

NMR analysis of the fractions was done on a Varian 600MHz spectrometer (CSIR, Pretoria, South Africa) operating at a proton NMR frequency of 600.13 MHz. All the M/W fractions were re-dissolved to a concentration of 15 mg/ml in a buffered mixture of CD_3OD (Sigma, USA) and KH_2PO_4 (Fluka, Gemany) - D_2O (Sigma, USA) solution, with the pH adjusted to pH 6 with NaOD (1M) (Sigma, USA). The internal standard trimethylsilane propionic acid sodium salt (0.1% TSP – Sigma, USA) was used for spectral referencing (0.00 ppm) of the M/W samples. All the samples (600 ul) were transferred to 5 mm NMR tubes and for each spectrum 64 scans were recorded with a spectral width of 14 ppm. Temperature was kept constant for each run at 25 °C and all ^{1}H NMR spectra were referenced to the respective internal standards and manually phased and baseline corrected (Whittaker smoother).

The ^{1}H NMR spectra were reduced to ASCII files using MestReNova 8.1.1 (Mestrelab Research, Spain). Normalisation was done by scaling the spectral intensities to 0.1% TSP and the region of 0.00 – 10.00 ppm was reduced to bins of 0.04 ppm in width. The region ranging from 3.28 – 3.36 ppm (residual MeOH) and 4.60 – 5.00 ppm (residual water) were removed prior to statistical analyses for the M/W samples.

With the processed NMR spectrum of fraction 6 (F6) showing the highest similarity with that of the activity profile (Chapter 3, Fig. 3.6) and showing characteristics of several known chlorogenic type compounds, a full characterisation and identification process was done using a Shimadzu liquid chromatography (20A) – ion trap – time of flight mass spectrometer (LC-IT-TOF) (Shimadzu, Japan).

4.2.5. Liquid chromatography – ion trap – time of flight mass spectrometry (LC-IT-TOF) characterisation.

For full characterisation of F6, a Shimadzu LC (20A)-IT-TOF (Shimadzu, Japan) was used with a Kinetix C18 reverse phase column (100 mm x 4.6 mm id, 2.6 µ, Phenomenex, USA) for complete characterization of F6. The solvent system used comprised of solvent A water (1% formic acid, Fluka, Germany) and solvent B acetonitrile (Sigma, USA) (1% formic acid, Fluka, Germany). Solvents were delivered at a total flow rate of 0.2 ml/min. The gradient profile was 20% B to 50% B linearly in 20 min, with a linear increase to 100% B in 2 min followed by 5 min of isocratic flow after which it was returned to 20% B at 28 min with another isocratic flow for 7 min to allow the column to equilibrate. Conditions for MS analysis (ESI^-) of F 6 included an interface voltage of -3.50 kV, nebulizing gas flow at 1.50 L/min, CDL (heated capillary) and heat block temperature of 200° as well as the detector voltage at 1.61 kV. For the tandem MS/MS analysis the CID energy as well as the collision gas (Argon gas) was set to 50%.

4.3. Results and Discussion

In this part of the study we continued with the NMR-based metabolomic screening described in Chapter 3, in which a “blue print” activity profile or contribution plot (Fig. 4.1 – replicated from Fig. 3.6) was obtained indicating specific regions associated with activity generated from several active *Helichrysum* extracts. The profile shows the regions of importance as 2.56 – 3.08 ppm, 5.24 – 6.28 ppm, 6.44 – 7.04 ppm and 7.24 – 8.04 ppm (bars protruding up) being directly correlated to the bio-activity of the anti-HIV *Helichrysum* extracts. This profile was generated from data, which was obtained by comparing active *Helichrysum* extracts’ NMR profiles against the

NMR profiles of the non-active *Helichrysum* extracts (Appendix, Chapter 7) to produce an Orthogonal Projections to Latent Structures (OPLS) scores plot (Chapter 3, Fig. 3.5). From the OPLS scores plot it was also possible to generate a loadings S-plot (Chapter 3, Fig. 3.7) which indicated the buckets that were responsible for the largest separation within the data. These buckets 2.76, 6.24, 6.44, 6.96, 7.28, 7.32 and 7.48 ppm (2.76 and 6.96 ppm were crucial for *H. populifolium*) were shown to be significant for an extract to be active. These regions could be indicative of several compounds (derivatives of each other) having these specific chemical shifts or it could be indicating a combination of compounds working together (synergistically) to achieve the observed activity. Special attention was thus given to these buckets in trying to identify a compound(s) that have similar signals to these buckets.

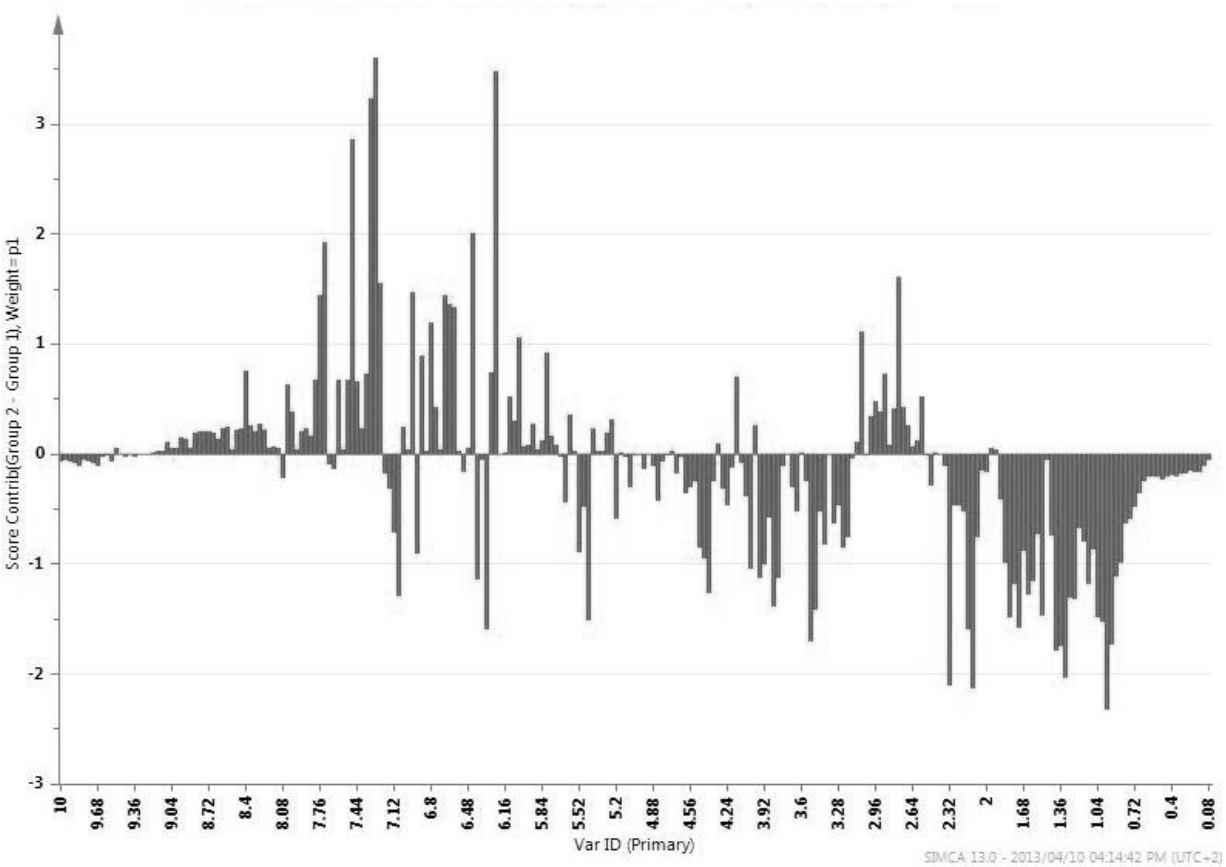

Fig. **4.1.** The contribution plot generated by comparing the active vs. non-active *Helichrysum* extracts. The buckets (positive bars) represent the specific regions of the *Helichrysum* NMR spectrum responsible for the activity. The buckets (negative bars) protruding down represent areas that are very prominent in non-active extracts (replicated from Chapter 3 due to repeated referencing in Chapter 4 and for easier comparison).

It was also assumed that areas falling outside of the above-mentioned regions could be discarded in the search for anti-HIV compounds, as these would most probably not contribute significantly to the activity.

After identifying the NMR regions of interest as well as the most active plant extract, *H. populifolium*, it was very important to isolate the active compounds relatively easy and in the shortest time period. Thus to stay within the objective of speeding up drug discovery, Sephadex LH-20 was used to quickly separate the active *H. populifolium* extract into chemically significantly different fractions. Sephadex was used due to its quick nature, being able to produce reproducible separation and low retention of compounds (reduced loss of total extract).

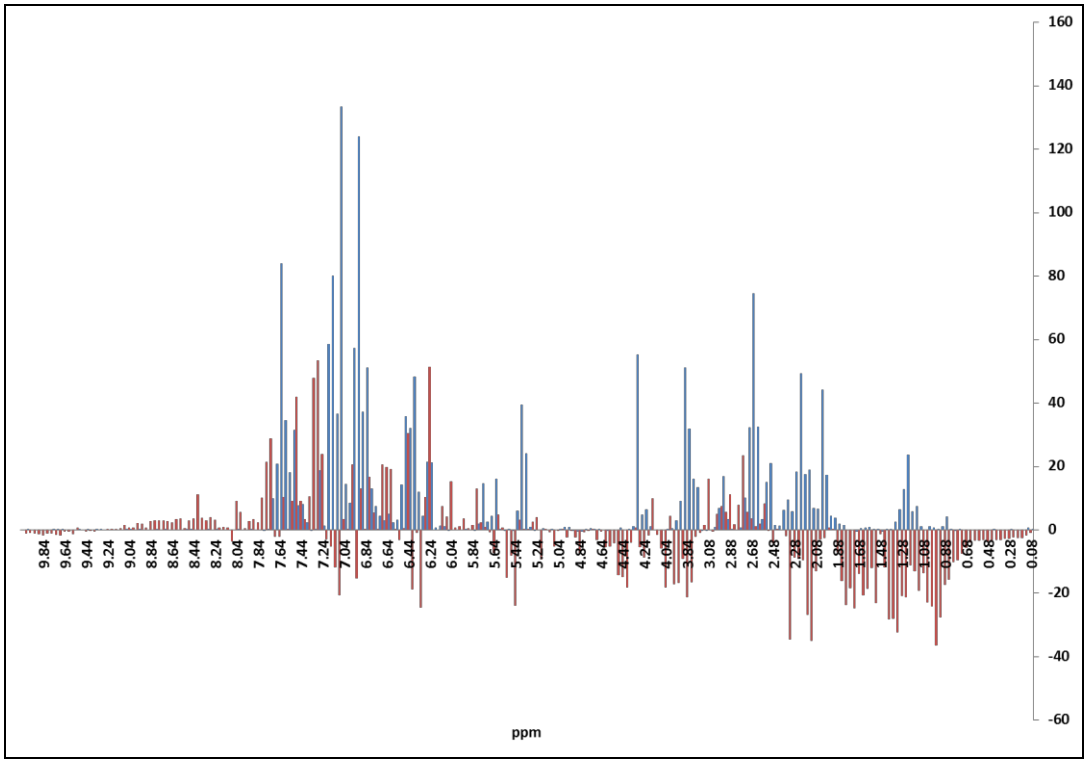

Fig. **4.2.** Illustration of the comparison of purified F6 (blue) of *H. populifolium* against the contribution plot (red) with the anti-HIV "blue print" activity profile.

When the NMR spectral data (Appendix, Chapter 7) of the fractions are compared to the contribution profile ("blue print") (Fig. 4.2), it shows that F6 (Fig. 4.3) is the most similar. By analysing F6 in more detail it was possible to identify three

regions that strongly correlated to the buckets and regions of the loading S-plot and the contribution plot, respectively. The chemical shifts 2.5 – 3 ppm could be indicative of the methylene protons at position 2 and 6 of the quinic acid (Fig. 4.4), where as the small signals between 4 – 6 ppm could be related to the protons at positions 3, 4 and 5 of the quinic acid which can be substituted by cinnamic acid. The 6.0 – 8.0 ppm region representing the aromatic and ethylene protons seems to be an important part of the activity profile as mentioned previously. In this region the coupling constant of 16Hz in the region of 6.0 – 6.5 ppm and 7.3 – 7.8 ppm are significant as these are normally associated with the ethylene protons of the double bond of the cinnamic acid of chlorogenic type compounds and which were very well represented in the contribution plot of the study. The region between 2.0 – 3.0 ppm is indicative of the number of cinnamic acids substituted to the quinic acid with higher chemical shifts being associated with the increased number of substitutions. Thus with the contribution plot showing that 2.5 – 3.0 ppm is of importance to the activity it could indicate that the activity could increase with the number of substitutions increasing.

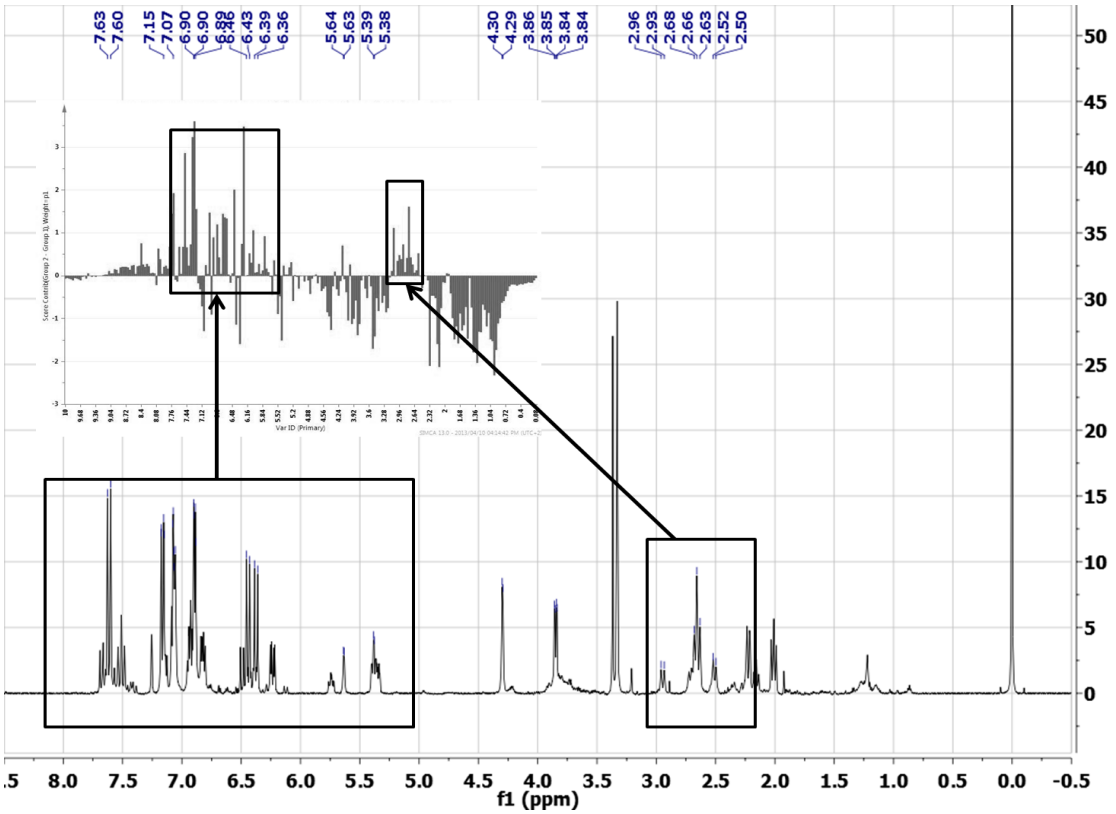

Fig. **4.3.** The NMR spectrum of fraction 6 correlating the best to all the regions of the activity profile (insert representing the contribution plot).

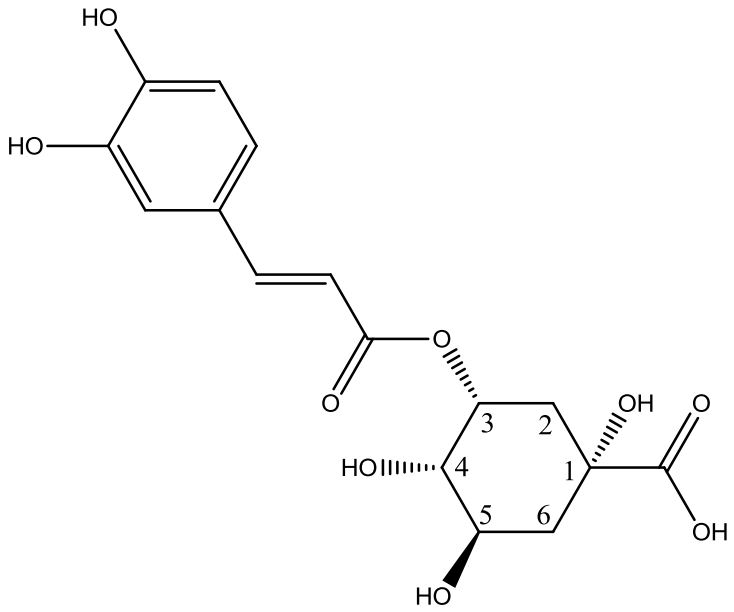

Fig. **4.4.** General structure (IUPAC numbering) of a quinic acid with a cinnamic acid substitution on postion 3, i.e. 3-chlorogenic acid.

Fraction 3 (Fig. 4.5) on the other hand showed chemical shifts correlating with that of chlorogenic acid, which was different in several regions when compared to F6 as well as the activity profile. As indicated above, the region between 2.5 – 3.0 ppm show the correlation to the number of cinnamic acids (di- or tri-substituted) substituted onto the quinic acid and activity, the mono substituted chlorogenic acid indicated chemical shifts (1.8 – 2.2 ppm) outside this region. The fact that chlorogenic acids' chemical shifts fall outside the regions of interest, as well as being reported to not have any significant activity against HIV (McDougall et al. 1998; Tamura et al. 2006), thus supports the validity of the activity profile as being able to show the specific characteristic important for the anti-HIV activity.

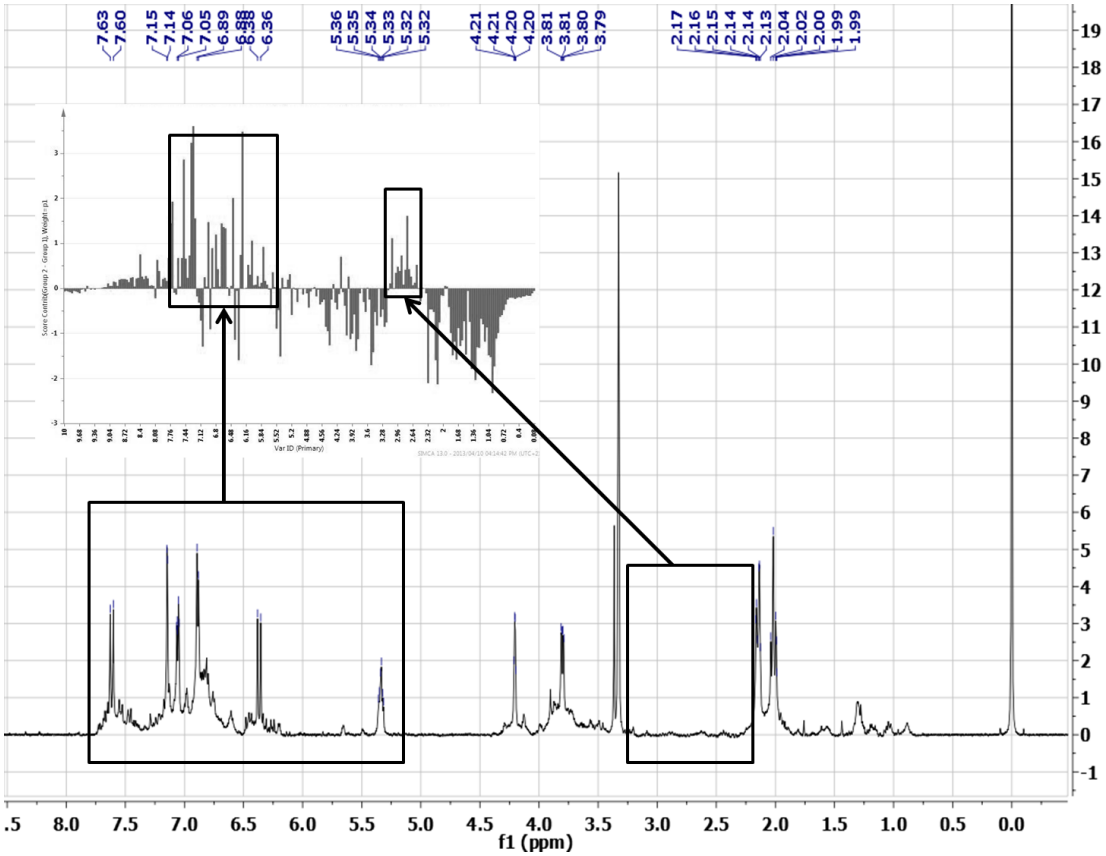

Fig. **4.5.** The NMR spectrum of fraction 3, with the correlation not fitting to the activity profile (contribution plot insert), especially in the region 2.5 – 3 ppm.

With F6 showing the highest similarity, it was selected for characterisation using liquid chromatography - ion-trap - time-of-flight mass spectrometry (LC-IT-TOF) analysis to identify the compounds possibly responsible for the activity. By making use of LC-IT-TOF, identification of the compounds of interest could be done relatively fast and thus supported the objective of this study to speed up drug discovery. This approach should identify and verify the active compounds with only a small quantity of fraction and without complete isolation needed of the specific compound of interest. With putative identification of the compounds it will be possible to evaluate further action on the fraction of interest. If the compounds are novel, further purification, isolation and structural elucidation will be required, but if the compounds are known, only identification and confirmation of the compounds will be necessary thus eliminating extensive purification and complete characterisation.

Five major compounds were observed in F6 and all of these were identified as being chlorogenic acid derivatives (Fig. 4.6). By making use of the a LC-IT-TOF, MS^4

fractionation was carried out to obtain the specific fractionation pattern with which the identification of the different chlorogenic type compounds within the fraction could be done similarly to that described in the studies of Clifford et al. (2005), Clifford et al. (2007), Karakose et al. (2011) and King et al. (1999).

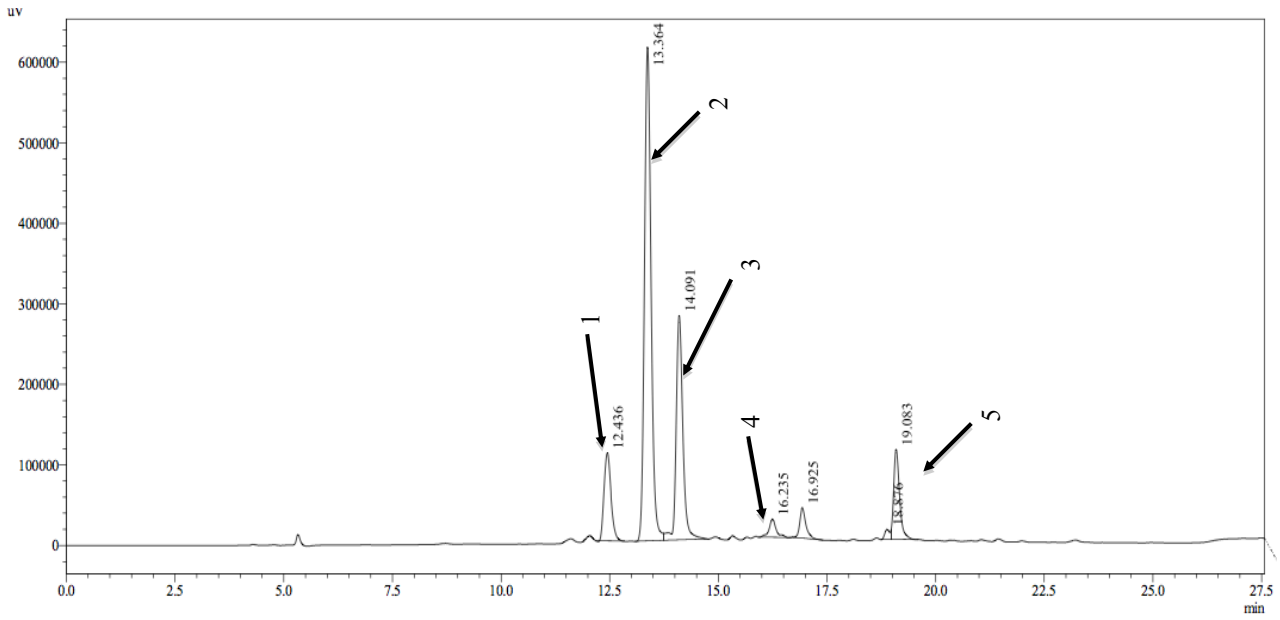

Fig **4.6.** The LC-IT-TOF chromatogram for fraction 6 with the five major chlorogenic type compounds identified (Peak 1; 3,4-DCQA (516 M_r), 2; 3,5-DCQA (516 M_r), 3; 4,5-DCQA (516 M_r), 4; 1,3,5-TCQA (678 M_r) and 5; 5-malonyl-1,3,4-TCQA or 3-malonyl-1,4,5-TCQA (764 M_r)).

The fractionation patterns (Table 4.2) indicated that three of the major compounds were the dicaffeoylquinic acids (DCQA) i.e. 3,4-DCQA (516 M_r), 3,5-DCQA (516 M_r) and 4,5-DCQA (516 M_r) (Fig 4.7 a,b and c respectively). The fragmentation pattern was used to differentiate between the DCQA's with cinnamic substitution at position 4, and those without. This was done by observing the MS^3 base peak of m/z ~ 173.0 which was always supported by the MS^4 fragments of m/z ~ 93.0 and m/z ~ 111.1. These are different to the fragments observed for the 3,5-DCQA as it has a distinct MS^3 base peak of m/z ~ 191.1 with subsequent support from the MS^4 base peaks of m/z ~ 85.0 and m/z ~ 127.0. Thus it was possible to unequivocally differentiate between 3,4-DCQA, 3,5-DCQA and 4,5-DCQA on the basis of the very specific MS^3 fragment ion of m/z ~ 173.0 which is diagnostic for position 4 (Clifford et al. 2005; Clifford et al. 2007; Gao et al. 2012).

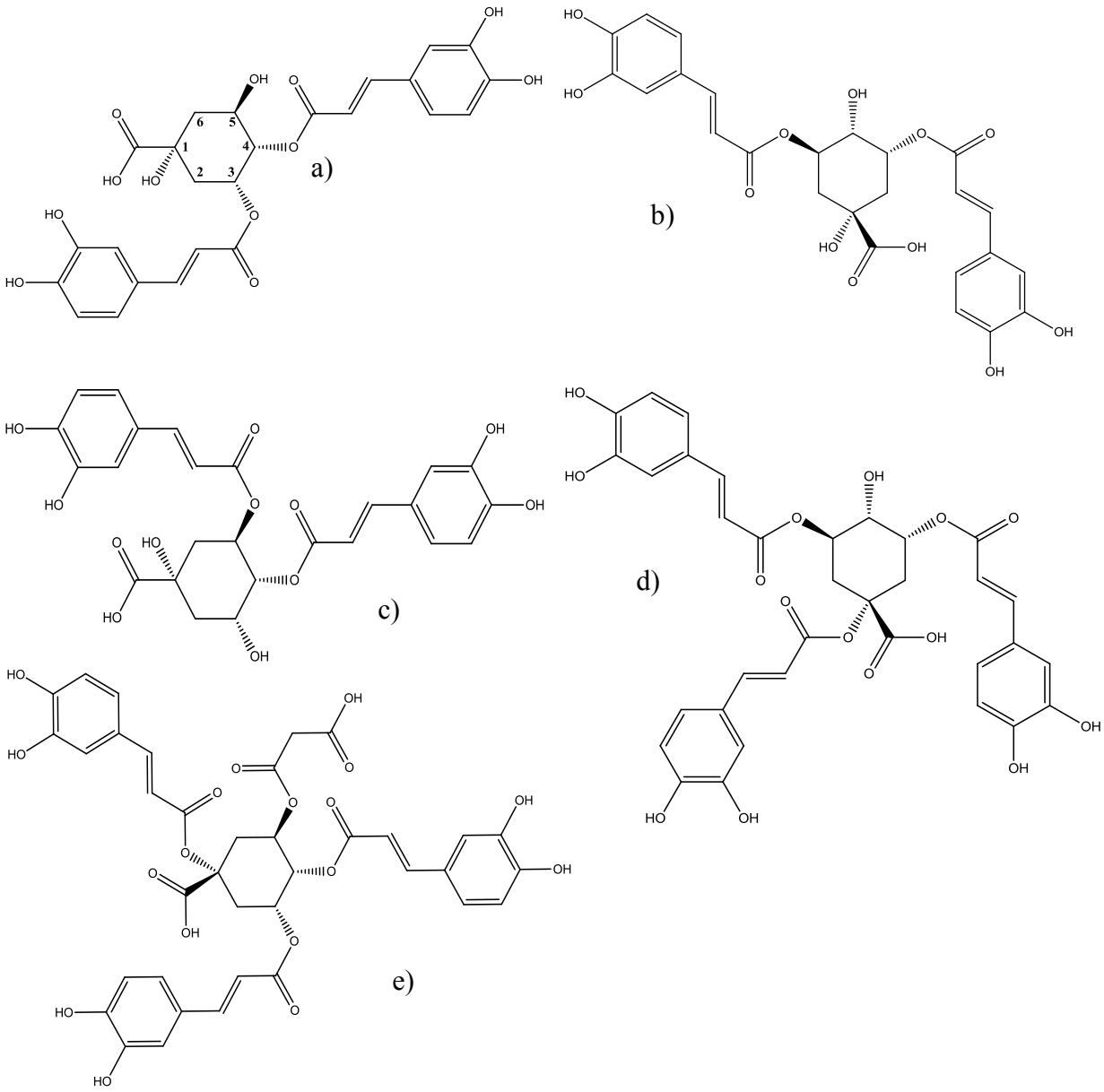

Fig **4.7.** The five anti-HIV active compounds putatively identified in the most active fraction of *H. populifolium*, a) 3,4-DCQA, b) 3,5-DCQA, c) 4,5-DCQA, d) 1,3,5-TCQA and e) 5-malonyl-1,3,4-TCQA.

Differentiating between the isomers with a substitution at position 4 is however a bit more difficult. It was possible to differentiate between these two isomers on the basis of some of the fragment ions that differed in their intensities. For 3,4-DCQA, the dehydrated MS^2 ion 335.1 was indeed present, as well as the

secondary MS^3 ion of 191.1, was more than 70 % higher than in the case of the 4,5-DCQA isomer, thus making it possible to differentiate to a certain extent between all three DCQA derivatives with the use of the hierarchical classification study of Gao et al. (2012) and Clifford et al. (2005).

The other two chlorogenic type compounds were putatively identified as tricaffeoylquinic acids (TCQA), the normal substituted TCQA (678 M_r), 1,3,5-TCQA and the other being either a 5-malonyl-1,3,4-TCQA or a 3-malonyl-1,4,5-TCQA (764 M_r) (Fig 4.6 d and e respectively). For the identification of the TCQA, the MS^2 ion of m/z ~ 497.1 clearly showed the “dehydrated” DCQA ion which could be linked to the easy loss of the C1 caffeoyl group with subsequent loss of H_2O. The MS^3 fragment pattern with the very prominent base peak ion of m/z ~ 335.1, is very indicative of the “dehydrated” form of the chlorogenic acid after another caffeoyl group (m/z ~ 162.0) has been lost. Even the hydrated fractionation pattern of the MS^3 and MS^4 ions with the respective base peaks of m/z ~ 353.1 and m/z ~ 191.1, reflect the substitution of 1,3,5-TCQA, which was confirmed with the results of the study conducted by Zhang et al. (2007).

The determination of the substitution of the malonyl-TCQA was more difficult as the substitution that is predicted in this study has to our knowledge never been characterised on the basis of MS^n fragmentation. The study of Zhang et al. (2007) characterised the 4-malonyl-1,3,5-TCQA up to a MS^3 level, thus not describing the fractionation of the chlorogenic acid component of the TCQA derivative. For the malonyl-TCQA derivative in this study the MS^2 fragmentation pattern indicated the malonic acid being fragmented at a higher rate compared to that of the 4-malonyl-1,3,5-TCQA, thus possibly indicating the malonic acid situated on a less stable C^n-caffeoyl, either on the C-5 or C-1 caffeoyl. The “dehydrated” DCQA fragment ion in MS^3 was one again indicative of the first fragmentation taking place at C-1 caffeoyl together with the removal of H_2O, thus providing the info suggesting the malonic acid substituted at the C-5 caffeoyl. Further fragmentation ions of MS^4 suggested a substitution at position 4 on the DCQA substructure, but the existence of a 4,5-DCQA substitution could possibly be rejected as the presence of a m/z ~ 335.1 ion is not associated with such a substitution (Clifford et al. 2005). These fragmentation

patterns thus led us to a preliminary identification of the malonyl-TCQA in the fraction with a configuration of 5-malonyl-1,3,4-TCQA.

With three compounds identified and another two putatively identified in F6, it was important to determine if they or similar compounds have been investigated previously for anti-HIV activity. The study of Tamura et al. (2006) and McDougall et al. (1998) reported anti-HIV activity (ED_{50}) of 3,4,5-TCQA, 3,4-DCQA, 3,5-DCQA and 4,5-DCQA as 1.15 µM, 12.0 µM, 2.0 µM and 4.0 µM (AZT 1µM) respectively. Several other DCQA's were also reported to have significant anti-HIV activity ranging from 2 to 7 µM (McDougall et al. 1998). This study also reported specific activity against the HIV integrase (HIV IN) for 3,4-DCQA, 3,5-DCQA and 4,5-DCQA at 0.71µM, 0.66µM and 0.30µM which supports the study of that also indicated significant activity of several DCQA's against HIV IN, ranging from 2µM to 12µM. It is thus clear that DCQA's and TCQA's are very potent inhibitors of the HI virus and more specifically targeting HIV IN.

In many of the Asteraceae family species the diversity and content of chlorogenic acid and dicaffeoylquinic acids are significant and as secondary metabolites are known to be important for UV protection, UV sensing and antiherbivory and protection against plant pathogens (Jaiswal et al. 2011). The presence of chlorogenic acids derivatives in the Asteraceae family (*Youngia japonica, Achillea millefolium, Arnica montana, Artemesia dracunculus, Cichorium intybus, Cnicus benedictus, Cynara scolymus, Echinops humilis, Inula helenium, Lactuca sativa, Petasites hybridus, Solidago virgaurea, Tanacetum parthenium,* etc.) is well known with several studies having investigated biological activities like antioxidant, anti-inflammatory, anti-HIV, anti-HBV, radical scavenging, inhibition of mutagenesis and carcinogenesis and for them being overall beneficial to human health (Fraisse et al. 2011; Gouveia and Castilho 2012; Jaiswal et al. 2011; Ooi et al. 2006).

Table **4.2:** MS^4 fragmentation data for the putatively identified di- and tricaffeoylquinic acids from fraction 6 of *Helichrysum populifolium* (based on fragmentation patterns in Clifford et al. 2005; Clifford et al. 2007; Gao et al. 2012; Karakose et al. 2011; King et al. 1999; Zhang et al. 2007).

Compound	Parent ion m/z	MS^2 base peak m/z	MS^2 secondary ions										MS^3 base peak m/z	MS^3 secondary ions								MS^4 base peak m/z	MS^4 secondary ions							
			m/z	int*	m/z	int*	m/z	int*	m/z	int*	m/z	int*		m/z	int*	m/z	int*	m/z	int*	m/z	int*		m/z	int*	m/z	int*	m/z	int*	m/z	int*
3,4-DCQA	515.1	353.1	335.1	8	317.1	4	299.1	7			203.0	10	173.0	191.1	48	179.0	57	135.0	4			93.0	111.0	22						
3,5-DCQA	515.1	353.1											191.1	179.0	44	173.0	6	135.0	8			85.0	173.0	15	127.0	73	111.1	18	93.0	21
4,5-DCQA	515.1	353.1			317.1	12	299.1	13	255.1	2	203.0	17	173.0	191.1	35	179.0	57	135.0	100			93.0	173.0	53	127.0	60	111.1	66		
1,3,5-TCQA	677.1	497.1	515.1	21	469.1	3	353.1	21	335.1	16			335.1	179.0	6							173.0	179.0	30	161.0	12	135.0	12		
5-malonyl-1,3,4-TCQA or 3-malonyl-1,4,5-TCQA	763.1	497.1	515.1	60	469.1	24	353.1	5	335.1	2	317.1	7	353.1	335.1	4	299.1	8	255.1	2	203.0	12	173.0	191.1	30	179.0	39	135.0	7		

* - intensity

This study showed that with NMR-based metabolomics, it is possible to screen complex extracts to identify the active chemical characteristics in such complex extracts, which can then be isolated and identified to eventually produce hits that could be used as in drug discovery. The presences of all five of these compounds that were identified by LC-IT-TOF have been reported for the first time in *H. populifolium* and support the activity of *H. populifolium* against HIV.

4.4. Conclusion

With the focus of increasing the competitiveness of natural products and with that especially plant products as viable sources for drug discovery in the future, new techniques need to be incorporated. It is also important that holistic techniques should be incorporated to take a broader view at as many metabolites, and their separate interaction, as possible in a single analysis to be able to obtain a bigger picture. Traditional bioassay-guided fractionation has been a very effective tool in drug discovery, but it has several limitations with the narrow focused analysis, being one of them. It was thus the objective of this study to show the potential of NMR-based metabolomics as a tool to guide the fractionation and purification steps of drug discovery and to then be able to speed up the identification and isolation of the most relevant constituents within the active extracts.

In this study we were able to use NMR-based metabolomic guided fraction to narrow down our search to one fraction (F6) that showed a pattern corresponding the best with that of the activity profile from the bio-active extracts. From this fraction, five compounds were identified and as literature revealed were known to have significant anti-HIV activity. The *H. populifolium* extract showed to contain large concentrations of these active chlorogenic type compounds, which could thus provide reason why the extract of *H. populifolium* has more potent activity against HIV.

Thus with a relative small amount of the active extract it was possible to accelerate the identification of the five caffeoylquinic acids derivatives in this study and was the first account of their presence in *H. populifolium*, thus adding to the

knowledge of other *Helichrysum* studies (Afolayan and Meyer 1997; Appendino et al. 2007; Bougatsos et al. 2003; Drewes and van Vuuren 2008; Lall et al. 2006; Lourens et al. 2004; Lourens et al. 2008; Mathekga and Meyer 1998; Meyer and Afolayan 1995; Meyer et al. 1996; Meyer et al. 1997; Van Puyvelde et al. 1989; van Vuuren et al. 2006) in providing a deeper understanding of the *Helichrysum* genus' chemistry and the workings that makes it so interesting and potentially of high value.

NMR-based metabolomics and metabolomics in general, can potentially be used as a screening and guided fractionation technique to scan plant extracts for a multitude of biological activity to be able to identify and finally isolate possible hit compounds that could potentially be used against harmful human pathogens. To our knowledge this was the first attempt to prove the use of NMR-based metabolomics as a tool to identify the anti-HIV characteristic in a complex extract and to use it to guide the fractionation and purification of a complex extract to identify the compounds responsible for its activity.

4.5. References

Afolayan, A. J., Meyer, J. J. M. (1997). The antimicrobial activity of 3,5,7-trihydroxyflavone isolated from the shoots of *Helichrysum aureonitens*. Journal of Ethnopharmacology 57, 177-181

Appendino, G., Ottino, M., Marquez, N., Bianchi, F., Giana, A., Ballero, M., Sterner, O., Fiebich, B. L., Munoz, E. (2007). Arzanol, an anti-inflammatory and anti-HIV-1 phloroglucinol alpha-pyrone from *Helichrysum italicum* ssp. *microphyllum*. Journal of Natural Products 70, 608-12 doi:10.1021/np060581r

Bougatsos, C., Meyer, J. J. M., Magiatis, P., Vagias, C., Chinou, I. B. (2003). Composition and antimicrobial activity of the essential oils of *Helichrysum kraussii* Sch. Bip. and *H. rugulosum* Less. from South Africa. Flavour and Fragrance Journal 18, 48-51 doi:10.1002/ffj.1152

Butler, M. S. (2004). The role of natural product chemistry in drug discovery. Journal of Natural Products 67, 2141-2153

Clifford, M. N., Knight, S., Kuhnert, N. (2005). Discriminating between the six isomers of dicaffeoylquinic acid by LC-MS^n. Journal of Agricultural and Food Chemistry 53, 3821-3832 doi:10.1021/jf050046h

Clifford, M. N., Wu, W., Kirkpatrick, J., Kuhnert, N. (2007). Profiling the chlorogenic acids and other caffeic acid derivatives of herbal chrysanthemum by LC-MS^n. Journal of Agricultural and Food Chemistry 55, 929-36 doi:10.1021/jf062314x

Drewes, S. E., van Vuuren, S. F. (2008). Antimicrobial acylphloroglucinols and dibenzyloxy flavonoids from flowers of *Helichrysum gymnocomum*. Phytochemistry 69, 1745-1749 doi: http://dx.doi.org/10.1016/j.phytochem.2008.02.022

Fraisse, D., Felgines, C., Texier, O., Lamaison, J.-L. (2011). Caffeoyl derivatives: major antioxidant compounds of some wild herbs of the Asteraceae family. Food and Nutrition 2, 181-192

Gao, L., Xu, X., Nan, H., Yang, J., Sun, G., Wu, H., Zhong, M. (2012). Isolation of cinnamic acid derivatives from the root of *Rheum tanguticum* Maxim. ex Balf. and its significance. Journal of Medicinal Plant Research 6, 929-931

Gouveia, S., Castilho, P. C. (2012). *Helichrysum monizii* Lowe: phenolic composition and antioxidant potential. Phytochemical Analysis 23, 72-83 doi:10.1002/pca.1326

Heyman, H. (2009). Metabolomic comparison of selected *Helichrysum* species to predict their antiviral properties. University of Pretoria

Jaiswal, R., Kiprotich, J., Kuhnert, N. (2011). Determination of the hydroxycinnamate profile of 12 members of the Asteraceae family. Phytochemistry 72, 781-790 doi:http://dx.doi.org/10.1016/j.phytochem.2011.02.027

Jamil, M., ul Haq, I., Mirza, B., Qayyum, M. (2012). Isolation of antibacterial compounds from *Quercus dilatata* L. through bioassay guided fractionation. Annals of Clinical Microbiology and Antimicrobials 11, 11

Karakose, H., Jaiswal, R., Kuhnert, N. (2011). Characterization and quantification of hydroxycinnamate derivatives in *Stevia rebaudiana* leaves by LC-MS^n. Journal of Agricultural and Food Chemistry 59, 10143-50 doi:10.1021/jf202185m

King, P. J., Ma, G., Miao, W., Jia, Q., McDougall, B. R., Reinecke, M. G., Cornell, C., Kuan, J., Kim, T. R., Robinson, W. E. (1999). Structure−activity relationships: Analogues of the dicaffeoylquinic and dicaffeoyltartaric acids as potent inhibitors of human immunodeficiency virus type 1 integrase and replication. Journal of Medicinal Chemistry 42, 497-509 doi:10.1021/jm9804735

Koehn, F. E., Carter, G. T. (2005). The evolving role of natural products in drug discovery. Nature Reviews Drug Discovery 4, 206-20 doi:10.1038/nrd1657

Lall, N., Hussein, A. A., Meyer, J. J. M. (2006). Antiviral and antituberculous activity of *Helichrysum melanacme* constituents. Fitoterapia 77, 230-232 doi: http://dx.doi.org/10.1016/j.fitote.2006.01.007

Lourens, A. C., Reddy, D., Baser, K. H., Viljoen, A. M., Van Vuuren, S. F. (2004). *In vitro* biological activity and essential oil composition of four indigenous South African *Helichrysum* species. Journal of Ethnopharmacology 95, 253-8 doi:10.1016/j.jep.2004.07.027

Lourens, A. C. U., Viljoen, A. M., Van Heerden, F. R. (2008). South African *Helichrysum* species: A review of the traditional uses, biological activity and phytochemistry. Journal of Ethnopharmacology 119, 630-652 doi: http://dx.doi.org/10.1016/j.jep.2008.06.011

Mathekga, A., Meyer, J. (1998). Antibacterial activity of South African *Helichrysum* species. South African Journal of Botany 64, 293-295

Mbah, J., Ngemenya, M., Abawah, A., Babiaka, S., Nubed, L., Nyongbela, K., Lemuh, N., Efange, S. (2012). Bioassay-guided discovery of antibacterial agents: in vitro screening of *Peperomia vulcanica*, *Peperomia fernandopoioana* and *Scleria striatinux*. Annals of Clinical Microbiology and Antimicrobials 11, 10

McDougall, B., King, P. J., Wu, B. W., Hostomsky, Z., Reinecke, M. G., Robinson, W. E. (1998). Dicaffeoylquinic and dicaffeoyltartaric acids are selective inhibitors of human immunodeficiency virus type 1 integrase. Antimicrobial Agents and Chemotherapy 42, 140-146

Meyer, J. J. M., Afolayan, A. J. (1995). Antibacterial activity of *Helichrysum aureonitens* (Asteraceae). Journal of Ethnopharmacology 47, 109-111 doi: http://dx.doi.org/10.1016/0378-8741(95)01261-B

Meyer, J. J. M., Afolayan, A. J., Taylor, M. B., Engelbrecht, L. (1996). Inhibition of herpes simplex virus type 1 by aqueous extracts from shoots of *Helichrysum aureonitens* (Asteraceae). Journal of Ethnopharmacology 52, 41-43 doi: http://dx.doi.org/10.1016/0378-8741(96)01387-6

Meyer, J. J. M., Afolayan, A. J., Taylor, M. B., Erasmus, D. (1997). Antiviral activity of galangin isolated from the aerial parts of *Helichrysum aureonitens*. Journal of

Ethnopharmacology 56, 165-169 doi: http://dx.doi.org/10.1016/S0378-8741(97)01514-6

Newman, D. J., Cragg, G. M. (2007). Natural products as sources of new drugs over the last 25 years. Journal of Natural Products 70, 461-477 doi:10.1021/np068054v

Newman, D. J., Cragg, G. M., Snader, K. M. (2003). Natural products as sources of new drugs over the period 1981-2002. Journal of Natural Products 66, 1022-37 doi:10.1021/np030096l

Ooi, L. S., Wang, H., He, Z., Ooi, V. E. (2006). Antiviral activities of purified compounds from *Youngia japonica* (L.) DC (Asteraceae, Compositae). Journal of Ethnopharmacology 106, 187-91 doi:10.1016/j.jep.2005.12.028

Phillipson, J. D. (2001). Phytochemistry and medicinal plants. Phytochemistry 56, 237-43

Raskin, I., Ribnicky, D. M., Komarnytsky, S., Ilic, N., Poulev, A., Borisjuk, N., Brinker, A., Moreno, D. A., Ripoll, C., Yakoby, N., O'Neal, J. M., Cornwell, T., Pastor, I., Fridlender, B. (2002). Plants and human health in the twenty-first century. Trends in Biotechnology 20, 522-31

Rates, S. M. (2001). Plants as source of drugs. Toxicon 39, 603-13

Tamura, H., Akioka, T., Ueno, K., Chujyo, T., Okazaki, K. i., King, P. J., Robinson Jr, W. E. (2006). Anti- human immunodeficiency virus activity of 3,4,5- tricaffeoylquinic acid in cultured cells of lettuce leaves. Molecular nutrition & food research 50, 396-400

Van Puyvelde, L., De Kimpe, N., Costa, J., Munyjabo, V., Nyirankuliza, S., Hakizamungu, E., Schamp, N. (1989). Isolation of flavonoids and a chalcone from *Helichrysum odoratissimum* and synthesis of helichrysetin. Journal of Natural Products 52, 629-33

van Vuuren, S. F., Viljoen, A. M., van Zyl, R. L., van Heerden, F. R., Başer, K. H. C. (2006). The antimicrobial, antimalarial and toxicity profiles of helihumulone,

leaf essential oil and extracts of *Helichrysum cymosum* (L.) D. Don subsp. cymosum. South African Journal of Botany 72, 287-290 doi: http://dx.doi.org/10.1016/j.sajb.2005.07.007

Zhang, Y., Shi, P., Qu, H., Cheng, Y. (2007). Characterization of phenolic compounds in *Erigeron breviscapus* by liquid chromatography coupled to electrospray ionization mass spectrometry. Rapid Communications in Mass Spectrometry 21, 2971-84 doi:10.1002/rcm.3166

Chapter 5

General Discussion

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With the need that has arisen to discover new chemical entities (NCE) for the treatment of many diseases especially viral diseases of which HIV is certainly the single most destructive viral infection needing attention, new drug discovering initiatives are imperative (Lozano et al. 2012; WHO 2012). It was thus the aim of this study to investigate a new method of drug discovery. The drug discovery process of antiviral constituents is very tedious, time consuming and also expensive as most of them are based on cellular bioassays. It would thus be very important to develop a new method that would be able to focus the process of fractionation, purification and isolation. Due to the rapid development of the analytical technologies e.g. LC-MS, GC-MS and NMR, new possibilities are arising more and more for these to be used to improve the drug development pipeline process.

This study was a follow up study from a previous NMR-based metabolomics study by Heyman (2009) in which 12 different *Helichrysum* species were tested for anti-HIV activity. In the current NMR-based metabolomics study the species list was extended to 30 species and one additional infraspecific species. The increase in the number of samples improved the statistical significance of the correlation study and subsequently provides a more specific activity profile with improved specificity regarding the chemical shifts related to the activity of *Helichrysum* water/methanol extracts against HIV.

It is very important to note that the plants selected for this study had a wide range of different morphologies, were collected in several very different geographical areas and have been associated with different traditional uses. With previous studies on the *Helichrysum* genus (Appendino et al. 2007; Bohlmann and Abraham 1979; Bohlmann and Zdero 1980; Bohlmann et al. 1979; Bougatsos et al. 2003; Heyman

2009; Lall et al. 2006; Lourens et al. 2008; Meyer and Afolayan 1995; Meyer et al. 1997; van Vuuren et al. 2006), it was clear that the chemistry of the genus is very interesting with the isolation of several rare and distinct metabolites like cannabinoid type compounds and several interesting phloroglucinols (Bohlmann and Hoffmann 1979; Bohlmann et al. 1984; Bohlmann and Suwita 1979; Drewes and van Vuuren 2008; Jakupovic et al. 1986; Jakupovic et al. 1989).

As metabolomics requires a broad and comprehensive analysis of as many metabolites as possible, the extensive indirect extraction procedure proposed by Kim et al. (2006) was used, where extractions were carried out with dichloromethane (DCM) (50%) and 1:1 methanol/water (50%) solutions. This was done to extract as many possible constituents from the plant material to obtain a comprehensive and holistic snapshot of what the composition was like in the extracts. The extraction then produced both non-polar (DCM) extracts and polar (methanol/water) extracts.

Both the non-polar and polar extracts of the 32 *Helichrysum* samples (64 extracts) were tested against HIV on a fill (live) virus model (Chapter 3). The live virus model gives a good reflection of the effect of all the constituents in the extract against all the targets of the virus. This holistic testing of the activity will decrease the need of extensive enzyme based bioassay screening and also reduce the time spend on bioassay analysis and therefore increase the effectiveness of the purification and isolation steps. Screening was initially done with all 64 extracts at 25 ug/ml and 2.5 ug/ml. This identified several extracts (22 extracts), which inhibited the virus at more than 50% at 25 ug/ml with low or not toxicity observed. This prompted the IC_{50} determination of these 22 extracts to determine more precisely the dose response and specific level of activity of them. Most of the DCM extracts showed significant, thus they were not selected for further analysis. From the 22 extracts, six methanol/water extracts were identified to be the most active with their IC_{50} values ranging from 12 – 21 ug/ml, with no toxicity detectable (Table 3.2). The methanol/water extract of *H. populifolium* was shown to be the most active polar extract at IC_{50} 12 ug/ml.

The next step of the study was to use NMR-based metabolomics to firstly identify the specific chemical characteristic that is shared between the active plant extracts and to also identify the distinct differences between the active and non-active plant extracts (Chapter 3). A proton NMR spectrum of each sample was generated to obtain an overview of the chemistry within the samples.

After processing the NMR data, it was then used in multivariate data analysis (MVA) (SIMCA+P, Umeå, Sweden). Principal component analysis (PCA) was used first to establish if the differences in activity could be explained by simply comparing the chemistry of all 32 M/W extracts against each other with out the inclusion of the biological activity factor. The PCA did not reveal any patterns related to the activity when comparing the 32 M/W extracts (Fig 3.4, 3.5), thus indicating the need for additional information into the dataset to assist the discriminant analysis when comparing the active and non-active extracts. At this point in the study we made use of a supervised MVA technique, Orthogonal Projections to Latent Structures – Discriminant Analysis (OPLS-DA) to explain the variation in our dataset. OPLS-DA as a supervised method separated the extracts into two groups with the inclusion of the bioactivity data (Fig 3.6). The inclusion of the bio-activity data shifted the focus of the discriminant analysis from the general chemical difference between all the extracts to the chemical differences associated solely with the bio-activity (secondary observation). By using the OPLS-DA data it was possible to select the active group cluster as well as the non-active group cluster and create a contribution plot. The contribution plot uses the loadings plots/bins data of each sample within the different clusters and indicates very specifically which chemical shifts (bins) are contributing, and to what extend, to the separation and differentiation of the actives from the non-actives. The active *Helichrysum* samples showed to be distinct in the regions 2.56 – 3.08 ppm, 5.24 – 6.28 ppm, 6.44 – 7.04 ppm and 7.24 – 8.04 ppm (Fig 3.7 - bars protruding up) and with further investigation, making use of the S-plot, the buckets that were shown to be crucial to the activity of a samples were those at 2.76, 6.24, 6.44, 6.96, 7.28, 7.32 and 7.48 ppm (2.76 and 6.96 being very crucial in *H. populifolium*). At this stage a specific characteristic could be identified that seemed to be a common factor between the active

extracts and which potentially could be linked to active metabolites within the active polar *Helichrysum* plant extract.

In this study the very low predictability of the OPLS-DA model was of concern. This was most probably caused by the large variation due to different collection sites, the diverse morphological groups (Hilliard 1983) used and the fact that 30 different *Helichrysum* species, together with one infraspecific species were used. The predictability could most likely be improved with the use of more biological replicates, which will ultimately give more data points to construct a more reliable predictive model. This will without doubt increase cost and time associated with the identification of NCE from natural products. It is thus very important to find a balance between cost and time of analysis as well as accuracy of the PCA or OPLS-DA model.

The second major objective of this study was to look into the possibility of NMR-based metabolomics to be used in a guided fractionation process, where the contribution pattern from the screening process could be used as a “blue print” in the search of the active constituents in the most active *Helichrysum* extract, *H. populifolium*. As the regions are not very specific to only one specific compound, it was noted that the regions could be due to several similar compounds responsible for the activity or maybe a synergistic interaction that was involved. It was thus important to take into consideration that these regions should be used as a guide in the fractionation process based on similarity to this specific profile.

For the fractionation one major aspect in this study had to be adhered to and that was to use a method of fractionation that would be easy and relative quick as the objective of reducing the time spend in identifying constituents as potential hits. It was thus decided to use Sephadex LH-20 (Sigma, USA) for the fractionation, as it is relatively fast, separates both on size and to a certain extend on polarity and it has very low retention of constituents. Fractionation was carried out on approximately six grams of *H. populifolium* extract, which was fractionated into 50 – 60 fractions. Based on their chemistry these were combined into seven major fractions. NMR analysis of all seven

fractions was done and the data was processed into bins to be comparable with the contribution plot profile of all the species.

The contribution plot is a representation of the bins/buckets in terms of their relative importance with regards to their activity against HIV. All seven fractions were then compared to the species activity contribution profile to establish the degree of similarity of the different fractions when compared to this activity profile. Fraction 3 revealed some similarities to the activity profile due to it containing a chlorogenic acid. The fact that the NMR spectra and contribution plot did not fit notably well was then also supported by literature; which revealed that chlorogenic acid (mono-substituted caffeoylquinic acid) does not have activity against HIV (McDougall et al. 1998; Tamura et al. 2006).

Fraction 6 (F6) was a much better fit with the contribution profile with most chemical shifts corresponding to the bins with the highest importance coefficient. With F6 showing the highest similarity, it was selected for characterisation using liquid chromatography - ion-trap - time-of-flight mass spectrometry (LC-IT-TOF) analysis to identify the compounds possibly responsible for the activity. By making use of LC-IT-TOF in the study we were able to identify the content of the fraction without extensive purification or even isolation. This would give us the opportunity to first establish if the compounds within the fraction being responsible for the activity are of interest and are not known already, before investing money and time in isolating the compounds for characterisation.

In F6 five major compounds were revealed, all being identified as being chlorogenic acid derivatives (Fig. 4.5). With the LC-IT-TOF analysis, it was possible to perform MS^4 fragmentations, which made it possible to use the known characteristic fragmentation patterns of caffeoylquinic acids to identify which derivatives were present in this specific fraction. The fragmentation patterns used in the studies of Clifford et al. (2005), Clifford et al. (2007), Karakose et al. (2011) and King et al. (1999) made it possible to identify several dicaffeoyl and tricaffeoylquinic acids. The fragmentation

patterns (Table 4.2) showed that three of the five chlorogenic type compounds were identified as being dicaffeoylquinic acids (DCQA) i.e. 3,4-DCQA (516 M_r), 3,5-DCQA (516 M_r) and 4,5-DCQA (516 M_r) (Fig 4.6 a,b and c respectively). With the fragmentation pattern it was possible to discriminate between the different substituted DCQA's due to the presence of specific fragmented ions and/or their intensities (Clifford et al. 2005; Gao et al. 2012).

Further putative identification revealed two more chlorogenic type compounds, i.e. tricaffeoylquinic acids (TCQA), one being a normal substituted TCQA (678 M_r), 1,3,5-TCQA and the other being either a 5-malonyl-1,3,4-TCQA or a 3-malonyl-1,4,5-TCQA (764 M_r) (Fig 4.6 d and e respectively). When comparing the fragmentation pattern to that of the study of Zhang et al. (2007) it was clear that the TCQA in this fraction was the 1,3,5-TCQA derivative. It was more difficult to identify the substitution of the malonyl-TCQA, due to the fact that the substitution, which is predicted in this study, has as far as we know never been characterised on the basis of MS^n fragmentation. The study of Zhang et al. (2007) characterised the 4-malonyl-1,3,5-TCQA up to a MS^3 level, thus not describing the fragmentation of the chlorogenic acid component of the TCQA derivative. For the malonyl-TCQA derivative in this study the MS^2 fragmentation pattern indicated the malonic acid being fragmented at a higher rate compared to that of the 4-malonyl-1,3,5-TCQA, thus it was possible to putatively identify a different substituted configuration i.e. 5-malonyl-1,3,4-TCQA.

The anti-HIV activity of three of the identified chlorogenic compounds as well as several other DCQA and TCQA derivatives have been reported previously. Published anti-HIV data of 3,4-DCQA, 3,5-DCQA and 4,5-DCQA revealed activity at 12.0 µM, 2.0 µM and 4.0 µM (AZT 1µM) respectively, as well as HIV integrase (HIV IN) activity at 0.71µM, 0.66µM and 0.30µM respectively. Similarly TCQA showed to have anti-HIV activity at levels as low as 1.15 µM against the HI virus. Several other DCQA derivatives had reported anti-HIV activity levels of between 2 to 7 µM (McDougall et al. 1998; Tamura et al. 2006) against the virus and between 2 µM and 12 µM against HIV IN (King et al. 1999). These results thus give a clear picture that DCQA's and TCQA's are

very potent inhibitors of the HI virus as well as specifically targeting HIV IN. Thus by using NMR-based metabolomics as a screening tool as well as a technique to guide the fractionation and purification process it was possible to identify the active constituents in the active *H. populifolium* extract. It is also noteworthy that all five of these compounds were identified for the first time to be present within *H. populifolium*.

This study showed the potential of a metabolomic approach in searching for NCE, and with that, NCE specifically targeted against viral pathogens as these pose several problems to the traditional bioassay-guided fractionation process. NMR-based metabolomics takes the chemical characteristics of both active and non-active extracts into consideration to create a profile that makes it possible for the fractionation process to identify the most relevant fraction to be investigated for potential bioactives. It was thus possible to use NMR-based metabolomics guided fractionation to speed up the discovery of the active compounds.

This study serves as a starting point of NMR-based metabolomic guided fractionation and more research should be done to prove that the guided fractionation can also work effectively when working in several different geographical areas, collected during different seasons as well as from different genera. New developments, both in the technologies like NMR, LC-MS, GC-MS etc., as well as the intricate field of multivariate data analysis, all make the prospects of NMR-based metabolomics and metabolomics in general, as a tool to be used in drug discovery, very exciting.

The phytochemistry of the *Helichrysum* genus is very interesting and also very diverse and will probably serve well as a genus for future research in this regard. With only a few species of the 245 species in South Africa having been investigated to some extend, there is still enormous potential for new discoveries of NCE for diseases and conditions from the *Helichrysum* genus. As was evident in this study, care needs to be taken to classify a species correctly (*H. pilosellum*). It must be said that the classification error made in this study alerted us to the possible influence that geographical distribution might be playing, thus making it worthwhile to include such investigation in future phytochemical studies.

5.2 References

Appendino, G., Ottino, M., Marquez, N., Bianchi, F., Giana, A., Ballero, M., Sterner, O., Fiebich, B. L. & Munoz, E. (2007). Arzanol, an anti-inflammatory and anti-HIV-1 phloroglucinol alpha-Pyrone from *Helichrysum italicum* ssp. *microphyllum.* Journal of Natural Products 70, 608-12 doi:10.1021/np060581r

Bohlmann, F. & Abraham, W. R. (1979). Neue, chlorsubstituierte thiophenacetylenverbindungen mit ungewöhnlicher struktur aus *Helichrysum*-arten. Phytochemistry 18, 839-842 doi: http://dx.doi.org/10.1016/0031-9422(79)80024-2

Bohlmann, F. & Hoffmann, E. (1979). Cannabigerol-ähnliche verbindungen aus *Helichrysum umbraculigerum*. Phytochemistry 18, 1371-1374 doi: http://dx.doi.org/10.1016/0031-9422(79)83025-3

Bohlmann, F., Misra, L. N. & Jakupovic, J. (1984). Weitere phloroglucin- und alpha-pyron-derivate aus *Helichrysum*-arten. Planta Medica 50, 174-6 doi:10.1055/s-2007-969664

Bohlmann, F. & Suwita, A. (1979). Weitere phloroglucin-derivate aus *Helichrysum*-arten. Phytochemistry 18, 2046-2049 doi: http://dx.doi.org/10.1016/S0031-9422(00)82740-5

Bohlmann, F. & Zdero, C. (1980). Neue phloroglucin-derivate aus *Helichrysum*-arten. Phytochemistry 19, 153-155 doi: http://dx.doi.org/10.1016/0031-9422(80)85038-2

Bohlmann, F., Zdero, C. & Ziesche, J. (1979). Neue flavone und phloroglucin-derivate aus *Helichrysum herbaceum* und *Helichrysum chrysargyrum*. Phytochemistry 18, 1375-1378 doi: http://dx.doi.org/10.1016/0031-9422(79)83026-5

Bougatsos, C., Meyer, J. J. M., Magiatis, P., Vagias, C. & Chinou, I. B. (2003). Composition and antimicrobial activity of the essential oils of *Helichrysum*

kraussii Sch. Bip. and *H. rugulosum* Less. from South Africa. Flavour and Fragrance Journal 18, 48-51 doi:10.1002/ffj.1152

Clifford, M. N., Knight, S. & Kuhnert, N. (2005). Discriminating between the six isomers of dicaffeoylquinic acid by LC-MS^n. Journal of Agricultural and Food Chemistry 53, 3821-3832 doi:10.1021/jf050046h

Clifford, M. N., Wu, W., Kirkpatrick, J. & Kuhnert, N. (2007). Profiling the chlorogenic acids and other caffeic acid derivatives of herbal chrysanthemum by LC-MS^n. Journal of Agricultural and Food Chemistry 55, 929-36 doi:10.1021/jf062314x

Drewes, S. E. & Van Vuuren, S. F. (2008). Antimicrobial acylphloroglucinols and dibenzyloxy flavonoids from flowers of *Helichrysum gymnocomum*. Phytochemistry 69, 1745-1749 doi: http: //dx.doi.org/10.1016/j.phytochem.2008.02.022

Gao, L., Xu, X., Nan, H., Yang, J., Sun, G., Wu, H. & Zhong, M. (2012). Isolation of cinnamic acid derivatives from the root of *Rheum tanguticum* Maxim. ex Balf. and its significance. Journal of Medicinal Plant Research 6, 929-931

Heyman, H. (2009). Metabolomic comparison of selected *Helichrysum* species to predict their antiviral properties. University of Pretoria

Hilliard, O. M. (1983). Flora of southern Africa: Asteraceae (Compositae). Inuleae. Gnaphaliinae (first part), National Botanical Institute.

Jakupovic, J., Kuhnke, J., Schuster, A., Metwally, M. A. & Bohlmann, F. (1986). Phloroglucinol derivatives and other constituents from South African *Helichrysum* species. Phytochemistry 25, 1133-1142 doi: http://dx.doi.org/10.1016/S0031-9422(00)81569-1

Jakupovic, J., Zdero, C., Grenz, M., Tsichritzis, F., Lehmann, L., Hashemi-Nejad, S. M. & Bohlmann, F. (1989). Twenty-one acylphloroglucinol derivatives and further constituents from south african *Helichrysum* species. Phytochemistry 28, 1119-1131 doi:http://dx.doi.org/10.1016/0031-9422(89)80195-5

Karakose, H., Jaiswal, R. & Kuhnert, N. (2011). Characterization and quantification of hydroxycinnamate derivatives in Stevia rebaudiana leaves by LC-MS^n. Journal of Agricultural and Food Chemistry 59, 10143-50 doi:10.1021/jf202185m

Kim, H. K., Choi, Y. H. & Verpoorte, R. (2006). Metabolomic Analysis of *Catharanthus roseus* Using NMR and Principal Component Analysis. In: Saito, K. R. & Dixon, L. Willmitzer (eds) Plant Metabolomics. Biotechnology in agriculture and forestry, Vol 57. Springer Berlin Heidelberg. pp. 261-276.

King, P. J., Ma, G., Miao, W., Jia, Q., McDougall, B. R., Reinecke, M. G., Cornell, C., Kuan, J., Kim, T. R. & Robinson, W. E. (1999). Structure−activity relationships: Analogues of the dicaffeoylquinic and dicaffeoyltartaric acids as potent inhibitors of human immunodeficiency virus type 1 integrase and replication. Journal of Medicinal Chemistry 42, 497-509 doi:10.1021/jm9804735

Lall, N., Hussein, A. A. & Meyer, J. J. M. (2006). Antiviral and antituberculous activity of *Helichrysum melanacme* constituents. Fitoterapia 77, 230-232 doi: http://dx.doi.org/10.1016/j.fitote.2006.01.007

Lourens, A. C. U., Viljoen, A. M. & Van Heerden, F. R. (2008). South African *Helichrysum* species: A review of the traditional uses, biological activity and phytochemistry. Journal of Ethnopharmacology 119, 630-652 doi: http://dx.doi.org/10.1016/j.jep.2008.06.011

Lozano, R., Naghavi, M., Foreman, K., Lim, S., Shibuya, K., Aboyans, V., Abraham, J., Adair, T., Aggarwal, R., Ahn, S. Y., AlMazroa, M. A., Alvarado, M., Anderson, H. R., Anderson, L. M., Andrews, K. G., Atkinson, C., Baddour, L. M., Barker-Collo, S., Bartels, D. H., et al. (2012). Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. The Lancet 380, 2095-2128

McDougall, B., King, P. J., Wu, B. W., Hostomsky, Z., Reinecke, M. G. & Robinson, W. E. (1998). Dicaffeoylquinic and dicaffeoyltartaric acids are selective inhibitors of

human immunodeficiency virus type 1 integrase. Antimicrobial Agents and Chemotherapy 42, 140-146

Meyer, J. J. M. & Afolayan, A. J. (1995). Antibacterial activity of *Helichrysum aureonitens* (Asteraceae). Journal of Ethnopharmacology 47, 109-111 doi:http://dx.doi.org/10.1016/0378-8741(95)01261-B

Meyer, J. J. M., Afolayan, A. J., Taylor, M. B. & Erasmus, D. (1997). Antiviral activity of galangin isolated from the aerial parts of *Helichrysum aureonitens*. Journal of Ethnopharmacology 56, 165-169 doi: http://dx.doi.org/10.1016/S0378-8741(97)01514-6

Tamura, H., Akioka, T., Ueno, K., Chujyo, T., Okazaki, K. i., King, P. J. & Robinson Jr, W. E. (2006). Anti–human immunodeficiency virus activity of 3,4,5–tricaffeoylquinic acid in cultured cells of lettuce leaves. Molecular nutrition & food research 50, 396-400

Van Vuuren, S. F., Viljoen, A. M., Van Zyl, R. L., Van Heerden, F. R. & Başer, K. H. C. (2006). The antimicrobial, antimalarial and toxicity profiles of helihumulone, leaf essential oil and extracts of *Helichrysum cymosum* (L.) D. Don subsp. *cymosum*. South African Journal of Botany 72, 287-290 doi: http://dx.doi.org/10.1016/j.sajb.2005.07.007

WHO (2012). 2012 UNAIDS Report on the Global AIDS Epidemic. Geneva: World Health Organization,

Zhang, Y., Shi, P., Qu, H. & Cheng, Y. (2007). Characterization of phenolic compounds in *Erigeron breviscapus* by liquid chromatography coupled to electrospray ionization mass spectrometry. Rapid Communications in Mass Spectrometry 21, 2971-2984 doi:10.1002/rcm.3166

Chapter 6

Acknowledgements

Chapter 6

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Chapter 7

Appendix

7.1 NMR spectra of the methanol/water plant extract

7. 1. 1. *Helichrysum acutatum*

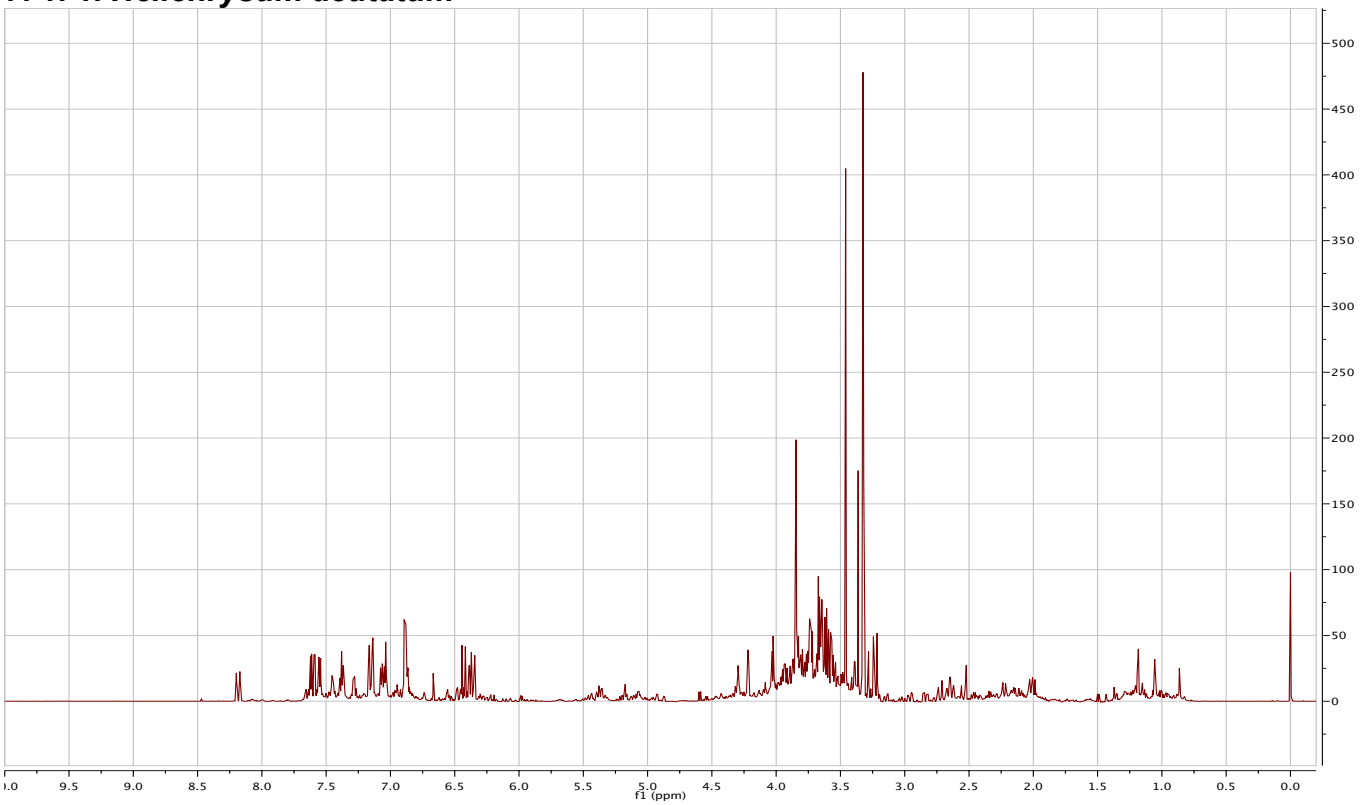

Fig 7. 1: NMR spectrum of the methanol/water plant extract of *H. acutatum* (600 MHz, Leiden University).

7. 1. 2. *Helichrysum allioides*

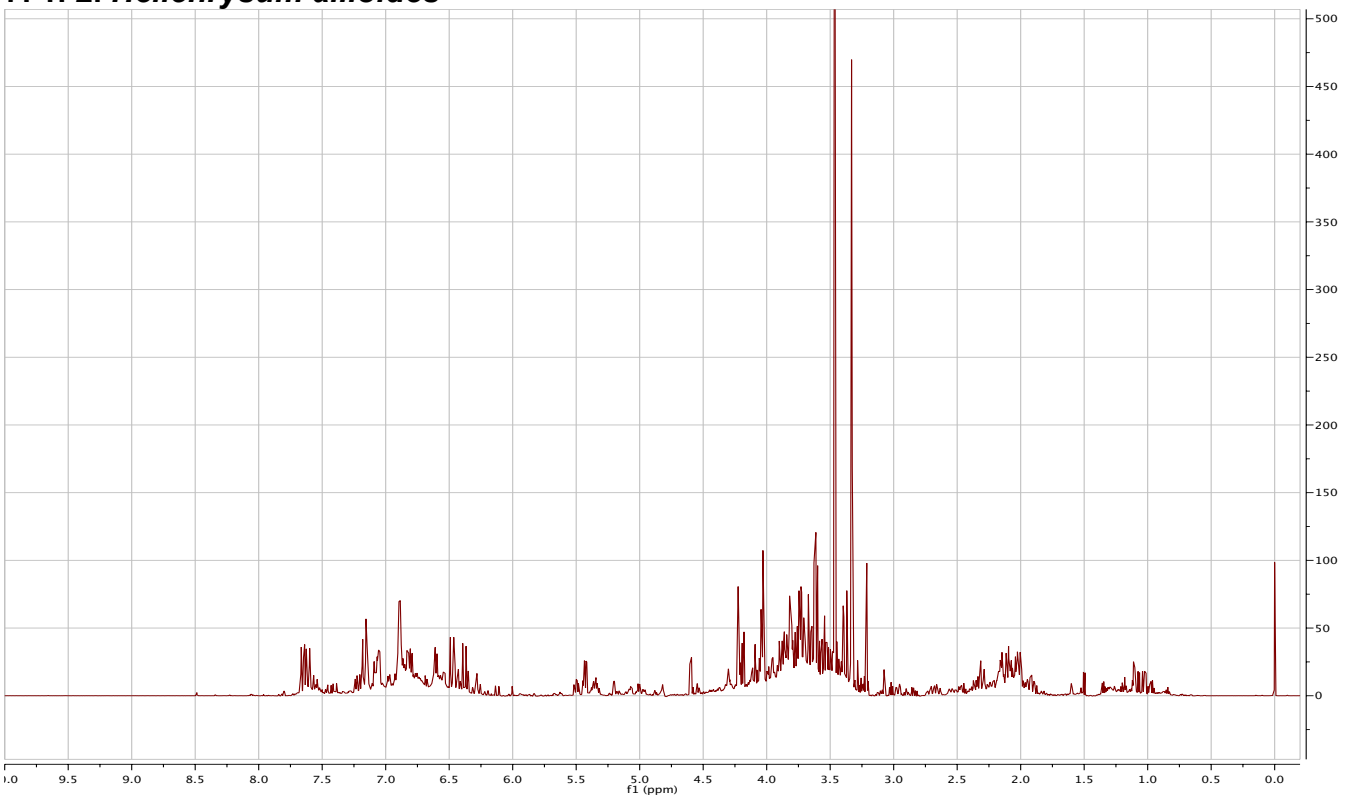

Fig 7. 2: NMR spectrum of the methanol/water plant extract of *H. allioides* (600 MHz, Leiden University).

7. 1. 3. *Helichrysum anomalum*

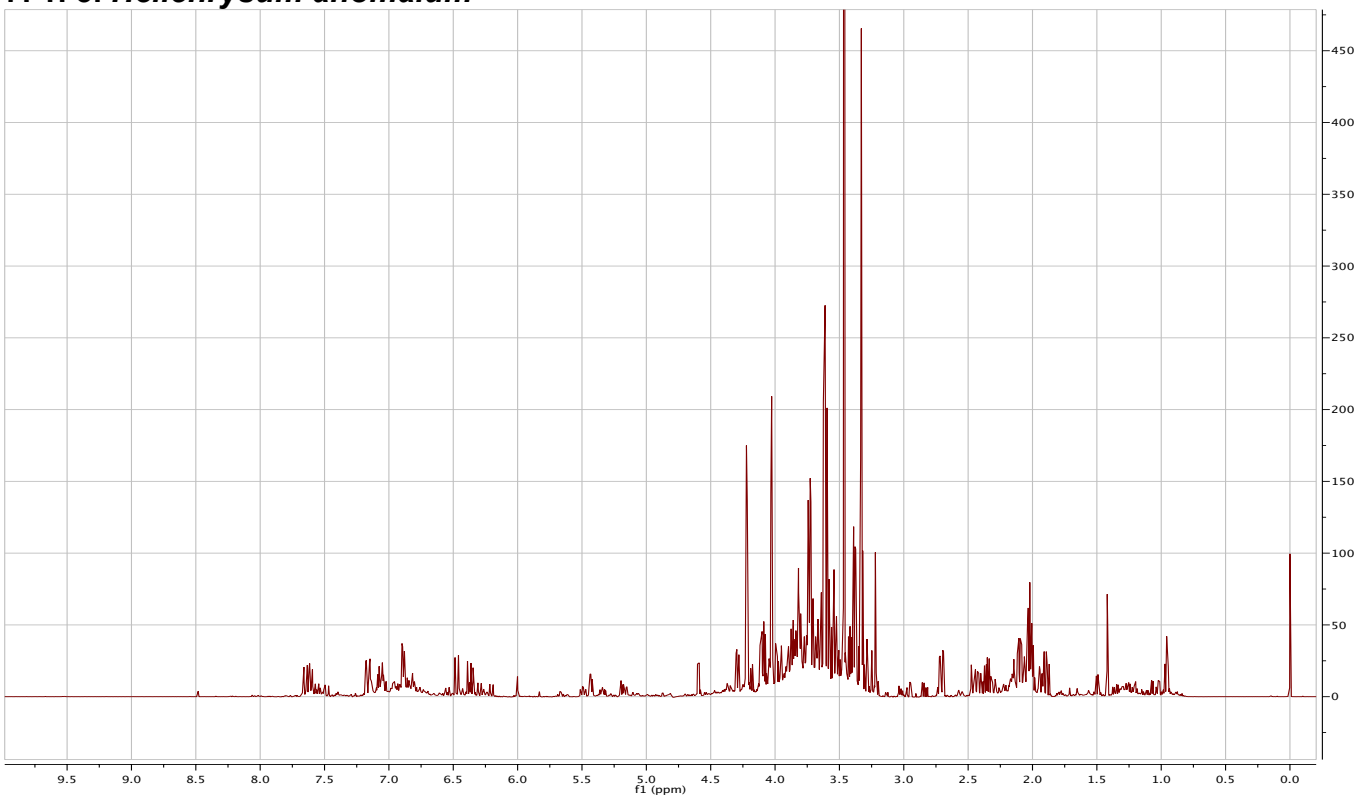

Fig 7. 3: NMR spectrum of the methanol/water plant extract of *H. anomalum* (600 MHz, Leiden University).

7. 1. 4. *Helichrysum appendiculatum*

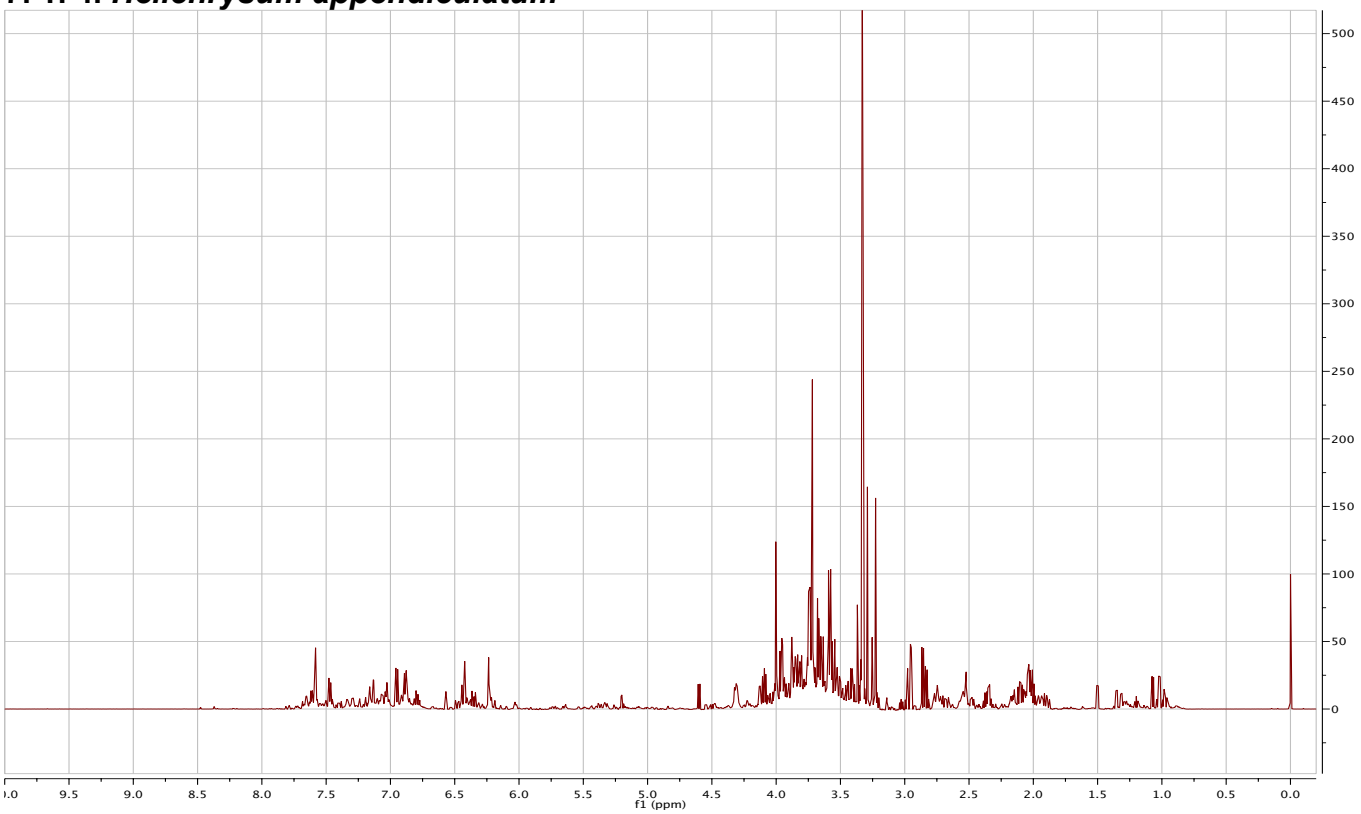

Fig 7. 4: NMR spectrum of the methanol/water plant extract of *H. appendiculatum* (600 MHz, Leiden University).

7. 1. 5. *Helichrysum aureonitens*

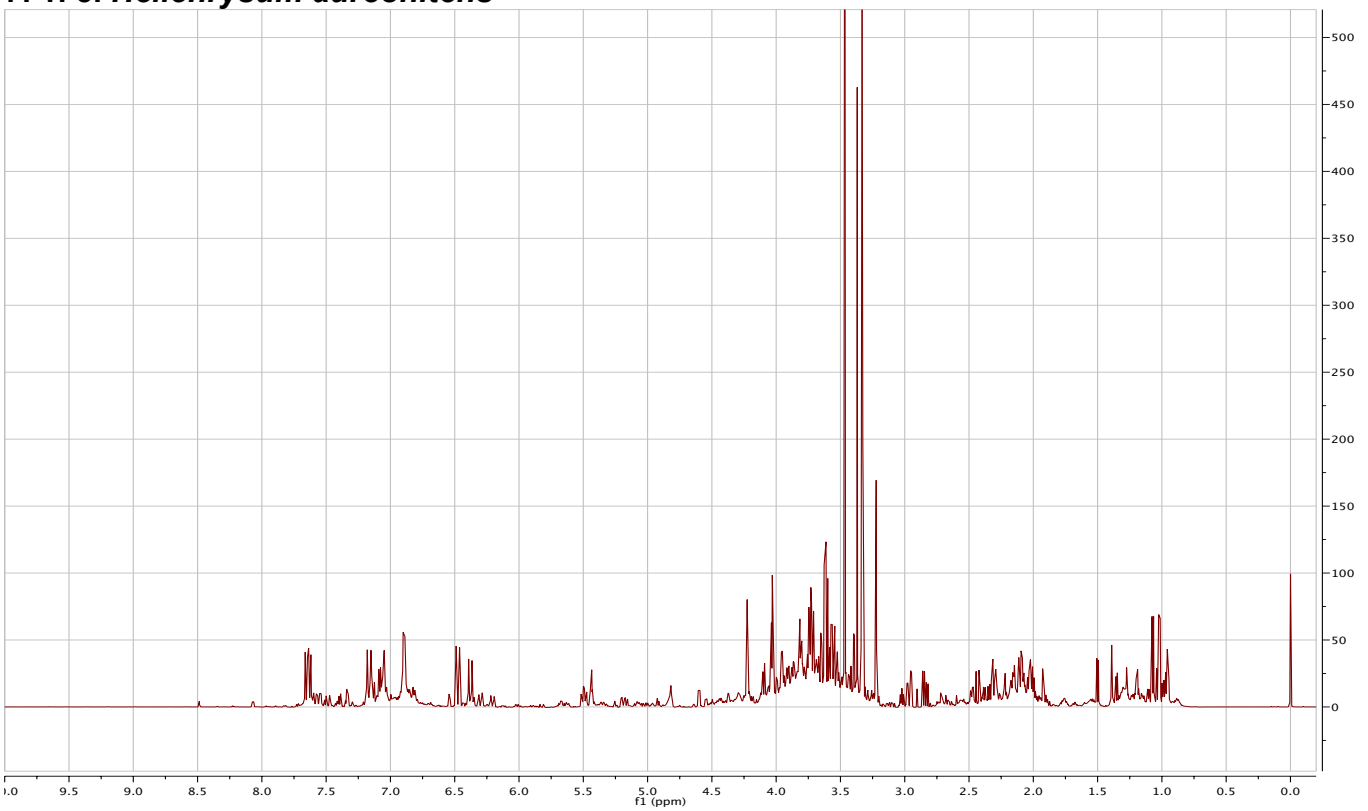

Fig 7. 5: NMR spectrum of the methanol/water plant extract of *H. aureonitens* (600 MHz, Leiden University).

7. 1. 6. *Helichrysum cephaloideum*

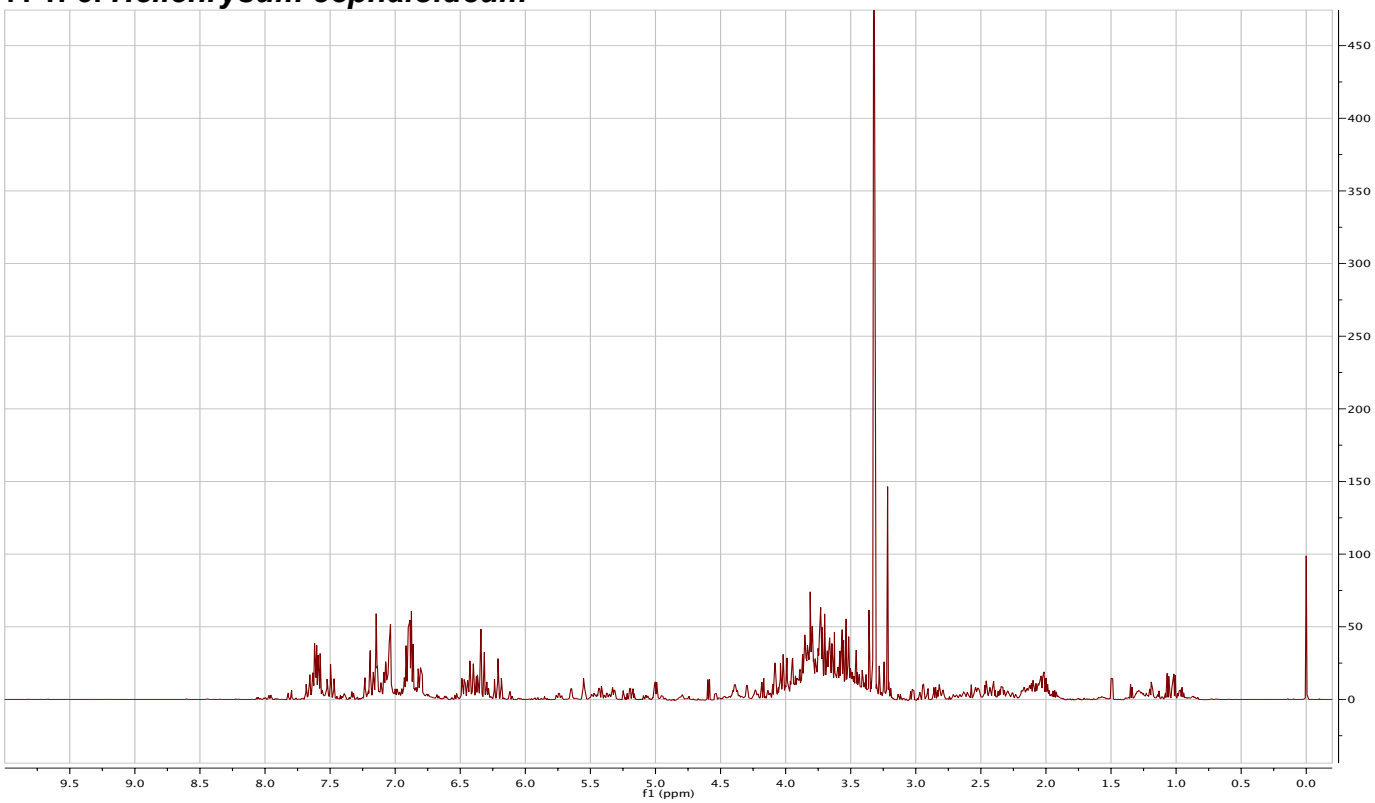

Fig 7. 6: NMR spectrum of the methanol/water plant extract of *H. cephaloideum* (600 MHz, Leiden University).

7. 1. 7. *Helichrysum chionosphaerum*

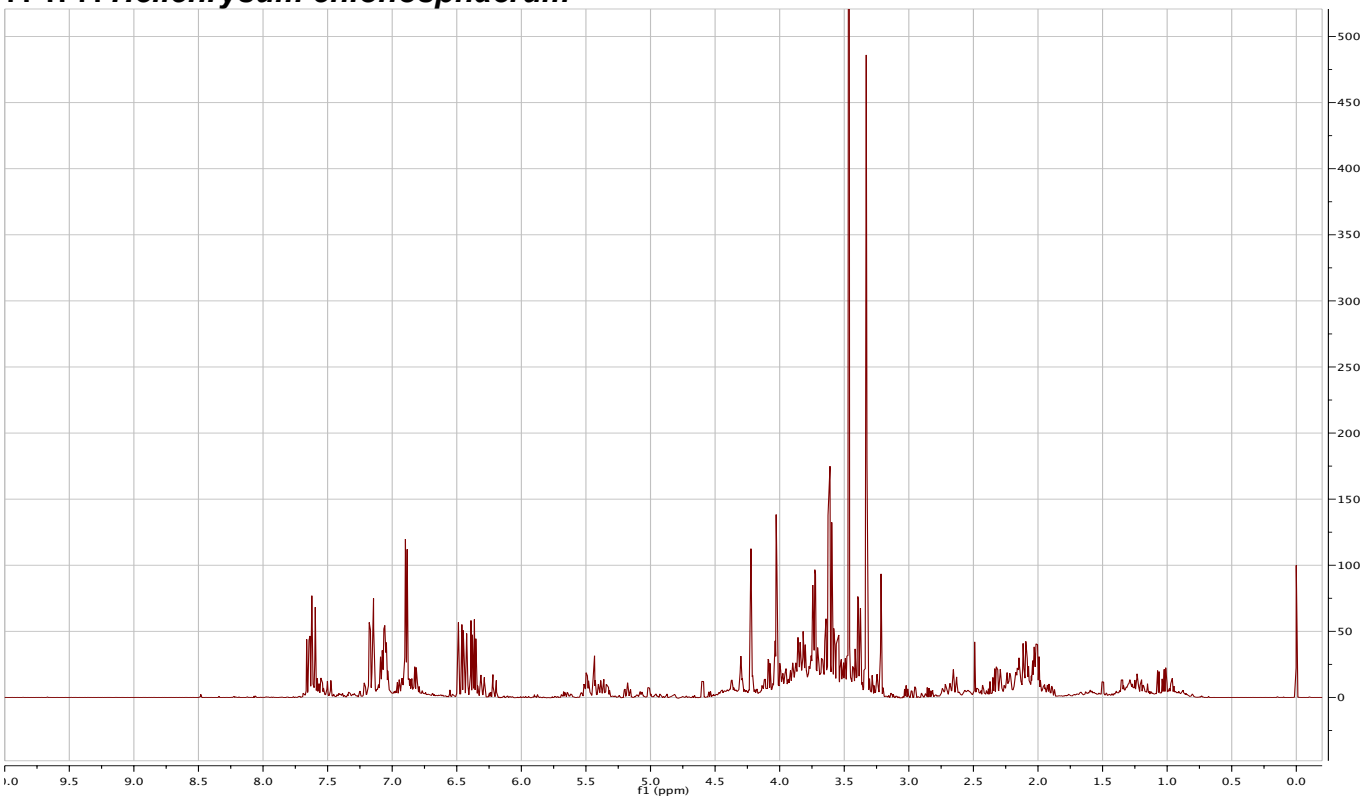

Fig. 7.7: NMR spectrum of the methanol/water plant extract of *H. chionosphaerum* (600 MHz, Leiden University).

7. 1. 8. *Helichrysum confertum*

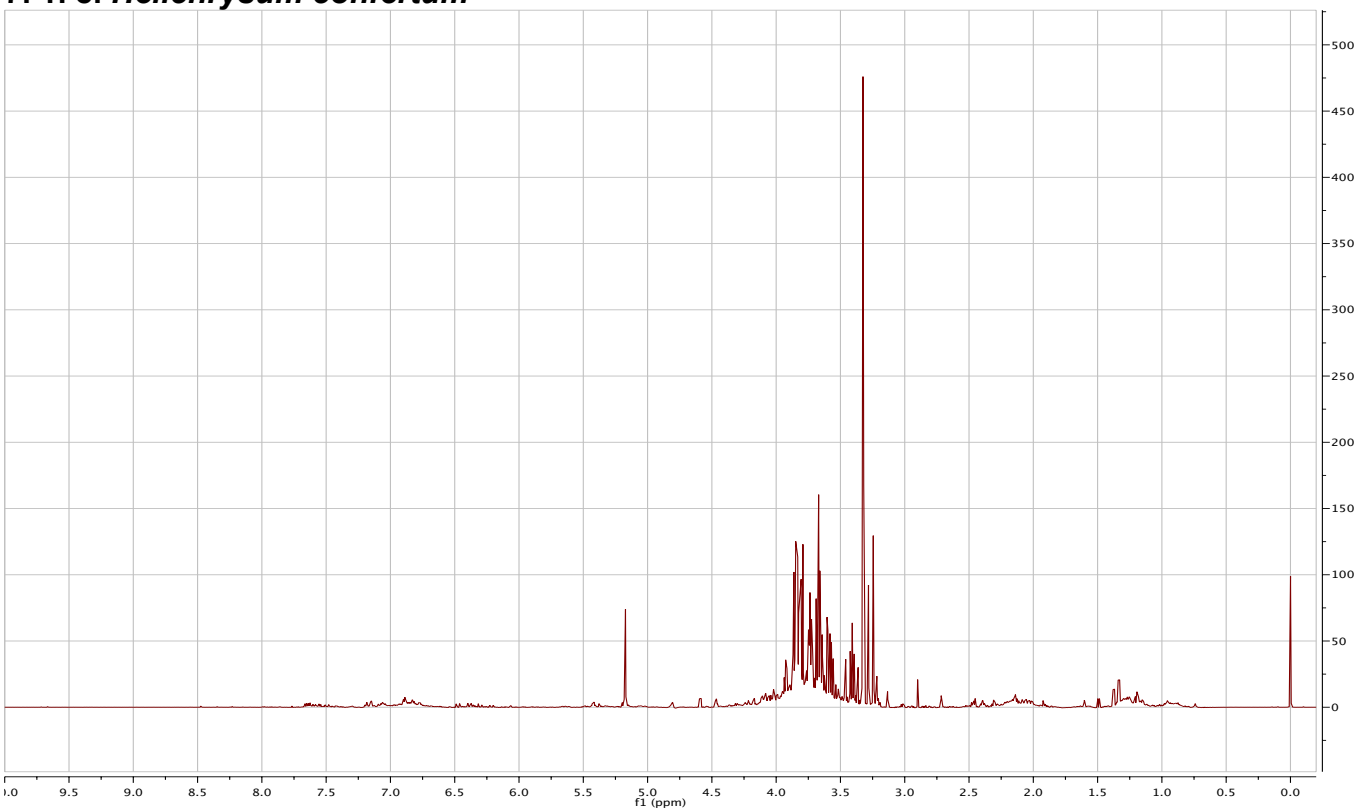

Fig. 7.8: NMR spectrum of the methanol/water plant extract of *H. confertum* (600 MHz, Leiden University).

7. 1. 9. *Helichrysum cymosum* subsp. *cymosum*

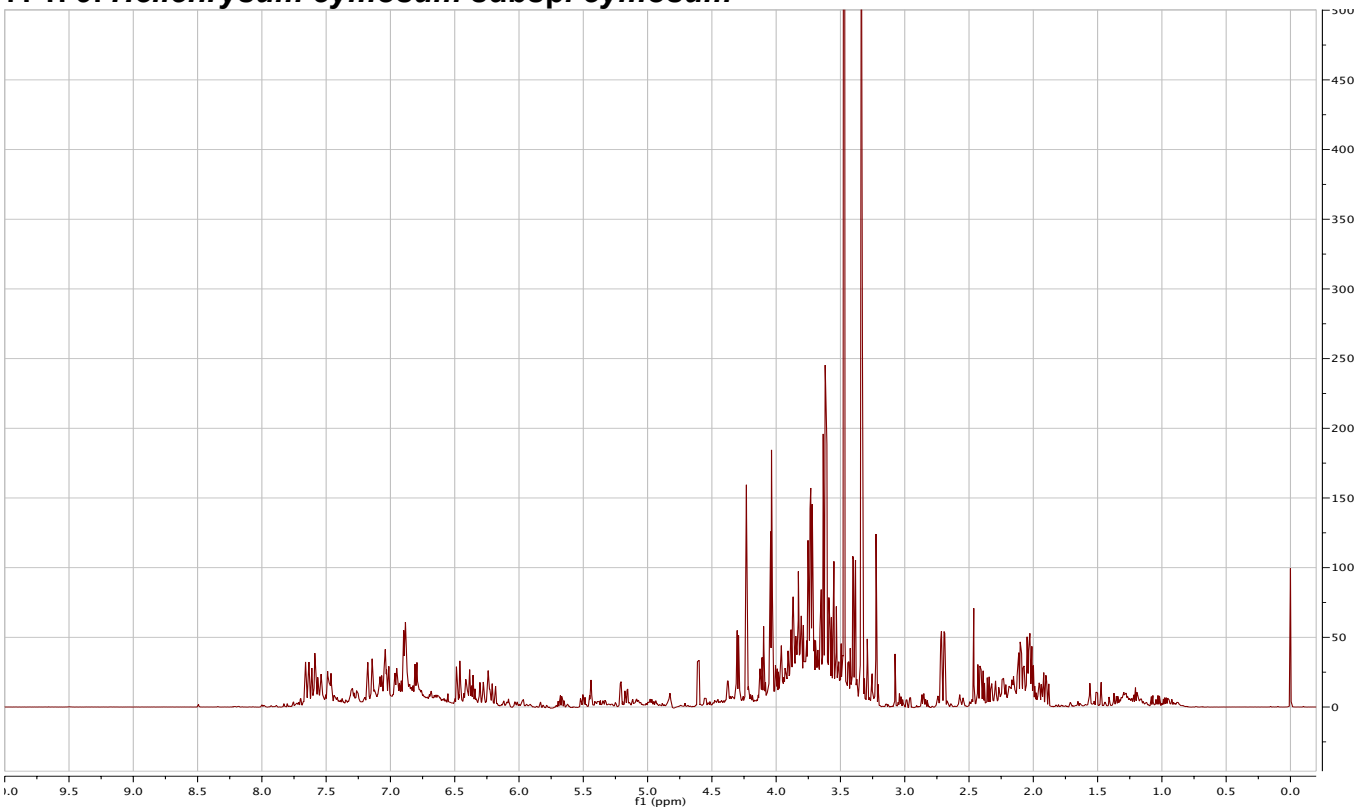

Fig. 7.9: NMR spectrum of the methanol/water plant extract of *H. cymosum* subsp. *cymosum* (600 MHz, Leiden University).

7. 1. 10. *Helichrysum cymosum* subsp. *clavum*

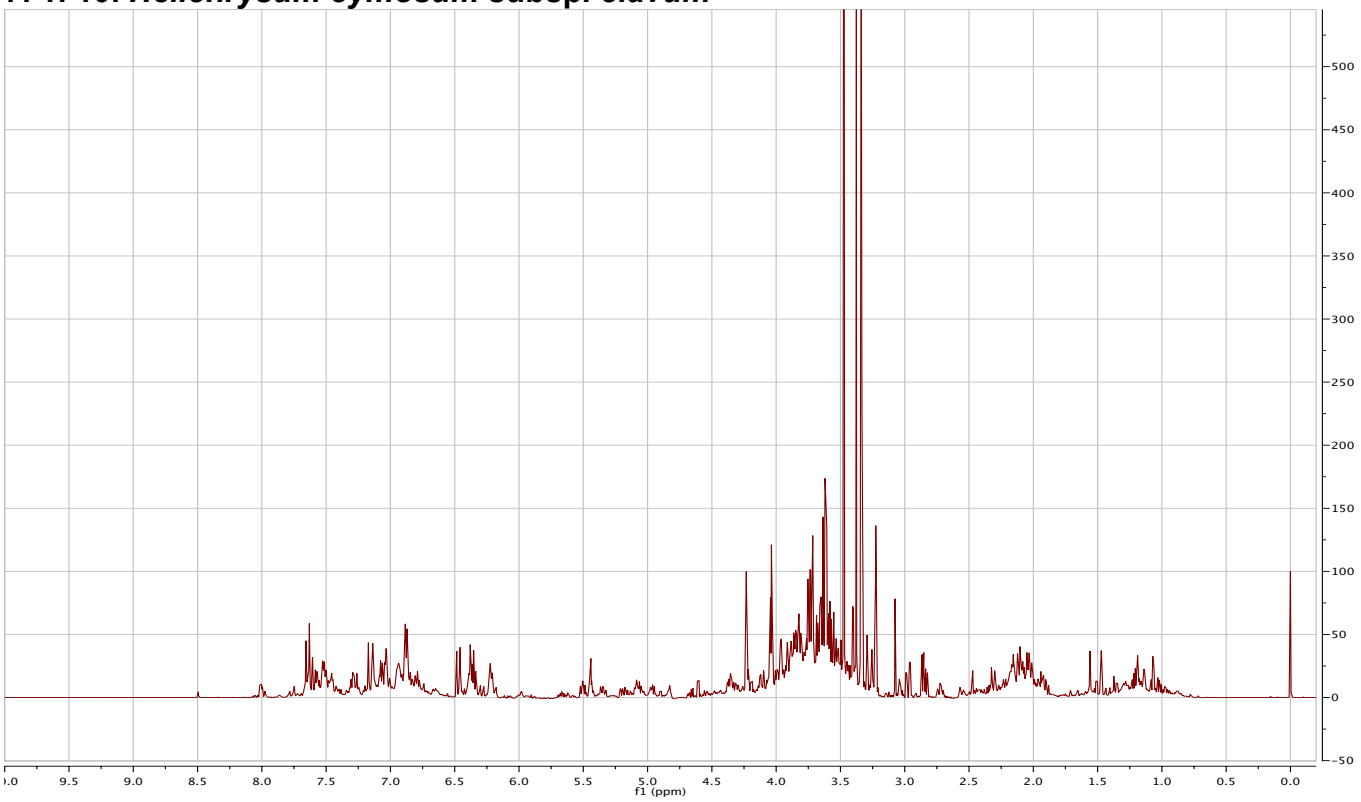

Fig. 7.10: NMR spectrum of the methanol/water plant extract of *H. cymosum* subsp. *clavum* (600 MHz, Leiden University).

7. 1. 11. *Helichrysum difficile*

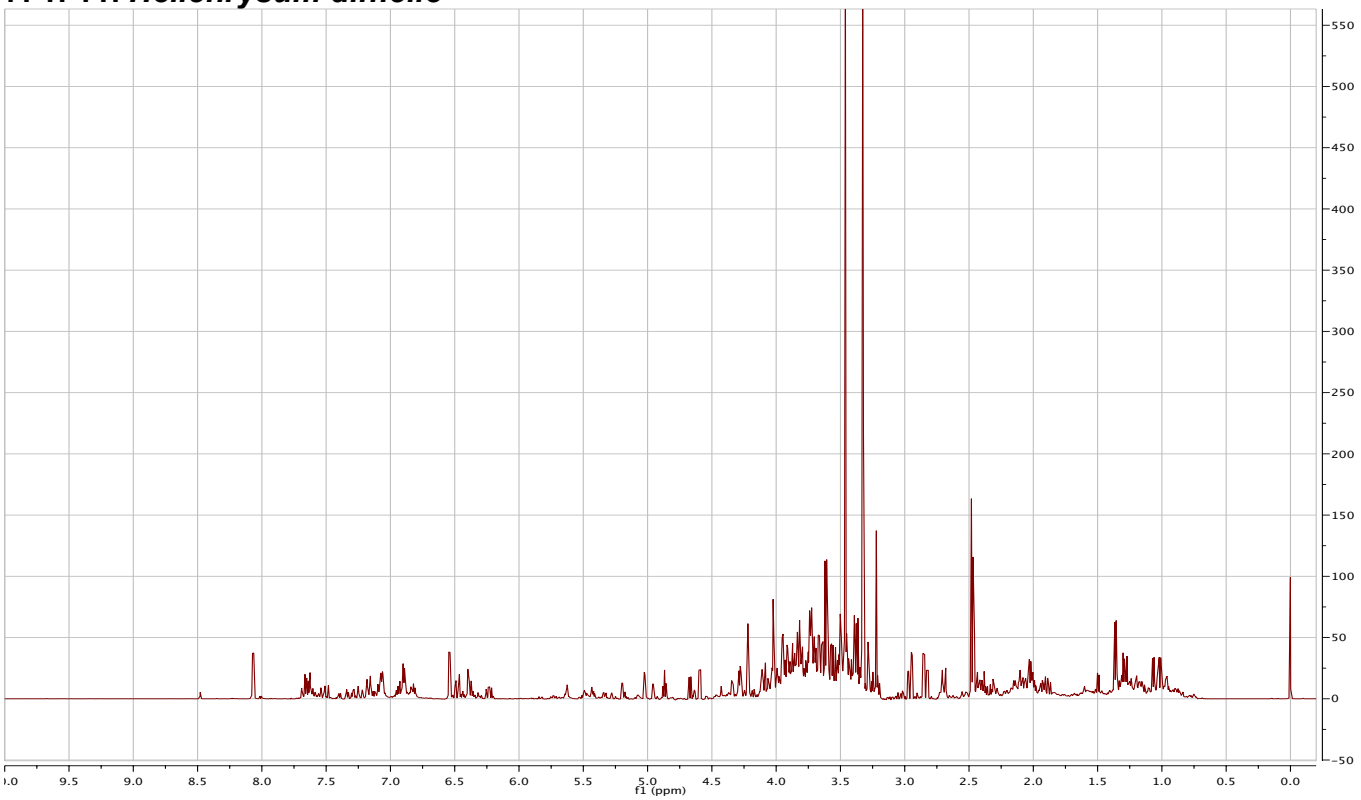

Fig. 7.11: NMR spectrum of the methanol/water plant extract of *H. difficile* (600 MHz, Leiden University).

7. 1. 12. *Helichrysum drakensbergense*

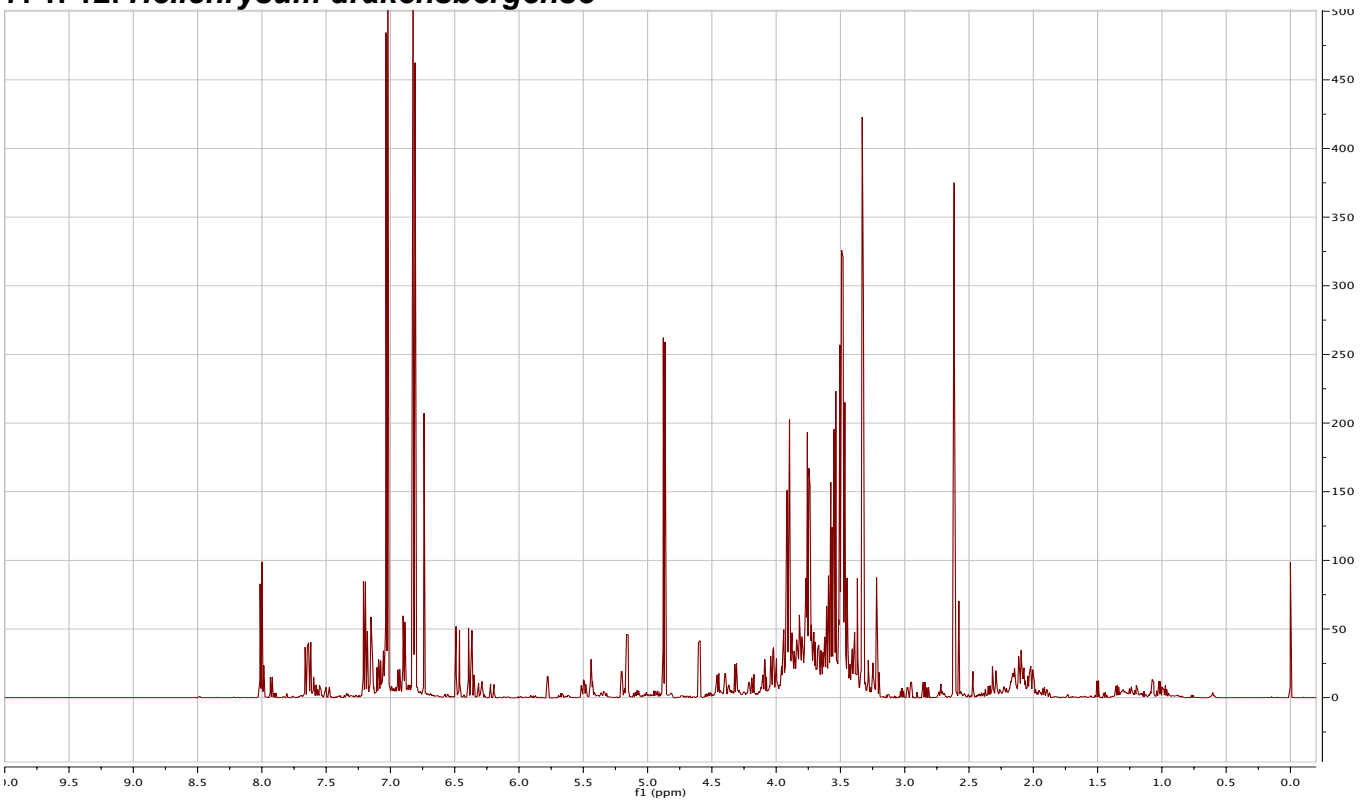

Fig. 7.12: NMR spectrum of the methanol/water plant extract of *H. drakensbergense* (600 MHz, Leiden University).

7. 1. 13. *Helichrysum herbaceum*

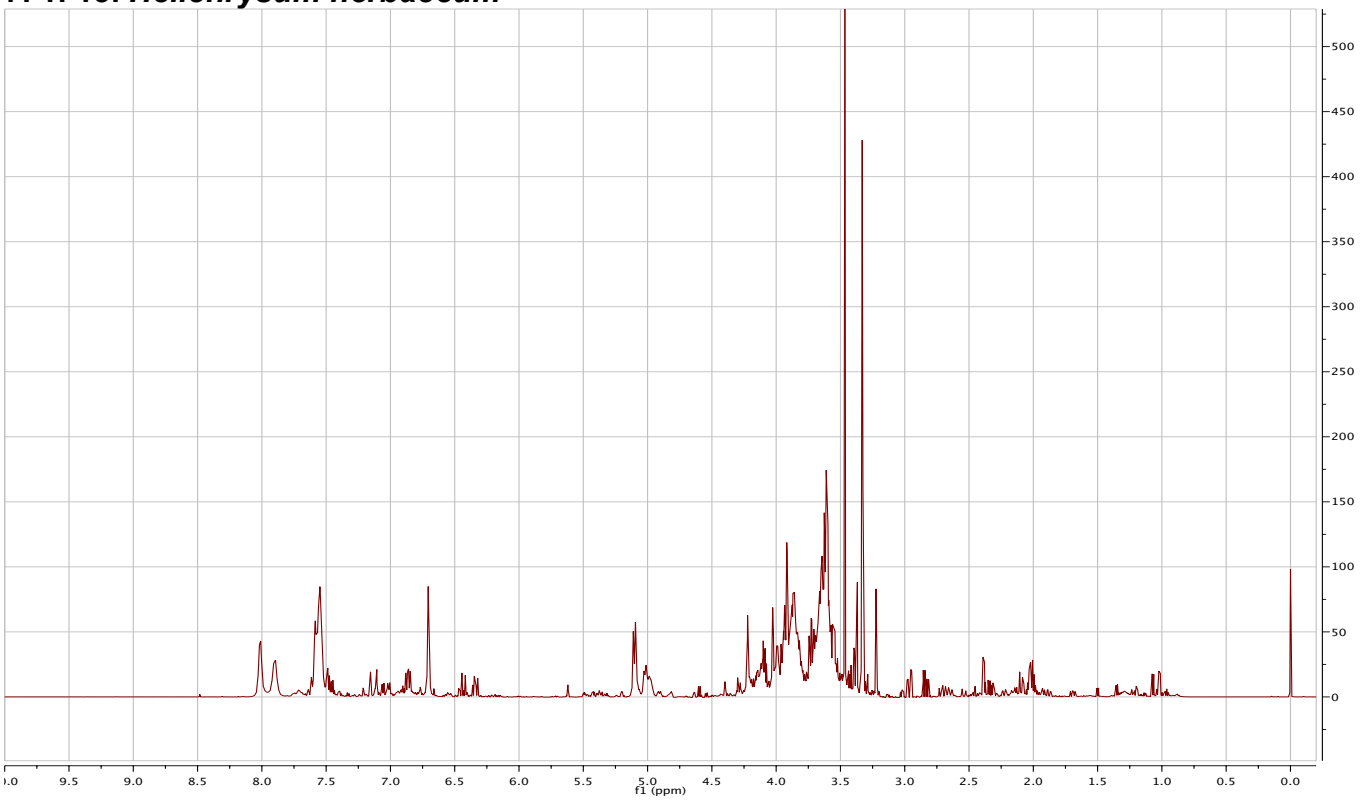

Fig. 7.13: NMR spectrum of the methanol/water plant extract of *H. herbaceum* (600 MHz, Leiden University).

7. 1. 14. *Helichrysum melanacme*

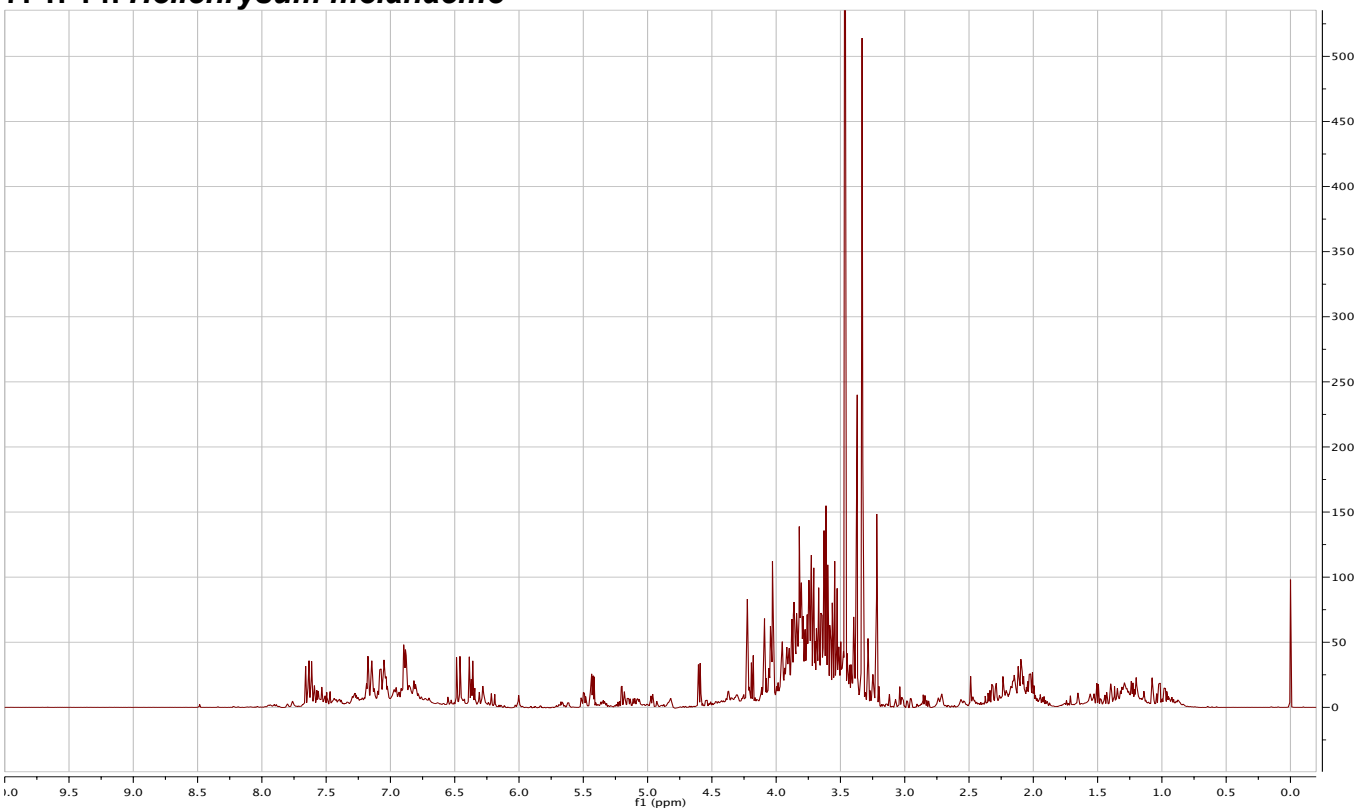

Fig. 7.14: NMR spectrum of the methanol/water plant extract of *H. melanacme* (600 MHz, Leiden University).

7. 1. 15. *Helichrysum miconiifolium*

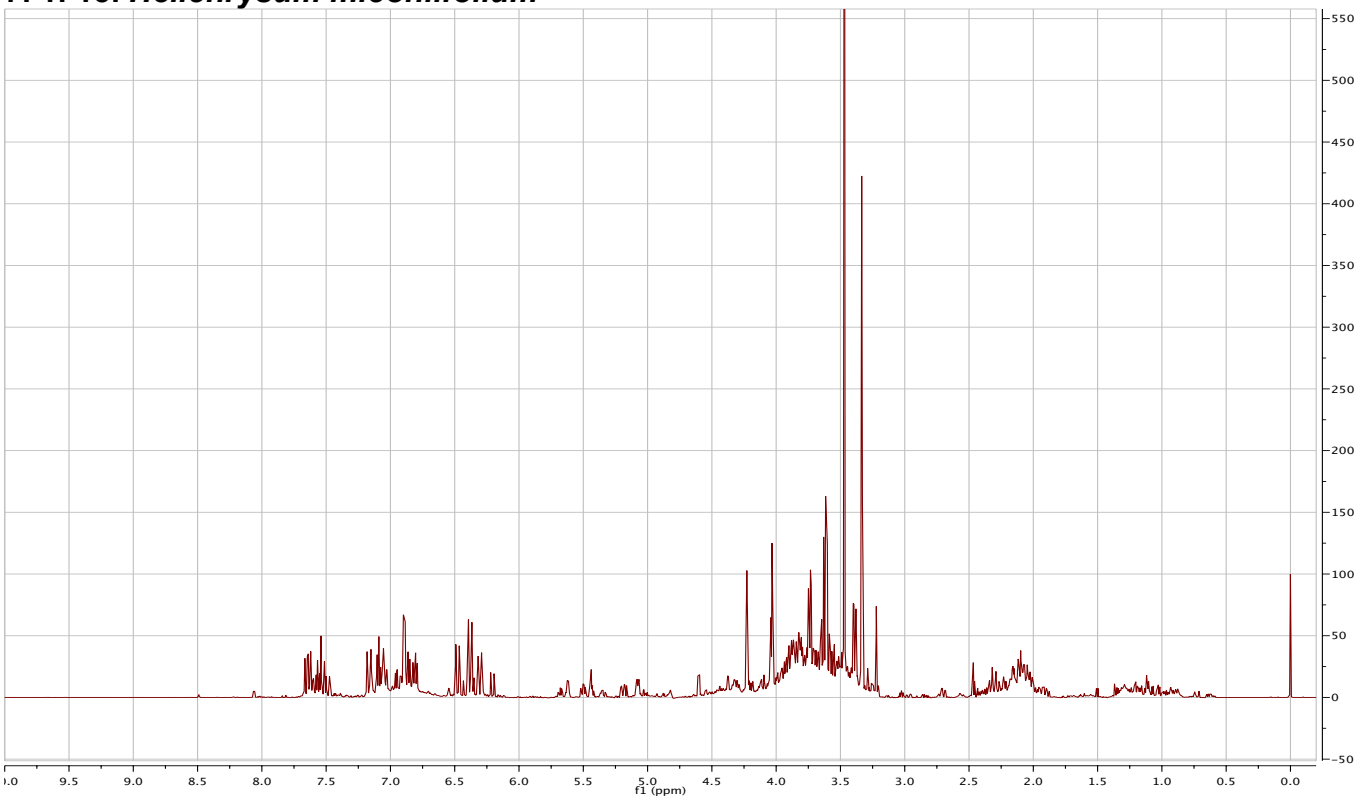

Fig. 7.15: NMR spectrum of the methanol/water plant extract of *H. miconiifolium* (600 MHz, Leiden University).

7. 1. 16. *Helichrysum natalitium*

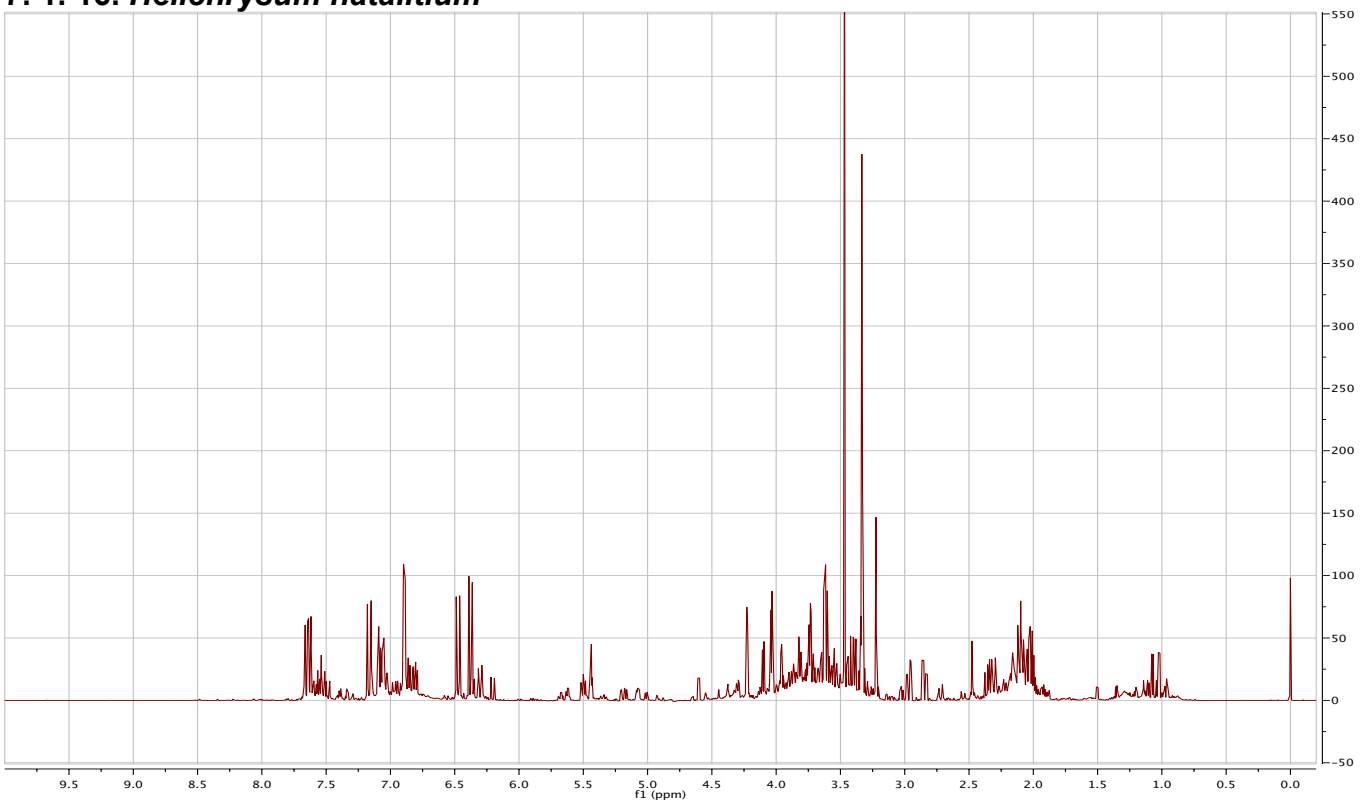

Fig 7.16: NMR spectrum of the methanol/water plant extract of *H. natalitium* (600 MHz, Leiden University).

7. 1. 17. *Helichrysum nudifolium* var. *nudifolium* (1)

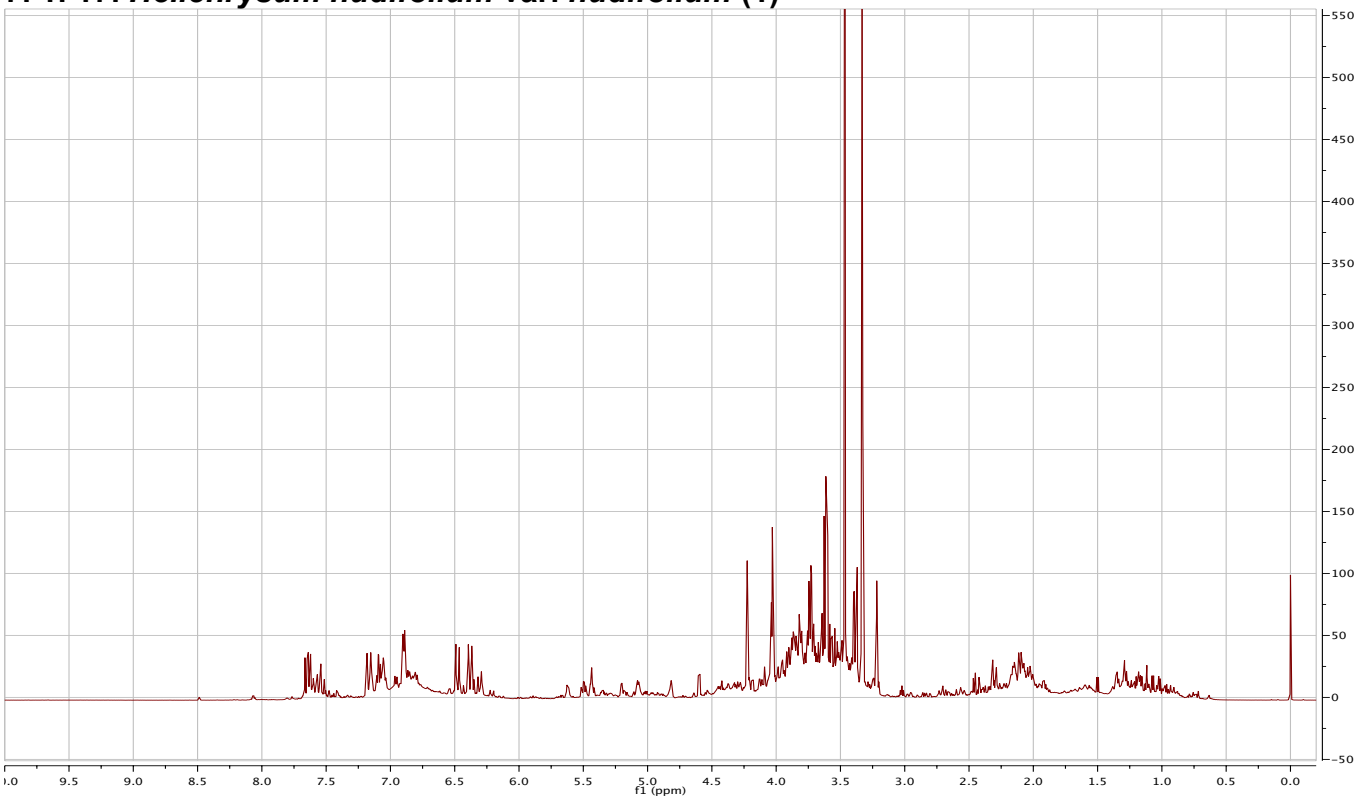

Fig 7.17: NMR spectrum of the methanol/water plant extract of *H. nudifolium* var. *nudifolium* (1) (600 MHz, Leiden University).

7. 1. 18. *Helichrysum odoratissimum*

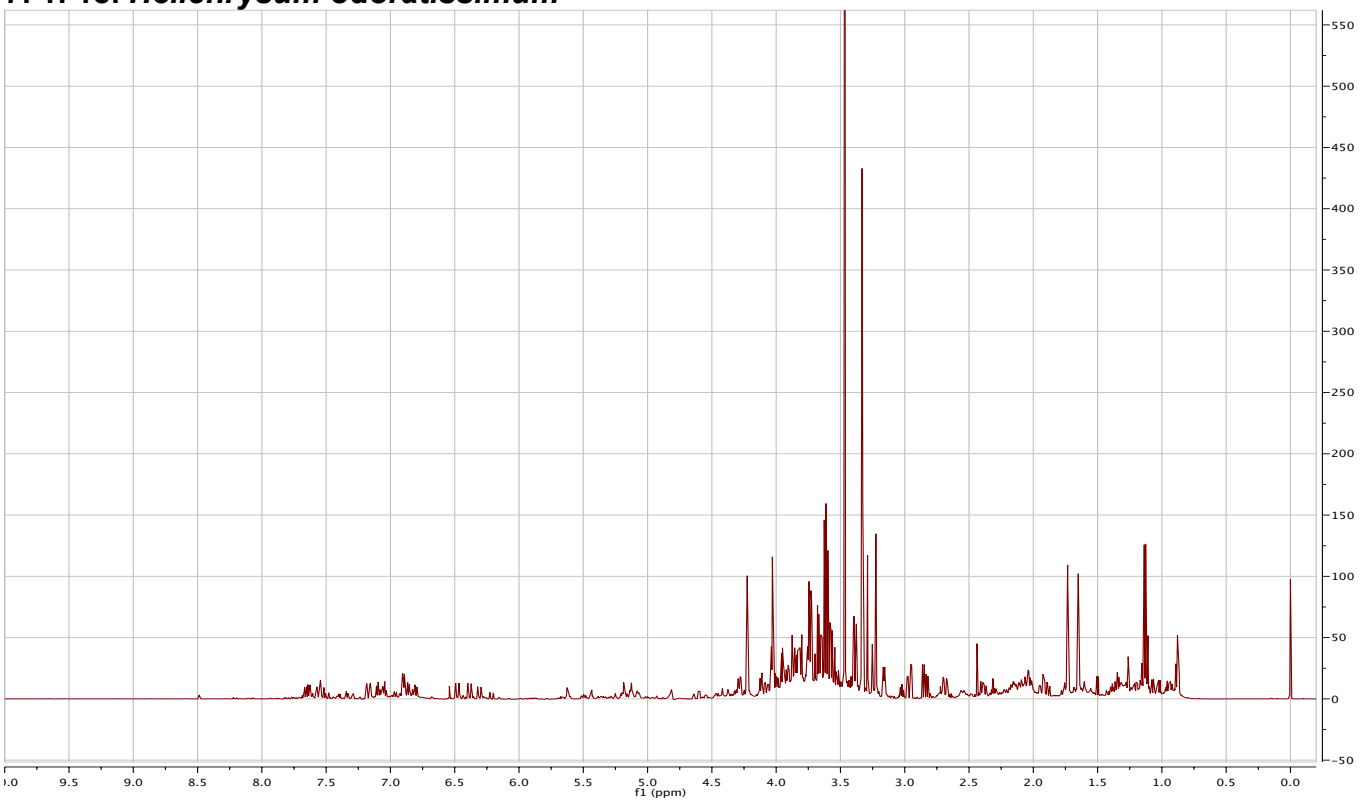

Fig 7.19: NMR spectrum of the methanol/water plant extract of *H. odoratissimum* (600 MHz, Leiden University).

7. 1. 19. *Helichrysum oreophilum*

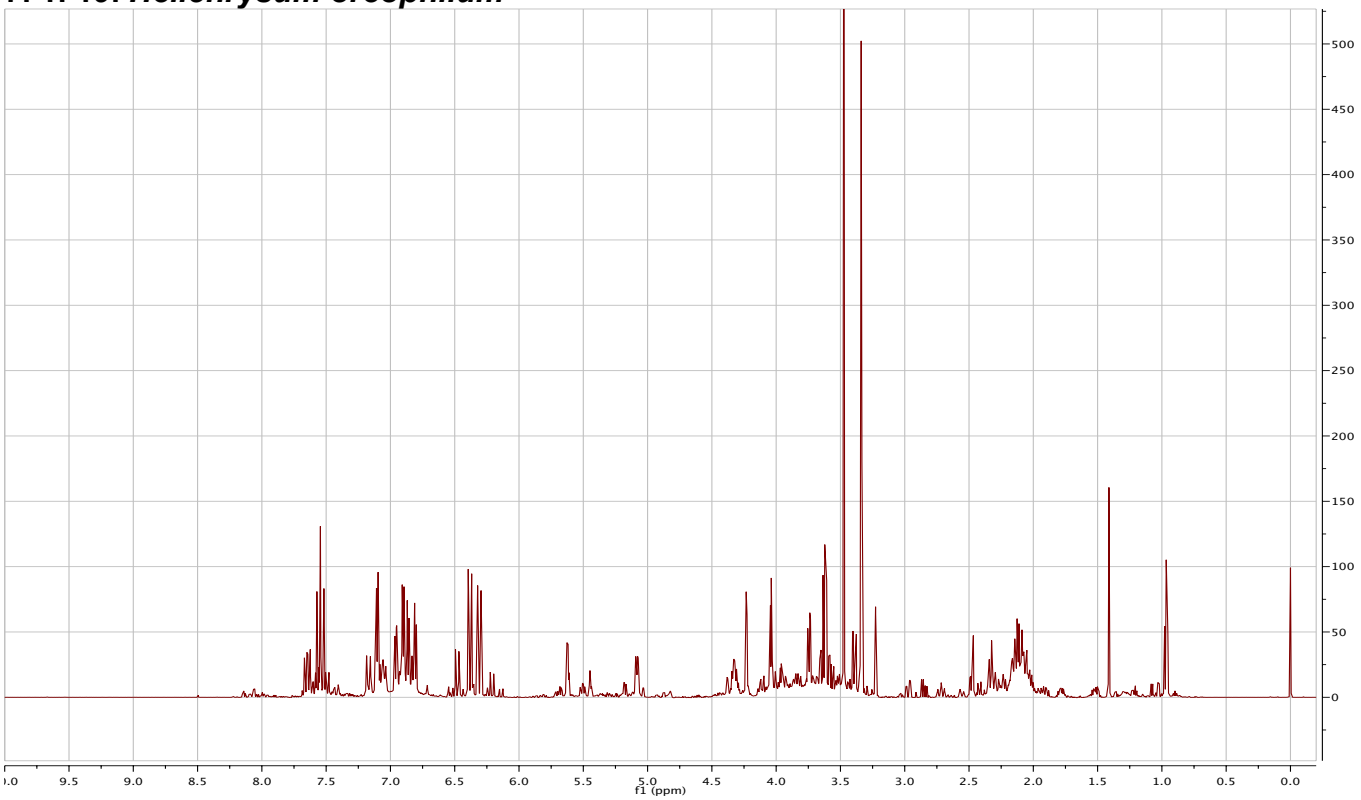

Fig 7.20: NMR spectrum of the methanol/water plant extract of *H. oreophilum* (600 MHz, Leiden University).

7. 1. 20. *Helichrysum oxyphyllum*

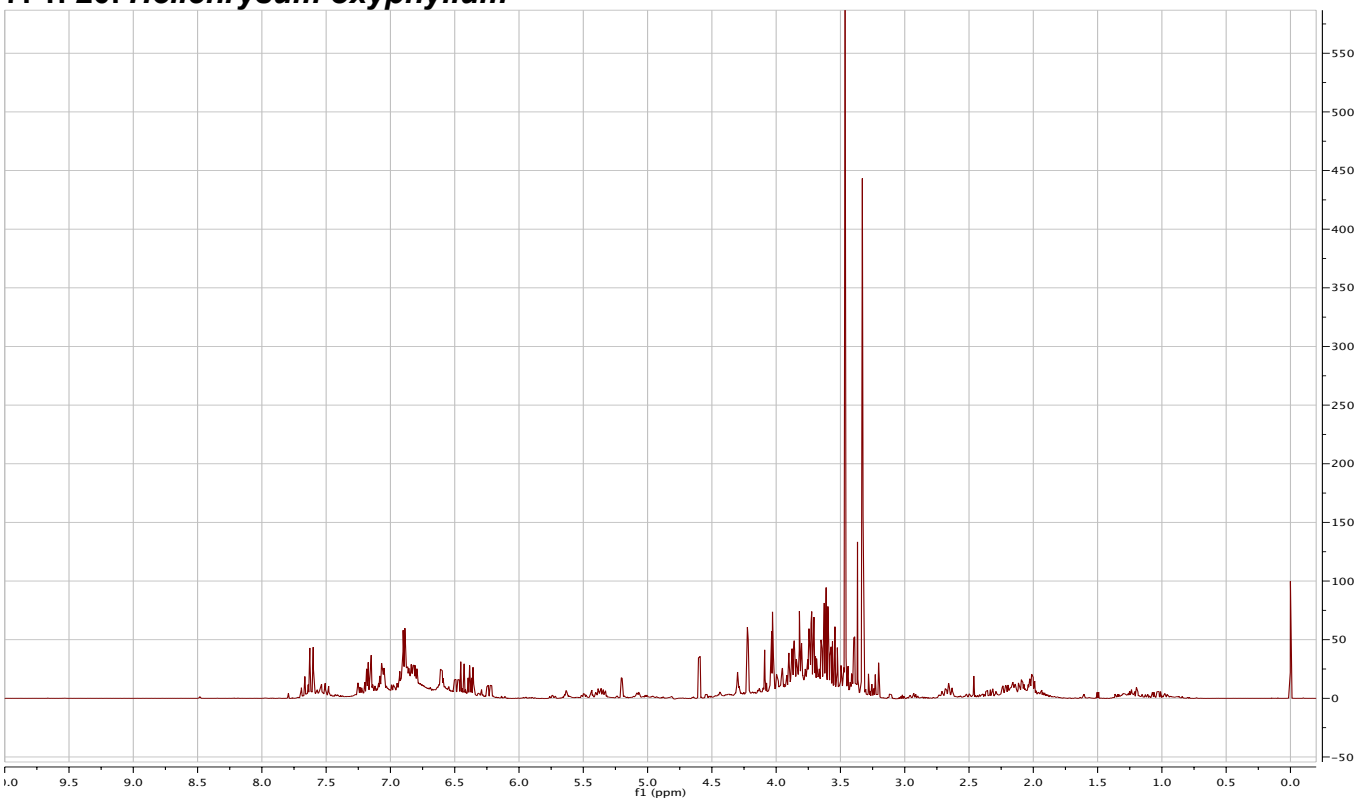

Fig 7.21: NMR spectrum of the methanol/water plant extract of *H. oxyphyllum* (600 MHz, Leiden University).

7. 1. 21. *Helichrysum pallidum*

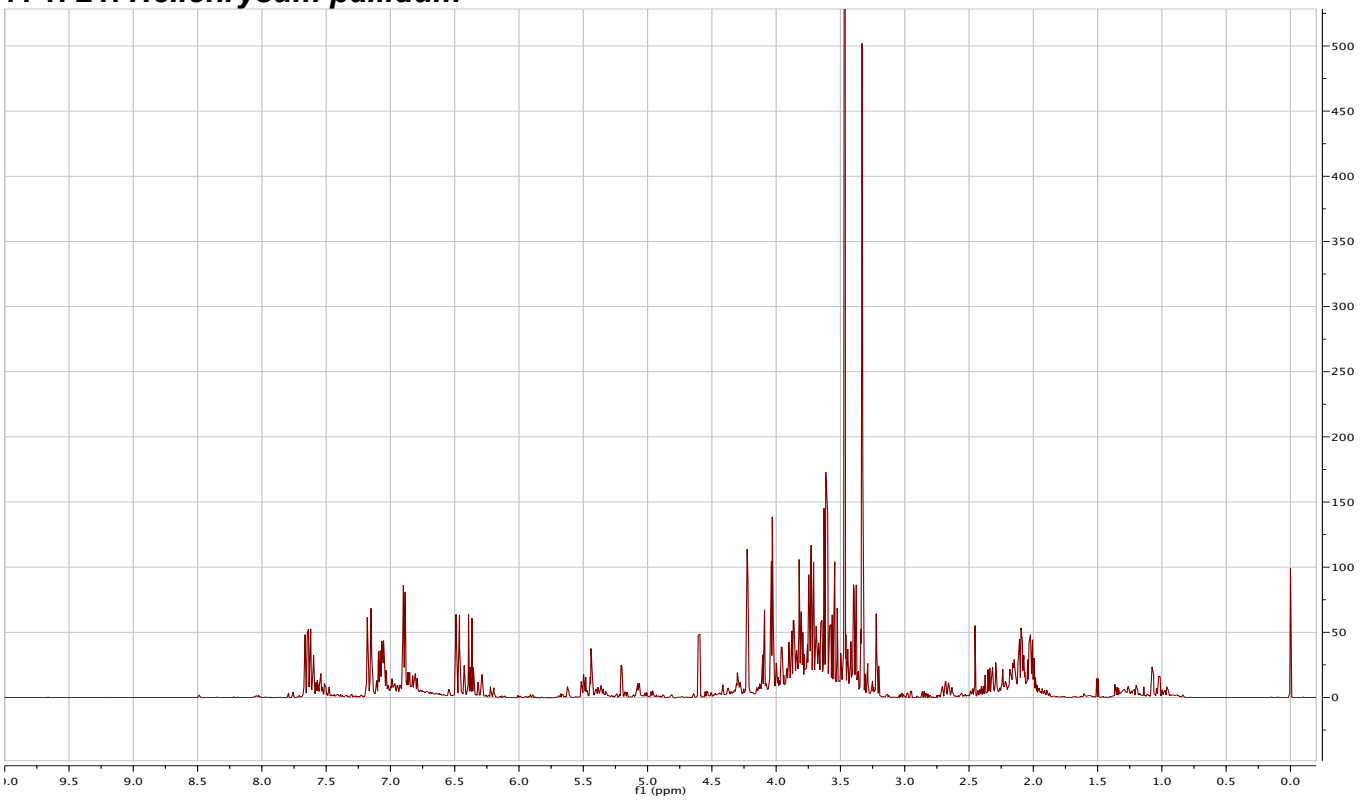

Fig 7.22: NMR spectrum of the methanol/water plant extract of *H. pallidum* (600 MHz, Leiden University).

7. 1. 22. *Helichrysum panduratum*

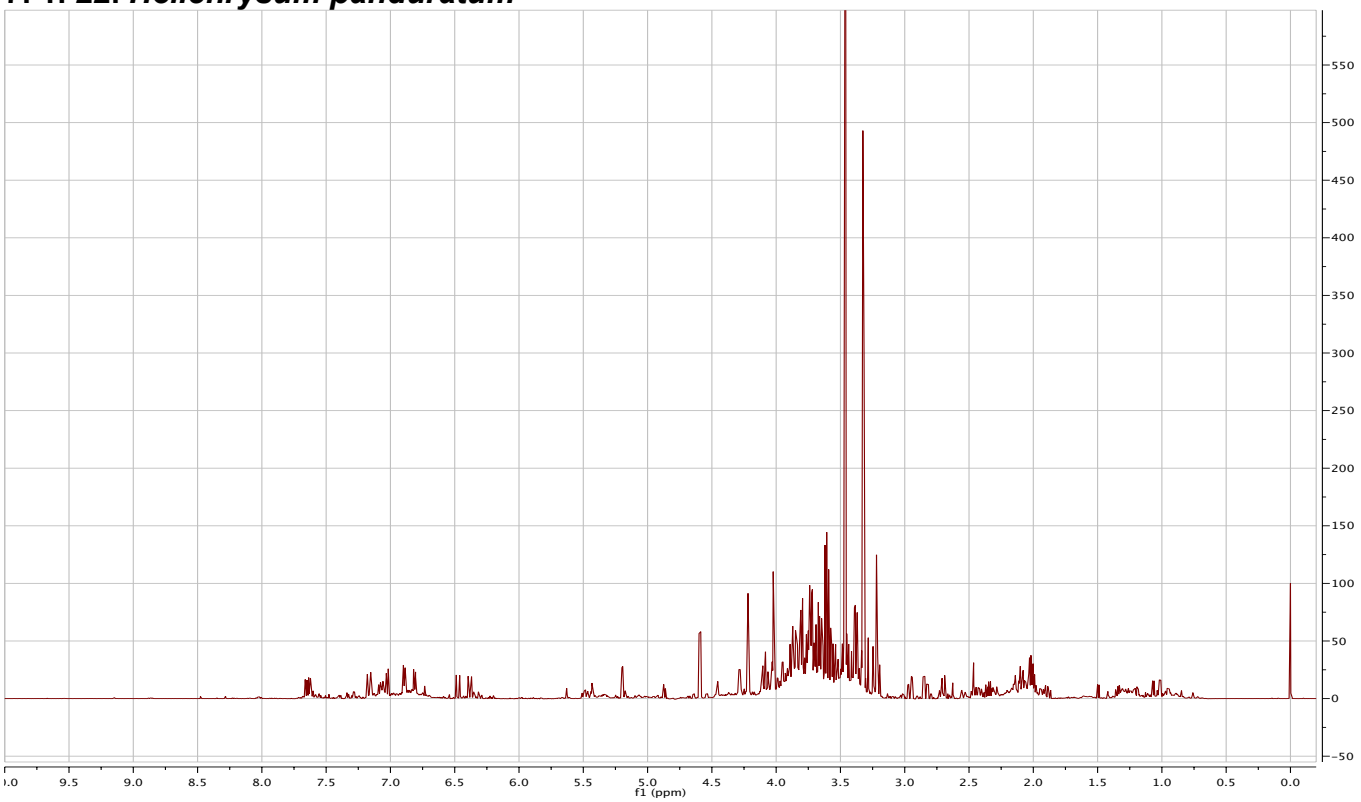

Fig 7.23: NMR spectrum of the methanol/water plant extract of *H. panduratum* (600 MHz, Leiden University).

7. 1. 23. *Helichrysum pannosum*

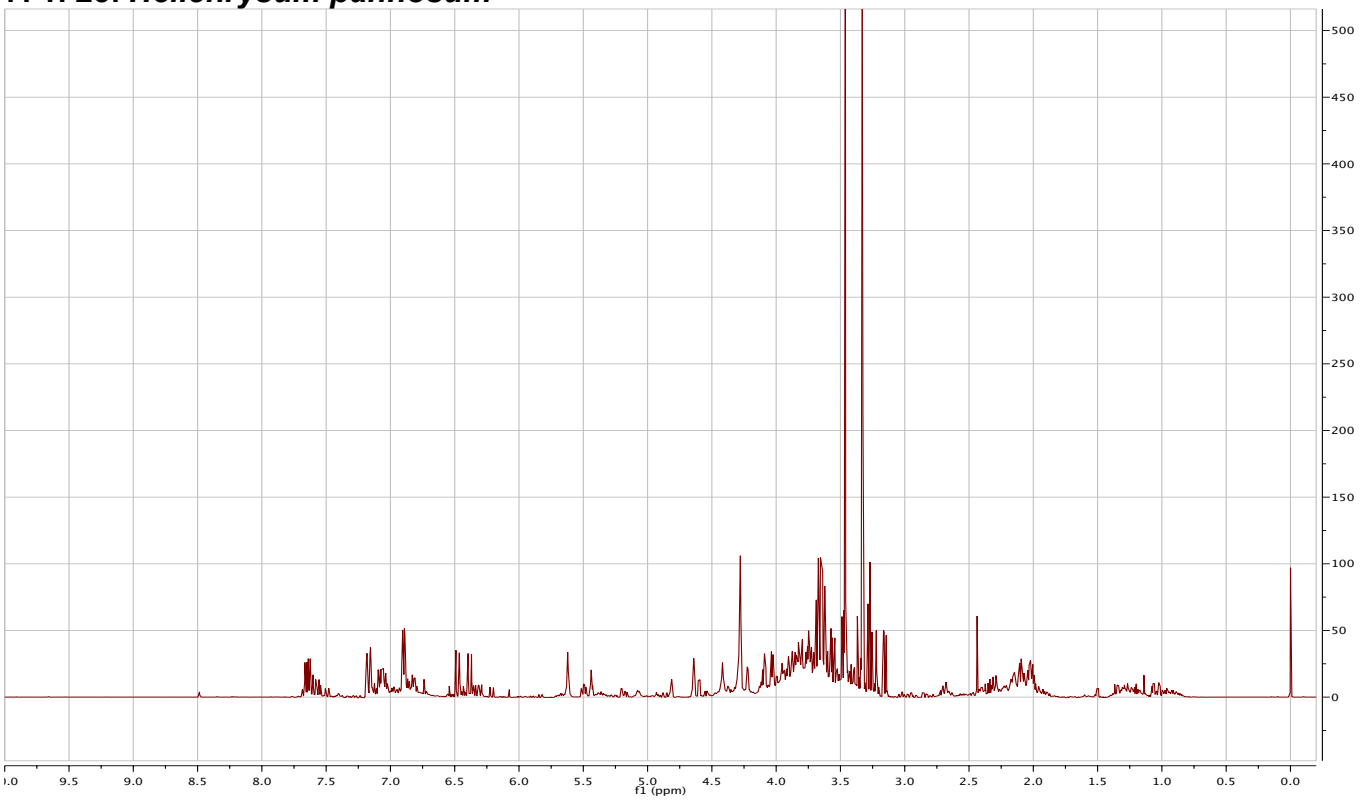

Fig 7.24: NMR spectrum of the methanol/water plant extract of *H. pannosum* (600 MHz, Leiden University).

7. 1. 24. *Helichrysum pilosellum*

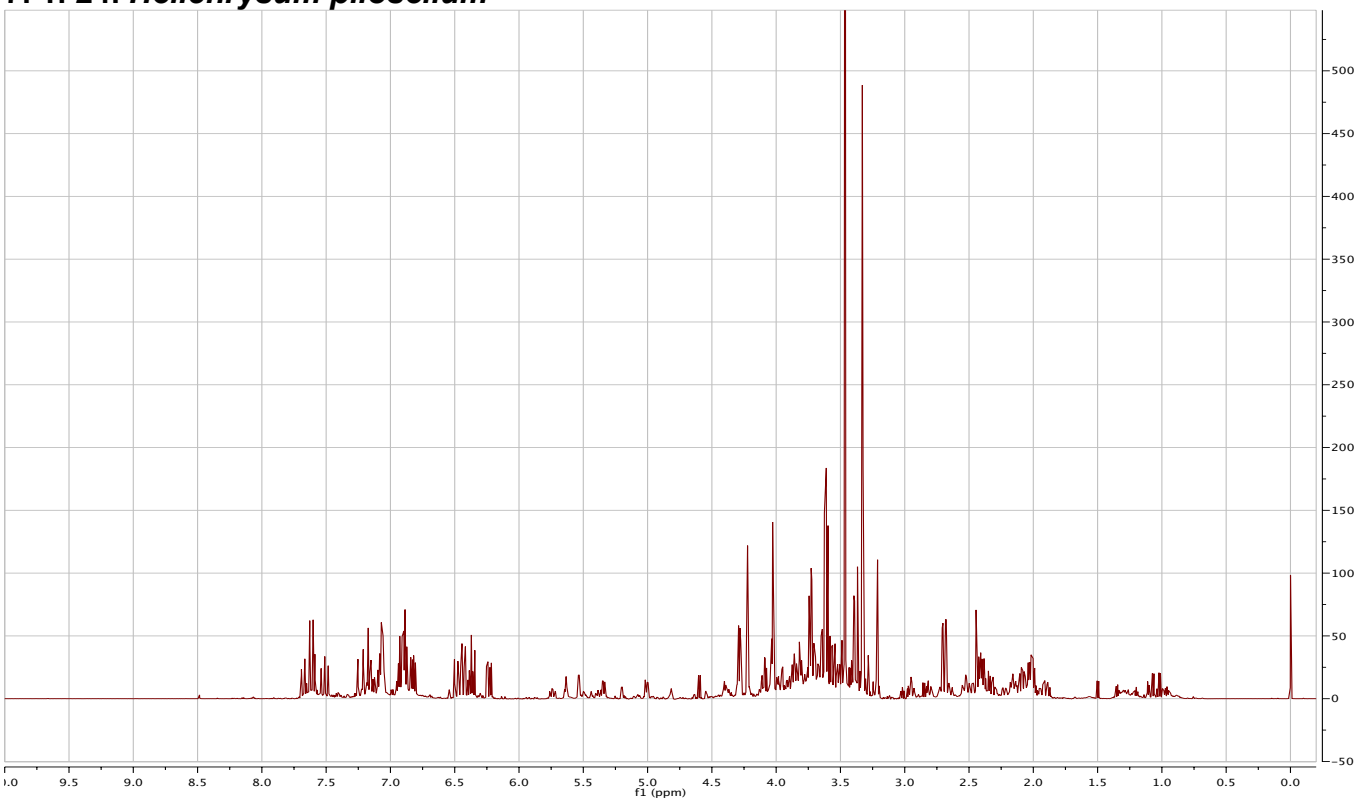

Fig 7.25: NMR spectrum of the methanol/water plant extract of *H. pilosellum* (600 MHz, Leiden University).

7. 1. 25. *Helichrysum pilosellum*†

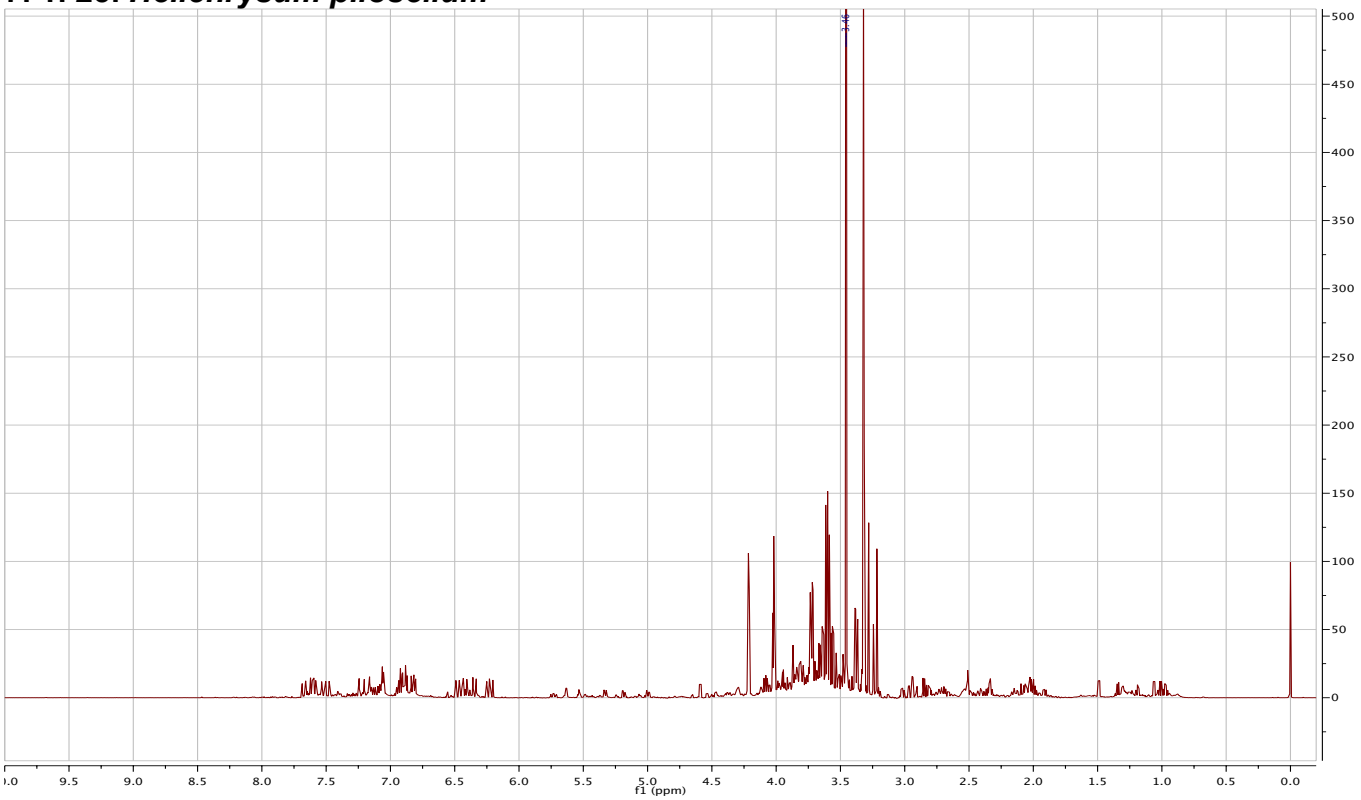

Fig 7.18: NMR spectrum of the methanol/water plant extract of *H. pilosellum* (HNUP) (600 MHz, Leiden University). († Reclassified later in the study)

7. 1. 26. *Helichrysum populifolium*

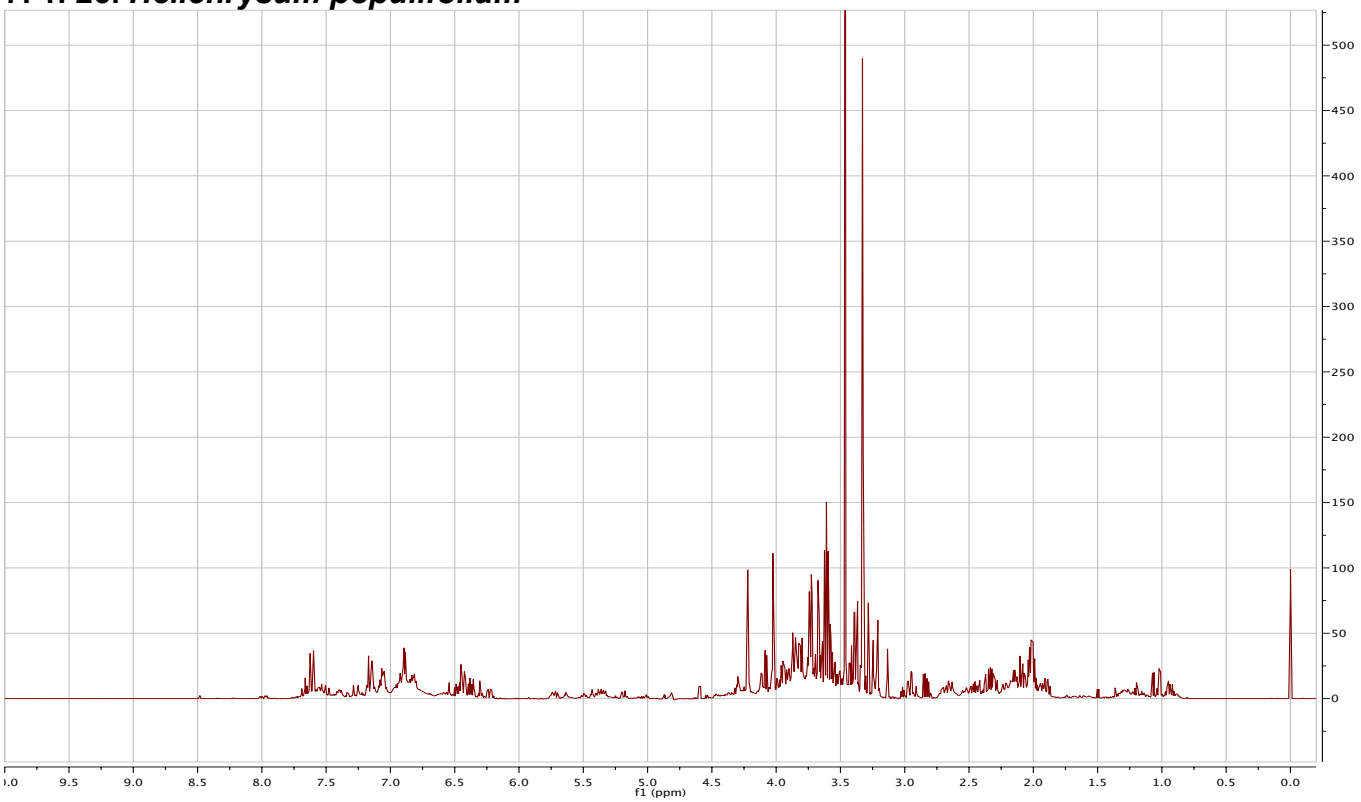

Fig 7.26: NMR spectrum of the methanol/water plant extract of *H. populifolium* (600 MHz, Leiden University).

7. 1. 27. *Helichrysum rugulosum*

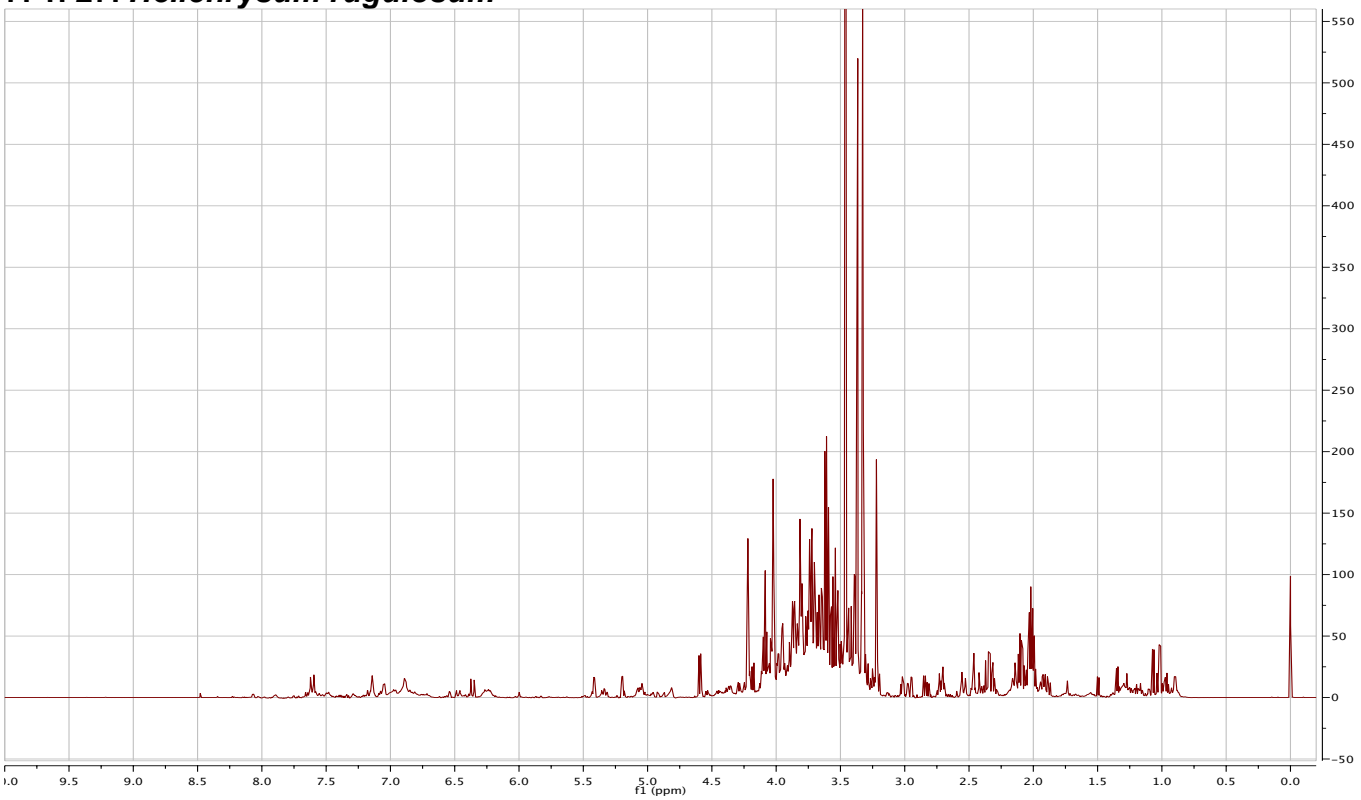

Fig 7.27: NMR spectrum of the methanol/water plant extract of *H. rugulosum* (600 MHz, Leiden University).

7. 1. 28. *Helichrysum splendidum* (1)

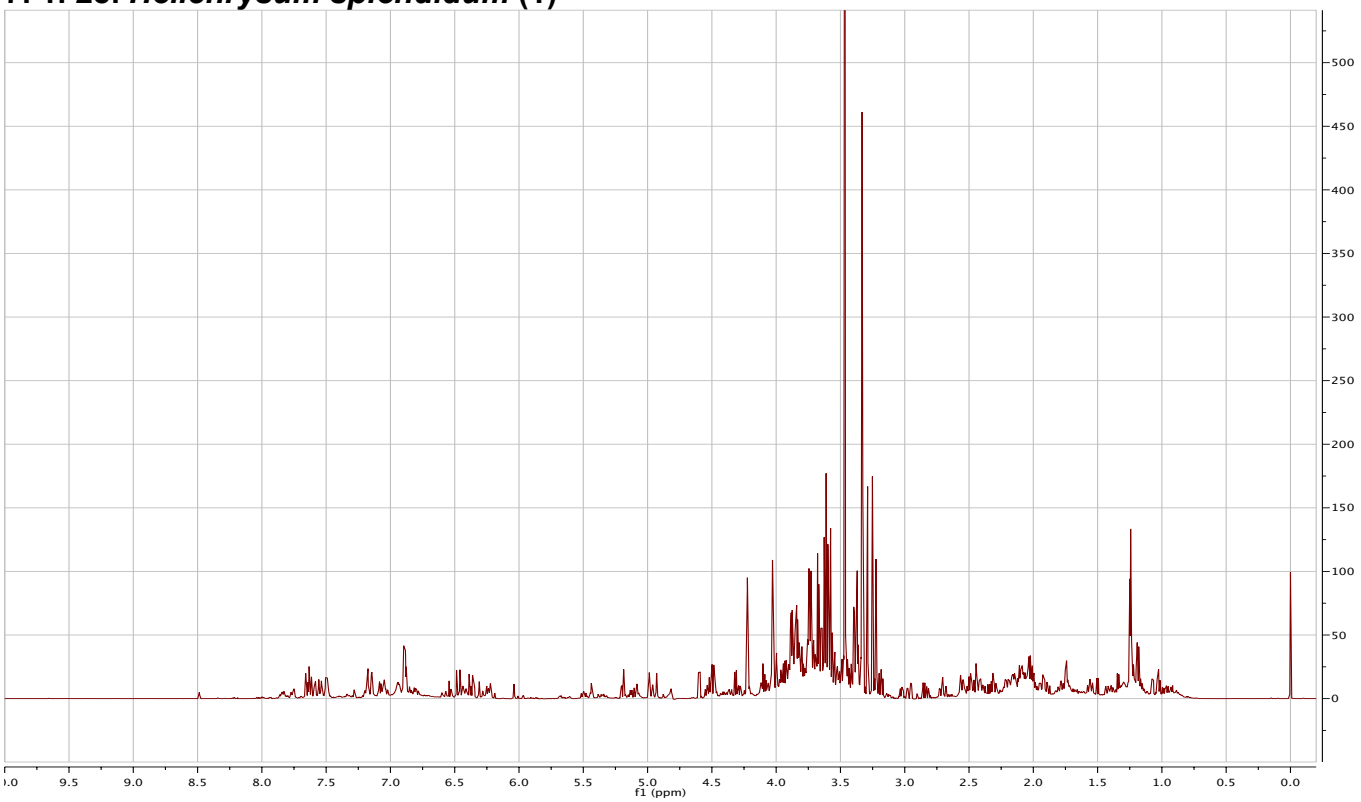

Fig 7.28: NMR spectrum of the methanol/water plant extract of *H. splendidum* (1) (600 MHz, Leiden University).

7. 1. 29. *Helichrysum subluteum*

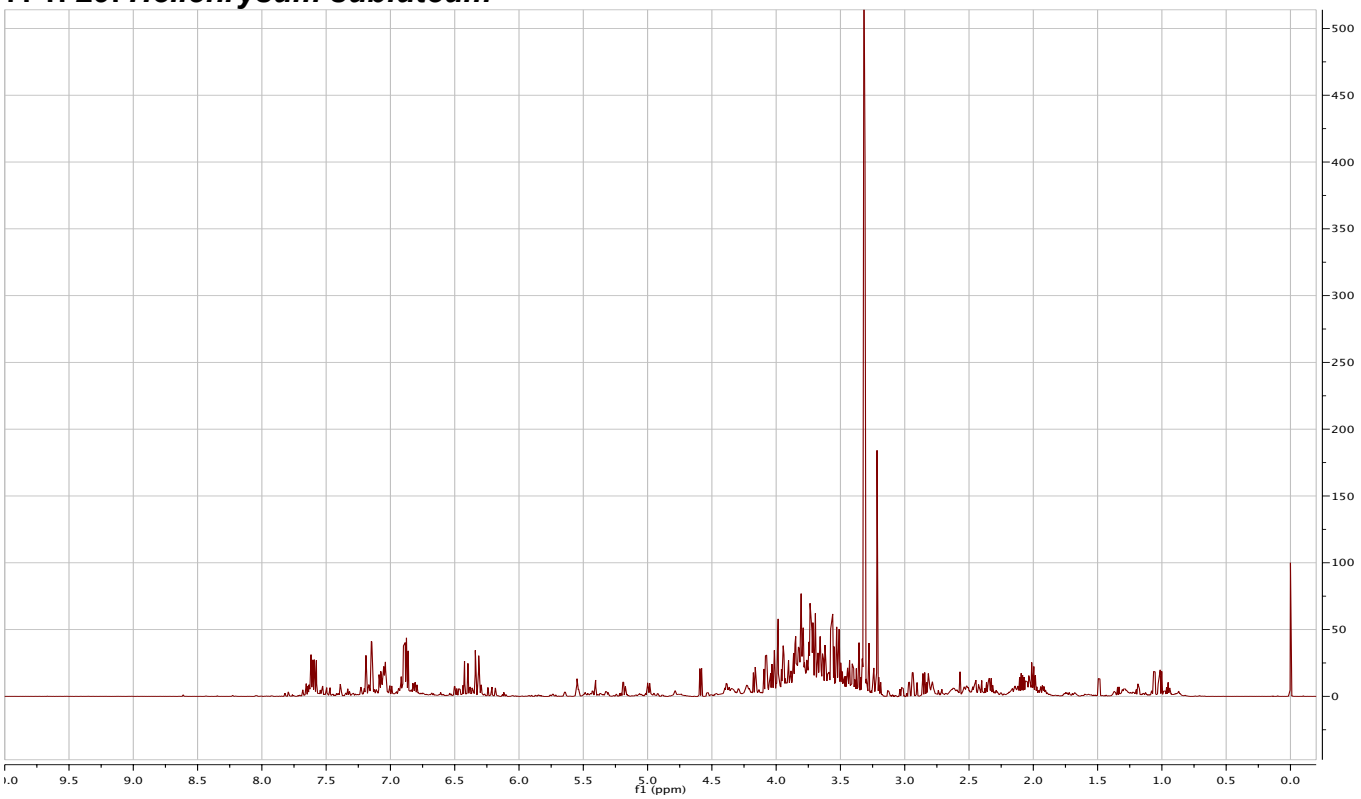

Fig 7.29: NMR spectrum of the methanol/water plant extract of *H. subluteum* (600 MHz, Leiden University).

7. 1. 30. *Helichrysum sutherlandii*

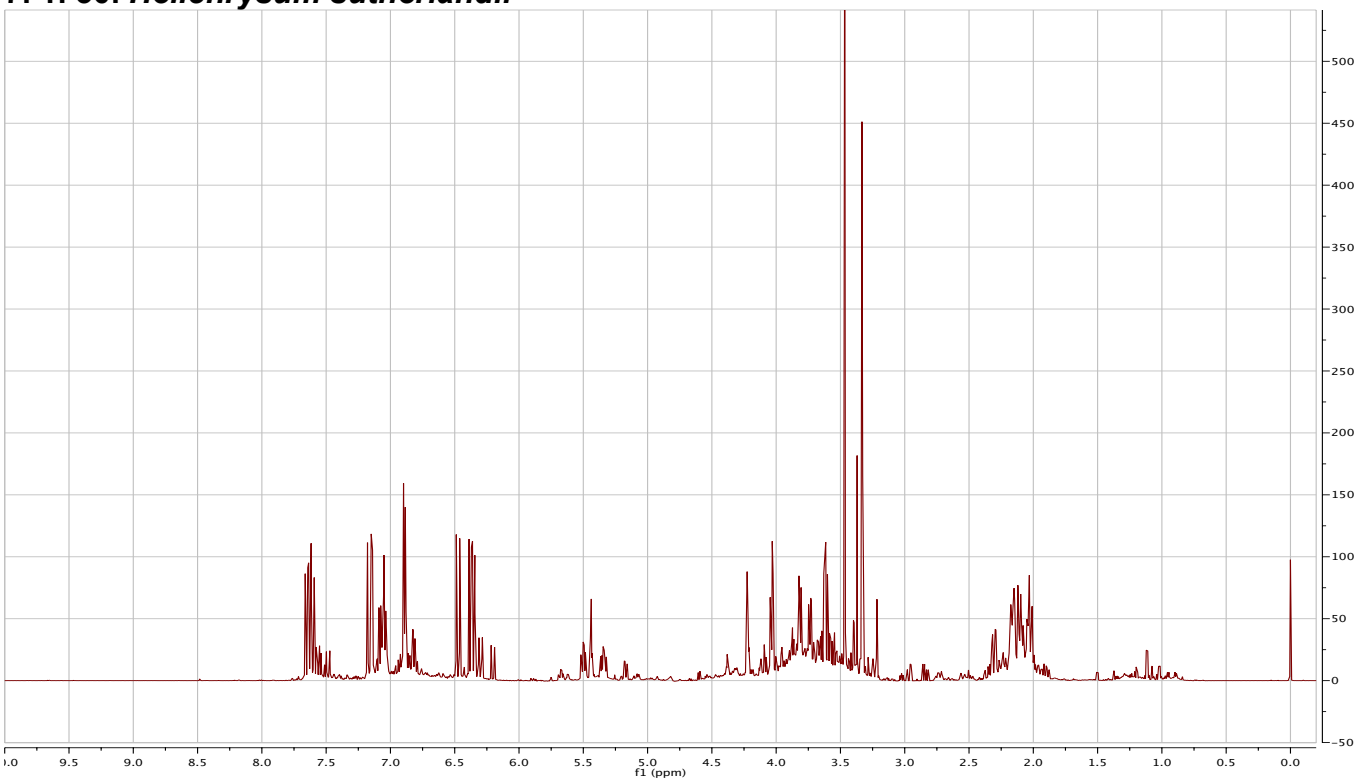

Fig 7.30: NMR spectrum of the methanol/water plant extract of *H. sutherlandii* (600 MHz, Leiden University).

7. 1. 31. *Helichrysum umbraculigerum*

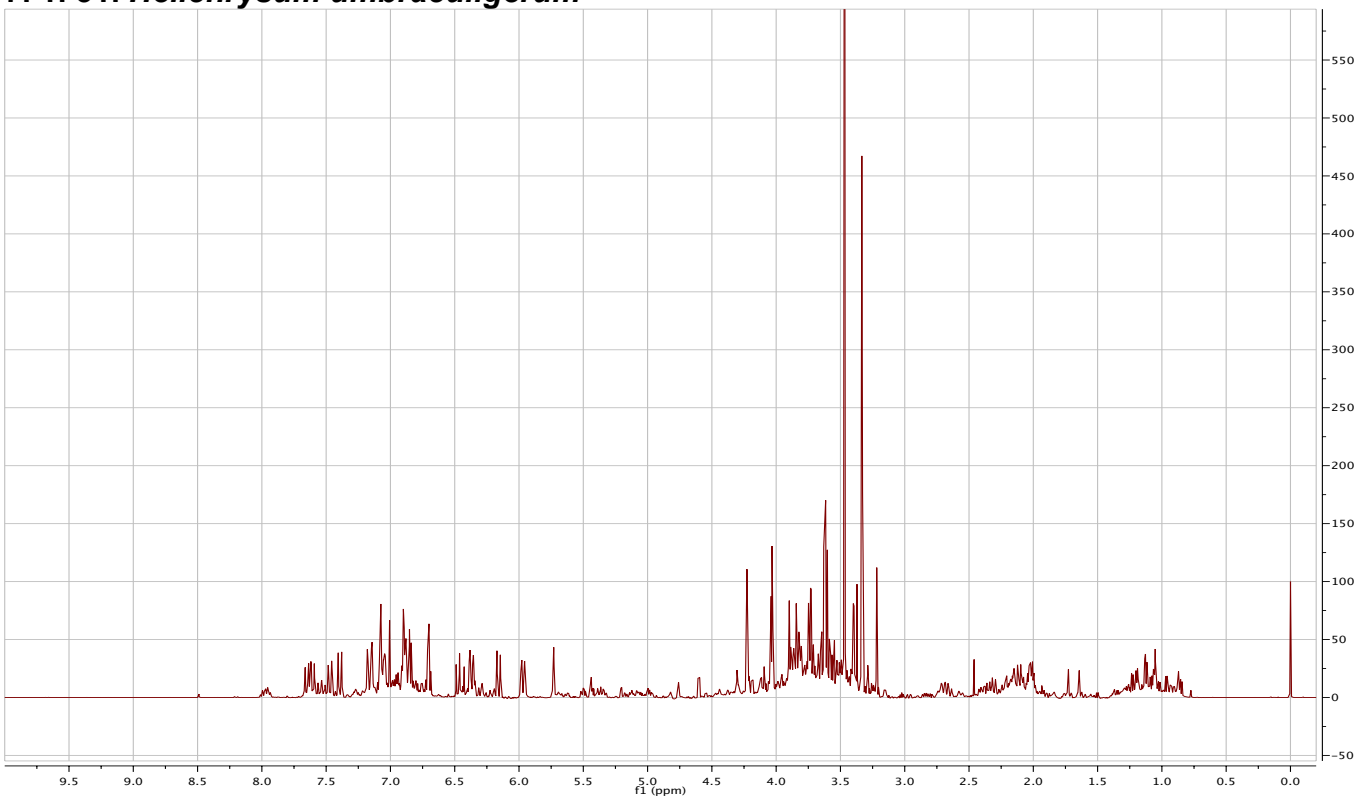

Fig 7.31: NMR spectrum of the methanol/water plant extract of *H. umbraculigerum* (600 MHz, Leiden University).

7. 1. 32. *Helichrysum vernum*

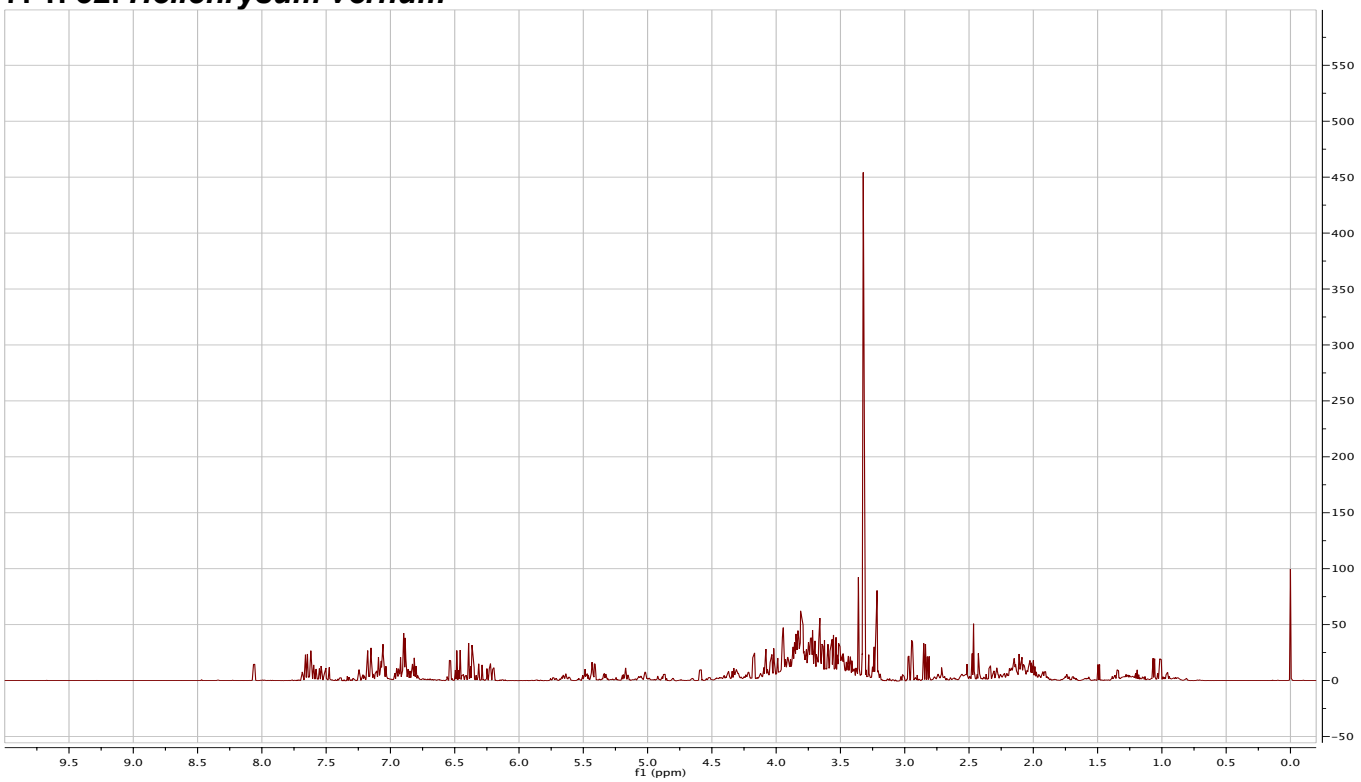

Fig 7.32: NMR spectrum of the methanol/water plant extract of *H. vernum* (600 MHz, Leiden University).

7.2 NMR spectra of the dichloromethane (DCM) plant extract

7. 2. 1. *Helichrysum acutatum*

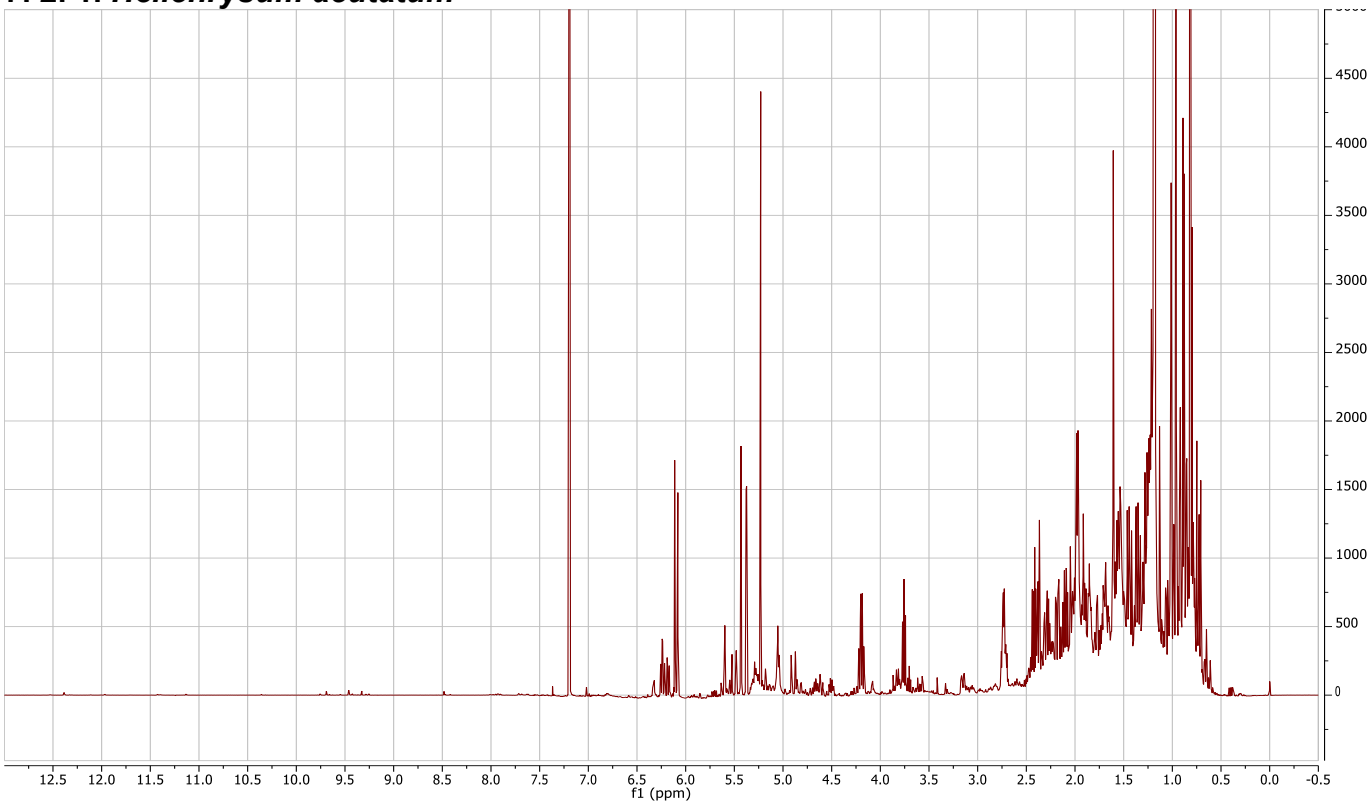

Fig 7. 33: NMR spectrum of the dichloromethane plant extract of *H. acutatum* (600 MHz, Leiden University).

7. 2. 2. *Helichrysum allioides*

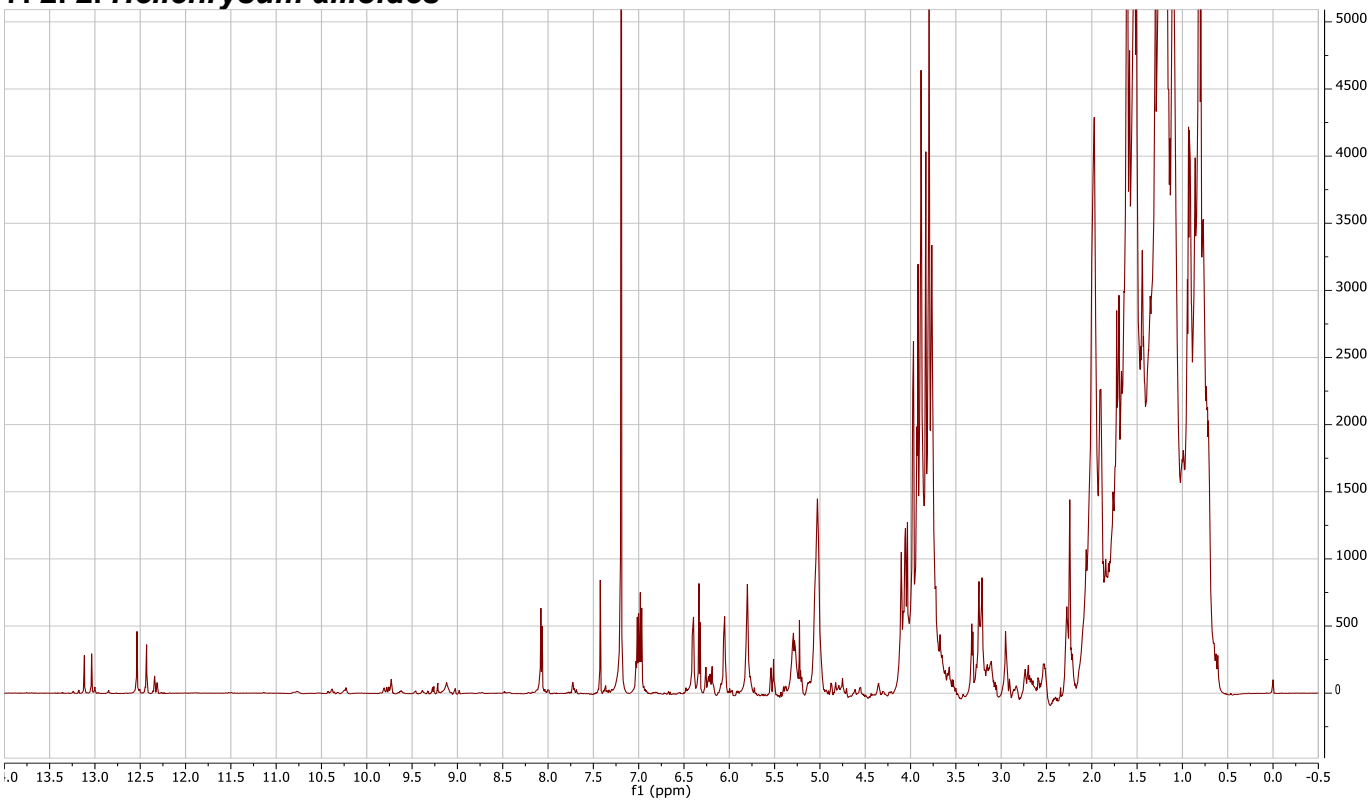

Fig 7. 34: NMR spectrum of the dichloromethane plant extract of *H. allioides* (600 MHz, Leiden University).

7. 2. 3. *Helichrysum anomalum*

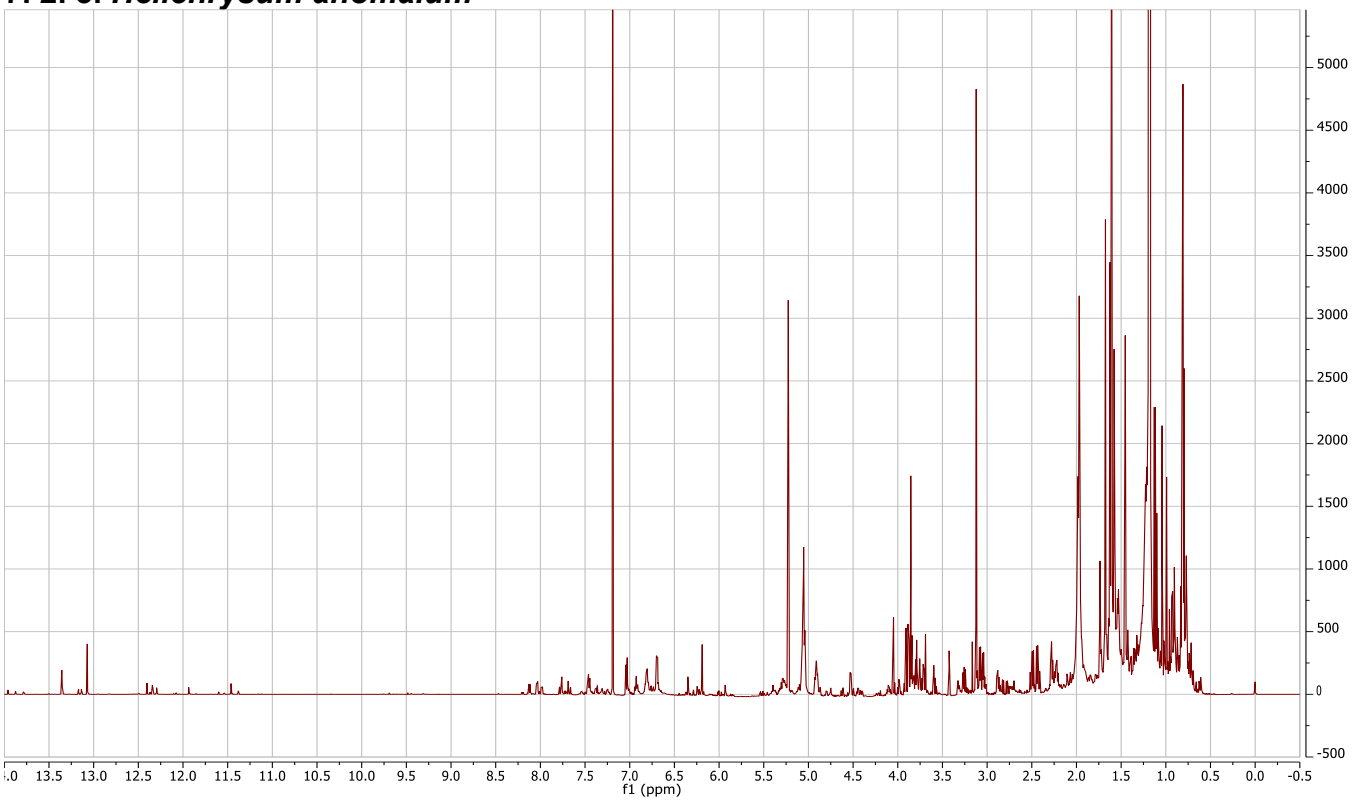

Fig 7. 35: NMR spectrum of the dichloromethane plant extract of *H. anomalum* (600 MHz, Leiden University).

7. 2. 4. *Helichrysum appendiculatum*

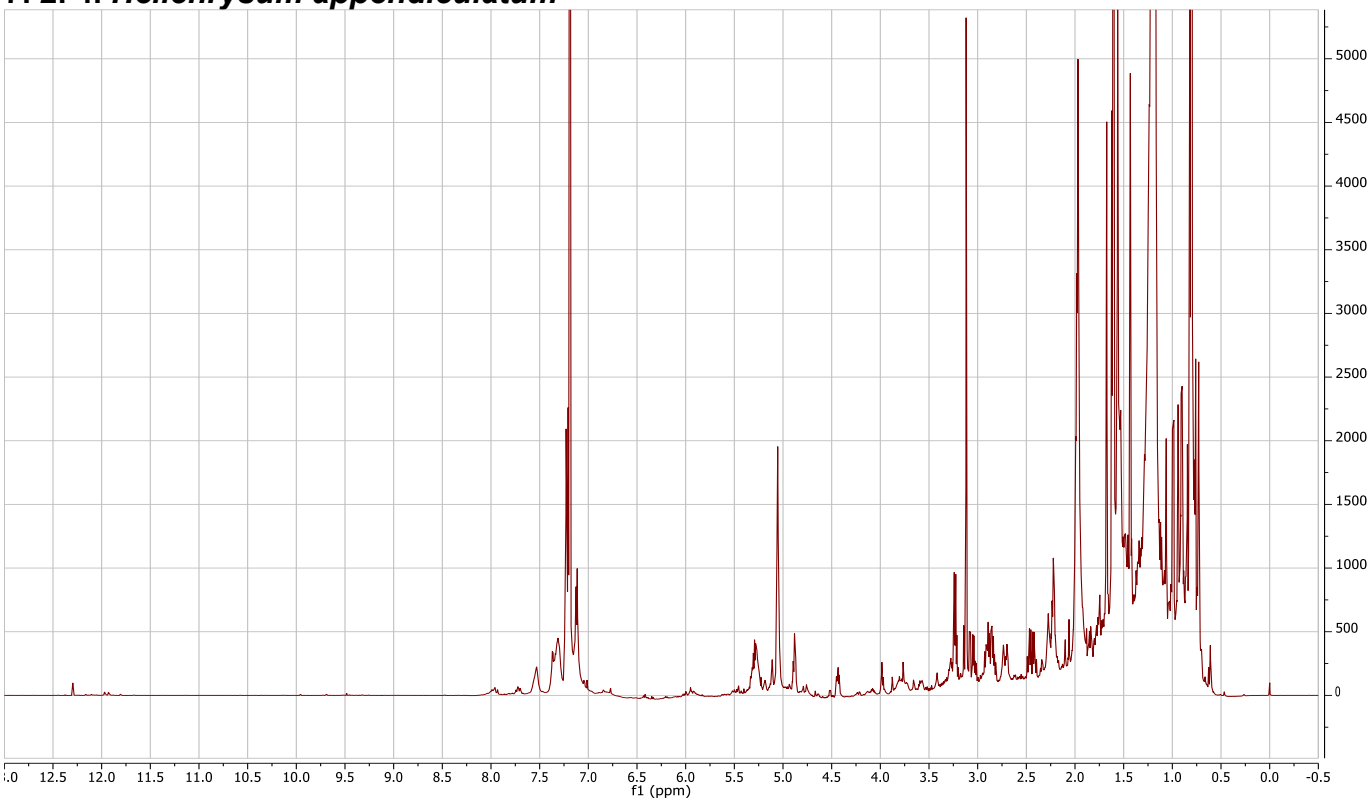

Fig 7. 36: NMR spectrum of the dichloromethane plant extract of *H. appendiculatum* (600 MHz, Leiden University).

7. 2. 5. *Helichrysum aureonitens*

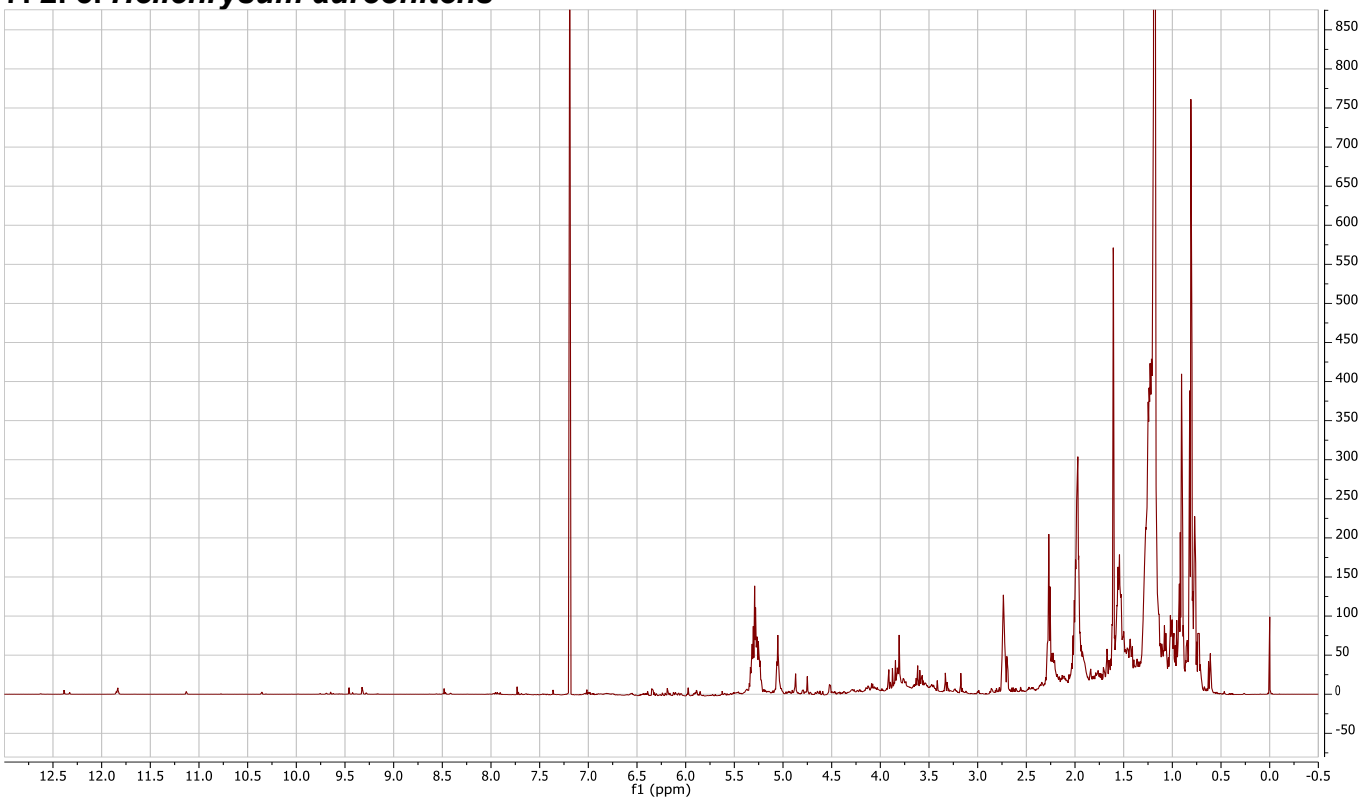

Fig 7. 37: NMR spectrum of the dichloromethane plant extract of *H. aureonitens* (600 MHz, Leiden University).

7. 2. 6. *Helichrysum cephaloideum*

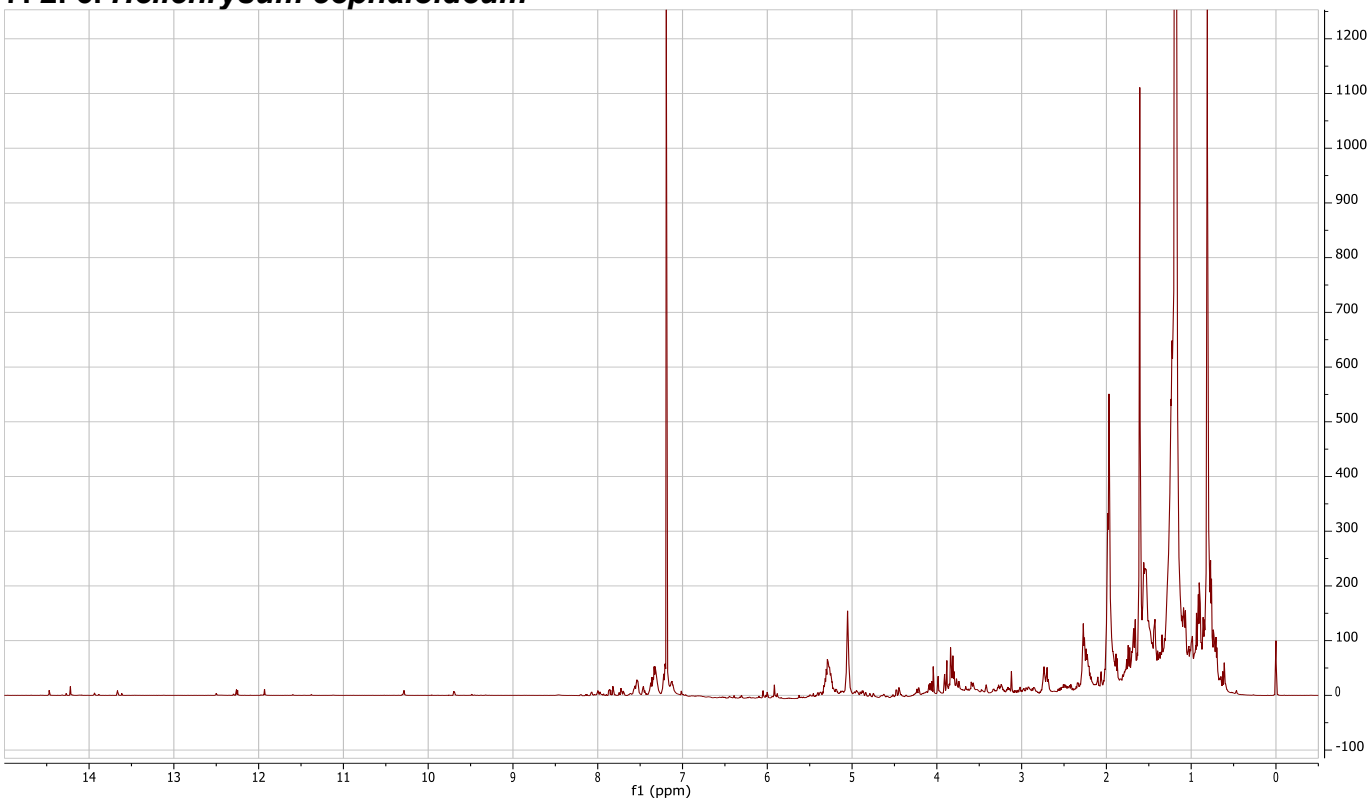

Fig 7. 38: NMR spectrum of the dichloromethane plant extract of *H. cephaloideum* (600 MHz, Leiden University).

7. 2. 7. *Helichrysum chionosphaerum*

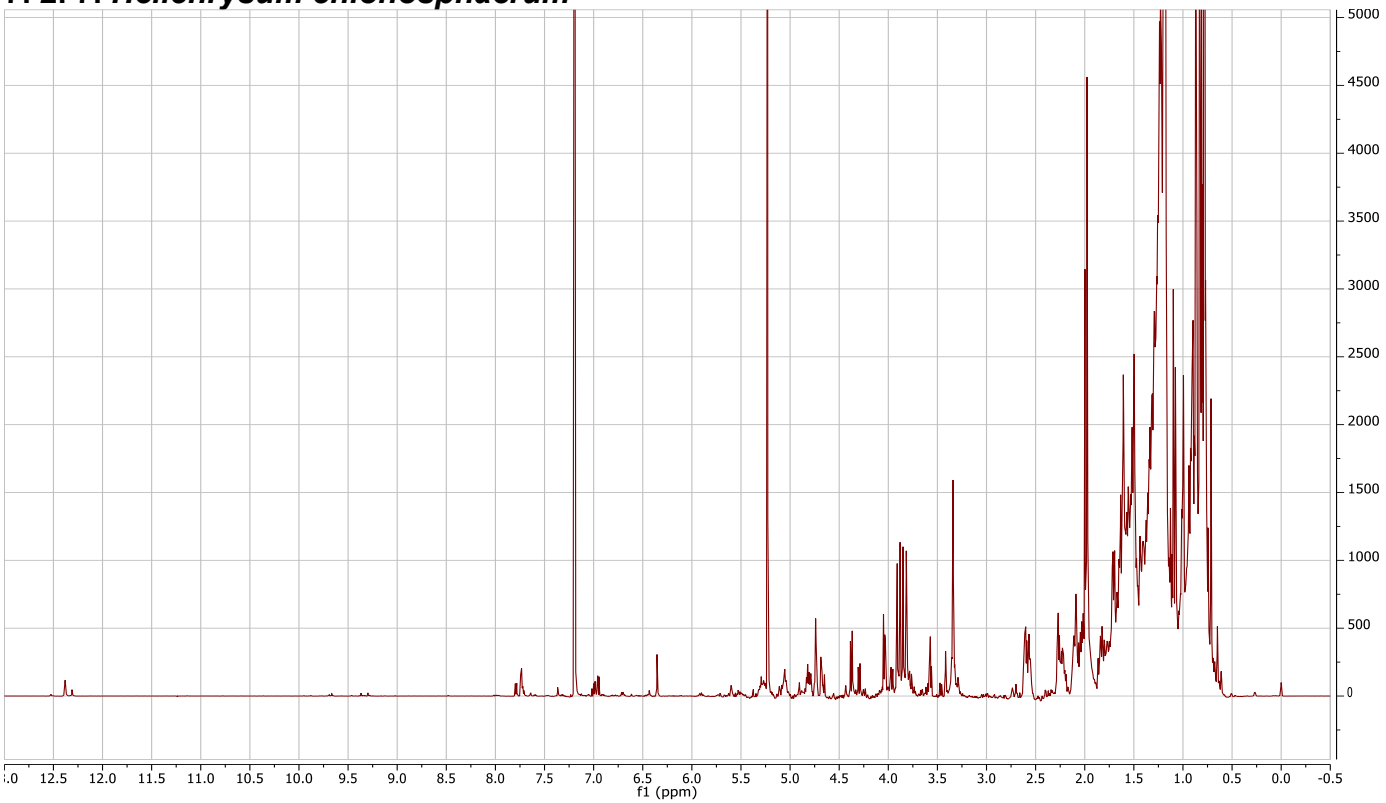

Fig. 7.39: NMR spectrum of the dichloromethane plant extract of *H. chionosphaerum* (600 MHz, Leiden University).

7. 2. 8. *Helichrysum confertum*

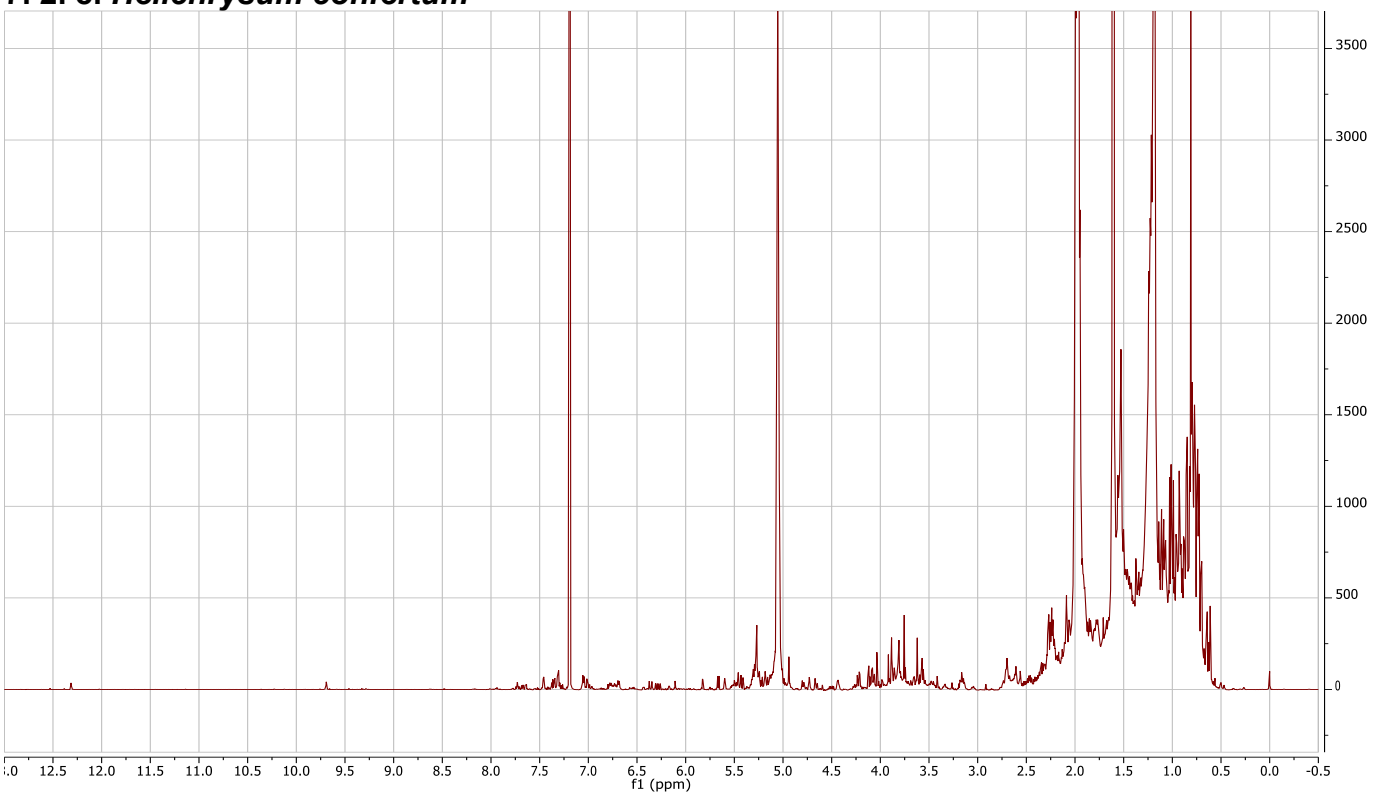

Fig. 7.40: NMR spectrum of the dichloromethane plant extract of *H. confertum* (600 MHz, Leiden University).

7. 2. 9. *Helichrysum cymosum* subsp. *cymosum*

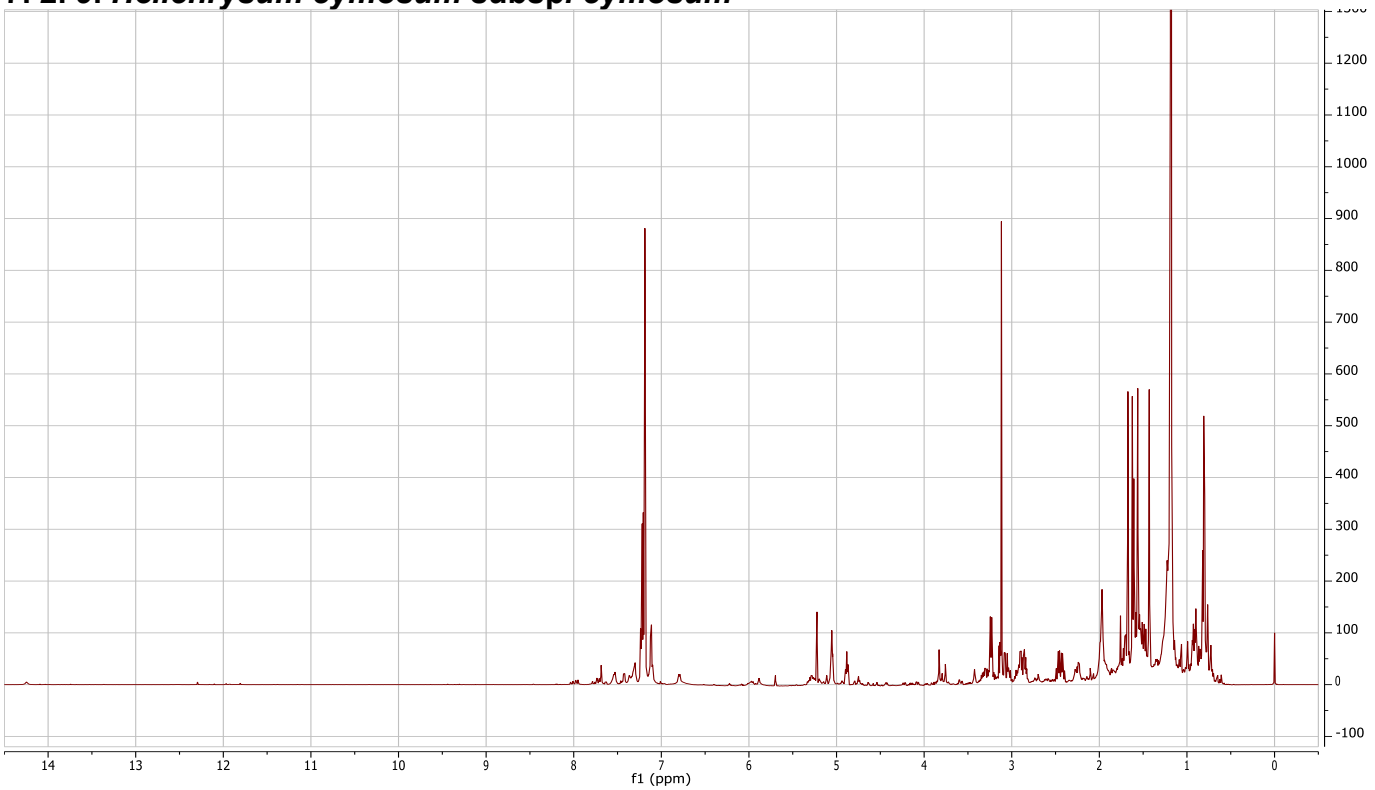

Fig. 7.41: NMR spectrum of the dichloromethane plant extract of *H. cymosum* subsp. *cymosum* (600 MHz, Leiden University).

7. 2. 10. *Helichrysum cymosum* subsp. *clavum*

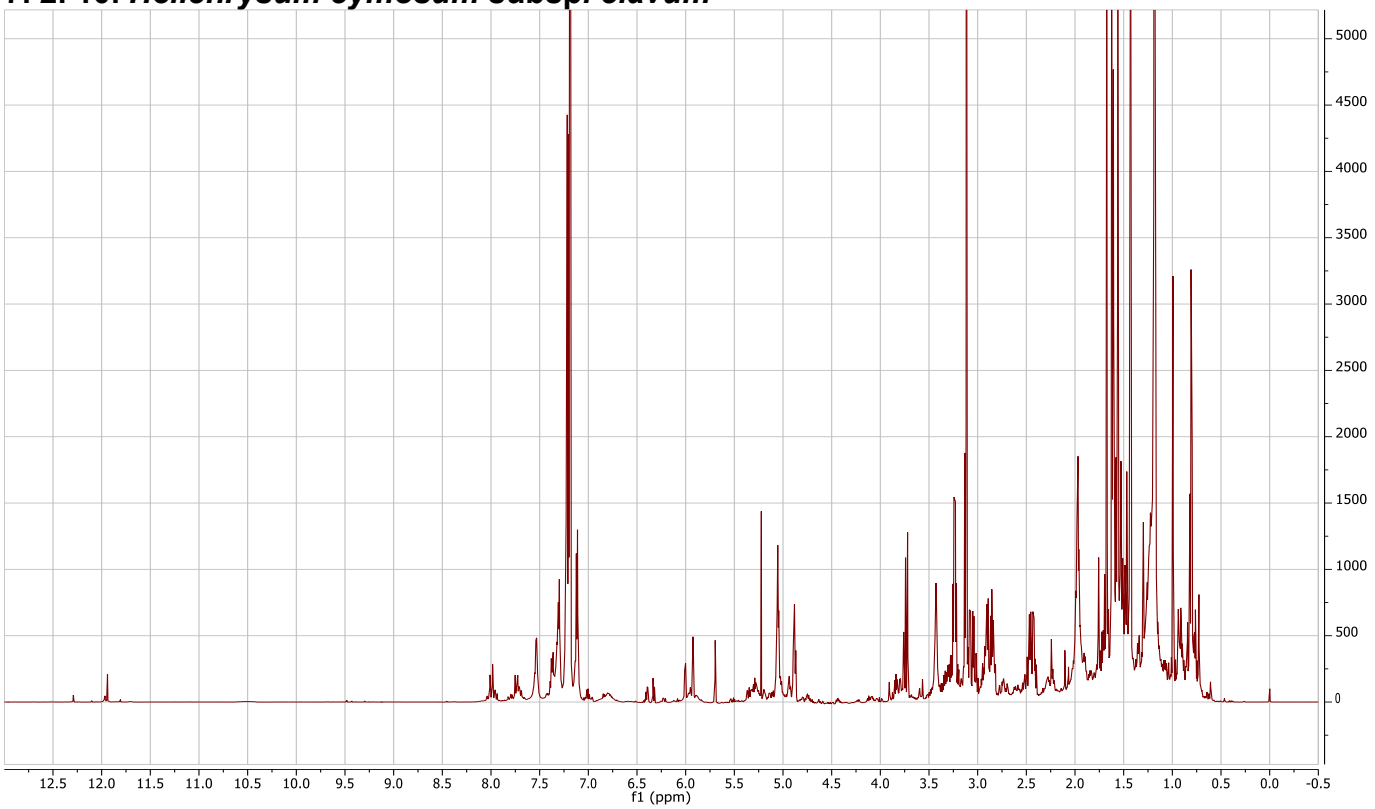

Fig. 7.42: NMR spectrum of the dichloromethane plant extract of *H. cymosum* subsp. *clavum* (600 MHz, Leiden University).

7. 2. 11. *Helichrysum difficile*

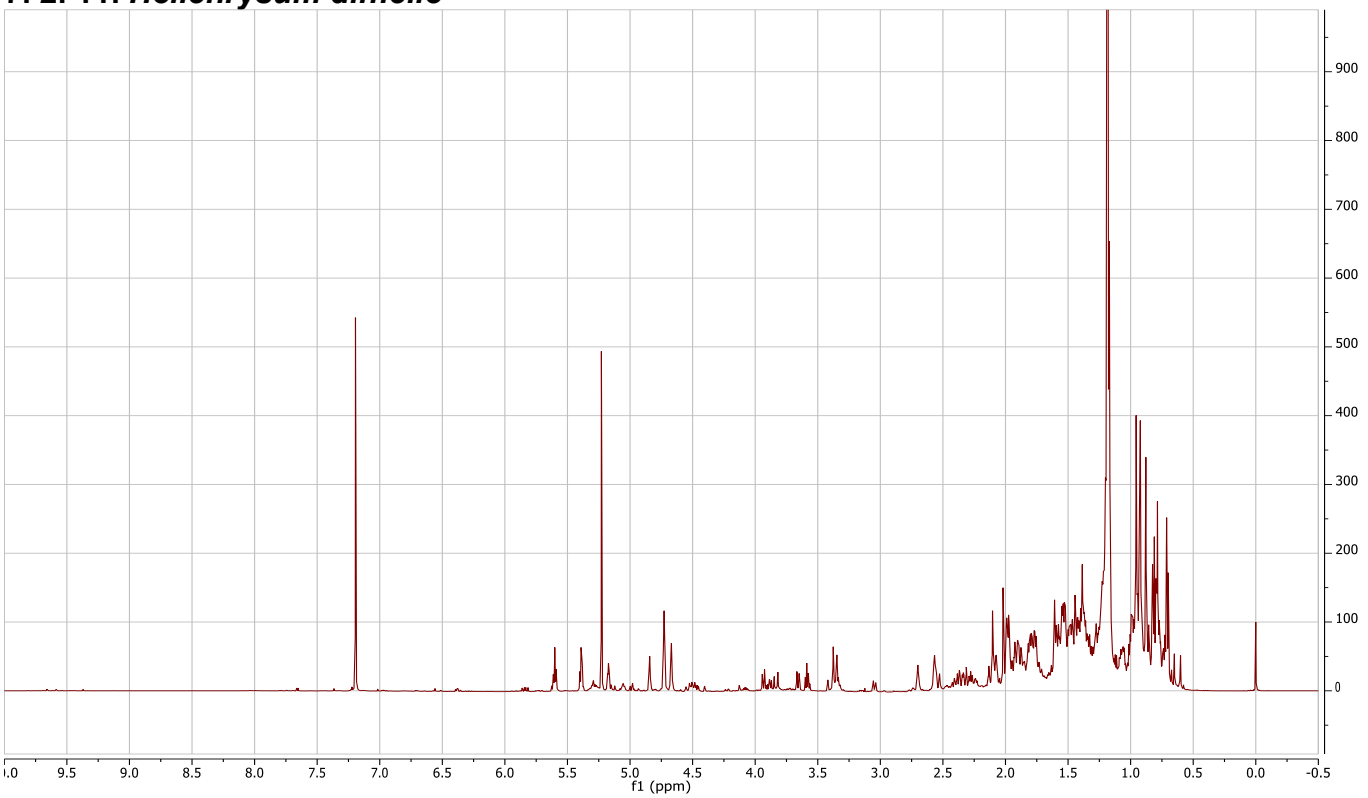

Fig. 7.43: NMR spectrum of the dichloromethane plant extract of *H. difficile* (600 MHz, Leiden University).

7. 2. 12. *Helichrysum drakensbergense*

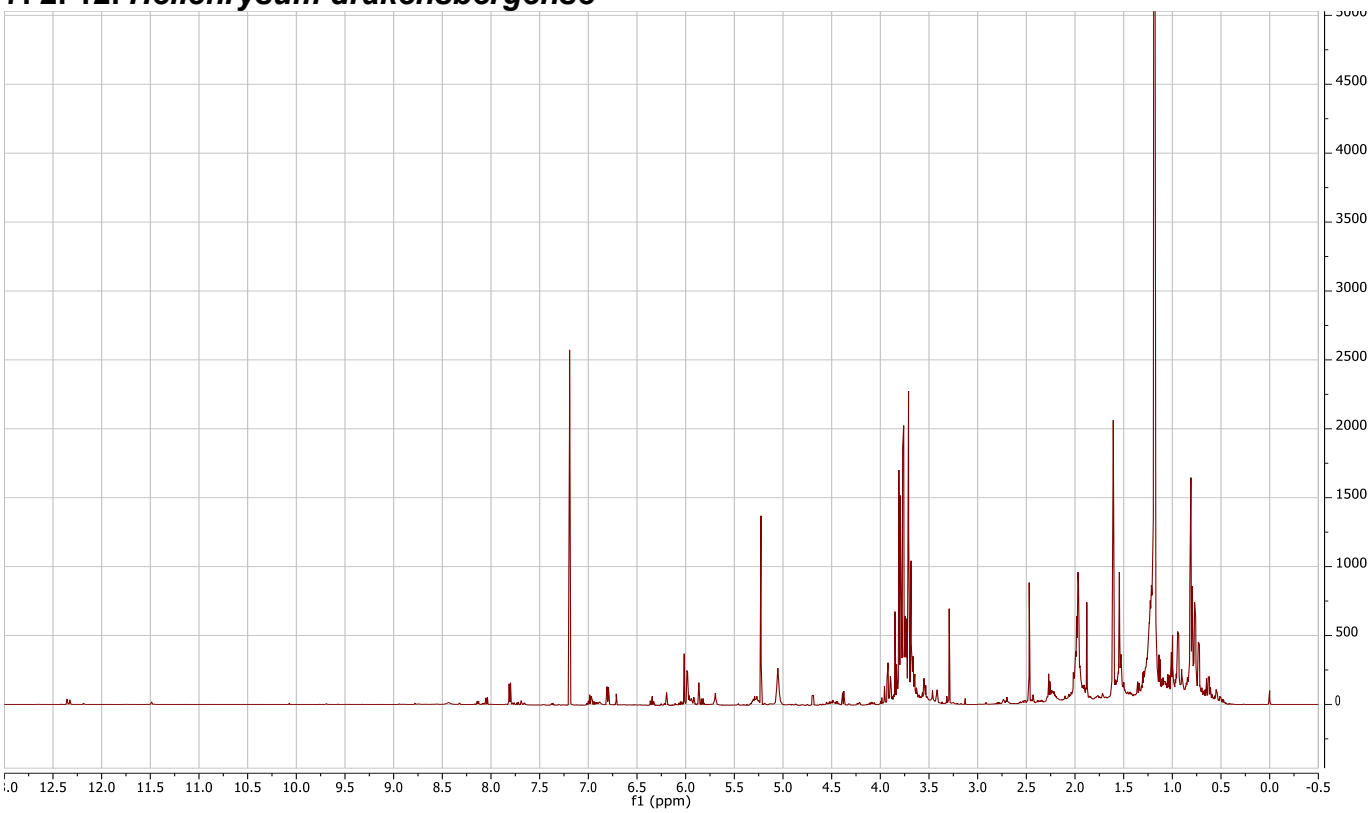

Fig. 7.44: NMR spectrum of the dichloromethane plant extract of *H. drakensbergense* (600 MHz, Leiden University).

7. 2. 13. *Helichrysum herbaceum*

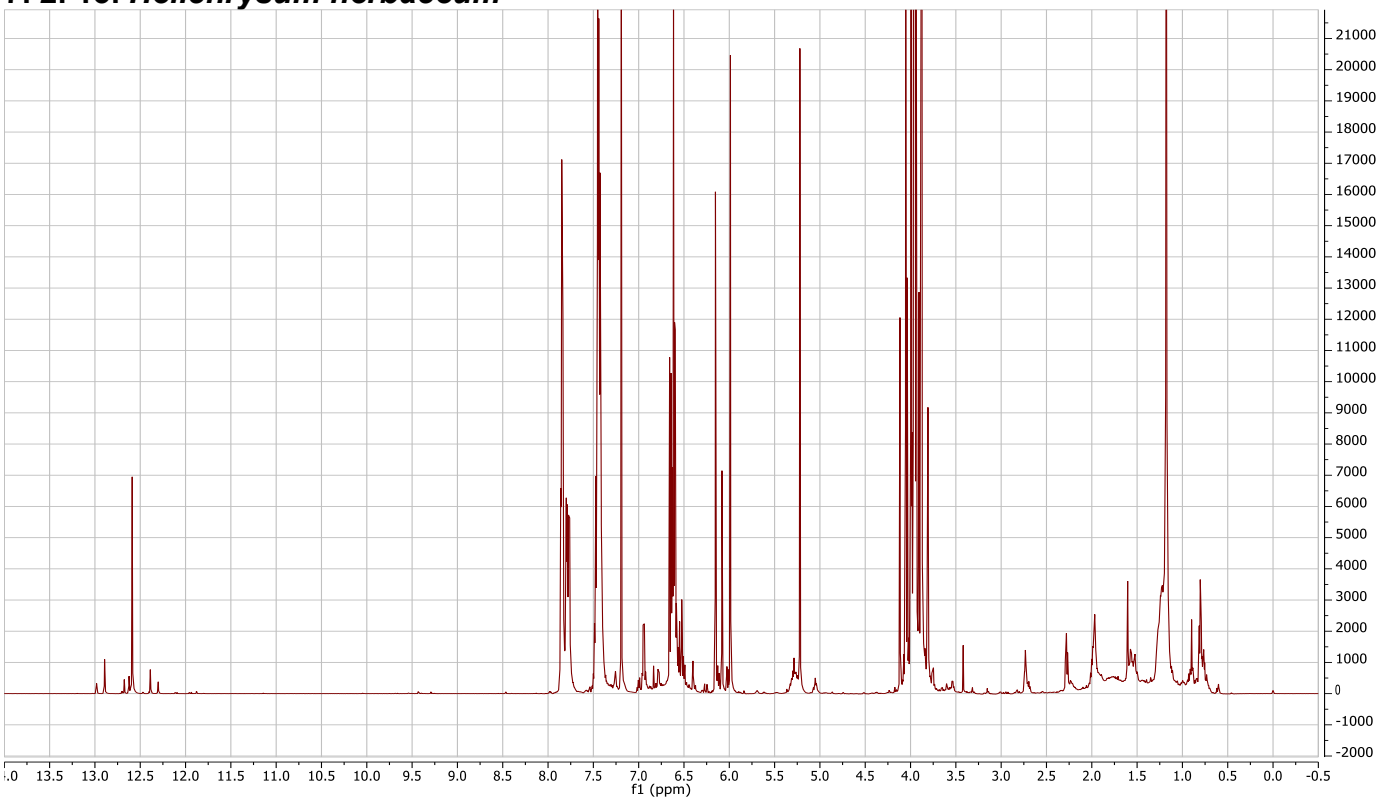

Fig. 7.45: NMR spectrum of the dichloromethane plant extract of *H. herbaceum* (600 MHz, Leiden University).

7. 2. 14. *Helichrysum melanacme*

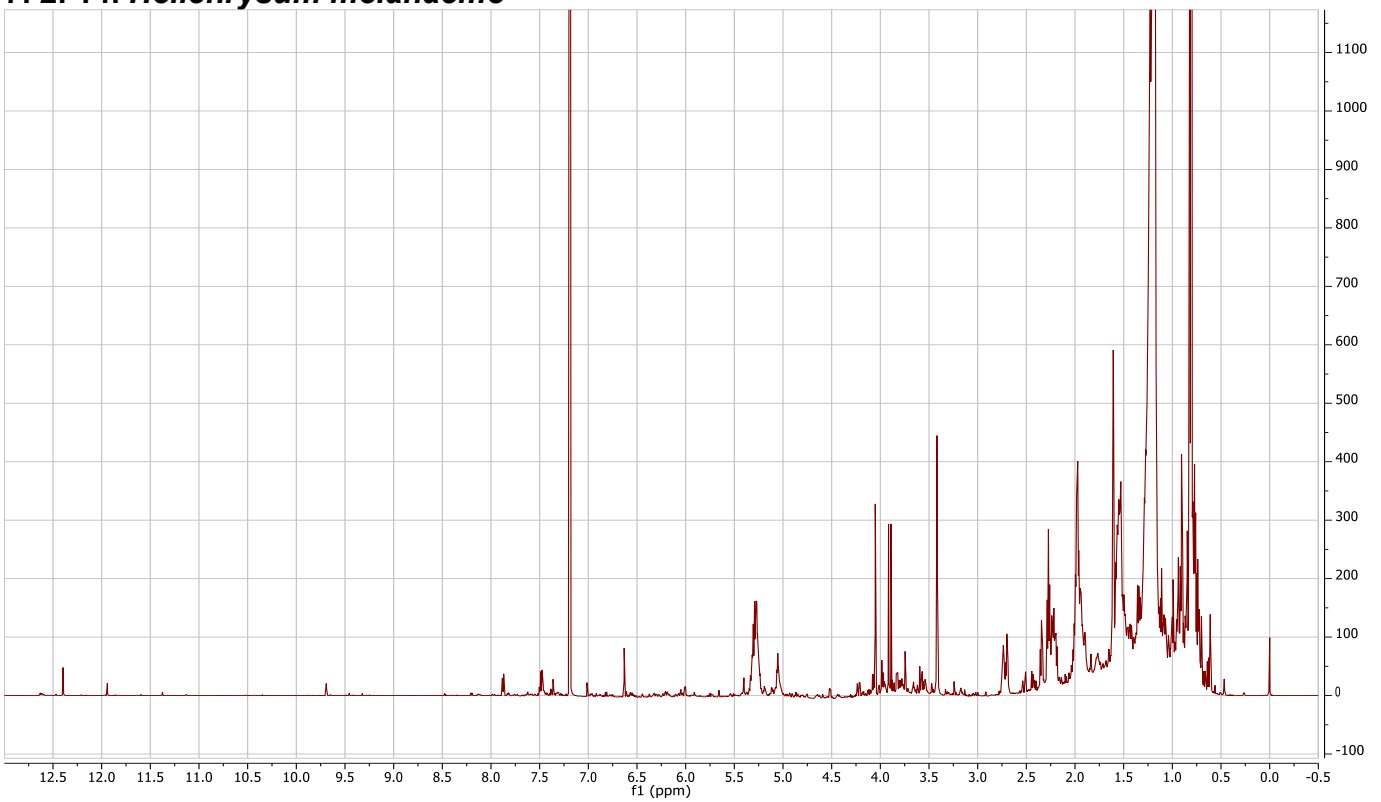

Fig. 7.46: NMR spectrum of the dichloromethane plant extract of *H. melanacme* (600 MHz, Leiden University).

7. 2. 15. *Helichrysum miconiifolium*

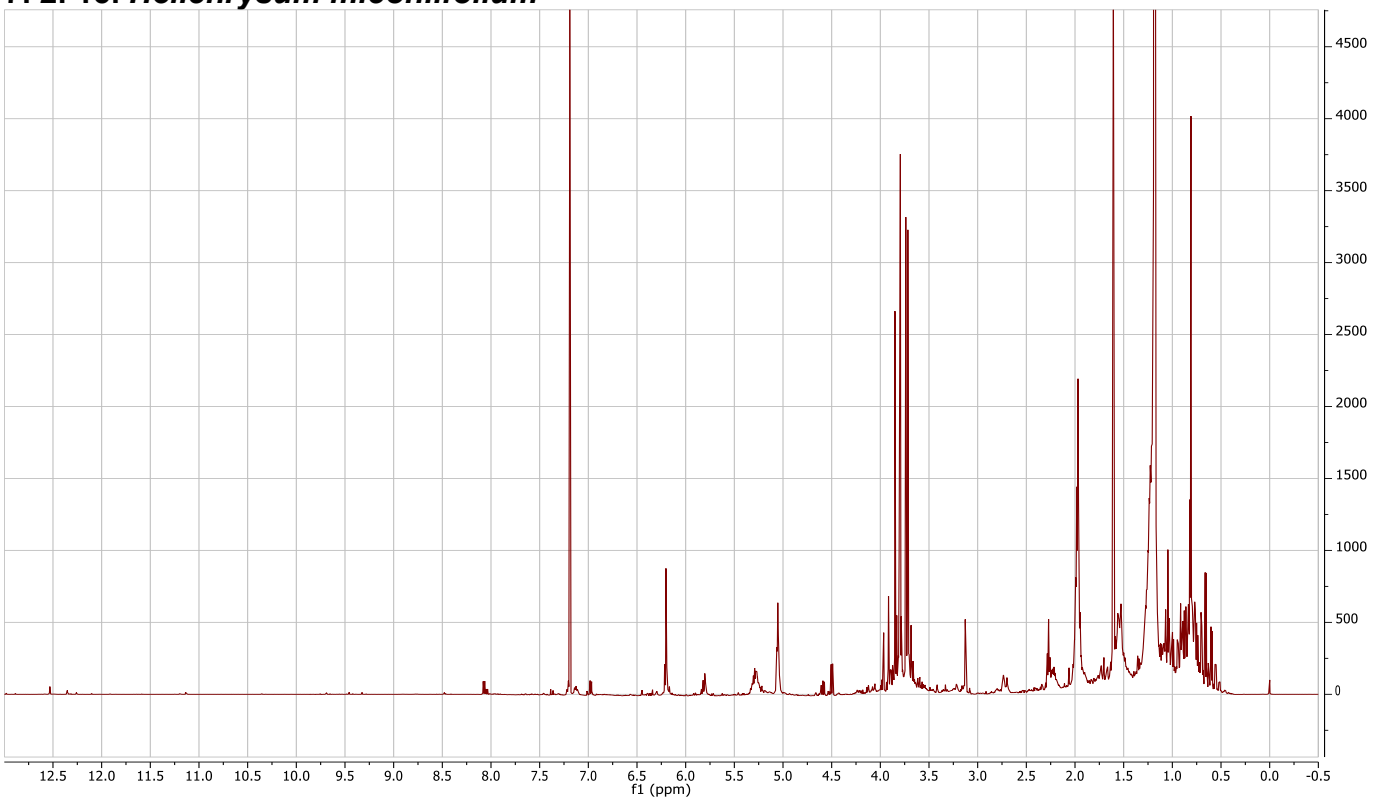

Fig. 7.47: NMR spectrum of the dichloromethane plant extract of *H. miconiifolium* (600 MHz, Leiden University).

7. 2. 16. *Helichrysum natalitium*

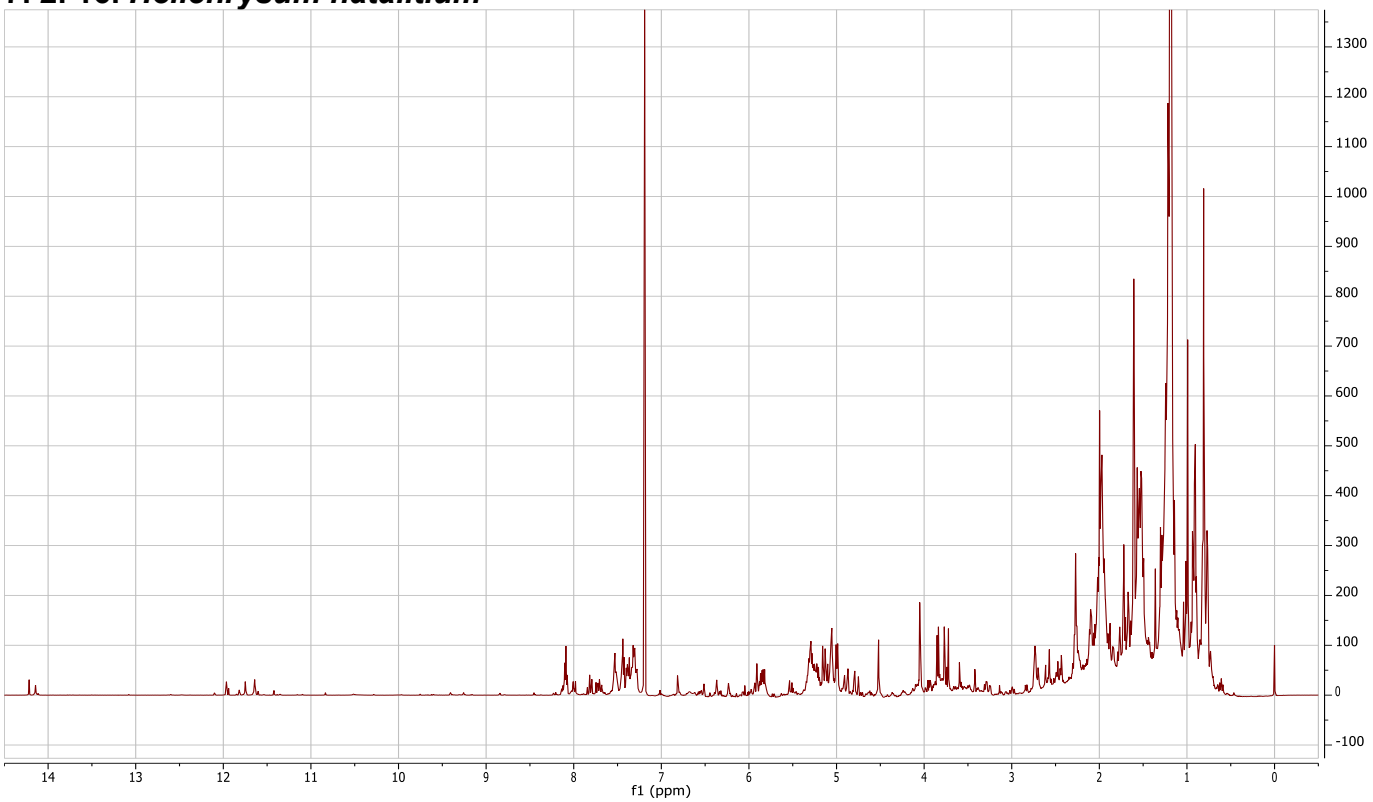

Fig 7.48: NMR spectrum of the dichloromethane plant extract of *H. natalitium* (600 MHz, Leiden University).

7. 2. 17. *Helichrysum nudifolium* var. *nudifolium* (1)

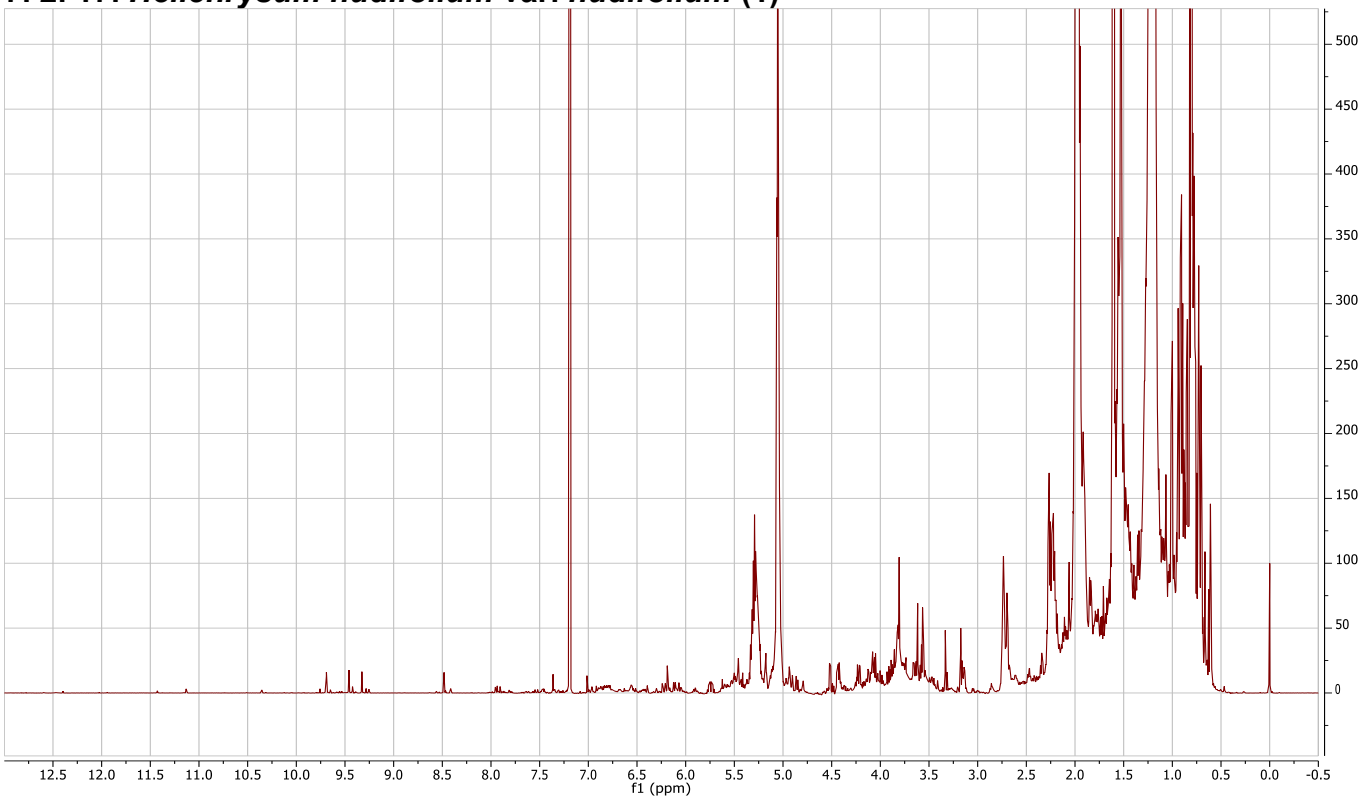

Fig 7.49: NMR spectrum of the dichloromethane plant extract of *H. nudifolium* var. *nudifolium* (1) (600 MHz, Leiden University).

7. 2. 18. *Helichrysum odoratissimum*

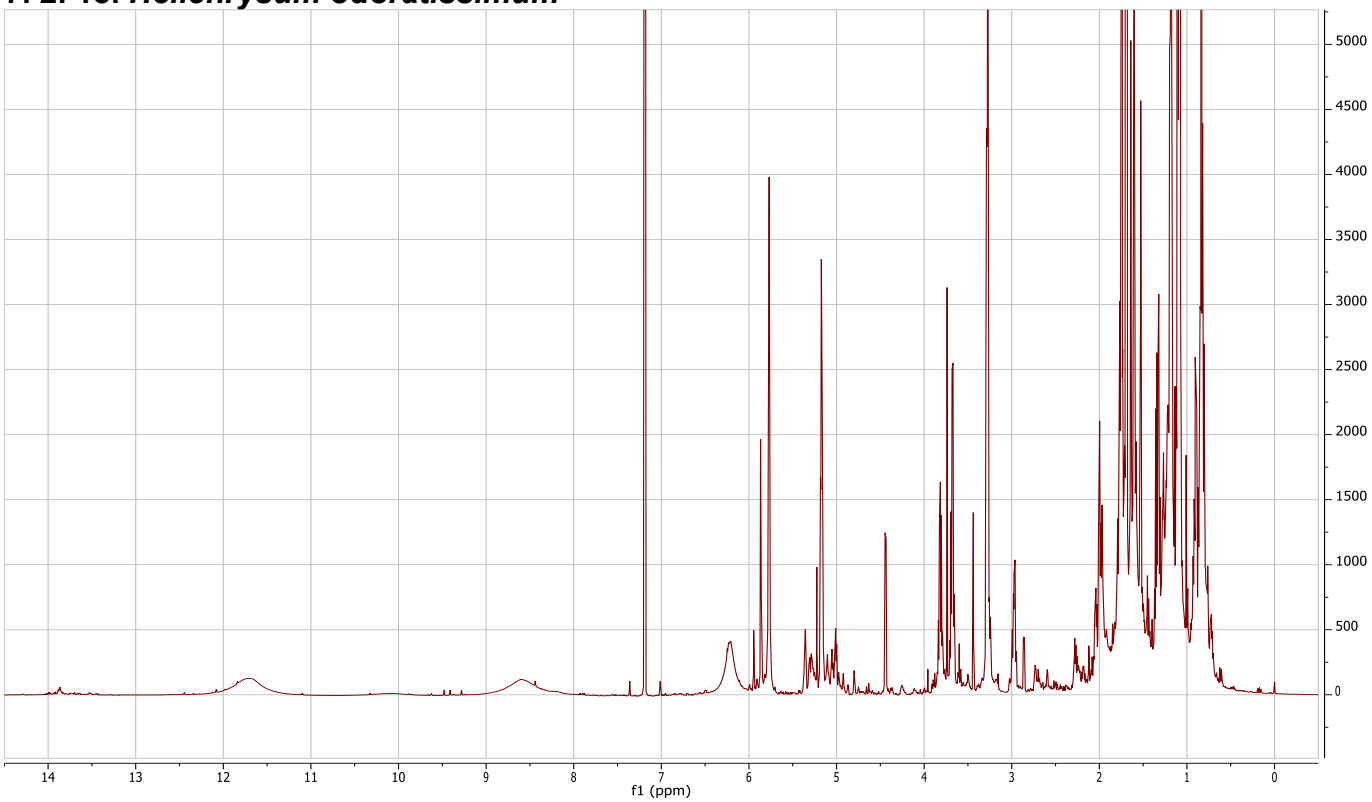

Fig 7.51: NMR spectrum of the dichloromethane plant extract of *H. odoratissimum* (600 MHz, Leiden University).

7. 2. 19. *Helichrysum oreophilum*

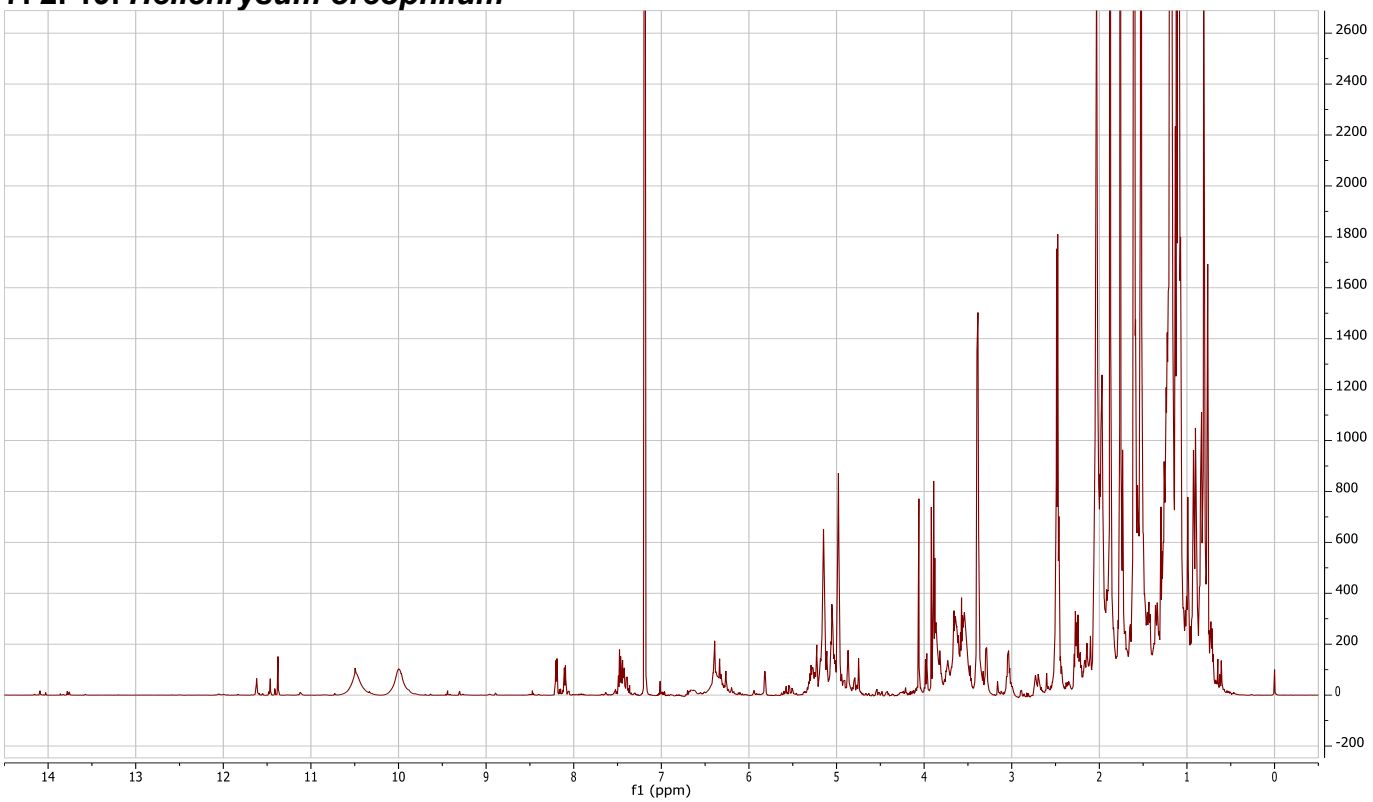

Fig 7.52: NMR spectrum of the dichloromethane plant extract of *H. oreophilum* (600 MHz, Leiden University).

7. 2. 20. *Helichrysum oxyphyllum*

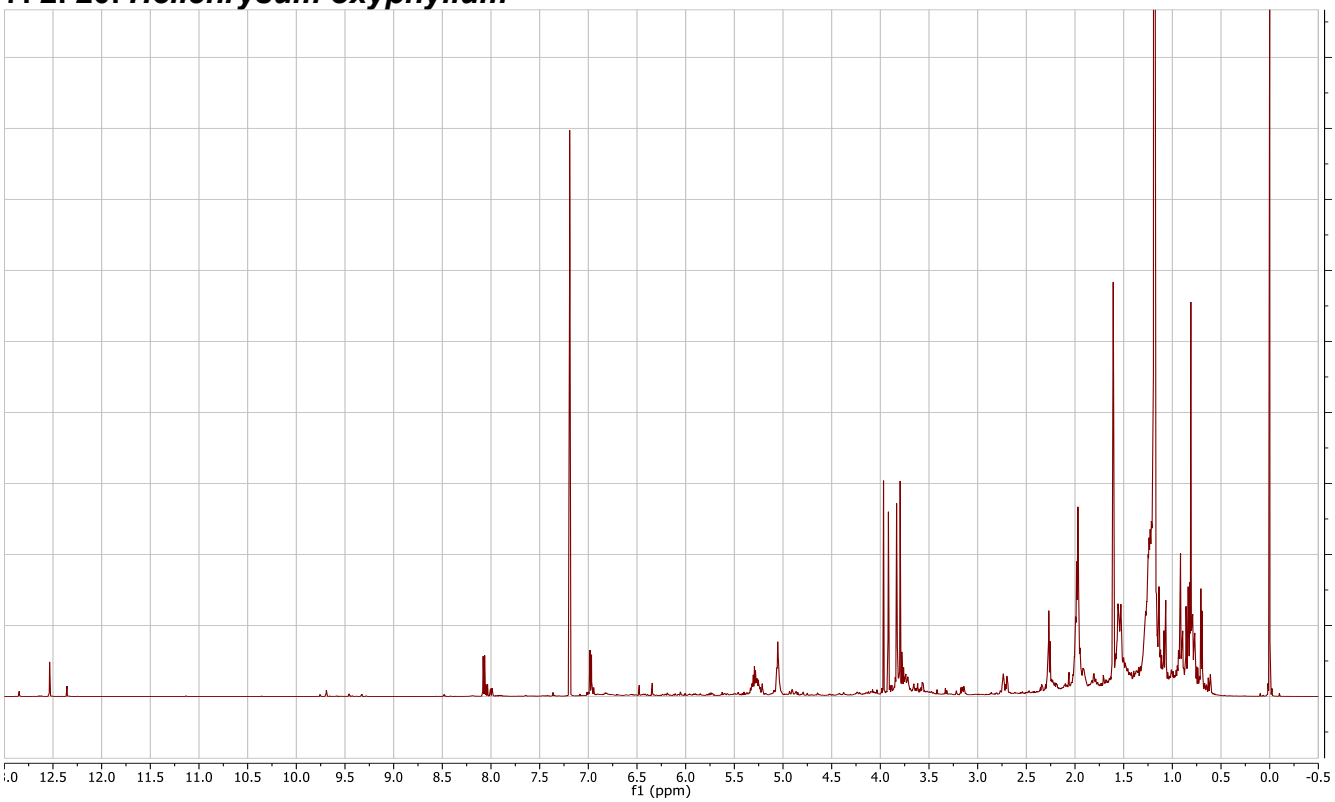

Fig 7.53: NMR spectrum of the dichloromethane plant extract of *H. oxyphyllum* (600 MHz, Leiden University).

7. 2. 21. *Helichrysum pallidum*

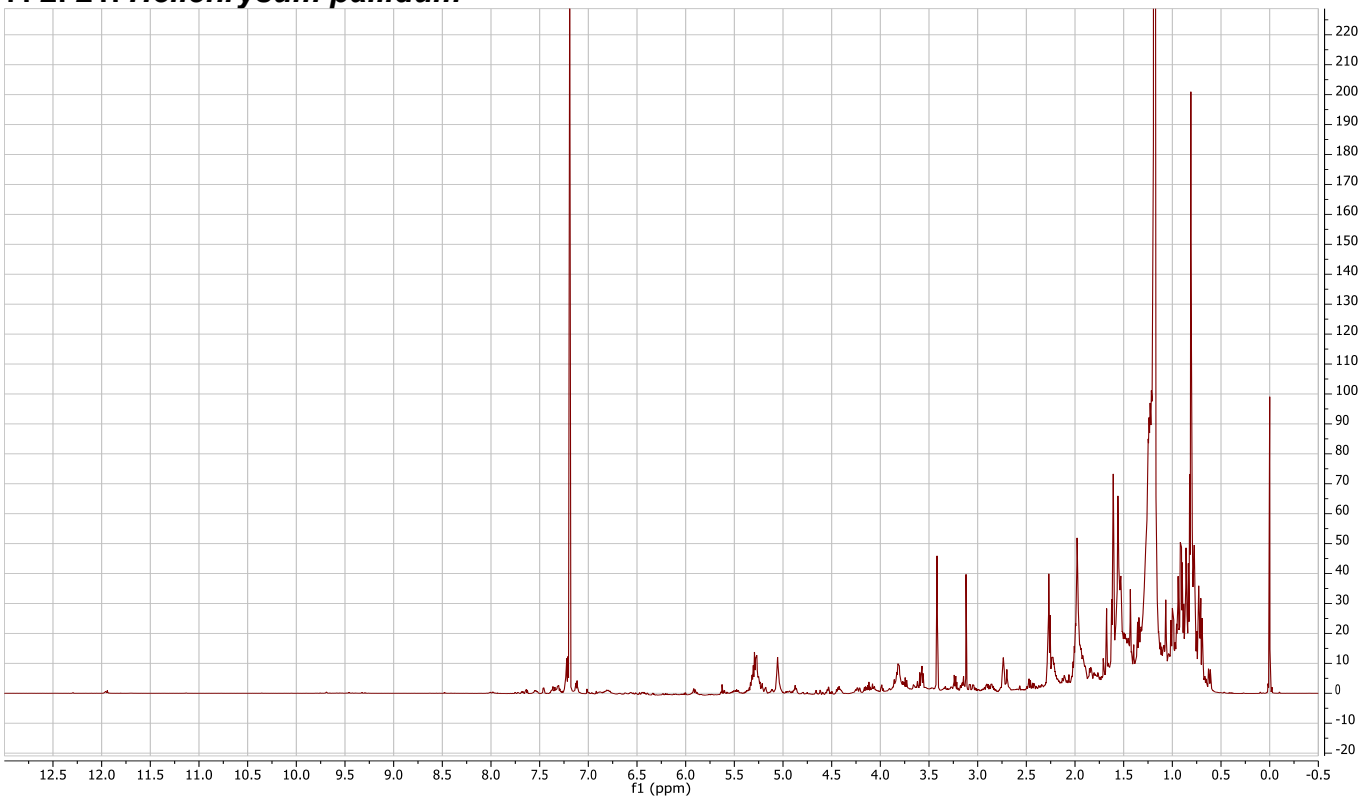

Fig 7.54: NMR spectrum of the dichloromethane plant extract of *H. pallidum* (600 MHz, Leiden University).

7. 2. 22. *Helichrysum panduratum*

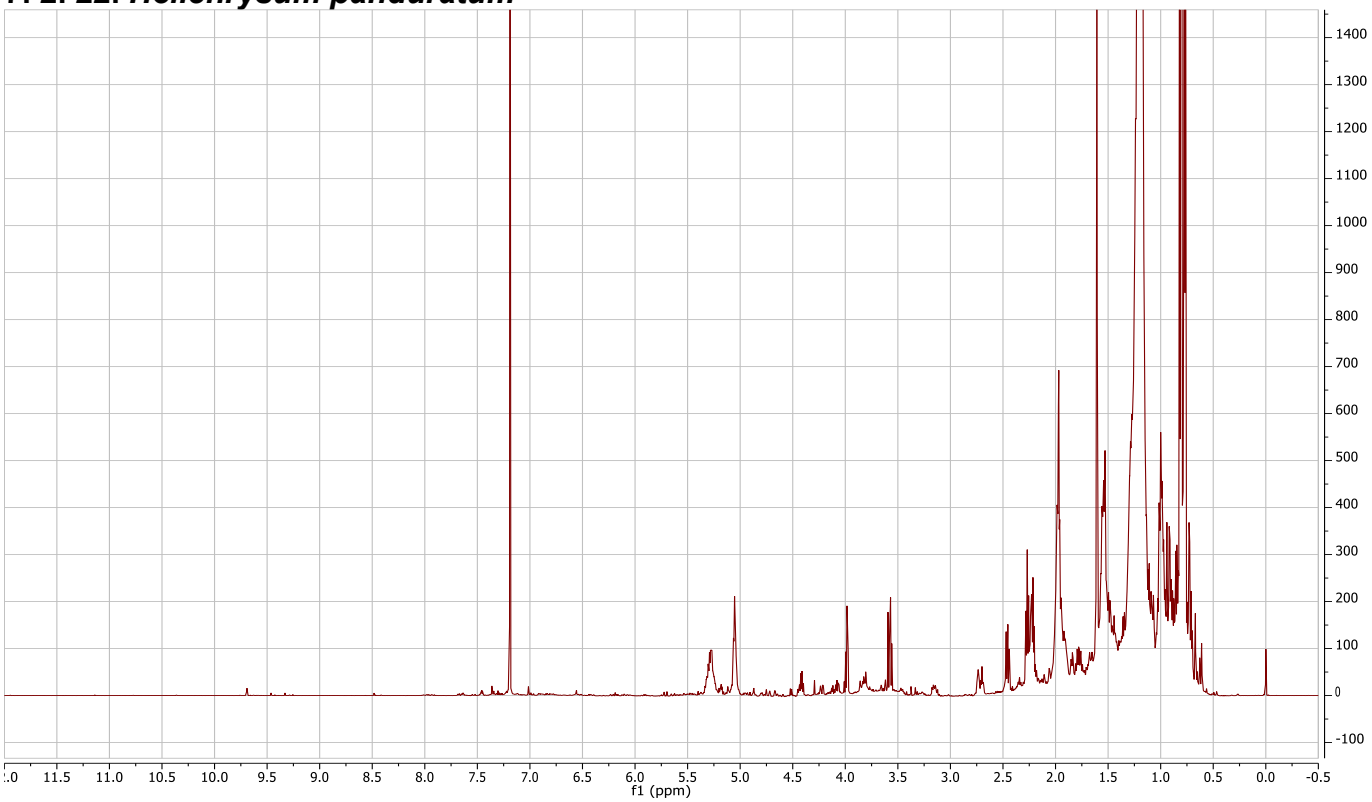

Fig 7.55: NMR spectrum of the dichloromethane plant extract of *H. panduratum* (600 MHz, Leiden University).

7. 2. 23. *Helichrysum pannosum*

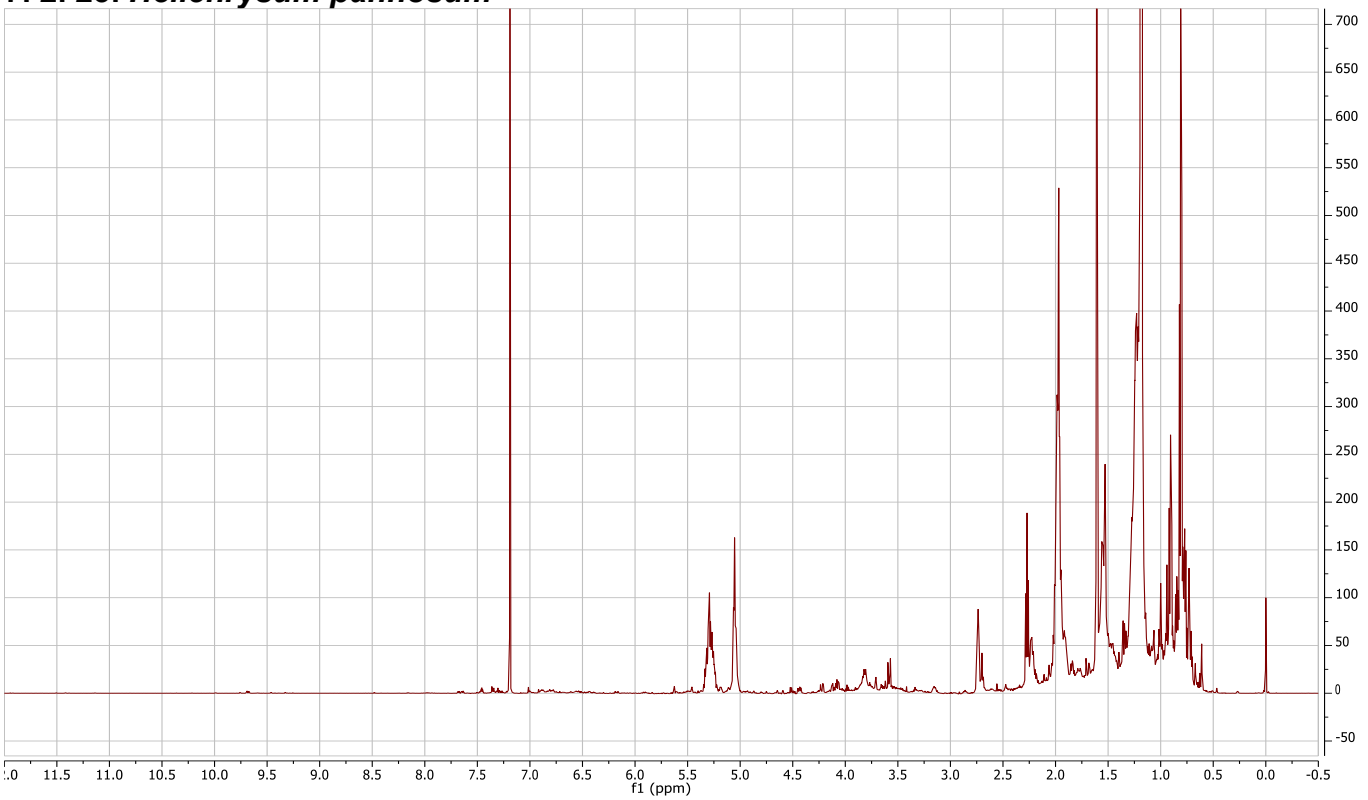

Fig 7.56: NMR spectrum of the dichloromethane plant extract of *H. pannosum* (600 MHz, Leiden University).

7. 2. 24. *Helichrysum pilosellum*

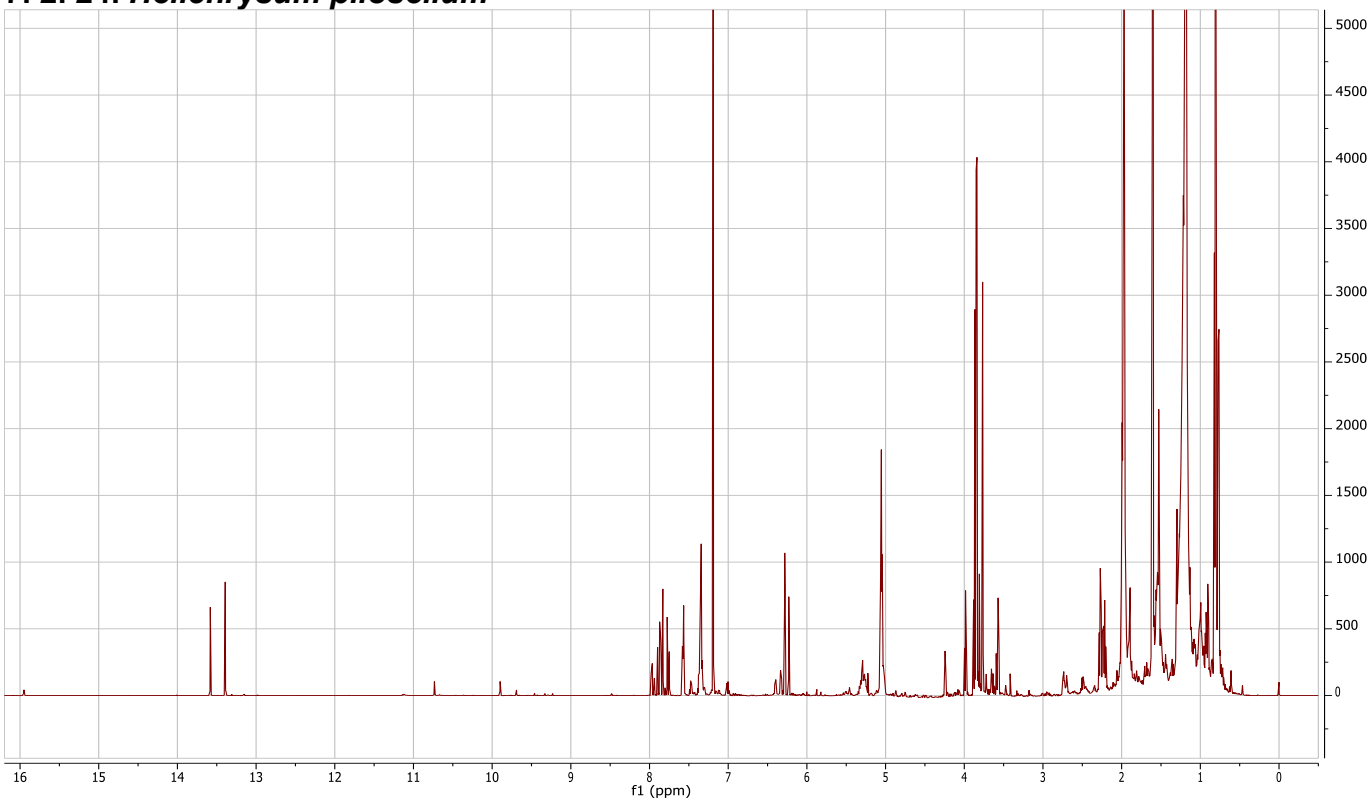

Fig 7.57: NMR spectrum of the dichloromethane plant extract of *H. pilosellum* (600 MHz, Leiden University).

7. 2. 25. *Helichrysum pilosellum*†

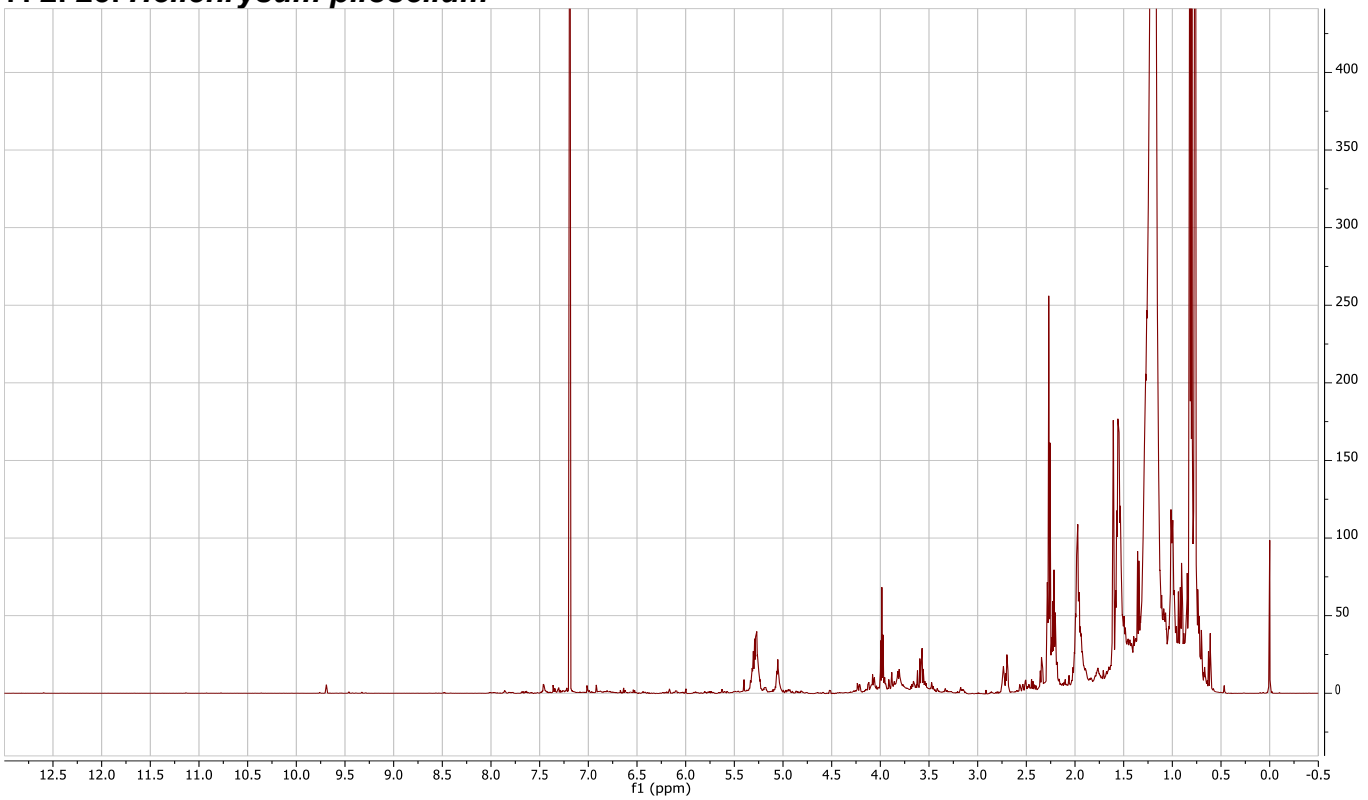

Fig 7.50: NMR spectrum of the dichloromethane plant extract of *H. pilosellum* (HNUP) (600 MHz, Leiden University). († Reclassified later in the study)

7. 2. 28. *Helichrysum populifolium*

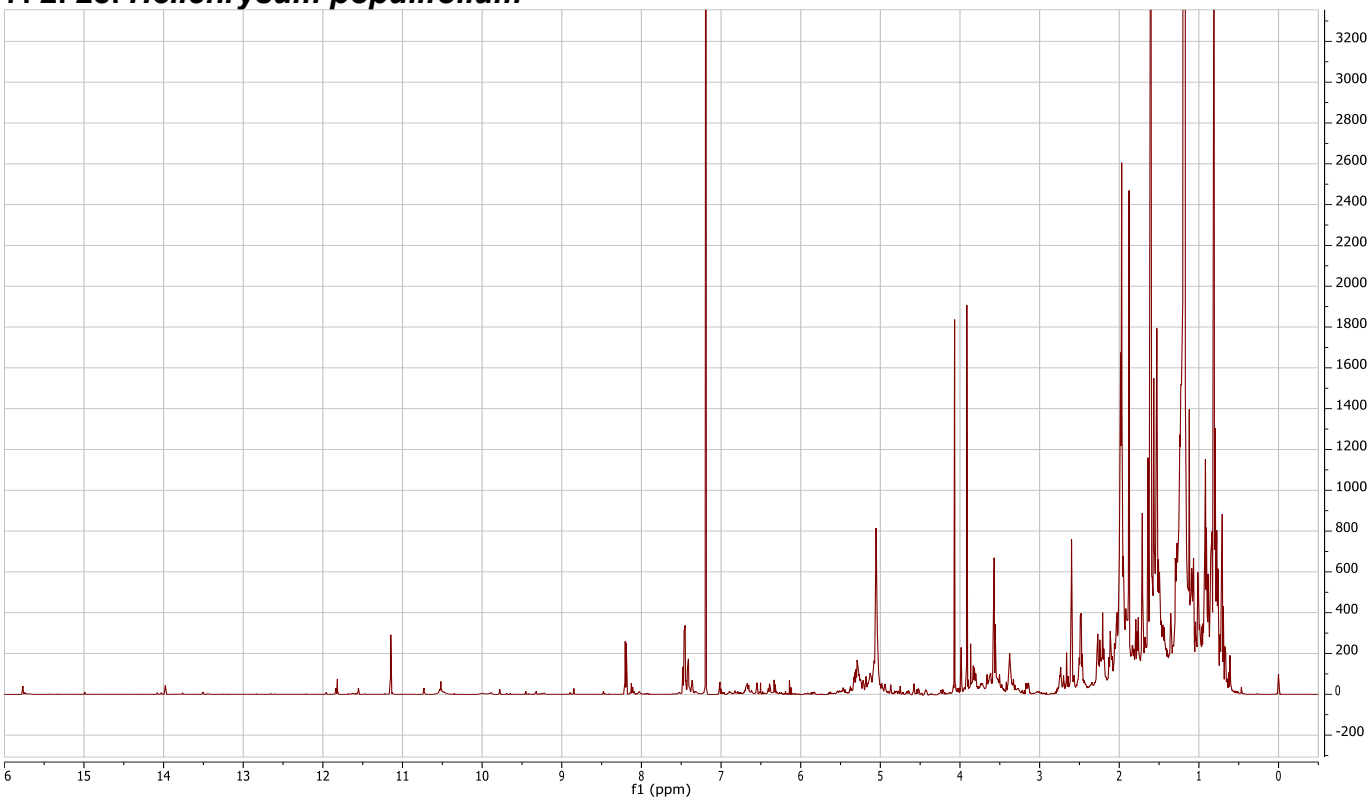

Fig 7.58: NMR spectrum of the dichloromethane plant extract of *H. populifolium* (600 MHz, Leiden University).

7. 2. 27. *Helichrysum rugulosum*

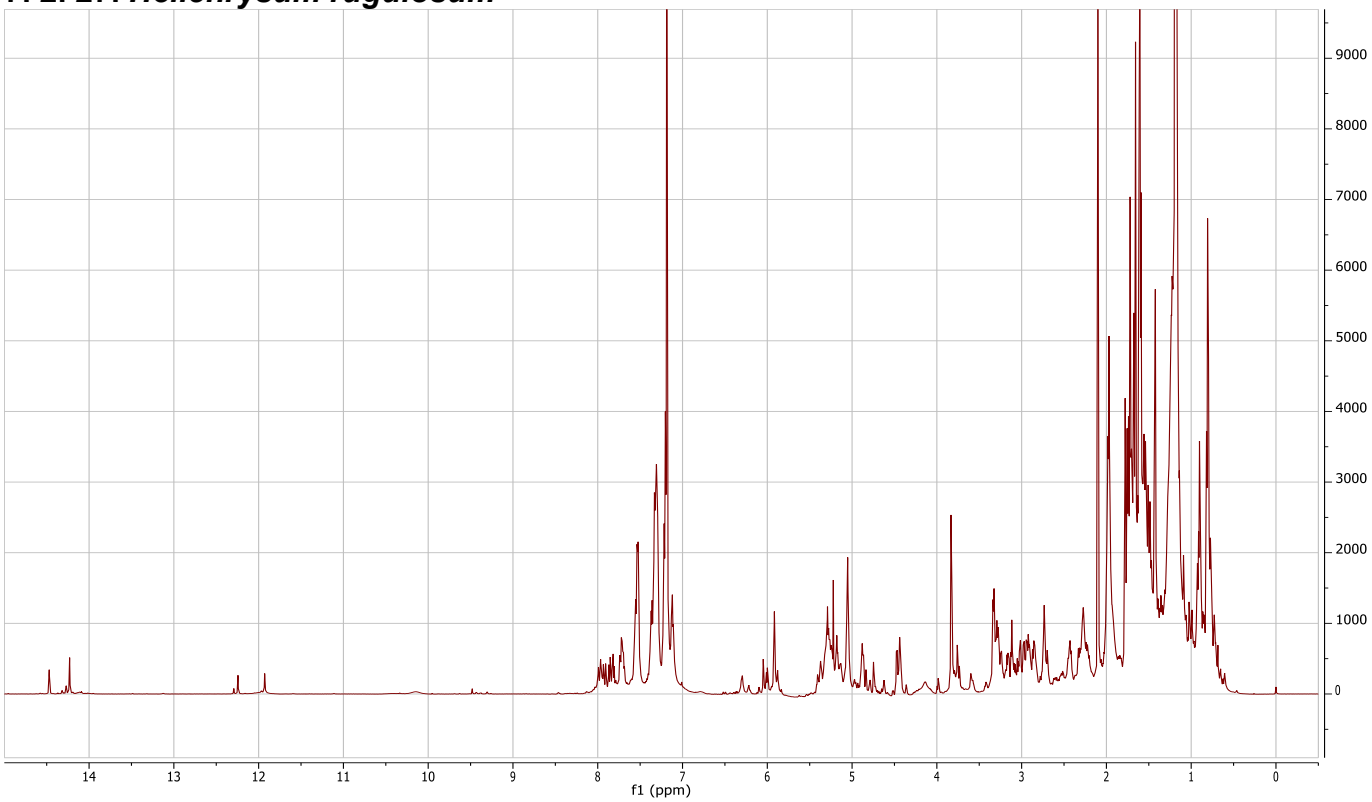

Fig 7.59: NMR spectrum of the dichloromethane plant extract of *H. rugulosum* (600 MHz, Leiden University).

7. 2. 28. *Helichrysum splendidum* (1)

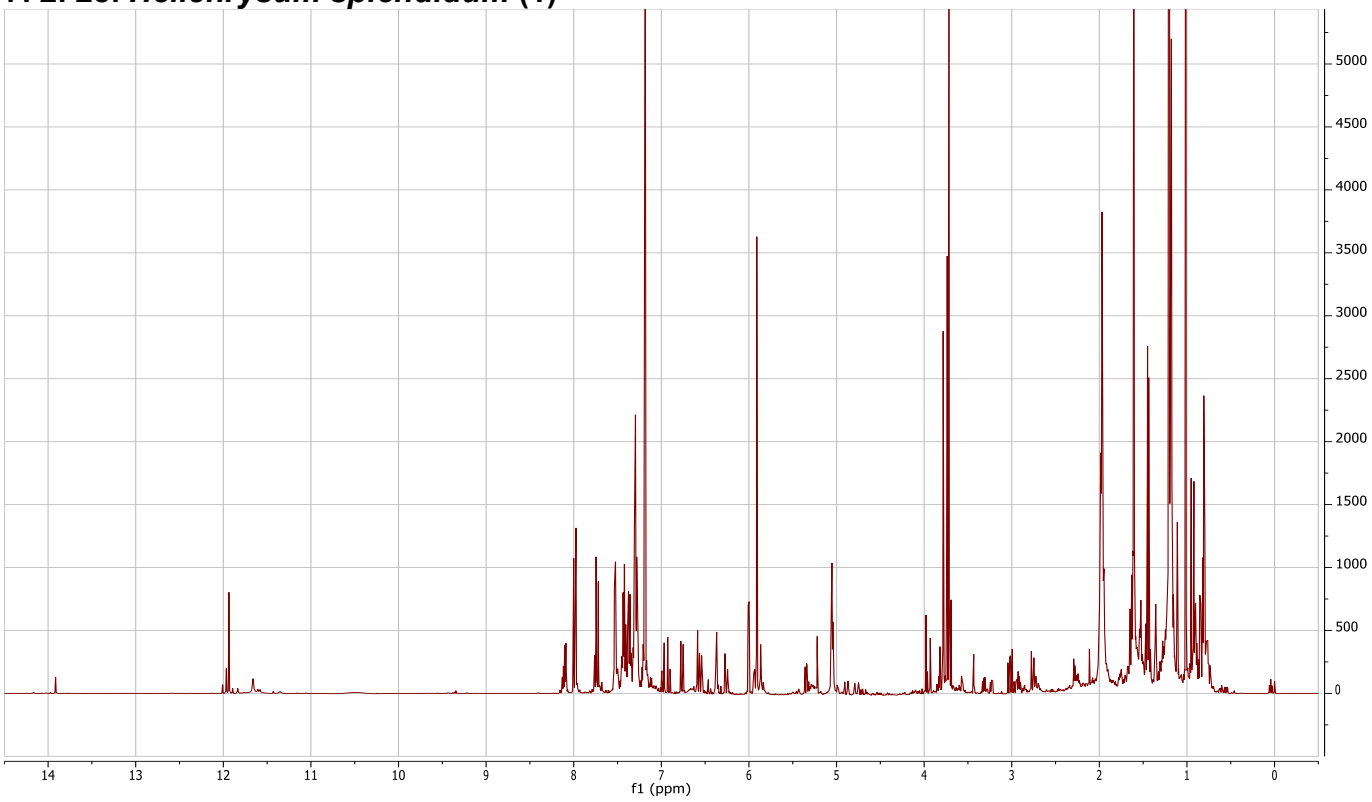

Fig 7.60: NMR spectrum of the dichloromethane plant extract of *H. splendidum* (1) (600 MHz, Leiden University).

7. 2. 29. *Helichrysum subluteum*

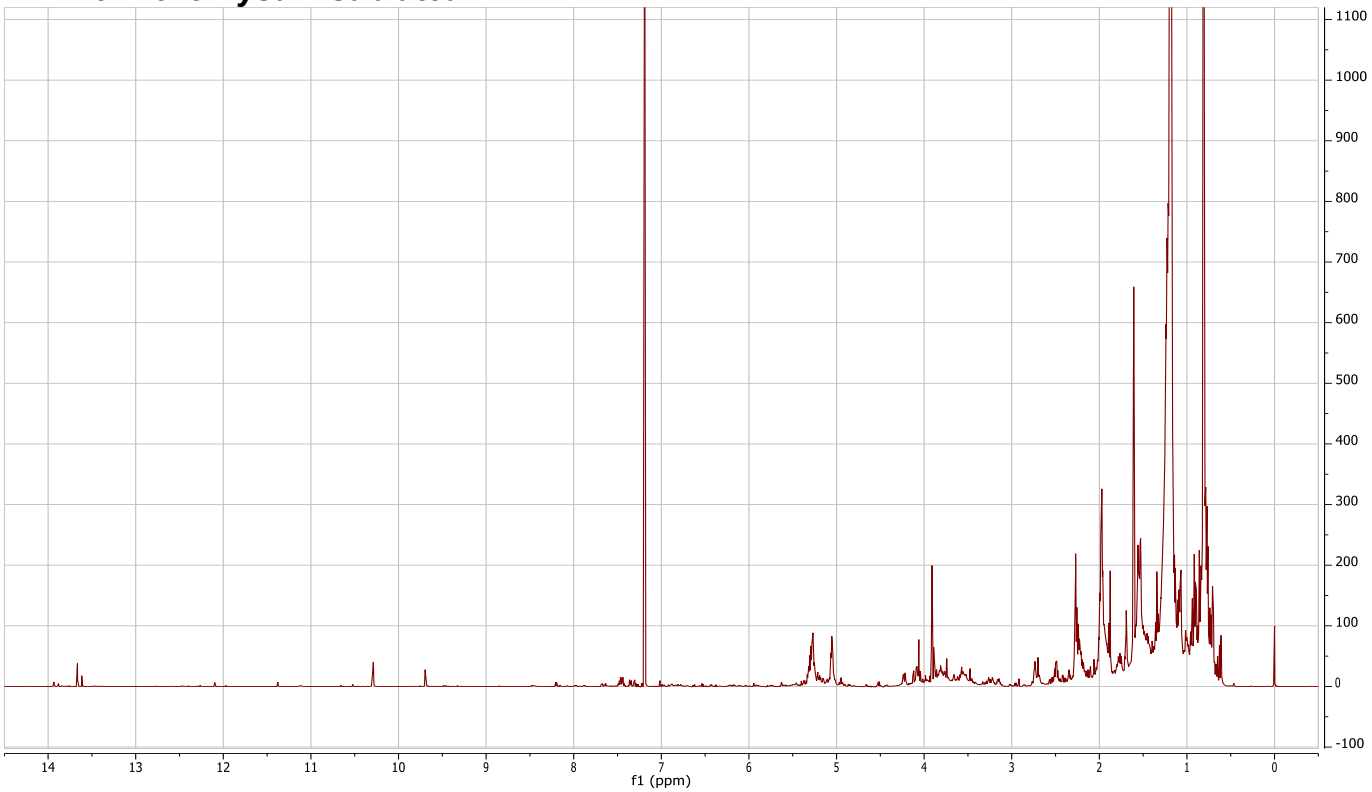

Fig 7.61: NMR spectrum of the dichloromethane plant extract of *H. subluteum* (600 MHz, Leiden University).

7. 2. 30. *Helichrysum sutherlandii*

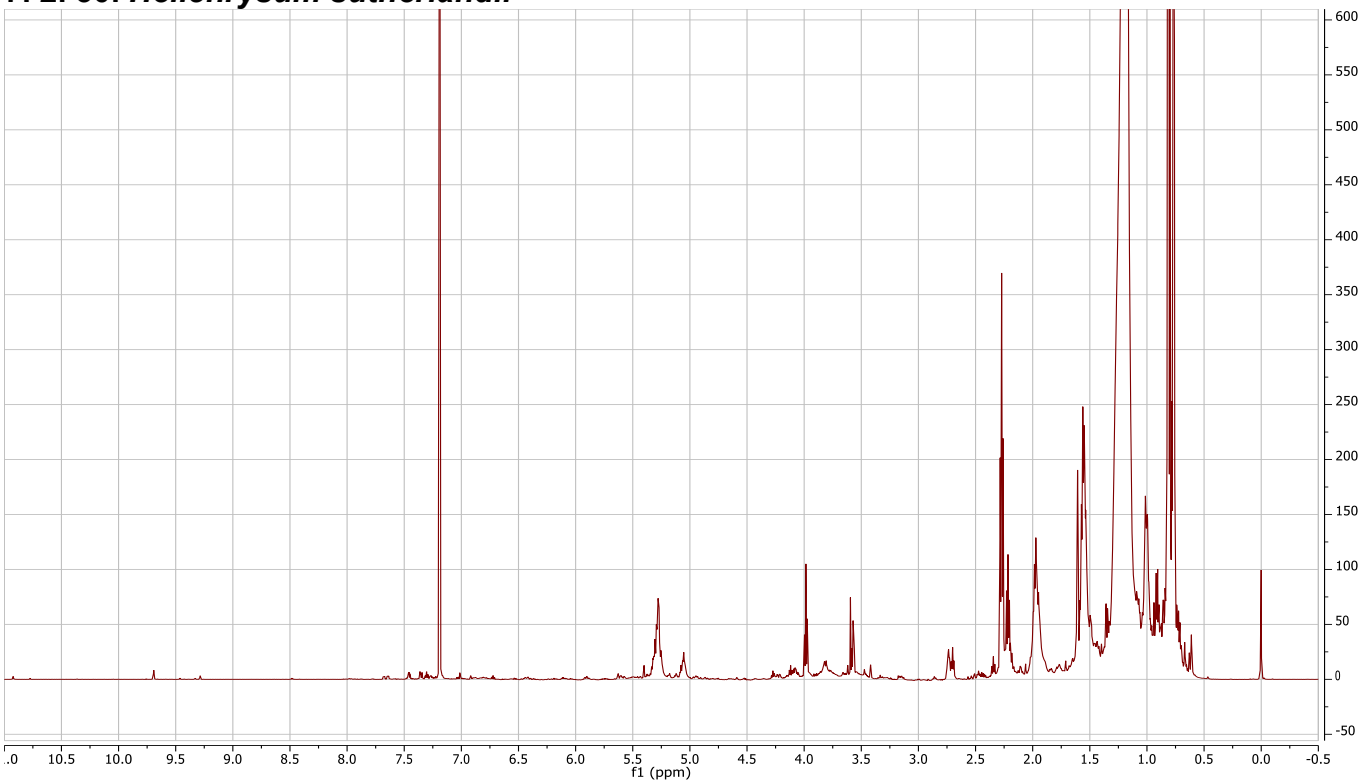

Fig 7.62: NMR spectrum of the dichloromethane plant extract of *H. sutherlandii* (600 MHz, Leiden University).

7. 2. 31. *Helichrysum umbraculigerum*

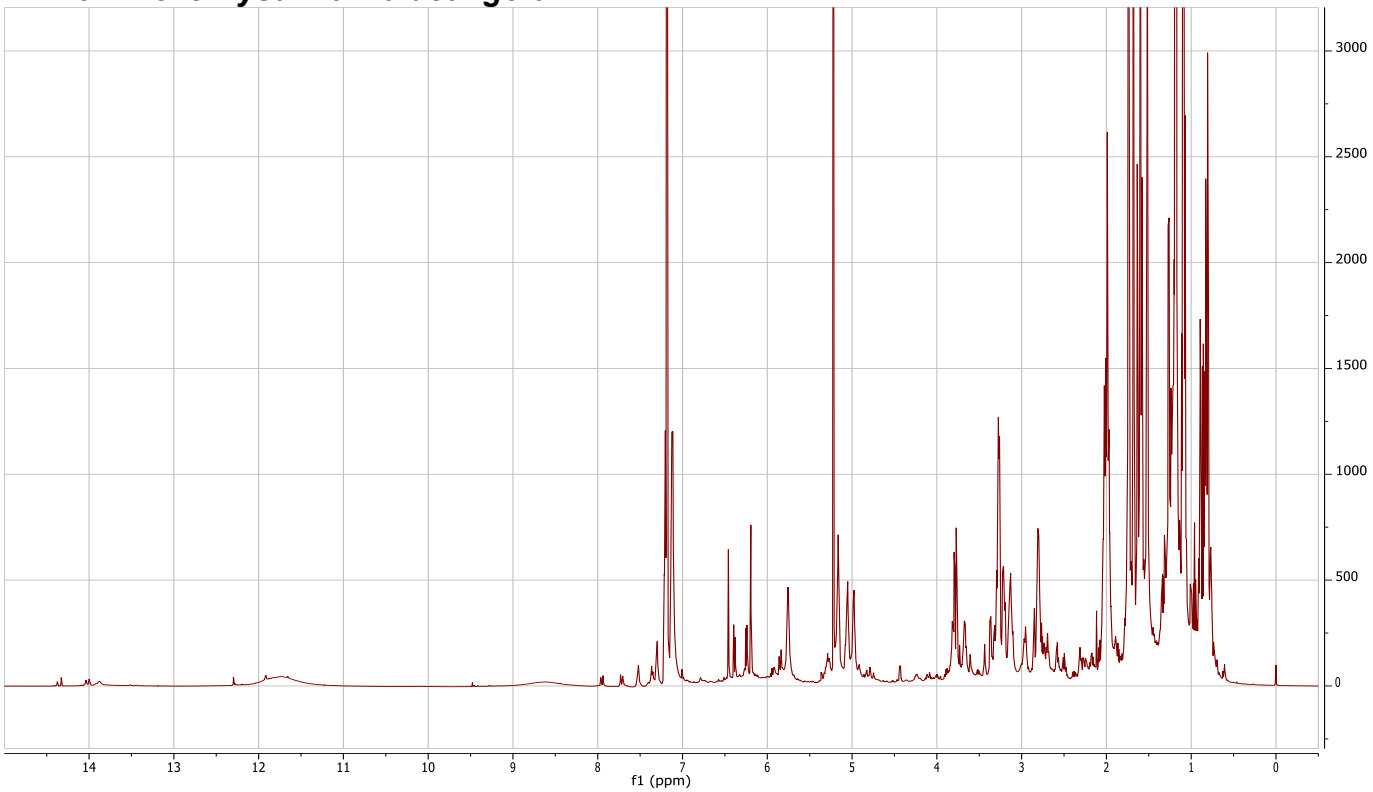

Fig 7.63: NMR spectrum of the dichloromethane plant extract of *H. umbraculigerum* (600 MHz, Leiden University).

7. 2. 32. *Helichrysum vernum*

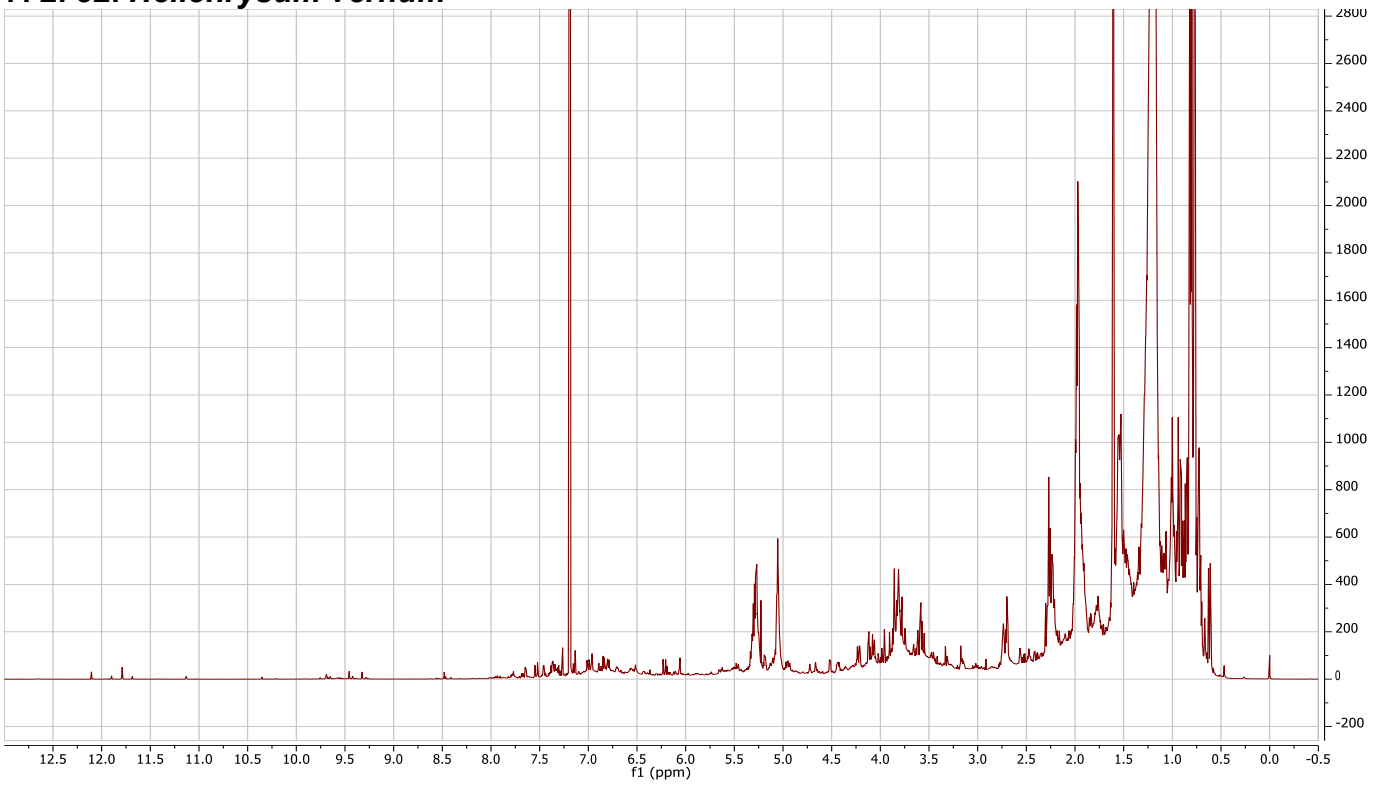

Fig 7.64: NMR spectrum of the dichloromethane plant extract of *H. vernum* (600 MHz, Leiden University).

7.3 NMR spectra of the Sephadex fractions

7. 3. 1. Fraction 1

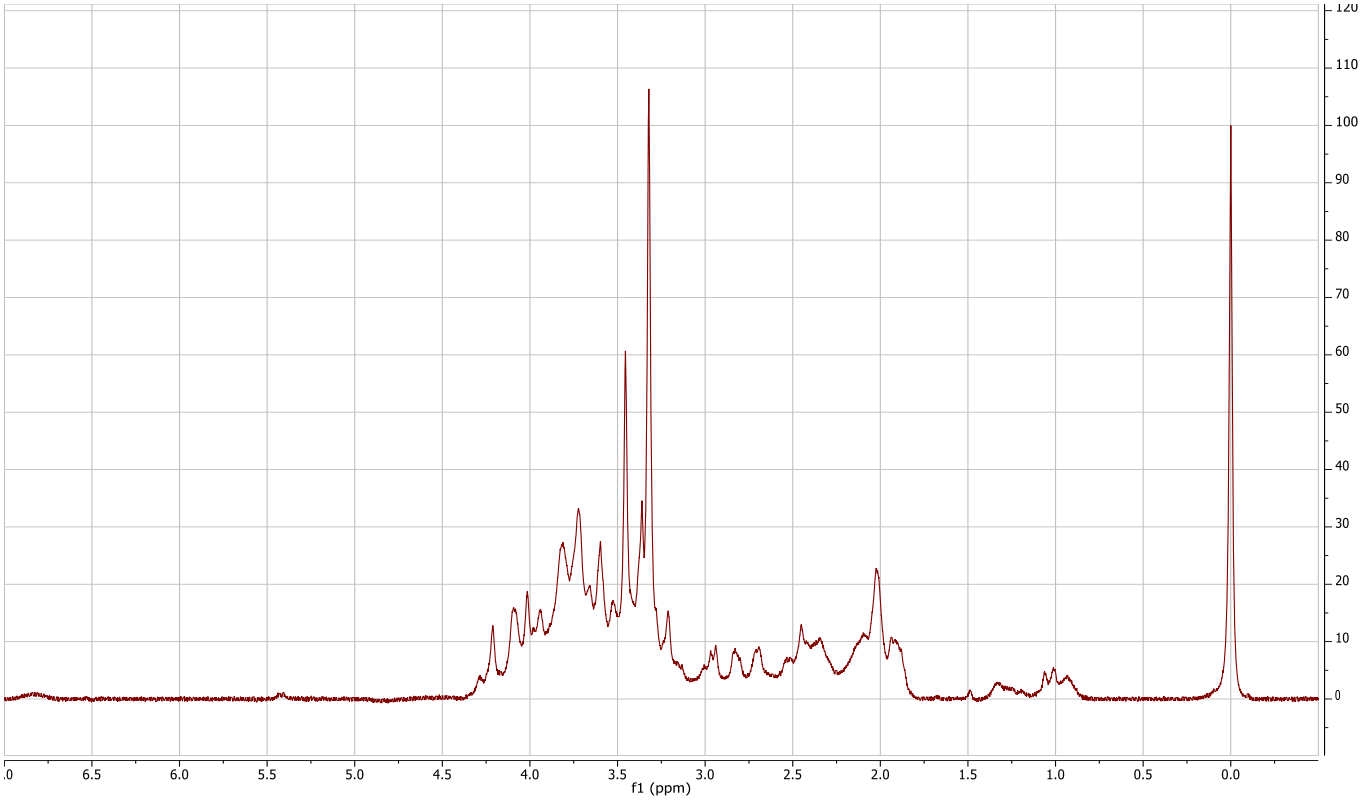

Fig 7.69: NMR spectrum of fraction 1 of the plant extract of *H. populifolium* (600 MHz, UNISA/CSIR).

7. 3. 2. Fraction 2

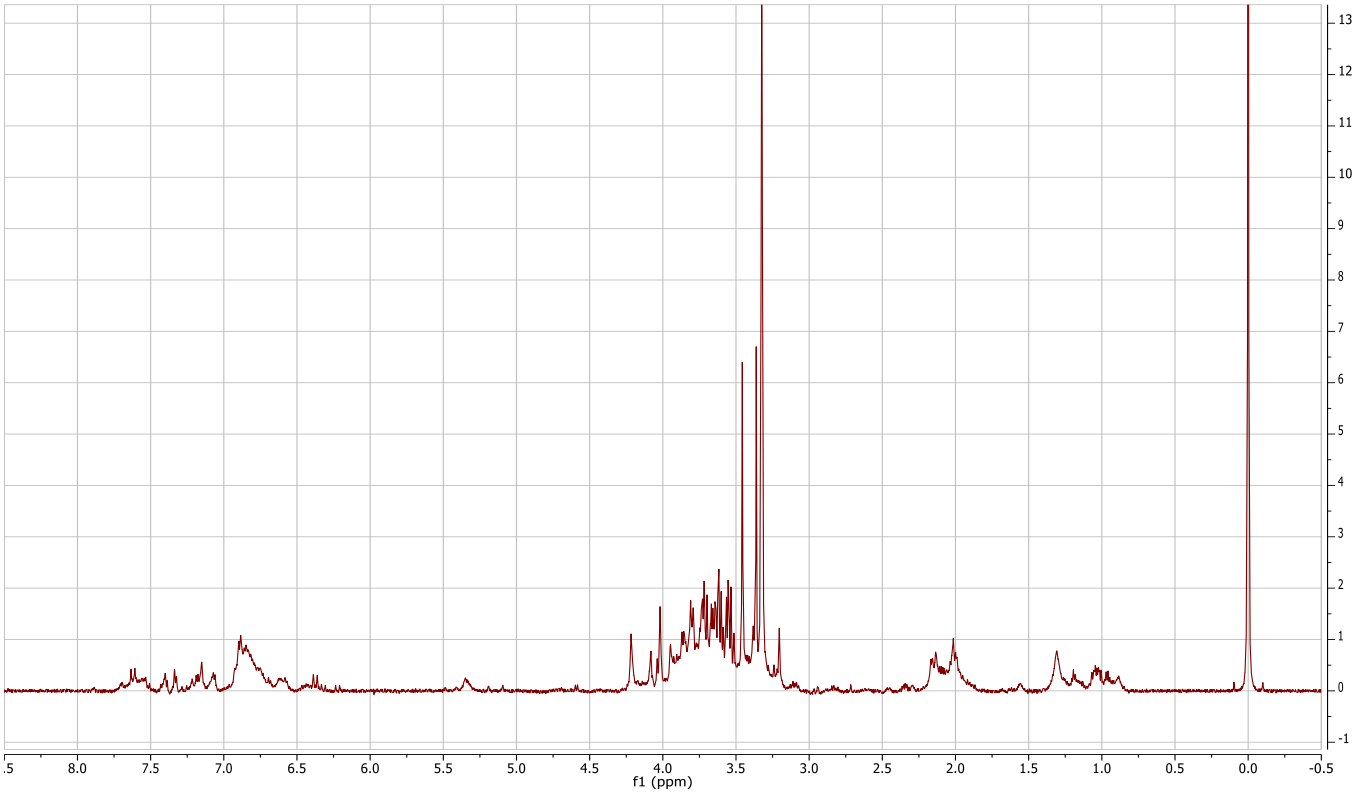

Fig 7.70: NMR spectrum of fraction 2 of the plant extract of *H. populifolium* (600 MHz, UNISA/CSIR).

7. 3. 3. Fraction 3

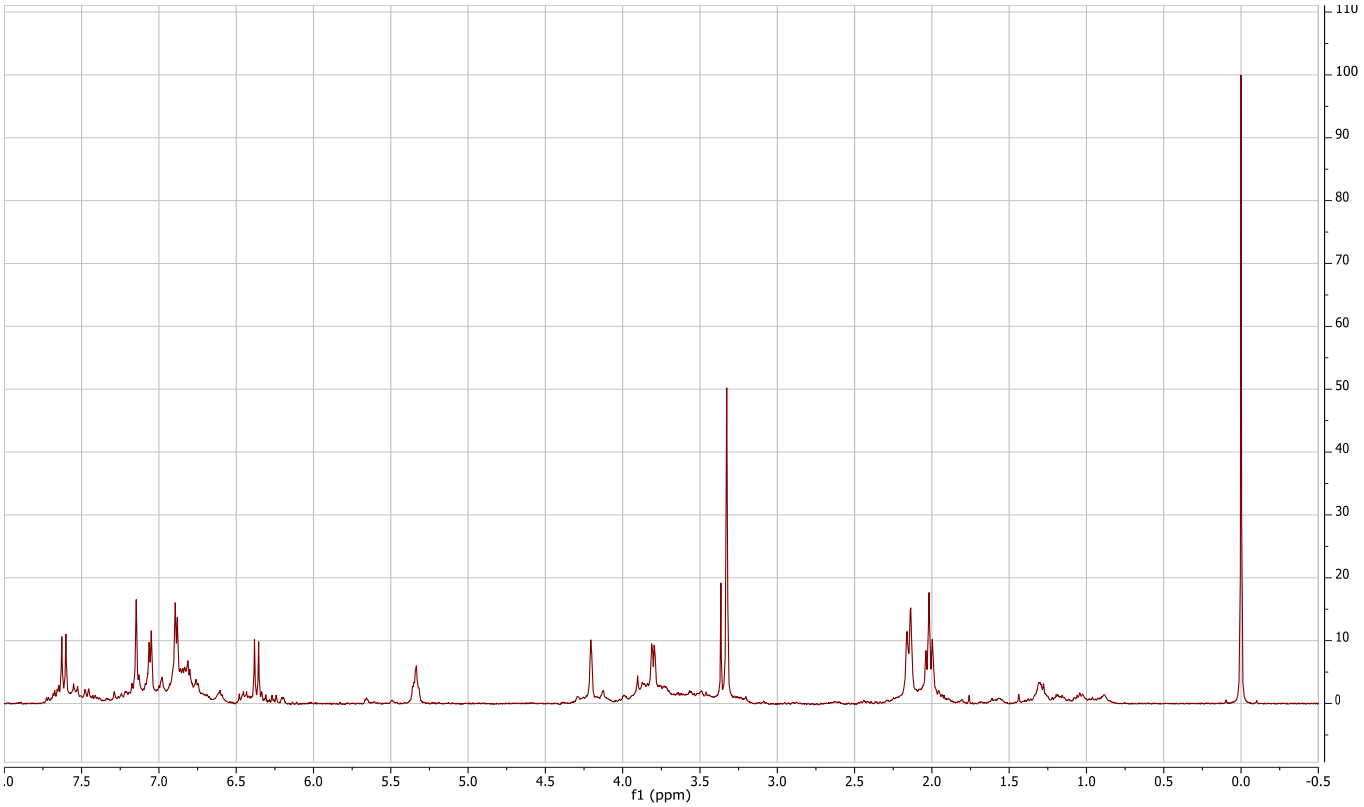

Fig 7.71: NMR spectrum of fraction 3 of the plant extract of *H. populifolium* (600 MHz, UNISA/CSIR).

7. 3. 4. Fraction 4

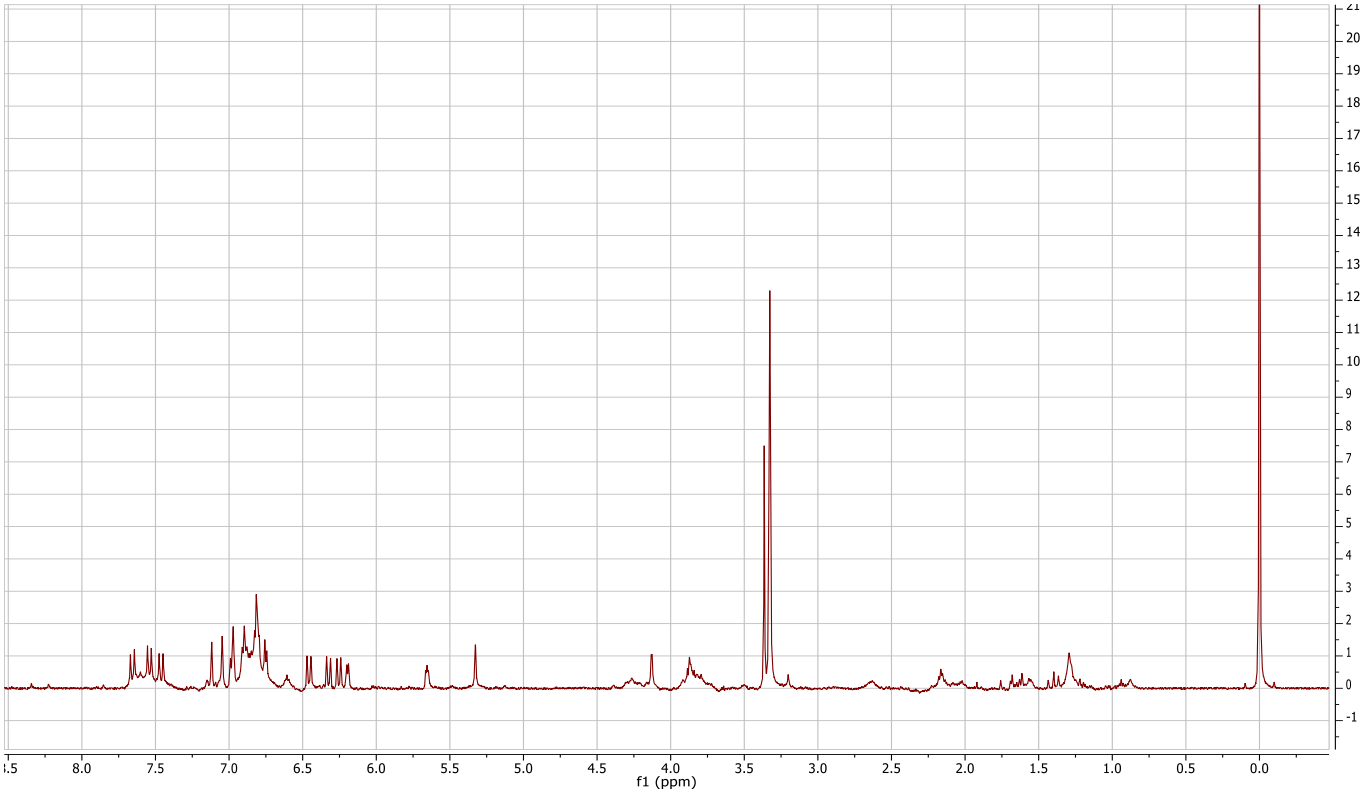

Fig 7.72: NMR spectrum of fraction 4 of the plant extract of *H. populifolium* (600 MHz, UNISA/CSIR).

7. 3. 5. Fraction 5

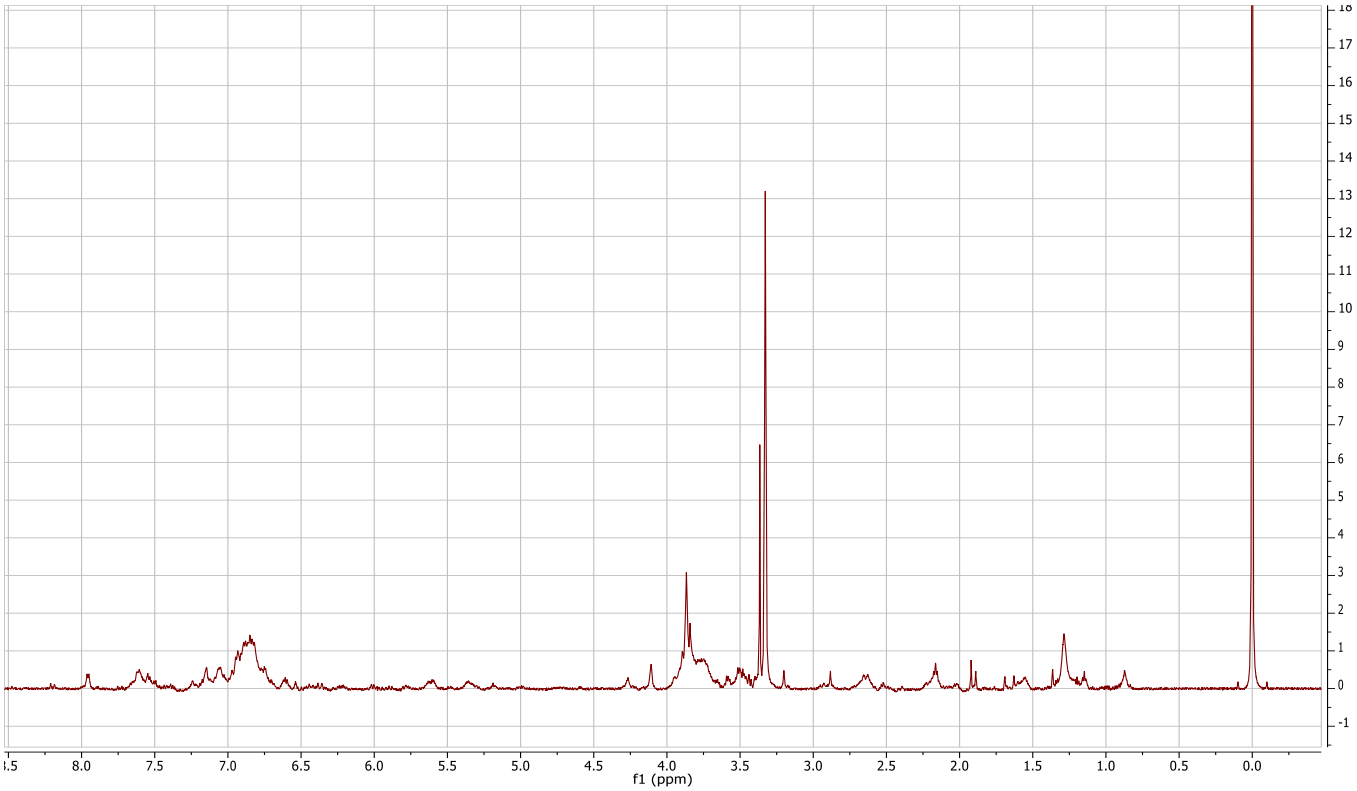

Fig 7.73: NMR spectrum of fraction 5 of the plant extract of *H. populifolium* (600 MHz, UNISA/CSIR).

7. 3. 6. Fraction 6

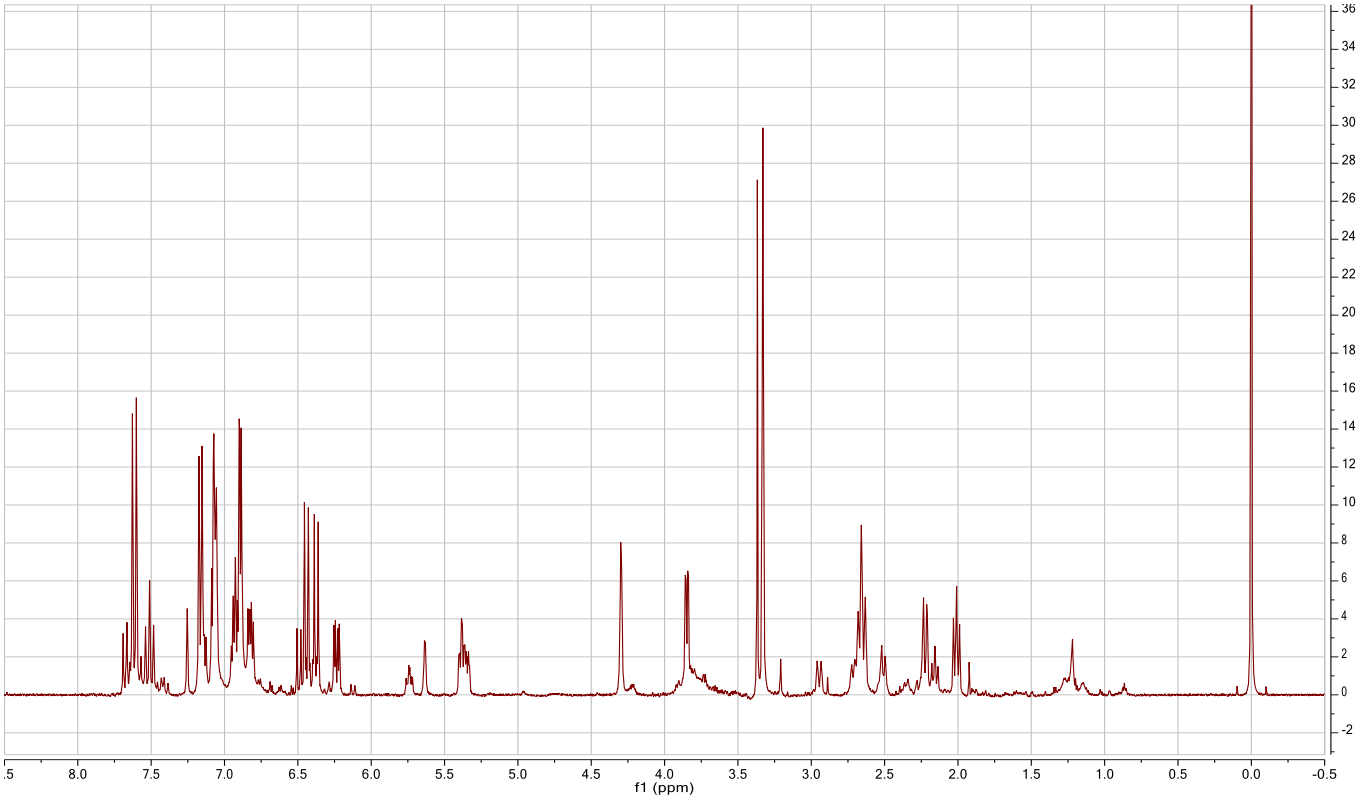

Fig 7.74: NMR spectrum of fraction 6 of the plant extract of *H. populifolium* (600 MHz, UNISA/CSIR).

7. 3. 6. Fraction 7

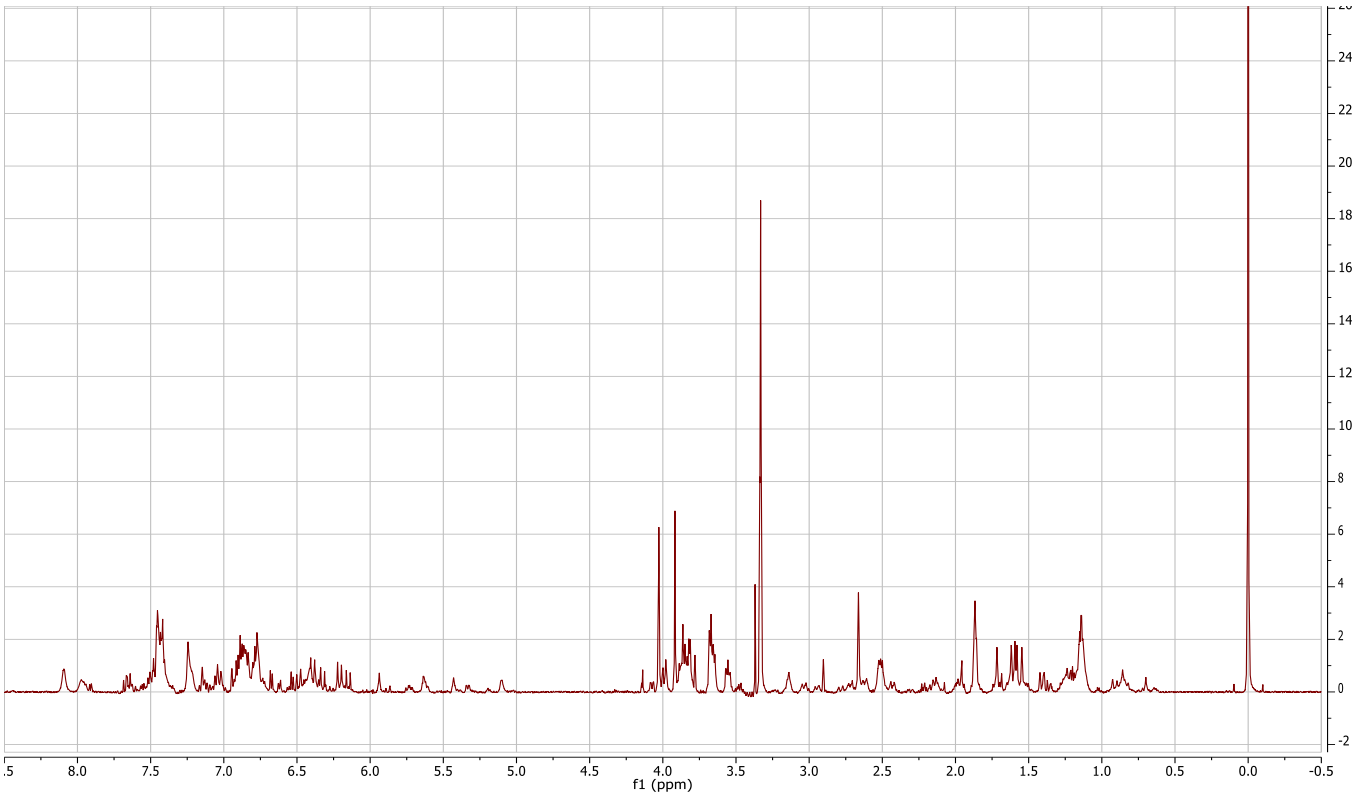

Fig 7.75: NMR spectrum of fraction 7 of the plant extract of *H. populifolium* (600 MHz, UNISA/CSIR).