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Metabolomic comparison of selected *Helichrysum* species to predict their antiviral properties

by

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Submitted in partial fulfilment of the requirements of the degree of Magister Scientiae

In the Faculty of Natural and Agricultural Sciences

Department of Plant Science

University of Pretoria

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Co-supervisor: Dr. V. Maharaj

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Abstract

From the *Helichrysum* genus 600 species occur in Africa of which 244 species are found in South Africa. The most commonly used *Helichrysum* species for medicinal purposes are *H. cymosum*, *H. odoratissimum*, *H. petiolare* and *H. nudifolium*. The medicinal uses include the treatment of coughs, colds, fever, infection, headaches, menstrual pain and are very popular for wound dressing. Previous published research has shown that *H. aureonitens* has antiviral properties against Herpes simplex virus type 1 (HSV-1). In this study, further investigation into the *Helichrysum* species was undertaken, to establish the active constituents responsible for anti-HSV activity using a metabolomics approach. The cytotoxicity of 12 *Helichrysum* species was investigated

and ranged from <3.125 µg/ml to 277.8 µg/ml on the vero cell line. The 12 *Helichrysum* species also showed various levels of antiviral activity against HSV, with both the water-methanol and chloroform extracts of *H. adenocarpum* subsp. *adenocarpum* being the most active extract at 25 µg/ml.

In this study the activity of *Helichrysum* species against HIV-1 RT was also investigated. *Helichrysum populifolium* was the most active extract, inhibiting the HIV-1 RT enzyme by 63.78 % at 200 µg/ml. The bioactivity data and the spectral nuclear magnetic resonance (NMR) data of al the *Helichrysum* species from this study was analysed using the SIMCA-P software to discriminate between the different species on the basis of their bioactivity and chemical composition. The samples did not group well on Principal Component Analysis (PCA) but did separate well using the Orthogonal Projection to Latent Structure – Discriminate Analysis (OPLS-DA) on the basis of their activity and NMR spectra data. From the OPLS scoring plots analysis, contribution plots were created which indicated regions responsible for the difference between the species, with these regions being investigated to identify the bioactive constituents. It was thus possible to use metabolomics to discriminate between samples on the basis of their activity and show that it could probably be used in future as a tool to identify active ingredients in medicinal plants and accelerate drug discovery.

Keywords: *Helichrysum*, *H. adenocarpum* subsp. *adenocarpum*, *Helichrysum populifolium*, metabolomics, Principal Component Analysis (PCA), nuclear magnetic resonance (NMR), Orthogonal Projection to Latent Structure – Discriminate Analysis (OPLS-DA),

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Chapter 1

Introduction

Chapter 1

Introduction

1.1 Background

Medicinal plants have played an important role in the primary healthcare system in South Africa and it is estimated that almost 80% of the black population currently consult traditional healers for treatment of the ailments (Jäger et al., 1996). The use of traditional medicine has been extended to more that just their use as traditional health care medicine, but is also today being used as alternative and complimentary medicine to the western medicine (Iwu and Wootton, 2002). Traditional medicines can potentially offer a rich and largely unexplored source of therapeutic leads for the pharmaceutical industry as was expressed by Corson and Crews (2007).

When studies on traditional medicine are conducted, three main fields play very important roles, namely Botany, Chemistry and Pharmacology. The study into the chemistry of plants is probably the most researched of these three fields and the results obtained have contributed tremendously in enhancing our knowledge of organic chemistry. On the other hand, the pharmacological field has not received the same attention. This in turn has led to many exciting compounds not being investigated as potential drugs. This is due to research not being followed up by experiments that help to understand the mode of action and the therapeutic specifications (Iwu and Wootton, 2002).

The importance of investigations into the discovery of new therapeutic agents from plants has been discussed extensively (Malone, 1983; Vlietinck and Van den Berghe, 1991). New therapeutic agents have delivered variability in the efficacy in treating

recurrent and chronic infections, and in immunodeficient patients. The other important fact to consider is that a number of plant extracts containing a number of bioactive compounds may be used to provide important combination therapies which affect multiple pharmacological targets but also at the same time providing clinical efficacy which are normally beyond single compound-based drugs capabilities (Williamson, 2001; Schmidt et al., 2007). These factors as well as the problem of prohibitive costs in developing countries have indicated the necessity to search for alternative drugs (Elanchezhiyan, 1993).

In a review study conducted by Newman and Cragg (2007) they reported that from 1981 to 2006 of the 1010 new chemical entities (NCE) used as novel drugs, 43 (4.3 %) were used as the natural product discovered (< 45 biological residue and are from all natural/biological sources), 232 (23 %) were derived from natural products and 47 (4.7 %) were total synthetical products but originated from a natural pharmacophore. In the review study it was also very clear that anti-infective drugs originated frequently from natural products or some form thereof. From the anti-infective drugs that have been produced, 75.5 % were not synthetic, of which 51.9 % were either natural products, derived from natural products, synthesised from natural products (pharmacophore) or natural product mimics. Examples of plants used for drug production are; *Digitalis* spp. for their digoxin content, *Papaver soniferum* which produces codeine and morphine, the cancer compounds, vincristrine and vinblasine, from *Catharanthus roseus*, *Cinchona* spp. contains quinine and quinidine, and *Atropa belladonna* as a source of atropine. These examples are only a few of the many plants that have been mined for their important and active compounds (Rate, 2001). Of the almost 250 000 species of plants, world wide only a small percentage has been investigated and the potential of natural products to contribute to the pharmaceutical industry is enormous.

It is impossible to calculate the exact impact that natural products have had on the human population. The facts that are known show a comprehensive role that natural products are playing and will keep on playing in the future. 25% of the modern medicines can be linked to natural products, be it directly or indirectly. Many of the

current drugs started out with a natural product which was then derivatised (Schmidt et al., 2007).

Research conducted in the medicinal plants field and especially on the antimicrobial activity front, has clearly become a progressive trend. This trend can mainly be attributed to advances made in laboratory techniques, scientific validation of the traditional uses of specific plants and the renewed interest in this field. All of these factors can now help bring traditional medicine to such a level of recognition that it can become an accepted alternative contingent to Western healthcare (Van Vuuren, 2008).

Natural products and traditional medicine have long been used to treat the symptoms of HSV-1 infections. Thus the research that has previously been done on HSV-1 has produced many pure compounds and standard extracts that have been isolated from natural sources or traditional herbs. Examples of pure compounds with anti-HSV-1 activity include, polysaccharides e.g. sulphate fucoidan isolated from seaweed *Stoechospermum marginatum*, polyphenols e.g. galangin and kaempferol isolated from various plants and terpenes e.g. andrographolide and neoandrographolide isolated from *Andrographis paniculata* (Xiang et al., 2008).

The potential of natural products and traditional medicine, as anti-viral agents is a valuable source for future development of new drugs. Thus future research is necessary to continue the search for a large number of potential lead compounds, but it is also very important to involve synthetic derivitation of the natural products to move beyond the initial identification of promising candidates *in vitro* (Mundinger and Efferth, 2008).

1.2 *Helichrysum* (Asteraceae)

From the *Helichrysum* genus 600 species occur in Africa of which 244 species are found in South Africa (Pooley, 2003). They are recorded over most parts of Southern Africa. The species display great morphological diversity and are morphologically divided into 30 groups. Species of the *Helichrysum* genus are aromatic perennial herbs

or shrublets with densely hairy or woolly leaves and persistent flower heads (Hilliard, 1983). The leaves and flowers are often pleasantly scented and have been reported to be used traditionally to fumigate a sick room or to invoke the goodwill of the ancestors (Pooley, 2003). The most commonly used species are *H. cymosum*, *H. odoratissimum*, *H. petiolare* and *H. nudifolium*. The medicinal uses include the treatment of coughs, colds, fever, infection, headaches, menstrual pain and they are very popular for wound dressing (Hutchings, 1996). *H. aureonitens* has been investigated extensively and has shown antiviral properties against herpes simplex virus type 1 (Meyer et al., 1996).

Previous research has lead to well documented antibacterial and antifungal activities of *Helichrysum* species, but antiviral, antimalaria, antimycobacterial and toxicity data is very limited. Some chemical classes such as acylphloroglucinols, diterpenes and flavonoids from the South African *Helichrysum* species have shown very promising antimicrobial activity, which may lead to further studies of these specific plants (Lourens et al., 2008).

1.2.1 Selected plants for this study

In the search for novel or pharmaceutical constituents from plant based sources, the selection of plants for extraction is usually done in one of two ways as described by Cox (1990); 1.) Targeted or focused selection, which can be subdivided into phylogenetic surveys (where close relatives of plants known to have useful constituents are sampled), ecological surveys (plants in a specific habitat with specific growth behaviour), or ethnopharmacological surveys (using traditional knowledge of specific plant species and their medicinal practises for the treatment of specific diseases) (Fransworth and Bingel, 1977); or 2.) Random selection, where the plants collected are not collected with any regard taken to taxonomic affinities, ethnobotanical background or other intrinsic qualities.

The species selected for this study are described in the following section according to their morphology, distribution, activity and in some instances some of their isolated constituents are also listed.

a) *H. acutatum* DC. (Group 21)

This plant is commonly thickly tomentose, but all degrees of development of woolly hairs occur and sometimes “wool” is either wanting or confined to the leaf margins. It ranges from southern KwaZulu-Natal (KZN) up through Swaziland, Mpumalanga and through to the northern parts of Limpopo. It growths in the grassland of KZN where it is widespread from near sea level to c. 1950 m above sea level. The flowering starts in September and continues up to January (Hilliard, 1983).

H. acutatum is widely used and sold as traditional medicine but no specific use has been reported (Lourens et al., 2008). The isolated constituents include flavonoid derivatives, terpenoids and a few others e.g. pinocembrin chalcone and pinocembrin (Bohlmann and Abraham, 1979). Pinocembrin chalcone and pinocembrin from *H. acutatum* have shown to have anti-staphylococcal activity.

b) *H. adenocarpum* subs. *adenocarpum* DC. (Group 28)

The leaves of this plant are radical cobwebby or woolly, involucral bracts ranging from crimson to scarlet or pink, or these colours on white, or rarely pure white and also with parti-coloured bracts. It occurs widespread through the eastern parts of South Africa. Recordings have been made from the eastern border of the Eastern Cape up through KZN, Mpumalanga, Swaziland, Free State (FS) and Limpopo. It is mainly found in grasslands on moist slopes or in moist depressions from sea level to c. 300m above sea level. The flowering period takes place between January and April (Hilliard, 1983).

H. adenocarpum is traditionally used to treat cases of vomiting and diarrhoea in children (Lourens et al., 2008). No biological activity has been reported. One of the chemical constituents isolated is a tridecapentaynediole derivative (Fig 1.1) (Chapman and Hall,

2009) and another constituents isolated by Bohlmann et al. (1980) was an acetylene derivative.

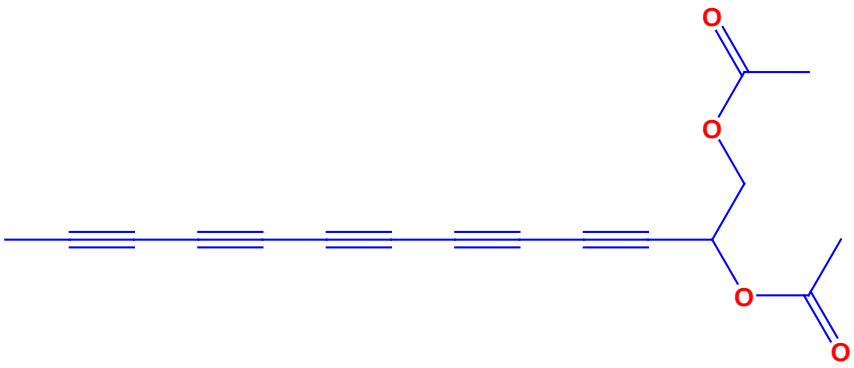


Fig 1.1: Acetic acid 1 - acetoxymethyl-dodeca - 2,4,6,8,10-pentaynyl ester

c) *H. appendiculatum* (L.F.) Less (Group 24)

This plant has leaves woolly on both surfaces and acute involucral bracts that are pale yellow, the outermost reddish. *H. appendiculatum* is distinguished from its allies by its dull, not glossy involucral bracts. It is distributed from the Western Cape through the Eastern Cape, KZN, FS, Swaziland, Lesotho, Mpumalanga and Limpopo. Flowering seasons starts December and ends February (Hilliard, 1983).

The traditional uses associated with *H. appendiculatum* are quite diverse. It is used in the treatment of respiratory conditions, infections and pain. It is also used to dress open wounds. Some antibacterial activity has been observed as well as antifungal activity on various microorganisms. Only terpenoids have been isolated form *H. appendiculatum* (Lourens et al., 2008).

d) *H. aureonitens* Sch. Bip (Group 8)

H. aureonitens has involucral bracts that are brownish, tawny, straw-coloured or yellow with leaves that are oblong or oblong-spathulate, obtuse or sub-acute, both surfaces of the leaves are appressed woolly and the heads are campanulate. Distribution of *H. aureonitens* ranges from the Eastern Cape through KZN, Lesotho, FS, Swaziland, Mpumalanga, Gauteng and Limpopo. These plants are very common and are visible

from a distance as grey patches in the grassland. The flowering season starts in September and ends in February (Hilliard, 1983).

Traditionally *H. aureonitens* is burnt as incense to invoke goodwill of the ancestors and to induce trances or it is used in a decoction to treat enuresis in children. It is also used as a topical treatment for skin infections especially against herpes zoster and other associated infections of herpes simplex (Hutchings et al., 1996; Meyer et al., 1996; Afolayan and Meyer, 1997; Pooley, 2003). A number of authors reported the antimicrobial activities of isolated constituents. Galangin isolated from *H. aureonitens* inhibited various Gram-positive bacteria as well as Gram-negative bacteria (Afolayan and Meyer, 1997). Cushnie et al. (2003) reported the activity of galangin against resistant *Staphylococcus aureus* strains being of significant low concentration. Galangin also displayed some anti-fungal activity according to Afolayan and Meyer (1997). These results support the use of *H. aureonitens* for infected skin which is usually caused by *S. aureus*.

e) *H. callicomum* Harv. (Group 2)

This small shrub has small leaves that are oblong to spathulate and both sides of the leaves are greyish felted. It also has involucral bracts that are not radiating, but are pale straw-coloured. *H. callicomum* ranges from the Eastern Cape also through KZN, FS, Lesotho, Gauteng, Limpopo and the North West, but not Swaziland. Flowering takes place between March and May (Hilliard, 1983).

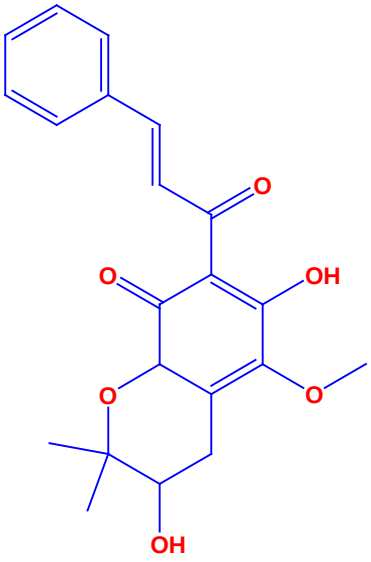
It is traditionally used to invoke goodwill from the ancestors and as a protective charm. It is also used as an ingredient in an enema for colic (Mathekga, 2001; Pooley, 2003). Isolated constituents include the following: flavanones, phloroglucinols, pyrones, diterpenes, terpenes and a monocyclic sesquiterpene (humulene) (Fig 1.2) (Bohlmann and Abraham, 1979a; Bohlmann et al., 1984).

Fig. 1.2: Humulene

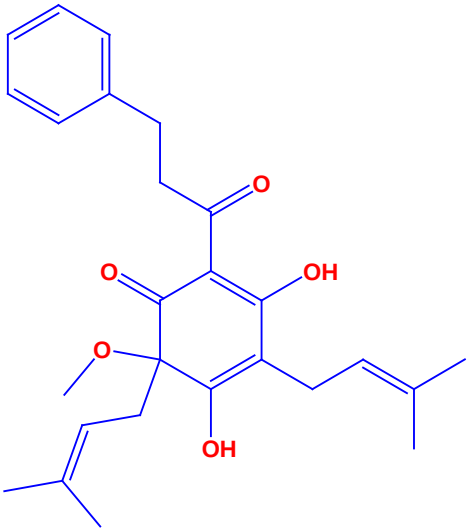
f) *H. cymocum* subsp. *cymosum* (L.) D. Don (Group 8)

This plant has involucral bracts that are brownish, tawny, straw-coloured or yellow. The tips of at least the inner involucral bracts are yellow. The leaves are linear-oblong to elliptic-oblong, acute or rarely acuminate, the upper surface of the leaves are clad in paper-like indumentums. The heads of this particular species have 6-20 flowers, with the fimbrils more than twice as long as the ovary. *H. cymosum* subsp*. cymosum* has been recorded from the Cape Point upwards through the Eastern Cape and KZN all along the coast. This plant occurs up to c. 1500m above sea level in the Cape mountains, but not more than 600m in KZN. It also often grows in moist places and can be seen growing in big clumps. Flowering takes place between September and April (Hilliard, 1983).

Three chemical constituents have been isolated from *H. cymocum* subsp. *cymosum* (Fig. 1.3). These are helihumulone an oil, a helichromanochalcone from the roots and the flavone derivative, 5-hydroxy-8-methoxy-7-prenyloxyflavanone (Bohlmann et al., 1979 (1); Chapman and Hall, 2009). Helihumulone, a phloroglucinol-derived compound, has also shown very significant anti-microbial activity, ranging from 125 µg/ml (*Staphylococcus aureus*) to 16 µg/ml (*Pseudomonas aeruginosa*) (Van Vuuren et al., 2006).



a)



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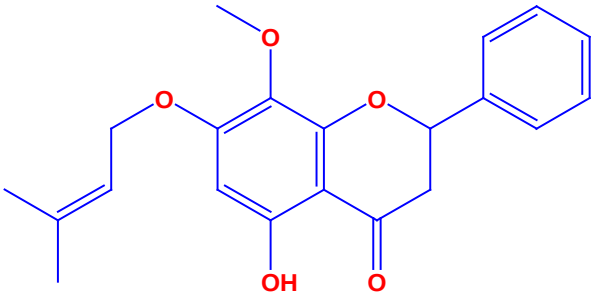


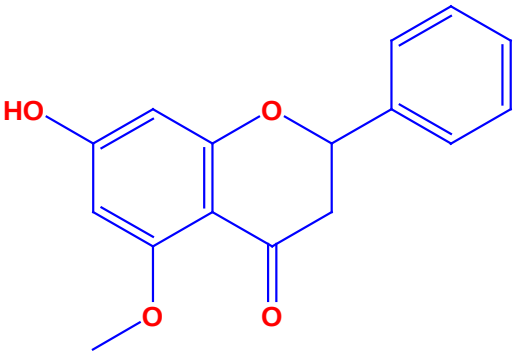
c)

Fig 1.3: a) helichromanochalcone, b) helihumulone and c) 5-hydroxy-8-methoxy-7-prenyloxyflavanone

g) *H. herbaceum* (Andr.) Sweet (Group 29)

The involucral bracts of this plant are yellow or yellow and brown. The heads are approximately 18 – 20 mm long and are loosely woolly. The distribution of *H. herbaceum* ranges from the Congo down to South Africa. In South Africa it can be found from the Western Cape through the Eastern Cape, KZN, Lesotho, FS and Limpopo. It has been recorded at sea level and up to c. 2500m above sea level. It can be found in grasslands as well as shrub communities; it will usually invade bare or disturbed areas. Usually flowering starts in October and will continue up to April (Hilliard, 1983).

Constituents isolated from *H. herbaceum* (Fig. 1.4) are mainly flavones with one flavanone (Bohlmann et al., 1979(2), Chapman and Hall, 2009).



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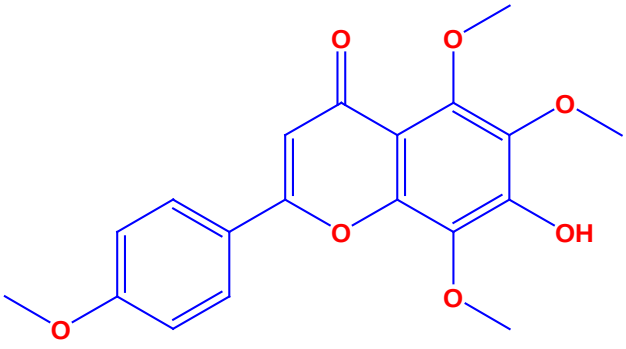

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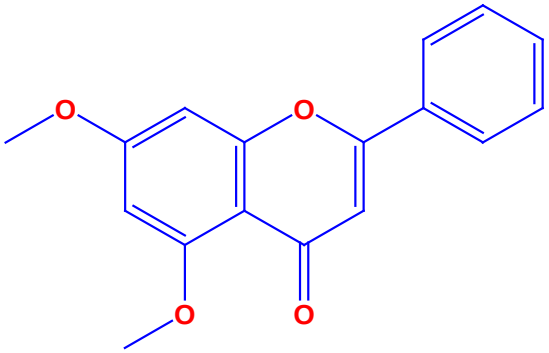

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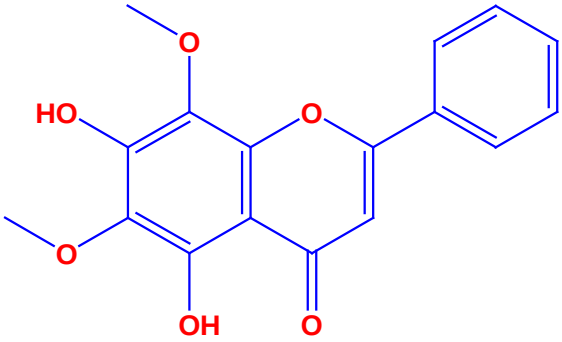

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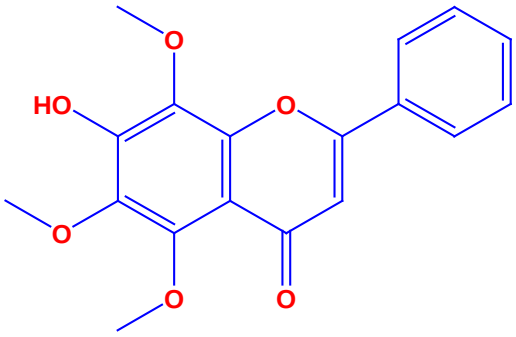

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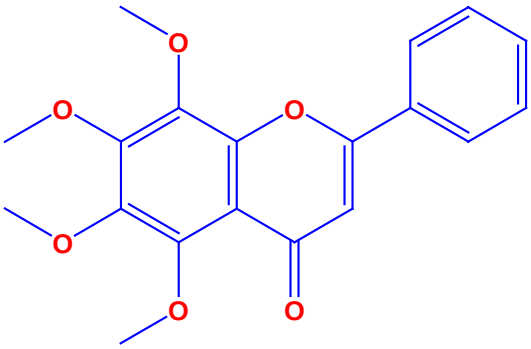


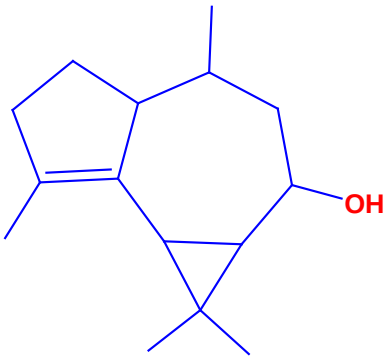
f)

Fig. 1.4: a) 7-hydroxy-5-methoxyflavanone (Alpinetin), b) 7-hydroxy-4',5,6,8-trimethoxyflavone, c) 5,7-dimethoxyflavone, d) 5,7-dihydroxy-6,8-dimethoxyflavone, e) 7-hydroxy-5,6,8-trimethoxyflavone and f) 5,6,7,8-tetramethoxyflavone

h) *H. nudifolium* var. *nudifolium* (L.) Less. (Group 23)

The flowering stems of this plant are leafy mainly in the lower half and are nude or with a few reduced leaves or bracts in the upper parts. The crown is not clothed in brown silky “wool”. Its leaves do not spread flat on the ground like some of the other species do. The upper leaf surface of *H. nudifolium* is variously hairy. The involucral bracts are mostly pale or lemon yellow and only the outermost are sometimes pale brown. It is widely distributed through southern Africa. In South Africa *H. nudifolium* can be found from the Western Cape right through to Limpopo with only the Northern Cape not included. This plant can be seen flowering between November and March (Hilliard, 1983).

H. nudifolium was thoroughly studied by Jakupovic et al. in 1986. They isolated 9 constituents from *H. nudifolium*, all of them isolated from oils (Fig 1.5).



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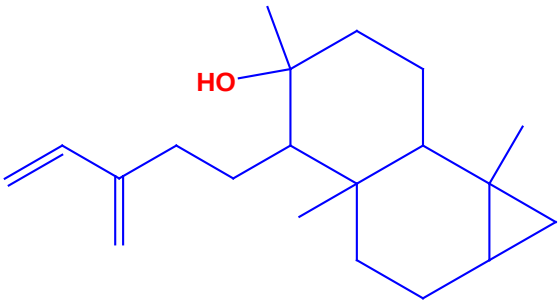

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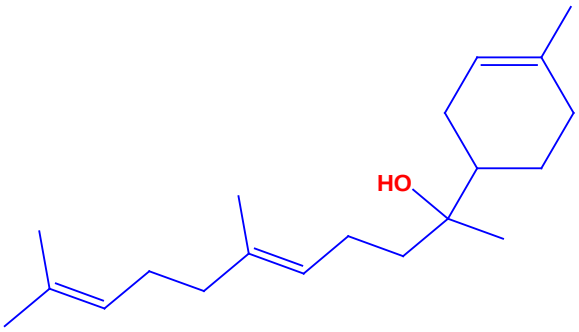
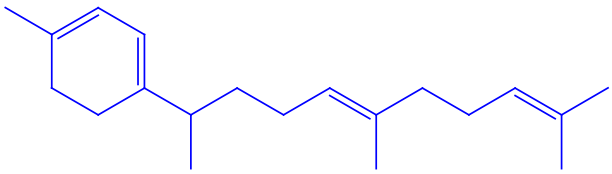


b)

Fig. 1.5 continued on next page: a) 8α-hydroxy-α-gurjunene, b) 8α-acetoxy-α-gurjunene and c) 13(16),14-gnaphaladien-8-ol.



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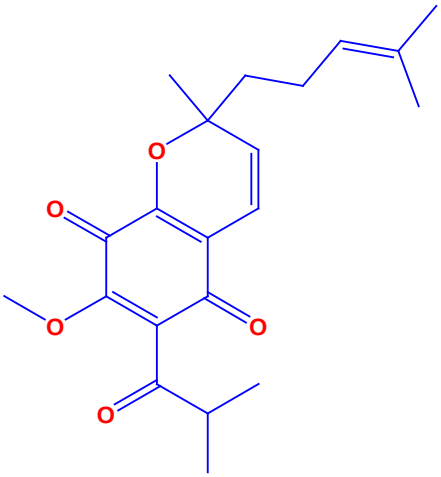

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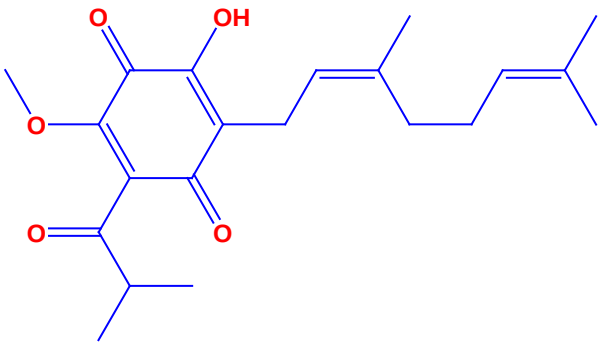

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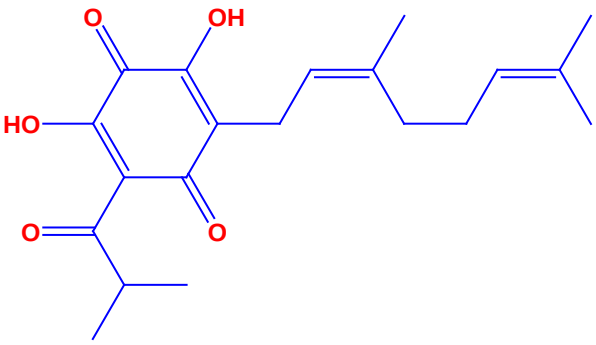

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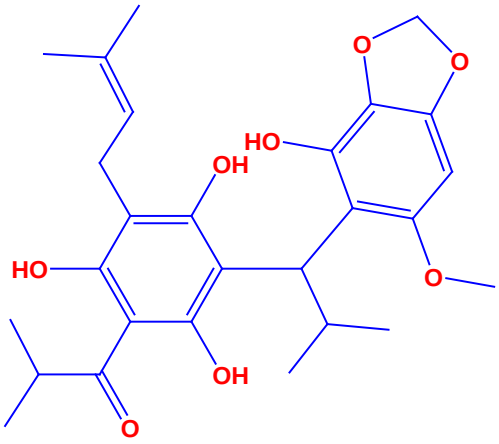

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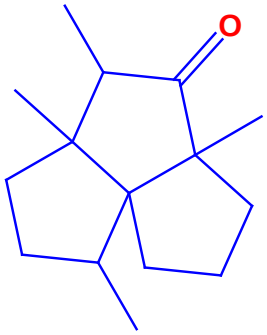

i)

Fig. 1.5 continued: d) 1-methyl-4-(1,5,9-trimethyl-4,8-decadienyl)-1,3-cyclohexadiene, e) helinudichromene quinone, f) helinudifolin, g) helinudiquinone (6-Me ether), h) helinudiquinone and i) isocomen-5-one

i) *H. panduratum* var. *panduratum* O. Hoffm. (Group 18)

The leaves of the species are panduriform and sessile and its involucral bracts are sub acute to acute. The flowers of the var. *panduratum* range from 27 – 48 and they are yellow as well as honey-scented. This species has been recorded in the Eastern Cape and KZN. It can be found from sea level to approx. 1200m above sea level. This species is often seen growing in large tangled clumps in high-rainfall areas, especially in mixed scrub-grassland near forest margins. The main flowering season is in December and January (Hilliard, 1983).

One thio-derivative (Fig 1.6) is known to be isolated from *H. panduratum* (Chapman and Hall, 2009).

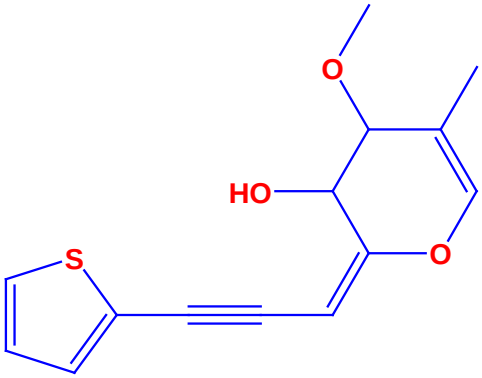

Fig 1.6: helipandurin

j) *H. populifolium* DC. (Group 16)

This species is a shrub, with its leaf blade being broadly ovate, acute or subrotund and obtuse, and its 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 make this a most distinctive species. It grows in large outcropping masses of rock or along the cliffs above gorges usually not higher than c. 800m above sea level. Flowering usually takes place between February and May (Hilliard, 1983). No records of any isolated compounds could be found for *H. populifolium*.

k) *H. psilolepis* Harv (Group 22)

This species is a shrub or tufted herb with small, linear or oblong/spathulate leaves. The leaf tips are obtuse to acute and both the leaf surfaces are greyish-white and woolly. Its involucral bracts are minutely radiating, yellow or sometimes golden-brown. The heads of this species are approx. 5 mm long. Areas were this species was recorded include, Eastern Cape, KZN, Lesotho, FS and Mpumalanga. Flowering usually takes place between November and January (Hilliard, 1983). There are no reports on chemical compounds isolated from *H. psilolepis.*

l) *H. rugulosum* Less. (Group 9)

In this species the tips of the inner involucral bracts are white, cream and rosy, but never bright yellow. The tips of the bracts are crisp with the heads being 3 – 5mm broad. It usually also has several to many in corymbose panicles. The stems are simple or sub-simple from a creeping stock. This species has been found in the Western Cape, Eastern Cape, KZN, FS, Lesotho, Swaziland, Mpumalanga, Gauteng, North West and Limpopo. It is usually found in poor stony or sandy grasslands, grows readily in overgrazed area and on the roadsides. Flowering takes place usually between December and March (Hilliard, 1983).

From *H. rugulosum* two similar flavanones were isolated (Fig 1.7). The difference being a methyl group at position 5 instead of an alcohol functional group (Chapman and Hall, 2009).

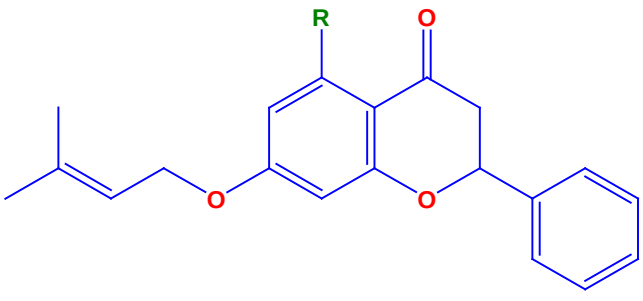

Fig 1.7: 5-hydroxy-7-prenyloxyflavanone (R = OH) and 5-methoxy-7-prenyloxyflavanone (R = Me)

1.3 Objectives of this study

- To ascertain the anti-herpes simplex virus (HSV) properties of selected *Helichrysum* species.

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

- To determine the differences in metabolite profile of the different *Helichrysum* species.

- To investigate the relationship between the metabolite profiles, anti-HSV and anti-HIV RT activity using nuclear magnetic resonance (NMR) - based metabolomics and identifying co-occurrence patterns and distinct differences between the different *Helichrysum* species.

1.3.1 *Hypotheses*

1. Some of the selected *Helichrysum* species have antiviral properties.
2. Metabolomics can be used to identify active constituents in complex plant extracts thereby accelerating the process of drug discovery.

1.4 Structure of thesis

This thesis was a study to investigate an easier and quicker way to correlate bio-activity to possible active constituents. Due to the herpes simplex cytopathic effect inhibition bioassay being time-consuming, analysing large quantities of plant samples through this

bioassay is not feasible. Faced with this problem, it was necessary to find a way to reduce the time and resources spent on the bioassay.

Setting up a database of analysed plants with a specific bioactivity was necessary, and using Chemometrics software in conjunction with NMR data and the bioactivity results was seen as a solution. NMR data of the *Helichrysum* spp. were analysed in conjunction with bioactivity results to set up a database. Though this is the beginning of the road, this study indicated that metabolomics might be used to identify functional groups in active compounds responsible for the biological activity of the *Helichrysum* spp.

Chapter 1: The introductory chapter consists of the general background of the *Helichrysum* spp. used in this study and the information on isolated constituents of the different species is explained in detail.

Chapter 2: This chapter entails the toxicity of the *Helichrysum* spp. *In vitro* cell cultures were used to determine the lowest concentration at which the extracts are toxic.

Chapter 3: This chapter covers the bioactivity of *Helichrysum* spp. extracts against the herpes simplex virus. The background of the virus, the cytopathic effect (CPE) bioassay and the results obtained testing on the virus are discussed.

Chapter 4: This chapter incorporates the basics of the HIV virus, the reverse transcriptase inhibition assay and the results obtained testing against the enzyme to establish the bioactivity of *Helichrysum* spp. extracts potentially against the human immunodeficiency virus.

Chapter 5: In this chapter, chemometric analyses using SIMCA - P software is investigated. The results from Chapters 2-4 are all used to search for co-occurrence

patterns and distinct differences. Metabolomics is also investigated in this chapter as a new tool for drug discovery.

Chapter 6: The general discussion and conclusions of the study are covered in this chapter.

Chapter 7: Acknowledgements are made in this chapter.

Chapter 8: This chapter includes all the nmr spectra used throughout this study.

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Chapter 2

Toxicity of *Helichrysum* spp.

Chapter 2

Toxicity of *Helichrysum* spp.

2.1 Introduction

Cytotoxicity tests are a very important part of drug discovery and development. These tests provide more information on the viability of a compound to be developed into a drug. These tests are obviously only contributing a small but important part in developing lead drugs from natural products. Various cell lines are used for these tests, i.e. liver, stomach, prostate as well as African green monkey kidney cells. For this study the African green monkey kidney cell line was used to establish the cytotoxicity of the plant extracts. These tests help in obtaining information on the dose-effect relationship which includes dose ranges for the use in humans (Yoo *et al*. 1998; Ren and Tang 1999). Toxicity tests have the advantage of being inexpensive and to a large extent very easy to carry out, compared to *in vivo* tests.

Before compounds or even extracts that have shown bioactivity can be considered for drug development, cytotoxicity tests should be carried out. The results from such tests could also help with the formulation of drugs and the prescribed dosage of a specific drug (Aviala *et al*., 1997).

The cell lines cytotoxicity method is not the only method that can be done to test for the toxic nature of biological active samples. The Brine shrimp lethality test (BST) which makes use of simple living organisms *in vivo*, is being used as a simple tool to guide screening and fractionation of biological active plant extracts. With the BST one of the simplest responses, lethality (one criterion: dead or alive), is monitored. This general bioassay correlates were well with cytotoxicity and other biological properties. Brine shrimp (*Artemia salina*) has also previously been used in various bioassay systems

especially in the general screening of bioactive substances in plant extracts (Pimentel Montanher et al., 2002).

2.2 Materials and Methods

2.2.1 Preparation of plant extracts

Plants were collected, 7-15g of plant material (leaves) were placed into liquid nitrogen and were then crushed into a fine powder. An indirect fractionation method was then used to separate the non-polar and polar metabolites. A solvent mixture of 50% water/methanol (1:1) and 50% chloroform was used to extract the metabolites. The plant material was then subjected to 1 min of vortexing and 5 min of sonication after which it was filtered (Whatman 42). The filtered extract was placed in a separating funnel, after which the non-polar and polar solvents phases were separated and collected. After collecting the different phases, the two fractions were then subjected to vacuum evaporation and evaporated until dry (Choi et al., 2004). The dry extract was weighed, after which it was dissolved in either water/methanol or chloroform to be stored as stock solution for later use on the bioassays as well as for the analysis on the NMR. Before use on the bioassays as well as for analytical analysis, the appropriate volume was removed from the stock solution and left to dry after which an appropriate solvent/solution for the specific bioassay or analysis was used to re-dissolve the extract. A total of 24 extracts, 12 water-methanol extracts and 12 chloroform extracts, were prepared for the study to be used throughout for all the bioassays and analyses.

2.2.2 Vero monkey kidney cells

Vero cells, from African green monkey kidney cells, were maintained in culture flasks in complete Minimum Essential Medium (Eagle) (MEM) (Highveld Biological (Pty) Ltd, Kelvin, South Africa) containing 1.5 g/L sodium bicarbobate, 2mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10 µg/ml penicillin, 10 µg/ml streptomycin

and 10% bovine serum at 37°C in a humidified atmosphere with 5 % CO_2. This was done every 2-3 days after it had formed a confluent monolayer. When the cells were sub-cultured the cells that attached to the culture flask were trypsinized (0.25% trypsin containing 0.01% EDTA) for 10 min at 37 in an atmosphere with 5% CO_2 and 95% humidity. The addition of complete medium to the cell suspension stopped the trypsinizing. About $1x10^5$ of the viable cells were then re-suspended in complete medium. In the outer wells of the 96-well plates, 200 µl of medium was dispensed to prevent the inner wells from drying out. All inner wells received 100 µl (1×10^5 cells) of the cell suspension. The plates were incubated overnight at 37°C in a humidified atmosphere with 5 % CO_2 (Zheng *et al*., 2001).

2.2.3 Cytotoxicity assay

For the cytotoxicity assay, the 24 plant extracts were prepared up to a stock concentration of 20 mg/ml which were subsequently diluted 50 times in MEM to a final concentration of 400 µg/ml. To determine the IC_{50} of the different plant extracts the solutions where then serially diluted to obtain a range of concentrations starting at 400.00 µg/ml with the lowest at 3.13 µg/ml. As a positive control Zearalenone was used as a reference throughout out the cytotoxicity testing.

Cytotoxicity was measured by the XTT method (Zheng *et al*., 2001) using the Cell Proliferation Kit II (Roche Diagnostics GmbH). The Vero cells were seeded at $1x10^5$ onto a microtitre plate and incubated for 24 hours to allow the cells to attach to the bottom of the plate. 100 µl solution of the plant extract stock solution were dispensed into cell containing wells in triplicate of the microtitre plates. Final concentrations were 3.13, 6.25, 12.5, 25.00, 50.00, 100.00, 200.00 and 400.00 µg/ml. Plates where then incubated for 48 hours at 37°C in a humidified atmosphere with 5% CO_2. The XTT reagent was added to a final concentration of 0.3mg/ml and again the cells were incubated for 1-2 hours. After incubation the absorbance of the colour complex was spectrophotometrically quantified using an ELISA plate reader, which measure the optical density at 490nm with a reference wavelength of 690 nm.

2.3 Results

The cytotoxicity of the 24 extracts was investigated prior to the efficacy testing for the main reason to establish the toxic concentrations of the different extracts.

The overall toxicity of the water-methanol (WM) plant extracts was generally low with the exception of *H. cymosum* subsp. *cymosum* which had an IC_{50} of 59.74 µg/ml (Table 2.1). *Helichrysum adenocarpum* subsp. *adenocarpum* (>200 µg/ml) is one of the plant extracts of interest due to its activity against the HSV-1 virus as will be discussed in the following chapter. *H. populifolium* (147.8 µg/ml), *H. herbaceum* (237.8 µg/ml), *H. appendiculatum* (217.8 µg/ml), *H. callicomum* (235.8 µg/ml) and *H. panduratum* (> 200 µg/ml) are also of interest due to their therapeutic indexes being greater than a factor of two against the HSV-1 virus (Table 2.1)(see Chapter 3, section 3.4).

Table 2.1: The IC_{50} values of the crude water-methanol plant extracts of *Helichrysum* spp. on Vero African green monkey kidney cells

Extract	IC_{50} (µg/ml) ± SD
H. acutatum	212.80 ± 0.04
H. adenocarpum subsp. a*denocarpum*	> 200.00
H. appendiculatum	217.80 ± 0.90
H. aureonitens	136.50 ± 0.06
H. callicomum	235.80 ± 0.03
H. cymosum subsp. *cymosum*	59.74 ± 0.14
H. herbaceum	237.80 ± 0.03
H. nudifolium var. *nudifolium*	138.40 ± 0.03
H. panduratum	> 200.00
H. psilolepis	277.80 ± 0.05
H. populifolium	> 200.00
H. rugulosum	147.80 ± 0.06
Zearalenone (Positive control)	1.33 ± 0.32

When compared to their activity (Chapters 3 & 4), most of the WM extracts showed lower toxicity levels than their efficacy levels. Further comparisons between the activity and toxicity will be discussed in later chapters where therapeutic indexes will be determined as well.

On the other hand, the results from the chloroform extracts show a different trend with most of the plant extracts showing higher toxicity, with the exception of *H. callicomum* at a moderate IC_{50} level of 94.71 µg/ml (Table 2.2). With the chloroform extracts the activity could directly be correlated back to the toxicity, thus the chloroform extract were active at their specific concentrations due to their toxic nature. The chloroform plant extracts are mostly toxic but due to the high activity on HSV-1 by *H. adenocarpum* subsp. *adenocarpum* with a therapeutic index of 2.45, the toxicity (61.22 µg/ml) needs to be kept in mind (Table 2.2). For the remaining chloroform plant extracts, no significant activity was recorded.

Table 2.2: The IC_{50} values of the crude chloroform plant extracts of *Helichrysum* spp. on Vero African green monkey kidney cells

Extract	IC_{50} (µg/ml) ± SD
H. acutatum	25.16 ± 0.11
H. adenocarpum subsp. *adenocarpum*	61.22 ± 0.06
H. appendiculatum	29.01 ± 0.12
H. aureonitens	< 3.13
H. callicomum	94.71 ± 0.07
H. cymosum subsp. *cymosum*	36.52 ± 0.27
H. herbaceum	49.93 ± 0.20
H. nudifolium var. *nudifolium*	< 3.13
H. panduratum	3.40 ± 0.06
H. psilolepis	27.07 ± 0.10
H. populifolium	2.99 ± 0.20
H. rugulosum	48.10 ± 3.36
Zearalenone (Positive control)	1.33 ± 0.32

2.4 Discussion and Conclusion

Toxicity data on the *Helichrysum* family is very limited and was usually done in conjunction with antiviral and antimalarial activities (Meyer et al., 1996; Meyer et al., 1997; Lall et al., 2006; Van Vuuren et al., 2006).

When comparing previous results from *H. cymosum* subsp. *cymosum* acetone extract, where the IC_{50} toxicity value was determined to be 172.01 µg/ml (Van Vuuren et al., 2006), it can be concluded that there is quite a variation in the results, compared to the current study. This can be attributed to various reasons such as the use of different extraction solvents, seasonal variations and geographical distribution. Studies conducted on *H. aureonitens* did not indicate any altered morphological or growth characteristics in cells at a very high concentration level of 8.44 mg/ml (Meyer et al., 1996) which is in contradiction to the results that we discovered in this study of *H. aureonitens* being toxic at 136.5 µg/ml and distinct morphological changes being observed at levels of 400 µg/ml on the Vero cells. It has to be taken into consideration how the extract was prepared for these bioassays. In the case of Meyer et al. (1996), the plant extracts were not homogenised and the plant material was extracted with boiling distilled water. The fact that the studies are not completely comparable highlights the reason for additional toxicity studies to be carried out to eliminate the variability between studies.

There is a clear indication that the non-polar plant extracts are more toxic than their polar counterparts (Tables 2.1 and 2.2). This can be due to various reasons, but the difficulties experienced in this study to effectively dissolve the non-polar chloroform plant extracts in a polar bioassay medium could explain these observed differences. Due to the nature of the bioassays used throughout the study all the tested samples needed to be dissolved in a mostly polar medium containing the cells. It was observed that the non-polar extracts precipitate out and settle mainly at the bottom of the wells in the plates. It could thus be speculated that the precipitate aggregates around the cells and thus could cause the cells to “suffocate” and thus causing the cells to die. Various measures were taken to ensure the non-polar extracts dissolved, however it still could not be achieved. It still is a major problem for the effective testing of non-polar plant extracts as well as non-polar pure compounds.

The toxicity of the WM plant extracts showed lower toxicity than those of the chloroform plant extracts. *Helichrysum adenocarpum* subsp. *adenocarpum, H. populifolium, H.*

herbaceum, H. appendiculatum, H. callicomum and *H. panduratum* show promise for further investigation due to their low toxicity and high bioactivity with *H. adenocarpum* subsp. *adenocarpum* being the best candidate.

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Chapter 3

Anti-herpes simplex virus bioactivity

Chapter 3

Anti-herpes simplex virus bioactivity

3.1 Introduction

The herpesviridae family contains more than 100 vertebrate viruses and is one of the oldest families of viruses known to infect humans. The first records of the herpes simplex virus (HSV) date back as early as 500 BC when it was first described by humans. The Greek scholars described the spreading lesions, associated with shingles (the clinical manifestation of Varicella-Zoster Virus (VZV), a.k.a. herpes zoster (HZV)), on their patients in 1398 using the word “herpes”, meaning “to creep”. This classification only came into use in the late 18th century and it was not until 1893, that person-to-person transmission of the HSV infection was recognised by Vidal. In 1919, Lowerstein confirmed the hypothesis from Vidal, by experimentally illustrating the infective nature of HSV. Between 1920 and 1930, research on HSV flourished. During this era, virologists for the first time established that HSV could infect more than just the cutaneous tissue and infections of HSV in the central nervous system were examined in detail. In 1988 the first extremely effective antiviral drug, acyclovir, was developed by Getrube Elion. Acyclovir is a very effective inhibitor of the viral DNA polymerase and is still used as a first-line antiviral drug to treat HSV types 1 and type 2 as well as VZV. During to the end of the 20th century and the beginning of the 21st century, Merck developed vaccines for the herpesviridae family, firstly in 1995 to prevent chickenpox and then in 2006 a vaccine against VZV to prevent shingles mainly in older individuals (Wildy, 1973; http://virus.stanford.edu/herpes/History.html, 2007).

HSV is a member of the viruses whose genome consists of a single large double-stranded DNA molecule (DNA virus). The range of diseases caused by HSV includes primary and recurrent infections of mucous membranes (e.g., herpes labialis, gingivostomatitis and genital HSV infections), keratoconjunctivitis, visceral HSV

infections in immuno-compromised hosts, neonatal HSV infections, HSV encephalitis, Kaposi's varicella-like eruption, and an association with erythema multiforme. The HSV type 1 is associated with non-genital infection and HSV type 2 is associated with genital infections (Whitley et al., 1998). The cardinal factors that may lead to an outbreak are direct sunlight, local skin trauma, menstruation, fever and stress. According to the National Institutes of Health (NIH) by the age of 50, 80% to 90% of people will harbour HSV-1, due to direct contact from someone close to them. Also in their study it was shown that 90% of the United States of America's population has been exposed to HSV-1 and just over 25 % of the population (ages 25-40) have been exposed to HSV-2 (http://stdmedicaltreatment.com).

3.2 Materials and Methods

Extracts used for the cytopathic effect (CPE) inhibition assay were prepared as described in Chapter 2 (2.2.1). With the CPE inhibition assay, the inhibitory concentration at 50% is determined. That is the concentration of antiviral agent that lowers the virus-induced CPE and the number of plaques formed by a given inoculum, by 50% (Contarela et al., 1999).

3.2.1 Vero monkey kidney cells

Vero cells were prepared and maintained as described in Chapter 2 (2.2.2).

3.2.2 Cytopathic effect (CPE) inhibition assay

For the antiviral activity bioassay, plant extracts were made up to a final concentration of 400 µg/ml in the row of wells. Subsequent doubling dilutions were added to the other rows of wells. The concentration test range was from 400 µg/ml to 3.13 µg/ml. The final concentration of the herpes simplex virus in the assay being used was 100 $TCID_{50}$/ml (tissue culture infective dose - the quantity of a cytopathogenic agent (virus) which will produce a cytopathic effect in 50 % of the cultures inoculated). To investigate the effect of the extracts on the viral absorption and subsequent replication in cell culture, 24-hour old monolayers of Vero cells in 96-well microtitre trays were

prepared and starved in serum-free MEM for 1 h at 37°C in 5% CO_2 in air in a humidified atmosphere. After starvation the serum-free MEM was withdrawn and 100µl (100 $TCID_{50}$) of virus and appropriate dilution of the extract were added to each of the six wells of the 96-well microtitre tray and the cell cultures were incubated at 37°C in 5% CO_2 in air in a humidified atmosphere. As positive and negative controls cells were infected with 100µl (100 $TCID_{50}$) virus in serum-free MEM and serum-free MEM respectively. Cells were examined daily for up to seven days, by light microscopy, for the appearance of a CPE. The absence of a CPE at a specific concentration of the compound is considered to be indicative of antiviral activity (Meyer et al., 1996).

3.3 Results

To assess the antiviral properties of the plant extracts, the correlation between the virus control and the virus infected cells treated with the plant extracts had to be made. The results of the anti-HSV of the water/methanol (WM) and chloroform plant extracts are given in Tables 3.1 and 3.2 and are expressed in µg/ml. The HSV infected cells treated with the plant extracts showed no CPE after one week at the levels indicated in Tables 3.1 and 3.2, compared to the virus control which showed extensive CPE after 36 – 48 hours.

For the CPE bioassay, acyclovir was used as positive control with an antiviral activity at 0.75 µg/ml. The WM plant extract of *H. adenocarpum* subsp. *adenocarpum* had the highest activity at 25 µg/ml. Three other WM extracts showed very good activity at 50 µg/ml i.e. *H. appendiculatum*, *H. callicomum* and *H. populifolium*.

The chloroform plant extracts showed very little activity, due to their high toxicity. The morphology of the cells was severely altered and extensive altered growth characteristics where observed using the CPE bioassay for most of the extracts except for *H. adenocarpum* subsp. *adenocarpum* and *H. aureonitens*. These two chloroform extracts showed activity at 25 µg/ml and 100 µg/ml respectively (Table 3.2).

Table 3.1: The inhibition of replication of 100$TCID_{50}$ herpes simplex virus type 1 on Vero African

green monkey kidney cells when the virus was inoculated simultaneously with various concentrations of the water-methanol extract of *Helichrysum* spp.. The lowest concentration at which no cytopathic effect was observed.

Extract	Cytopathic effect (µg/ml)
H. acutatum	200
H. adenocarpum subsp. a*denocarpum*	25
H. appendiculatum	50
H. aureonitens	100 *
H. callicomum	50
H. cymosum subsp. *cymosum*	400 *
H. herbaceum	100
H. nudifolium var. *nudifolium*	100 *
H. panduratum	50 *
H. psilolepis	200
H. populifolium	50
H. rugulosum	na
DMSO (negative control) (highest level used)	0.20%
Acyclovir (positive control)	0.753

* - slightly toxic
n/a - not active

Table 3.2: The inhibition of replication of 100TCID$_{50}$ herpes simplex virus type 1 on Vero African green monkey kidney cells when the virus was inoculated simultaneously with various concentrations of the chloroform extract of *Helichrysum* spp.. The lowest concentration at which no cytopathic effect was observed.

Extract	Cytopathic effect (µg/ml)
H. acutatum	na
H. adenocarpum subsp. a*denocarpum*	25 *
H. appendiculatum	na
H. aureonitens	100 *
H. callicomum	na
H. cymosum subsp. *cymosum*	na
H. herbaceum	na
H. nudifolium var. *nudifolium*	na
H. panduratum	na
H. psilolepis	na
H. populifolium	na
H. rugulosum	na
DMSO (negative control) (highest level used)	0.20%
Acyclovir (positive control)	0.753

* - slightly toxic
n/a - not active

3.4 Discussion and Conclusion

The observed toxicity of the chloroform extract was very prominent once again in this bioassay. With this bioassay the morphological change of the Vero cells were observed over a period of seven days using a light microscope, where infection with the HSV-1 virus resulted in spherically shaped cells and cells that have swollen and then cluster in small groups together. Toxicity on the other hand was also very easily identified by disrupted monolayers of the Vero cells and cells that lost all of their growth characters and their morphology and appeared only as black dots when observed under the light microscope. It was thus easy to separate HSV-1 CPE from the general cytotoxicity of the plant extracts.

Very little data is available on HSV activity of *Helichrysum* spp. plant extracts and *Helichrysum* spp. plant derived constituents. The data available was a previous study conducted by Meyer et al. 1996 were the activity of *H. aureonitens* (water extract) was at 1.35 mg/ml. In this current study lower concentrations were tested (as little as 3.13 µg/ml) to determine the lowest active concentration of the different *Helichrysum* species. The highest activity observed with the WM plant extracts was that of *H. adenocarpum* subsp. *adenocarpum* at 25 µg/ml and then *H. appendiculatum*, *H. callicomum* and *H. populifolium* at 50 µg/ml (Table 3.1). These are relatively good results for plant extracts which could indicate potential for increased potency with isolated pure compounds. This was seen with the isolated constituent from *H. aureonitens*, galangin (Meyer et al., 1997) which had activity at 12 µg/ml against HSV-1, which was a significant increase in activity from their 1.35 mg/ml activity observed in the original extract. The activity observed for *H. aureonitens* (WM) in this study was at 100 µg/ml, quite significantly more potent than previously recorded, this could most probably be due to the different extraction methods used. The increase in activity observed between the two extraction methods could indicate on the presence of more active constituents or additional active compounds in the water-methanol extracts when compared to the homogenised, boiled water extracts.

The chloroform plant extracts showed mainly the morphological changes associated with toxicity and not CPE. The toxic effects could be observed after 24 hours. The only

activity observed was that of *H. adenocarpum* subsp. *adenocarpum* and *H. aureonitens* at 25 µg/ml and 100 µg/ml respectively (both slightly toxic). These are relative good results for plant extracts but the doubt did arise on the accuracy of the non-polar extracts' results. As indicated in the previous chapter, the problem of solubility arose with the CPE bioassay once again. Ensuring the solubility of the chloroform extracts in the polar medium was not possible. The chloroform extracts did precipitate out, thus raising the question again on the effect of the precipitate on the living cells. It was thus speculated that the precipitate (as mentioned in Chapter 2) could possibly have "suffocated" the vero cells and thus the cells died and no definite conclusion could have been made as to the effect of the plant extracts on the activity against the HSV-1.

Taking the cytotoxicity and the anti-herpes activity data into account it is possible to calculate the therapeutic indexes (TI) of the plant extracts. The TI is determined by dividing the toxicity value by the efficacy value. The TI values of the WM extracts of *H. appendiculatum*, *H. callicomum* and *H. populifolium* were 4.35, 4.72 and 2.95, respectively, which were significantly higher than the other plant extracts and thus making these plant extracts prime candidates for further investigations.

Work done by Orhan et al. (2009) on edible plants from the Asteraceae (not *Helichrysum* species), Caryophyllaceae, Lamiaceae, Polygonaceae, Scrophulariaceae and Crassulaceae families, hexane plant extracts showed similar ranges of activity when compared to the chloroform extracts of *H. adenocarpum* subsp. *adenocarpum* and *H. aureonitens*, 25 µg/ml and 100 µg/ml respectively (Table 3.2). The highest activity obtained in their study was 50 µg/ml and the lowest being at 200 µg/ml, but it has to be highlighted that the extract solvent used in their study was hexane.

For any extract to be considered noteworthy it should have antimicrobial activity at concentrations lower than 1 mg/ml (Gibbons, 2004; Rios and Reico, 2005). This was achieved with all of the plant extracts in our study, but cytotoxicity was the determining factor in this study. The testing done with the plant extracts was guided by the cytotoxicity of the plant extracts, firstly to establish a concentration testing range for the CPE bioassay and not to do unnecessary testing at too high levels of concentration of the plant extract. Some of the WM extracts showed high cytotoxicity, but most of the

chloroform extracts were very toxic and thus did not indicate any activity greater than their toxicity.

Further analyses with these plant extracts were done using metabolomics and nuclear magnetic resonance (NMR) (Chapter 5) to investigate the constituent(s) that was/were responsible for the high activity in the most potent extracts.

3.5 References

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Chapter 4

Anti-human immunodeficiency virus bioactivity

Chapter 4

Anti-human immunodeficiency virus bioactivity

4.1 Introduction

The acquired immunodeficiency syndrome (AIDS) is still a threatening disease world-wide and was branded as the pandemic of the second half of the 20th century (Meek et al., 1990). The identification of the retrovirus, human immunodeficiency virus (HIV), as the causative agent of AIDS, has been important in boosting research for new antiviral agents and new modalities of antiviral chemotherapy (Balzarini et al., 1986; Sarin, 1988). Many research approaches are currently aimed at the development of novel agents to inhibit the replication of HIV through various targets. The main targets in current research are the inhibition of HIV reverse transcriptase (RT), HIV protease (PR), membrane fusion and HIV integrase (Meek et al., 1990). However, the rapid appearance of HIV strain resistance to currently available drugs and also the rapid spread of the AIDS epidemic, suggests that effective and durable chemotherapy of the HIV disease will require the use of innovative combinations of drugs having different mechanisms of anti-HIV activity (Tantillo et al., 1994; Balzarini et al., 1996; Lipsky, 1996). Alongside the development of resistance, the acceptable levels of toxicity, high cost, unavailability and lastly the lack of curative effect are the main short comings of the current HIV/AIDS drugs (Pomerantz and Horn, 2003). This has thus created a great need to search for and develop new and different anti-HIV candidates. Fortunately, ongoing searches for these candidates from plants and natural products are considerable (Kinghorn, 1995).

AIDS is a syndrome which is responsible for the dysfunction of the immune system, which leads to the immune system being targeted by opportunistic infections of bacteria, fungal, protozoan or viral aetiology. There are thus many possibilities in which herbal extracts can be beneficial in the HIV/AIDS condition, this could be due to the obvious inhibition of

HIV replication but as well as boosting the immune system or inhibiting several other opportunistic infections. To date there is very limited data available on the potential therapeutic effects of South African medicinal plants on HIV infections (Bessong et al., 2005).

4.2 Materials and Methods

The *Helichrysum* plant extracts that were used for the HIV RT bioassay were prepared as described in Chapter 2 (2.2.1).

4.2.1 Reverse Transcriptase bioassay (RT)

This bioassay was performed using a colorimetric reverse transcriptase assay (Roche, Germany). The recombinant HIV-1 reverse transcriptase (HIV-1 RT) was diluted with a 200 ng/ml lysis buffer supplied with the kit. Water-methanol (WM) extract stocks were prepared in DMSO to a concentration of 6 mg/ml, these where then ten times diluted with lysis buffer. Each test well contained 20 µl of diluted recombinant HIV-1 RT (4-6 ng), 20 µl of diluted extract, and 20 µl of reaction mixture. The final extract concentration in each of the wells was 200 µg/ml. Negative control wells contained 40 µl of lysis buffer and 20 µl reaction mixture. Two positive controls were made; the first one contained 20 µl diluted recombinant HIV-1 RT, 20 µl of lysis buffer containing 10% DMSO (chloroform plant extracts positive control was made up of 1:1, 10% DMSO : Peanut oil), and 20 µl of reaction mixture, the second one did not include the 10% DMSO, only 20 µl of lysis buffer was added. After adding al the reactants the wells of the microtitre plate was covered and sealed (plastic cover included in the kit). After incubating for 1 hour at 37°C, the wells were washed five times with 250 µl of washing buffer per well for 30 seconds each. After washing, the washing buffer was removed carefully, and 200 µl of and-DIG-POD (anti-digoxigenin-peroxidase) working solution was added into each well. After covering the plate, it was then incubated again for 1 hour at 37°C. The wells were then once again washed (as explained before). The washing buffer was then carefully removed from the wells and 200 µl of the chemiluminescent peroxidase substrate luminol/4-iodophenol was added to the wells. The mixtures were then incubated at room temperature for a further

30 minutes after which the absorbencies of the samples were measured at 405 nm (reference wavelength set at 492 nm). The percentage inhibitory activities of the extracts were then calculated, in reference with the DMSO positive control.

$$\text{Percent RT inhibition} = 100 - \frac{A^{405nm} - A^{492nm}\ (\text{Extract})}{A^{405nm} - A^{492nm}\ (\text{PC})} \times 100$$

Adriamycin, an anticancer drug and also an inhibitor of viral reverse transcriptase was used as a positive drug control (Goud et al., 2003). The assay was carried out in triplicate. The active extracts were further tested with the above mentioned method, but at three different concentrations (200 µg/ml, 100 µg/ml and 50 µg/ml)

4.3 Results

The WM plant extracts showed limited RT inhibition. Inhibition was restricted to only *H. populifolium* which inhibited HIV-1 RT by 63.870 %. Minor activities where detected from *H. appendiculatum* and *H. herbaceum* at 4.409 % and 5.039 % respectively (Table 4.1 and Fig. 4.1).

The positive drug control (PDC), Adriamycin showed nearly complete inhibition of the HIV-1 RT in all three repetitions of the bioassay at 0.1 µg/ml.

With the chloroform plant extracts showed less activity. *Helichrysum herbaceum* and *H. panduratum* had inhibitory activities at 24.81 % and 10.77 %, respectively. *Helichrysum psilolepis* to a lesser extent had inhibitory activity at 8.46 % (Table 4.2 and Fig 4.2).

The WM extract of *H. populifolium* and the chloroform extracts of *H. herbaceum* and *H. panduratum* were further tested on the HIV RT dose response assay. *H. populifolium* still showed some inhibition activity of 35.59 % at 100 µg/ml and the inhibition was dose related.

Table 4.1: HIV-1 Reverse transcriptase (RT) inhibition of the water-methanol extracts from *Helichrysum* spp. at 200 µg/ml.

Controls & Extracts	% Inhibition ± SD
H. acutatum	-19.685 ± 0.007
H. adenocarpum subsp. a*denocarpum*	-15.906 ± 0.004
H. appendiculatum	4.409 ± 0.012
H. aureonitens	-6.772 ± 0.017
H. callicomum	-52.913 ± 0.006
H. cymosum subsp. *cymosum*	-67.087 ± 0.026
H. herbaceum	5.039 ± 0.020
H. nudifolium var. *nudifolium*	-7.402 ± 0.016
H. panduratum	-11.496 ± 0.009
H. populifolium	63.780 ± 0.009
H. psilolepis	-23.780 ± 0.020
H. rugulosum	-16.378 ± 0.007
Positive control (- DMSO)	0.000 ± 0.018
Positive control (+DMSO)	6.480 ± 0.007
Andriamycin (positive drug control)	99.808 ± 0.004

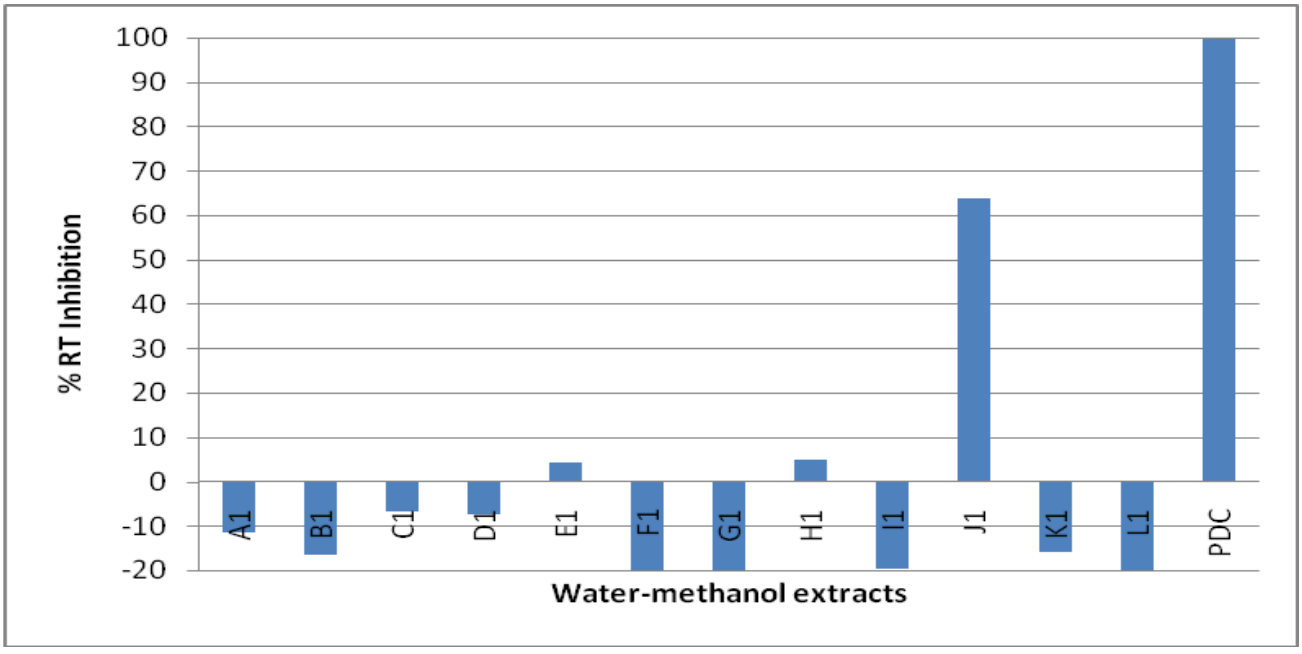

Fig 4.1: Percentage (%) RT inhibition of the water-methanol extracts at 200 µg/ml. *H. panduratum* (A1), *H. rugulosum* (B1), *H. aureonitens* (C1), *H. nudifolium* var. *nudifolium* (D1), *H. appendiculatum* (E1), *H. callicomum* (F1), *H. psilolepis* (G1), *H. herbaceum* (H1), *H. acutatum* (I1), *H. populifolium* (J1), *H. adenocarpum* (K1) and *H. cymosum* subsp. *cymosum* (L1) and the positive drug control (Andriamycin).

Table 4.2: HIV-1 Reverse transcriptase (RT) inhibition of the chloroform extracts from *Helichrysum* spp. at 200µg/ml.

Controls & Extracts	% Inhibition ± SD
H. acutatum	-1.346 ± 0.034
H. adenocarpum subsp. a*denocarpum*	-115.000 ± 0.031
H. appendiculatum	-5.865 ± 0.028
H. aureonitens	-72.692 ± 0.013
H. callicomum	-7.596 ± 0.019
H. cymosum subsp. *cymosum*	-70.577 ± 0.026
H. herbaceum	24.808 ± 0.028
H. nudifolium var. *nudifolium*	-6.923 ± 0.018
H. panduratum	10.769 ± 0.013
H. populifolium	-28.654 ± 0.022
H. psilolepis	8.462 ± 0.016
H. rugulosum	-58.077 ± 0.024
Positive control (- DMSO)	0.000 ± 0.025
Positive control (+DMSO + Peanut Oil)	4.407 ± 0.012
Andriamycin (positive drug control)	98.978 ± 0.025

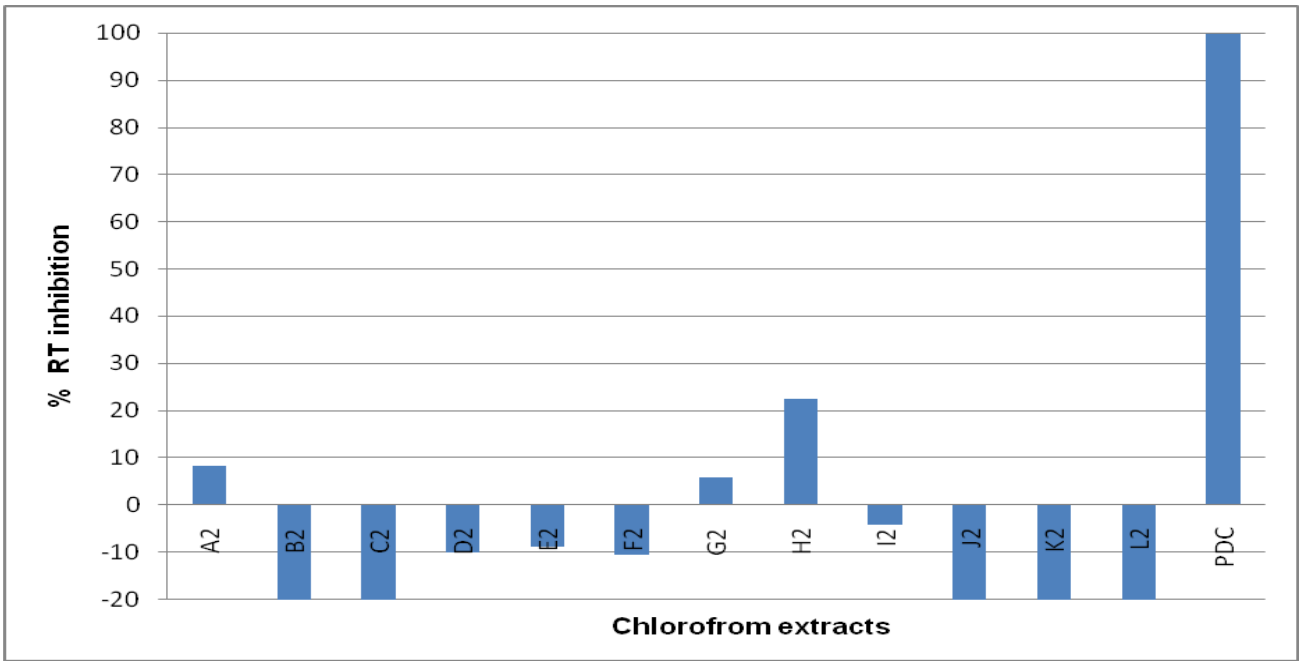

Fig 4.2: Percentage (%) RT inhibition of the chloroform extracts at 200 µg/ml. *H. panduratum* (A2), *H. rugulosum* (B2), *H. aureonitens* (C2), *H. nudifolium* var. *nudifolium* (D2), *H. appendiculatum* (E2), *H. callicomum* (F2), *H. psilolepis* (G2), *H. herbaceum* (H2), *H. acutatum* (I2), *H. populifolium* (J2), *H. adenocarpum* (K2), *H. cymosum* subsp. *cymosum* (L2) and the positive drug control (Andriamycin).

Table 4.3: HIV-1 Reverse transcriptase (RT) inhibition of the promising extracts at different concentrations.

Controls & Extracts	µg/ml	% Inhibition ± SD
Helichrysum populifolium (Water/methanol)	200	60.847 ± 0.007
	100	35.593 ± 0.010
	50	2.712 ± 0.005
Helichrysum herbaceum (Chloroform)	200	28.983 ± 0.017
	100	9.831 ± 0.017
	50	-36.610 ± 0.017
Helichrysum panduratum (Chloroform)	200	6.102 ± 0.008
	100	-1.186 ± 0.012
	50	-11.864 ± 0.012
Positive control (- DMSO)		0.000 ± 0.009
Positive control (+DMSO)		6.480 ± 0.007
Positive control (+DMSO + Peanut Oil)		4.407 ± 0.012
Andriamycin (positive drug control)		98.978 ± 0.025

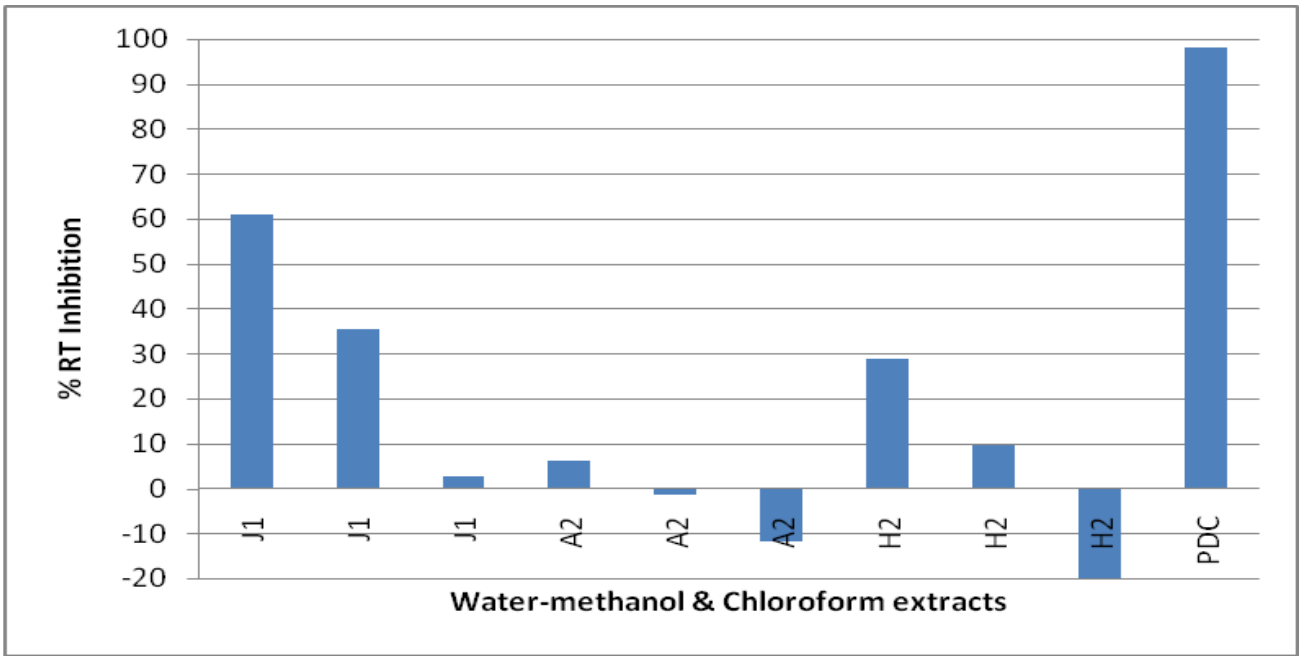

Fig 4.3: Percentage (%) RT inhibition at three different concentrations (200, 100 and 50 µg/ml) of *H. populifolium* (J1) water/methanol extract, *H. panduratum* (A2) chloroform extract, *H. herbaceum* (H2) chloroform extract and the positive drug control (Andriamycin).

4.4 Discussion and Conclusion

Only a few *Helichrysum* species have previously been tested against the HIV-1 virus (Appendino et al., 2007; Drewes and Van Vuuren, 2008) and even less against the reverse transcriptase enzyme. In this study we established that three extracts, one WM and two chloroform, showed some activity against the HIV-1 RT. The WM extract of *H. populifolium* inhibited the HIV-1 RT by 63.87 % at 200 µg/ml and the chloroform extracts of *H. herbaceum* and *H. panduratum* had inhibitory activities of 24.81 % and 10.77 % at 200 µg/ml (Table 4.1, Fig. 4.1, Table 4.2 and Fig. 4.2), respectively.

These three extracts where tested further at different concentrations (200, 100 and 50 µg/ml). The dose response results correlated well with the different concentrations.

The chloroform extracts once again showed difficulties in solubility and precipitation was frequently observed. This could have the effect that the reaction site at the bottom of the wells could have been affected by compounds precipitating out and thus inhibiting the enzyme to react sufficiently with the bioassay. In order to limit the precipitation of the chloroform extracts they were dissolved not only with DMSO, but 50% peanut oil was added to the solution mixture to decrease the polarity of the solution and “help” the non-polar extracts to dissolve. A positive control of 1:1 10% DMSO and peanut oil was also added to the bioassay to determine the effect of peanut oil on the HIV-1 RT. The DMSO and peanut oil mixture had a 4.41 % inhibition on the RT, which had to be considered when determining the chloroform extract activity.

Previous results such as that of Ali et al. (2002) showed HIV-1 RT inhibition activity of various plant extracts with the best inhibition being that of a 66 µg/ml concentration of *Combretum hartmannianum* at 99.7 %. These extracts where done using only methanol which supports a finding from Bessong et al. (2005) which showed that methanol plant extracts from nine South African medicinal plants showed better inhibition than the water extracts of the HIV-1 RT. In the study of Woradulayapinij et al. in 2005, water extracts showed better HIV-1 RT inhibitory activity compared to ethanol extracts. The inhibitory activity of the water extracts at 200 µg/ml ranged from 13.39 % up to 98.95 % compared to the ethanol extracts which at 200 µg/ml ranged from 11.21 % up to 50.31 %.

Woradulayapinij et al. (2005), mentioned that at 200 µg/ml a % inhibitory ratio (%IR) of more than 90 % can be classified as high activity, an %IR of 50 - 89 % can be classified as moderate and from 49 % and lower was seen as low activity. When comparing the HIV-1 RT inhibitory activity obtained in this study, it can be deducted that the activity of *H. populifolium* is only moderate at 63.87 %.

HIV-RT is only a single target for HIV and when plant extracts are being analysed, additional screening against other HIV targets have to be considered.

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Chapter 5

Metabolomic analyses of bioactivity and NMR spectral data

Chapter 5

Metabolomic analyses of bioactivity and NMR spectral data

5.1 Introduction

Metabolomics is one of few new emerging technologies that are enabling both high–yield and high–throughput capacities for data acquisition. This results in a more comprehensive approach to evaluate holistic *in vivo* functions. The wealth of data at gene and protein level is usually not sufficient, due to it ignoring the cellular dynamic metabolic status. Information on the metabolite levels must be included for a more complete picture, thus the need for metabolomics (Reo, 2002).

Nuclear magnetic resonance (NMR) is considered to be the most suitable technique for the purpose of quality evaluation and differentiation due to its non-destructive and non-selective nature, and with the fact that it reveals a significant amount of information (Tarachiwin et al., 2008). Metabolomics with the use of NMR could be a very reliable technique for the investigation of antiviral components in the *Helichrysum* species. The NMR spectra contain a wealth of accurate qualitative and quantitative information on the components of a sample (Reo, 2002).

The assignment of all the resonances in a ^{1}H NMR spectrum from a complex mixture is not a trivial task, thus it is very important when dealing with complex biological samples to investigate enough samples to account for the biological as well as the experimental variation within each group of samples (Box et al., 1978). Metabolomic studies generate large data matrices where the underlying systematic variation is difficult to assess by manual inspection of the spectra, especially if the variations are very subtle e.g. genetic modifications and drug treatment results. All these factors promote the use of statistical analysis of NMR data in metabolomic studies (Hedenström et al., 2008).

The information gathered from NMR in combination with the chemometrics information obtained by pattern recognition techniques such as principal component analysis (PCA) and orthogonal projection to latent structure-discriminate analysis (OPLS-DA) provides reliable and conclusive information about the chemical composition in crude samples (Tarachiwin et al., 2008).

Principal component analysis is a pattern recognition technique use for “unsupervised” and “unbiased” methods of data analysis. Especially with complex NMR spectra where enormous amounts of data are produced PCA is incorporated to check this data and reduce volume and complexity to a suitable level of information content (Lindon et al., 2001).

Orthogonal Projection to Latent Structure – Discriminate Analysis (OPLS-DA) is an extension to the supervised partial least square to latent structure – discriminate analysis (PLS-DA) regression method with the extended feature of the orthogonal signal correction (OSC) filter. The OPLS-DA method is well suited for classification of data that have multi-collinear and noisy variables which is common for many types of biological data. The OPLS-DA algorithm will model the discriminatory components and the Y orthogonal components separately, which results in a model that is much easier to interpret compared to the standard PLS-DA model (Bylesjö et al., 2006).

This study was a combination of non-selective ^{1}H NMR-based metabolomics with the use of multivariate analysis by PCA and OPLS-DA to discriminate between the *Helichrysum* species with regards to their biological activity differences. Future work will involve building a predictive model to predict the biological activities of unknown *Helichrysum* or other plant samples.

5.2 Materials and Methods

5.2.1 Sample preparation for ^{1}H NMR analysis

For the NMR analysis plant extracts were prepared as described in Chapter 2 (2.2.1). For the NMR analysis, 25 mg of plant extract was obtained and removed from stock solution and left to dry. The 25 mg of plant extract was then dissolved in deuterated solvents. For the analysis on the water-methanol (1:1) plant extract, the extracts were dissolved with KH_2PO_4 buffer in deuterated oxide (D_2O) (pH 6.0) with trimethylsilypropionic acid sodium salt (TSP) as an internal reference standard. The chloroform plant extracts were dissolved in deuterated methanol (CD_3OD) and tetramethylsilane (TMS) as the internal reference standard (Choi et al., 2004).

5.2.2 NMR analysis

The NMR analysis was performed on a 600 MHz Bruker spectrometer (Leiden University, Netherlands). For each sample 128 scans were performed after which the spectra were Fourier transformed. The spectra were reference to the internal standards TMS for the chloroform plant extracts and TSP for the water-methanol plant extracts. The spectral intensities were reduced to integrated regions (buckets). These buckets are 0.04 ppm in width and include the region from -0.1 ppm to 10.0 ppm. The residual chloroform (7.28 ppm to 7.24 ppm) regions in the chloroform extracts and water (5.1 ppm to 4.5 ppm) regions in the water-methanol extracts were excluded (Choi et al., 2004).

5.2.3 Multivariate statistical analysis of NMR and bioassay data

The NMR data was automatically reduced to ASCII files using AMIX (v. 3.8, Bruker Biospin). The files were imported in to Microsoft Excel files and transferred to SIMCA-P (12.0 Umetrics, Umeå, Sweden) for PCA and OPLS analysis. Spectral intensities were scaled down to TMS for the chloroform extract and to TSP for the water/methanol extracts (Choi et al., 2004). Orthogonal projection to latent structure discrimination analysis (OPLS-DA) is a supervised recognition method that is used to explain the separation among groups of observations. In simple terms, OPLS uses information in

the **Y** matrix to decompose the **X** matrix into blocks of structured variation correlated to and orthogonal to **Y**, respectively (Bylesjö et al., 2006).

5.3 Results

5.3.1 Scoring plots analysis

In this study the aim was to discriminate between the different *Helichrysum* species on the basis of their activity and their chemical composition. Multivariate analysis is used to compare complex sets of NMR spectra by sorting data sets into categories without overall metabolite assignments (Tarachiwin et al., 2008). In this study, PCA and OPLS, the former an unsupervised and the latter a supervised pattern recognition method were used to elucidate the discrimination among the *Helichrysum* species. It was applied to 12 *Helichrysum* species, of which there were 12 water-methanol plant extracts and 12 chloroform plant extracts. NMR spectra (Chapter 8) were divided into buckets which were then analysed with the multivariate software. After scoring plots and contribution plots have been created and specific regions on the NMR spectra have been identified as the area of interest, it is then possible to use these regions in combination with the original spectra to possibly identify the specific active constituent. The activity categories of the different bioassays were classified as described in table 5.1.

Cytotoxicity

When applying the PCA scoring plots no obvious clustering could be observed that correlates with the cytotoxicity of both the water-methanol and chloroform extracts (Fig 5.1 and 5.2).

On the other hand when applying the supervised pattern recognition method, OPLS scoring plots showed very good clustering on the basis of the cytotoxicity (Fig 5.3 and Fig 5.4). To investigate what the reasons for the clustering are, further studies were done using contribution plots which discriminated between the different toxic clusters (Section 5.3.2).

Table 5.1: Clustering categories for statistical analysis

Category	Water-methanol	IC_{50} (µg/ml)	Chloroform	IC_{50} (µg/ml)
Cytotoxicity	HT	≤ 100	HT	< 20
	MT	> 100 - <200	MT	≥ 20 - < 50
	LT	≥ 200	LT	≥ 50
		Lowest conc. with no CPE effect (µg/ml)		**Lowest conc. with no CPE effect (µg/ml)**
HSV	VHA	≤ 25	VHA	≤ 25
	HA	> 25 - ≤ 50	HA	> 25 - ≤ 50
	MHA	> 50 - ≤ 100	NA	> 200 (or toxic)
	MA	> 100 - ≤ 200		
	NA	> 200 (or toxic)		
		% inhibition		**% inhibition**
HIV RT	HA	≥ 60	HA	≥ 60
	MA	≥ 20 - < 60	MA	≥ 20 - < 60
	LA	0 - < 20	LA	0 - < 20

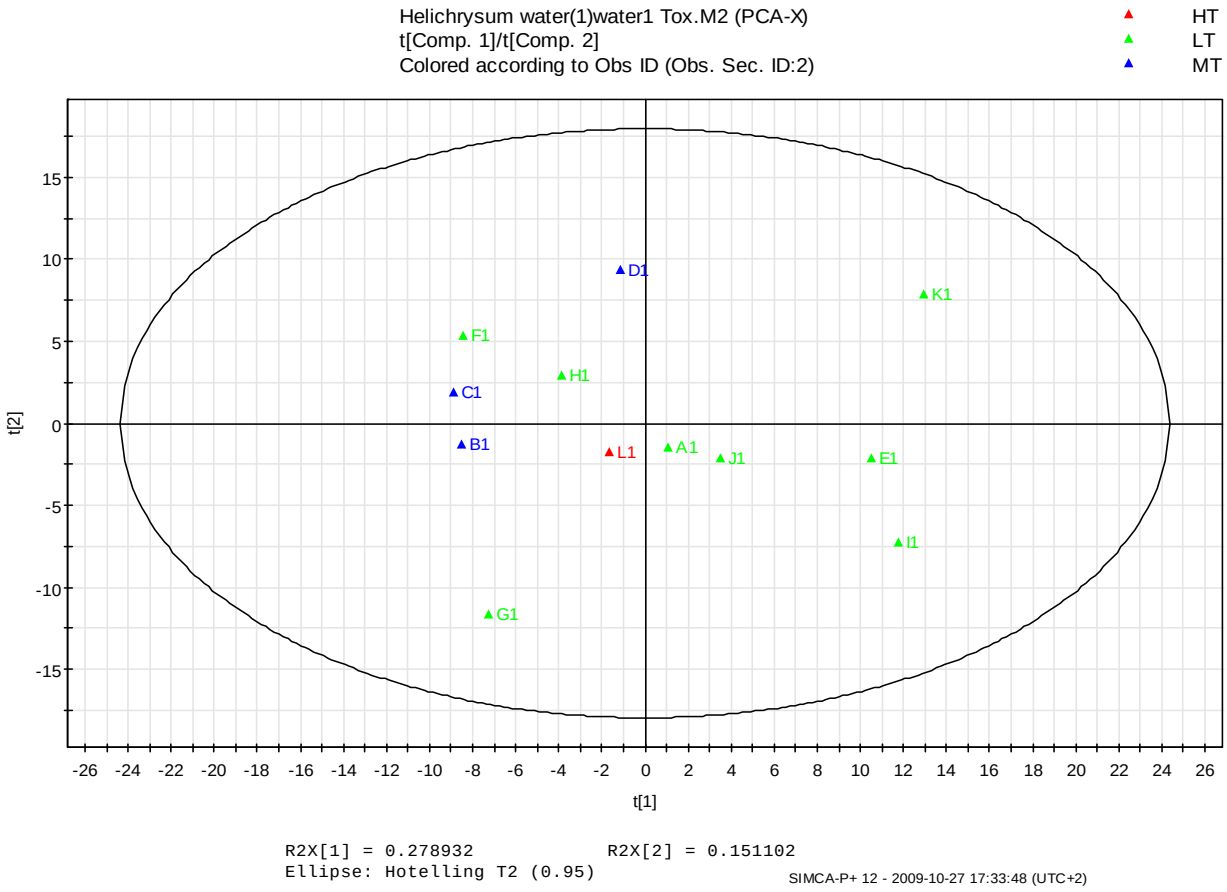

Fig 5.1: PCA scoring plot of the water-methanol extracts' cytotoxicity on vero cells. *H. panduratum* (A1), *H. rugulosum* (B1), *H. aureonitens* (C1), *H. nudifolium* var. *nudifolium* (D1), *H. appendiculatum* (E1), *H. callicomum* (F1), *H. psilolepis* (G1), *H. herbaceum* (H1), *H. acutatum* (I1), *H. populifolium* (J1), *H. adenocarpum* (K1) and *H. cymosum* subsp. *cymosum* (L1). (HT – high toxicity, MT – medium toxicity, LT – low toxicity)

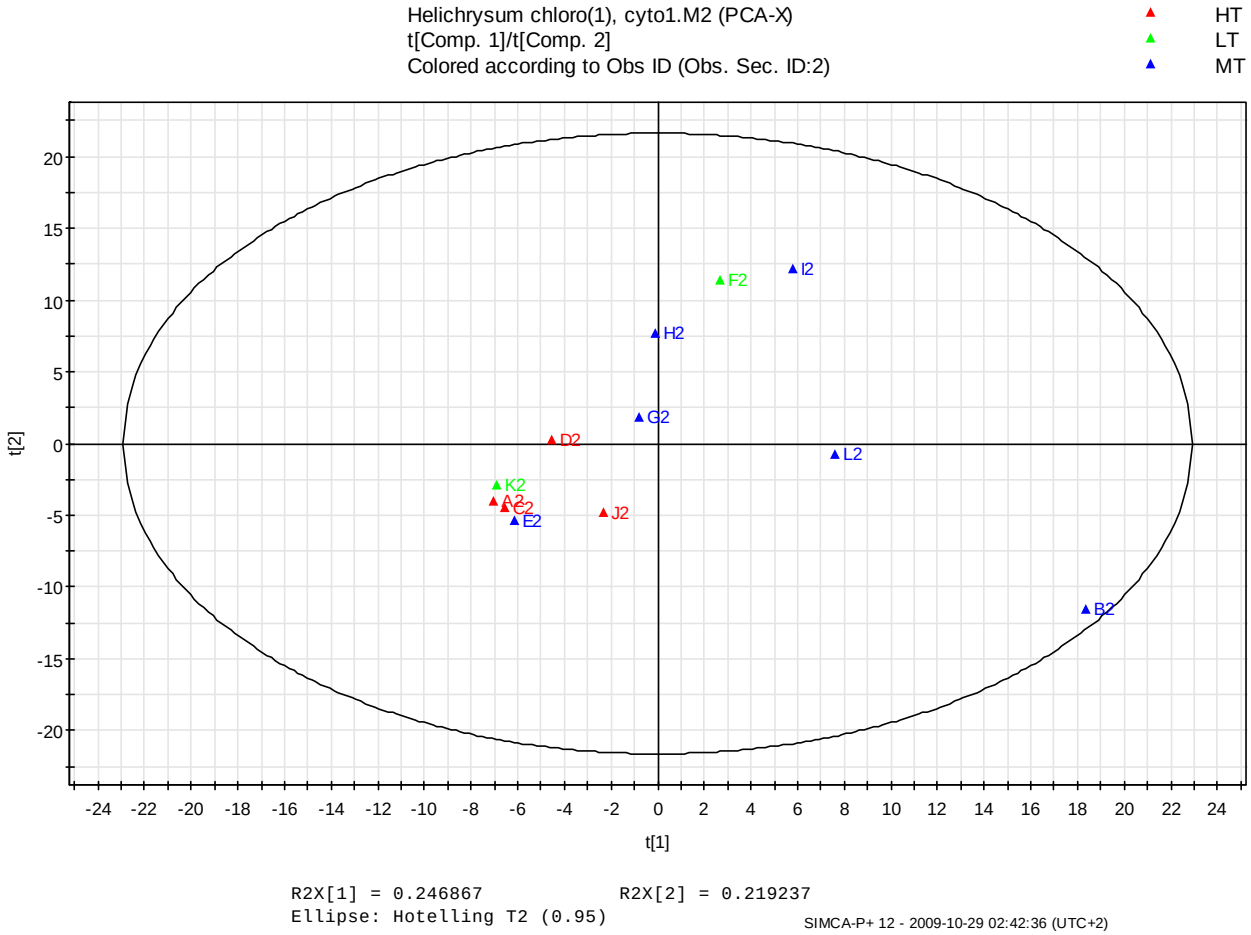

Fig 5.2: PCA scoring plot of the chloroform extracts' cytotoxicity on vero cells. *H. panduratum* (A2), *H. rugulosum* (B2), *H. aureonitens* (C2), *H. nudifolium* var. *nudifolium* (D2), *H. appendiculatum* (E2), *H. callicomum* (F2), *H. psilolepis* (G2), *H. herbaceum* (H2), *H. acutatum* (I2), *H. populifolium* (J2), *H. adenocarpum* (K2) and *H. cymosum* subsp. *cymosum* (L2). (HT – high toxicity, MT – medium toxicity, LT – low toxicity).

It is interesting to notice in Fig. 5.3 that two different clusters of samples group very closely together (LT and HT, Table 5.1). The dotted line could possibly also be seen as a cluster but once again it will contain both LT and HT samples. This could possibly indicate that there was some error in the analysis (cytotoxicity) of either the LT samples or the HT sample and that the specific toxicity assigned to the samples was incorrect. Good discrimination is seen between the MT and the LT/HT clusters and the contributing intervals were analysed in Fig 5.13.

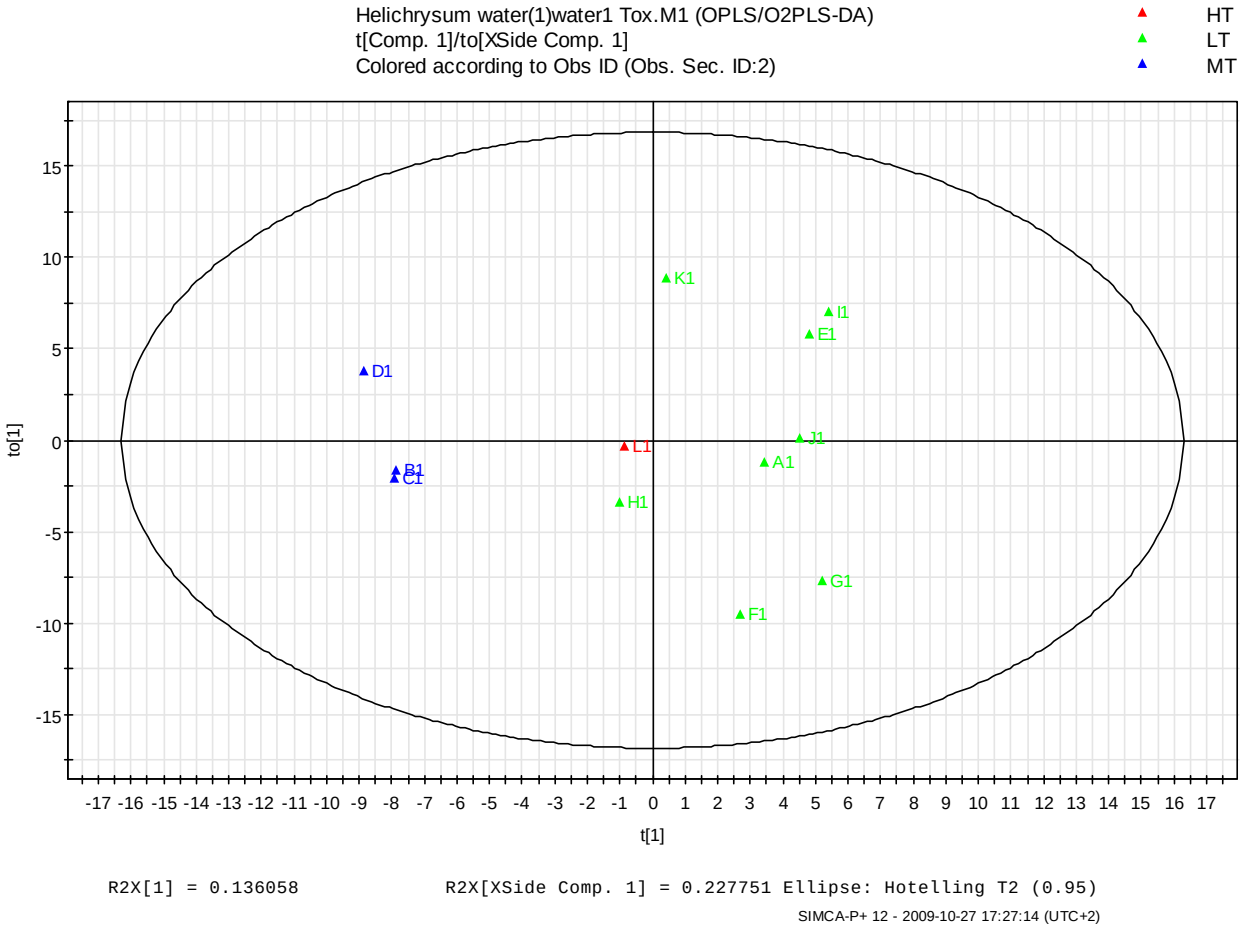

Fig 5.3: OPLS scoring plot of the water-methanol extracts' cytotoxicity on vero cells. *H. panduratum* (A1), *H. rugulosum* (B1), *H. aureonitens* (C1), *H. nudifolium* var. *nudifolium* (D1), *H. appendiculatum* (E1), *H. callicomum* (F1), *H. psilolepis* (G1), *H. herbaceum* (H1), *H. acutatum* (I1), *H. populifolium* (J1), *H. adenocarpum* (K1) and *H. cymosum* subsp. *cymosum* (L1). (HT – high toxicity, MT – medium toxicity, LT – low toxicity)

The OPLS cytotoxicity scoring plot of the chloroform extract (Fig. 5.4) showed three clusters. It is interesting to observe the relative position of the LT cluster to the HT cluster. There is possibly a small difference in the overall chemical composition between the LT samples and the HT samples, but the difference in the chemical composition that exists would most probably be the reason for the levels of toxicity. The MT cluster separates well from the two other clusters indicating definite differences in chemical composition between the samples in the different cluster. A contribution plot was analysed (Fig. 5.14 see section 5.3.2) to establish the differences in the MT and LT clusters

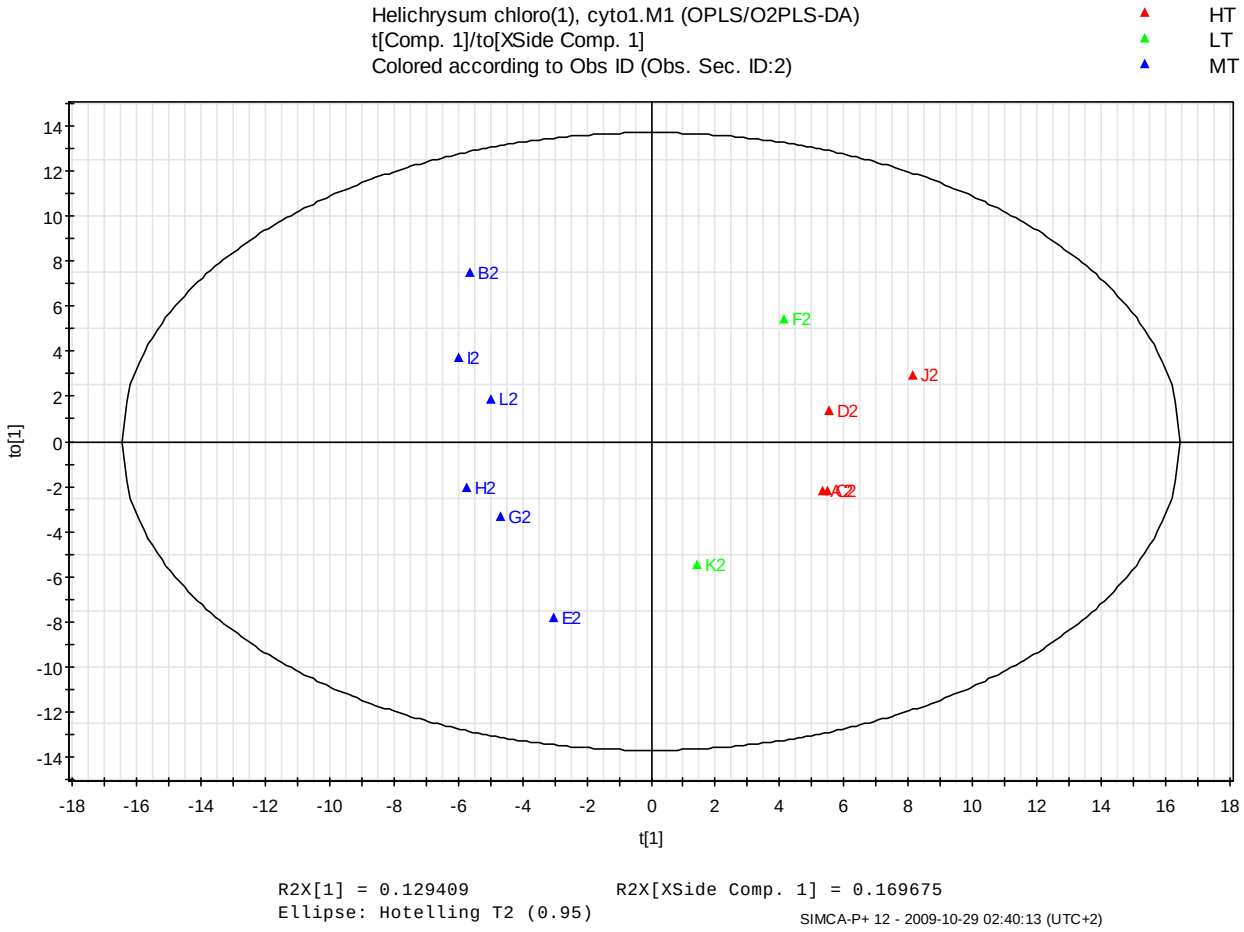

Fig 5.4: OPLS scoring plot of the chloroform extracts' cytotoxicity on vero cells. *H. panduratum* (A2), *H. rugulosum* (B2), *H. aureonitens* (C2), *H. nudifolium* var. *nudifolium* (D2), *H. appendiculatum* (E2), *H. callicomum* (F2), *H. psilolepis* (G2), *H. herbaceum* (H2), *H. acutatum* (I2), *H. populifolium* (J2), *H. adenocarpum* (K2) and *H. cymosum* subsp. *cymosum* (L2). (HT – high toxicity, MT – medium toxicity, LT – low toxicity)

Anti-herpes simplex virus activity

No PCA clustering patterns were observed with the activity against HSV (Fig 5.5 and 5.6). Random distribution of the samples with no specific discrimination based on their activity was observed. Thus it was decided to only use the supervised pattern recognition method, OPLS for the analyses.

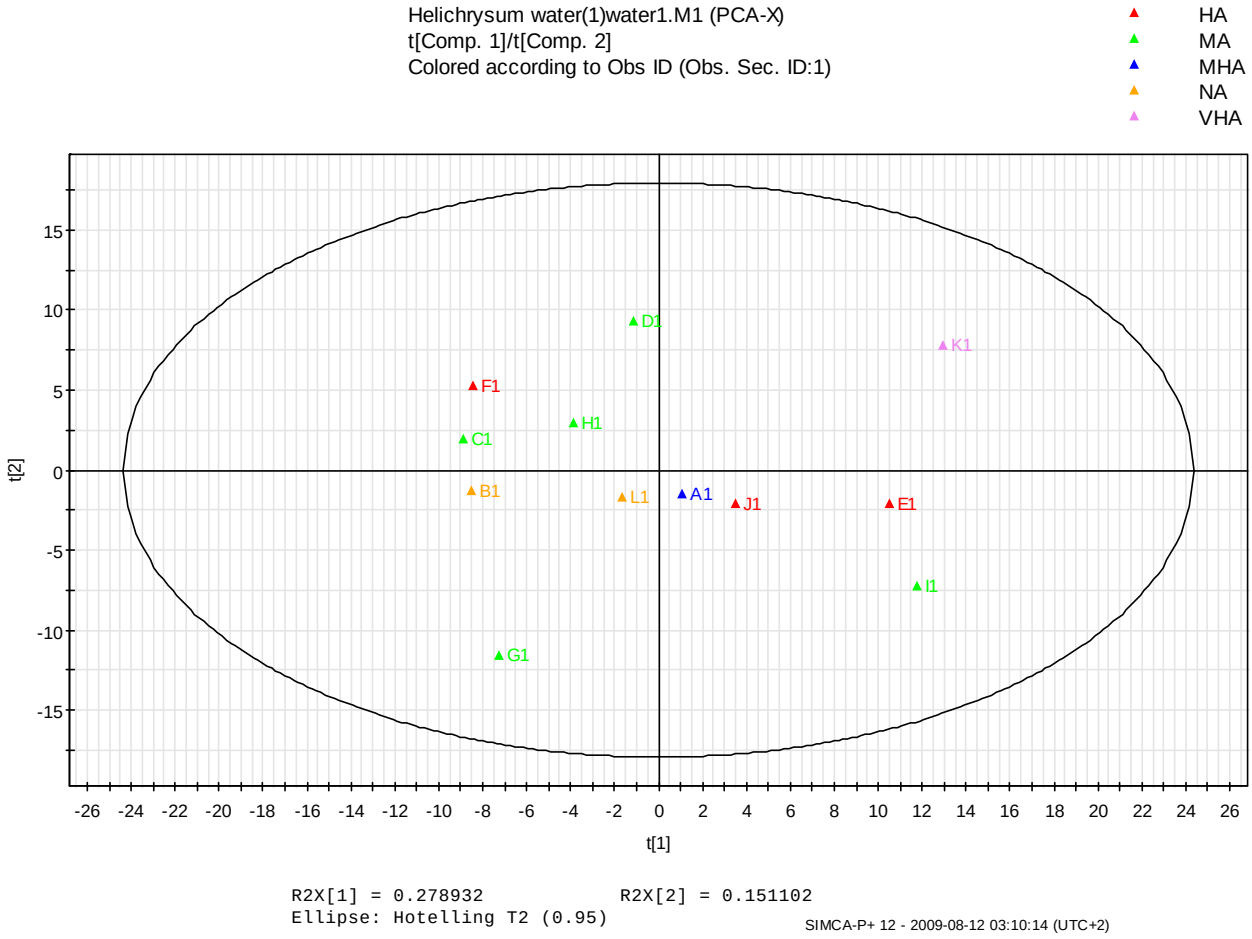

Fig 5.5: PCA scoring plot of the water-methanol extracts' activity against HSV. *H. panduratum* (A1), *H. rugulosum* (B1), *H. aureonitens* (C1), *H. nudifolium* var. *nudifolium* (D1), *H. appendiculatum* (E1), *H. callicomum* (F1), *H. psilolepis* (G1), *H. herbaceum* (H1), *H. acutatum* (I1), *H. populifolium* (J1), *H. adenocarpum* (K1) and *H. cymosum* subsp. *cymosum* (L1). (VHA – very high activity, HA – high activity, MHA – medium to high activity, MA – medium activity, NA – no activity)

The OPLS scoring plot in Fig 5.7 indicated good separation between the extracts with the very high activity (VHA cluster) and those with high activity and medium to high activity (HA and MHA clusters) as well as those with medium activity and no activity (MA and NA clusters). It is interesting to observe the distance between the VHA cluster (K1) sample and that of the remaining samples. The reason for the significant discrimination between the different samples will be investigated in the following section with the help of contribution plots.

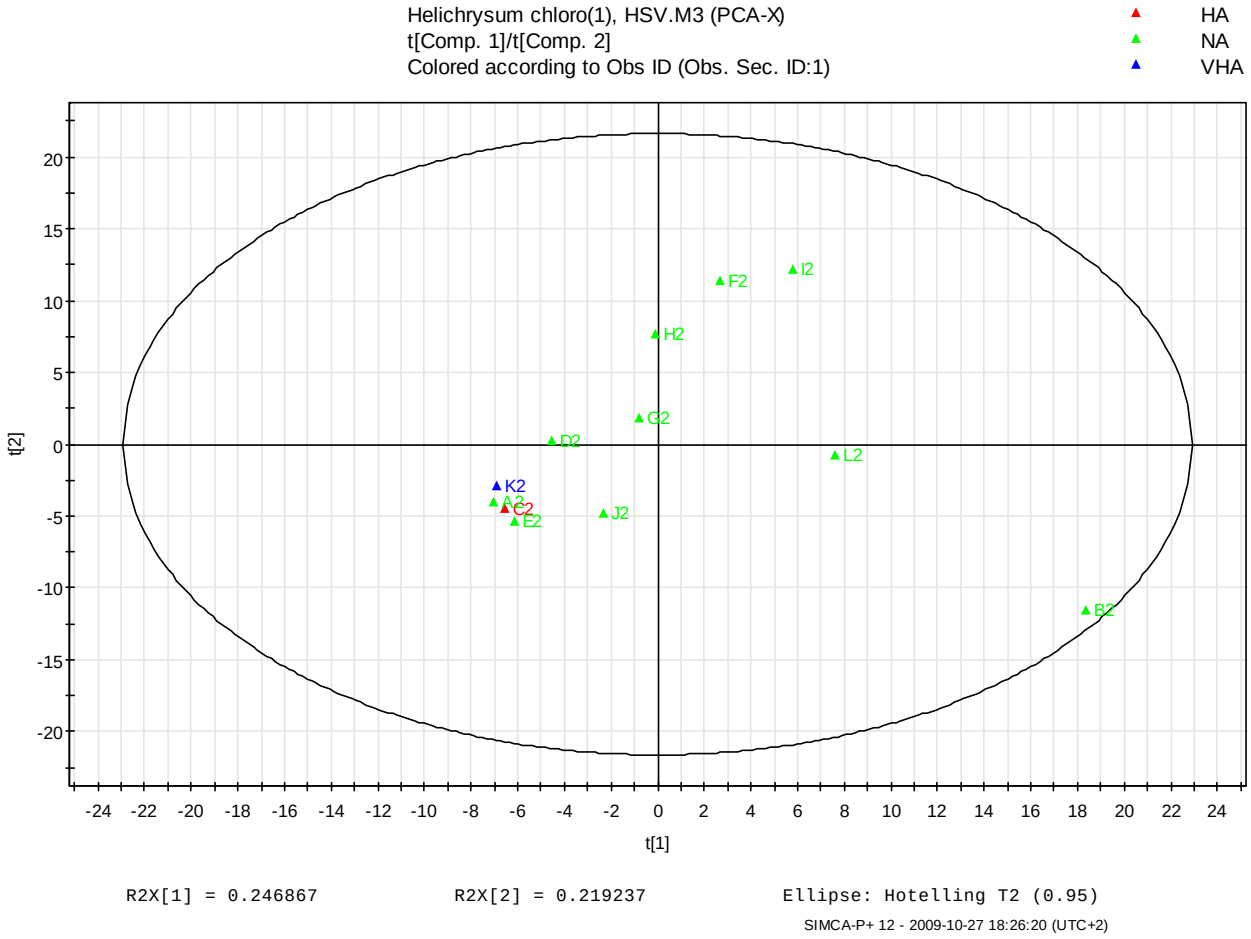

Fig 5.6: PCA scoring plot of the chloroform extracts' activity against HSV. *H. panduratum* (A2), *H. rugulosum* (B2), *H. aureonitens* (C2), *H. nudifolium* var. *nudifolium* (D2), *H. appendiculatum* (E2), *H. callicomum* (F2), *H. psilolepis* (G2), *H. herbaceum* (H2), *H. acutatum* (I2), *H. populifolium* (J2), *H. adenocarpum* (K2) and *H. cymosum* subsp. *cymosum* (L2). (VHA – very high activity, HA – high activity, NA – no activity)

The separation on the y-axis, must also be taken into account for the complete analyses of the group of samples. The variation between the samples in a specific group could be indicative of different levels of the chemical constituents in the samples or the distribution of similar chemical constituents in the different samples and not attributed to the activity. As an example, sample I1 (*H. appendiculatum*) (Fig. 5.7) as a medium active sample separates from other medium active samples in the y-axis (D1,G1 etc.). These samples do group together on the x-axis due to their activity but separate on the y-axis due to other factors, which are not in the scope of this study.

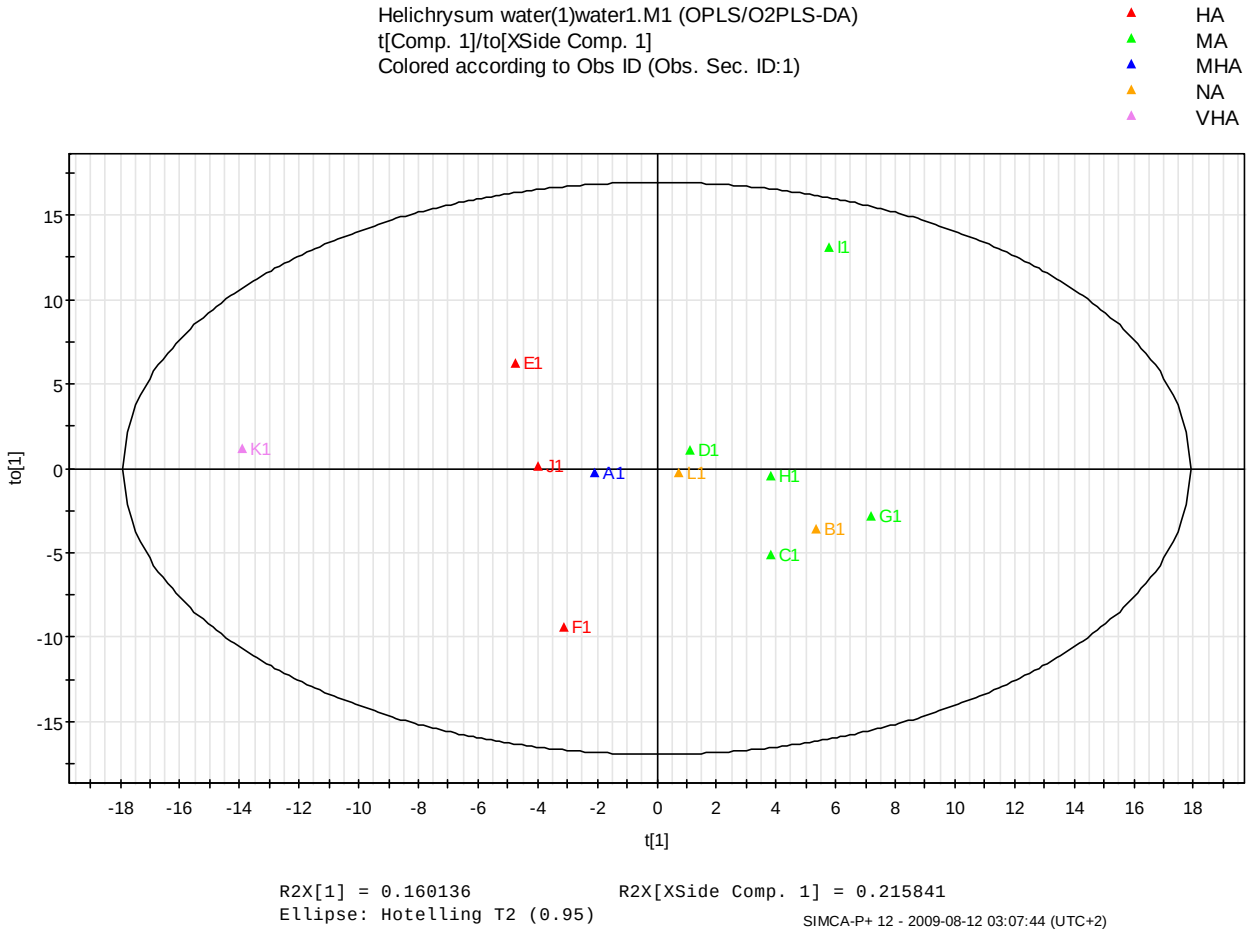

Fig 5.7: OPLS scoring plot of the water-methanol extracts' activity against HSV. *H. panduratum* (A1), *H. rugulosum* (B1), *H. aureonitens* (C1), *H. nudifolium* var. *nudifolium* (D1), *H. appendiculatum* (E1), *H. callicomum* (F1), *H. psilolepis* (G1), *H. herbaceum* (H1), *H. acutatum* (I1), *H. populifolium* (J1), *H. adenocarpum* (K1) and *H. cymosum* subsp. *cymosum* (L1). (VHA – very high activity, HA – high activity, MHA – medium to high activity, MA – medium activity, NA – no activity)

In Fig 5.8 good separation is once again observed, with the HA cluster and VHA cluster grouping together and the NA cluster group grouping very well together. When comparing the clustering of the VHA/HA cluster of Fig. 5.8 to the clustering in Fig. 5.7 it is easier to see that the grouping differs and that in Fig. 5.8 the VHA and HA samples have similar properties which are not the same in Fig. 5.7. The large variation within the NA cluster is once again visible and indicated the variation in chemical composition of the individual samples within the cluster. This variations also don't contribute to the difference in activity.

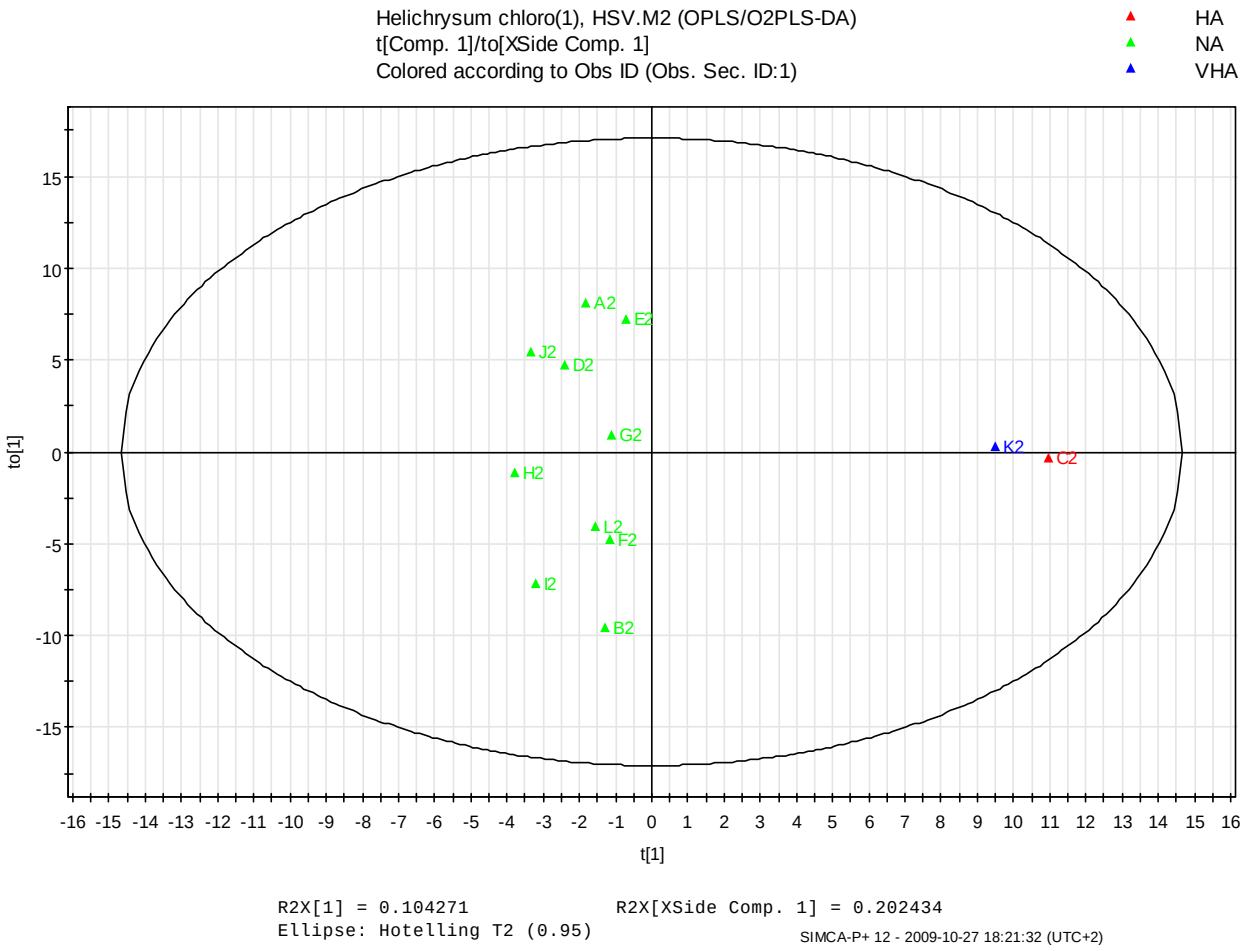

Fig 5.8: OPLS scoring plot of the chloroform extracts' activity against HSV. *H. panduratum* (A2), *H. rugulosum* (B2), *H. aureonitens* (C2), *H. nudifolium* var. *nudifolium* (D2), *H. appendiculatum* (E2), *H. callicomum* (F2), *H. psilolepis* (G2), *H. herbaceum* (H2), *H. acutatum* (I2), *H. populifolium* (J2), *H. adenocarpum* (K2) and *H. cymosum* subsp. *cymosum* (L2). (VHA – very high activity, HA – high activity, NA – no activity)

Anti-human immunodeficiency virus reverse transcriptase activity

The next two scoring plots (Fig. 5.9 and Fig. 5.10) do not show any discrimination between the samples on the basis of their anti-HIV RT activity.

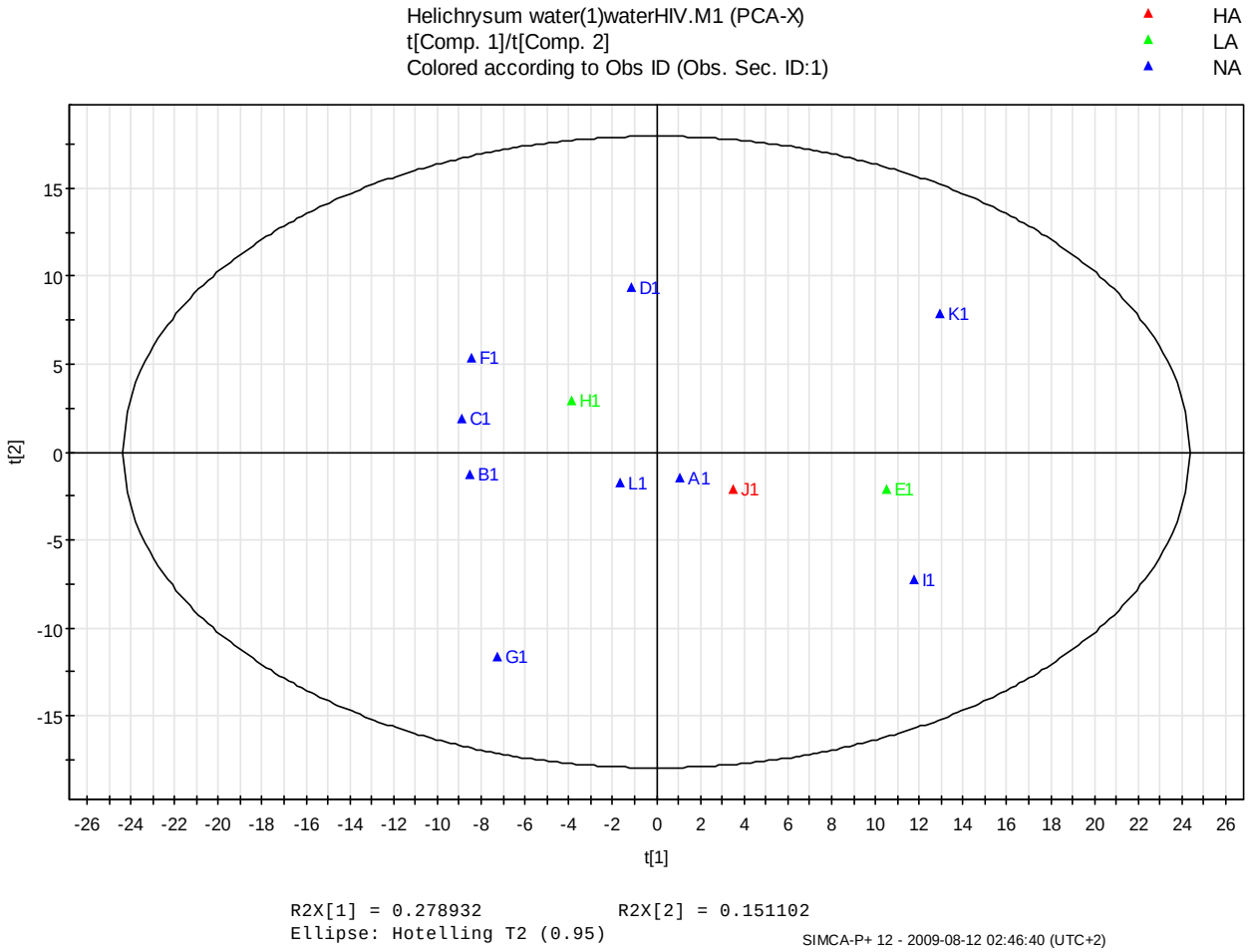

Fig 5.9: PCA scoring plot of the water-methanol extracts' activity against HIV-1 RT. *H. panduratum* (A1), *H. rugulosum* (B1), *H. aureonitens* (C1), *H. nudifolium* var. *nudifolium* (D1), *H. appendiculatum* (E1), *H. callicomum* (F1), *H. psilolepis* (G1), *H. herbaceum* (H1), *H. acutatum* (I1), *H. populifolium* (J1), *H. adenocarpum* (K1) and *H. cymosum* subsp. *cymosum* (L1). (HA – high activity, LA – low activity, NA – no activity)

The OPLS scoring plot of HIV-1 RT of the water-methanol extracts showed three clusters (Fig 5.11). The HA cluster and LA cluster do separate from the NA cluster and the reason for this will be explained in the following section (5.3.2).

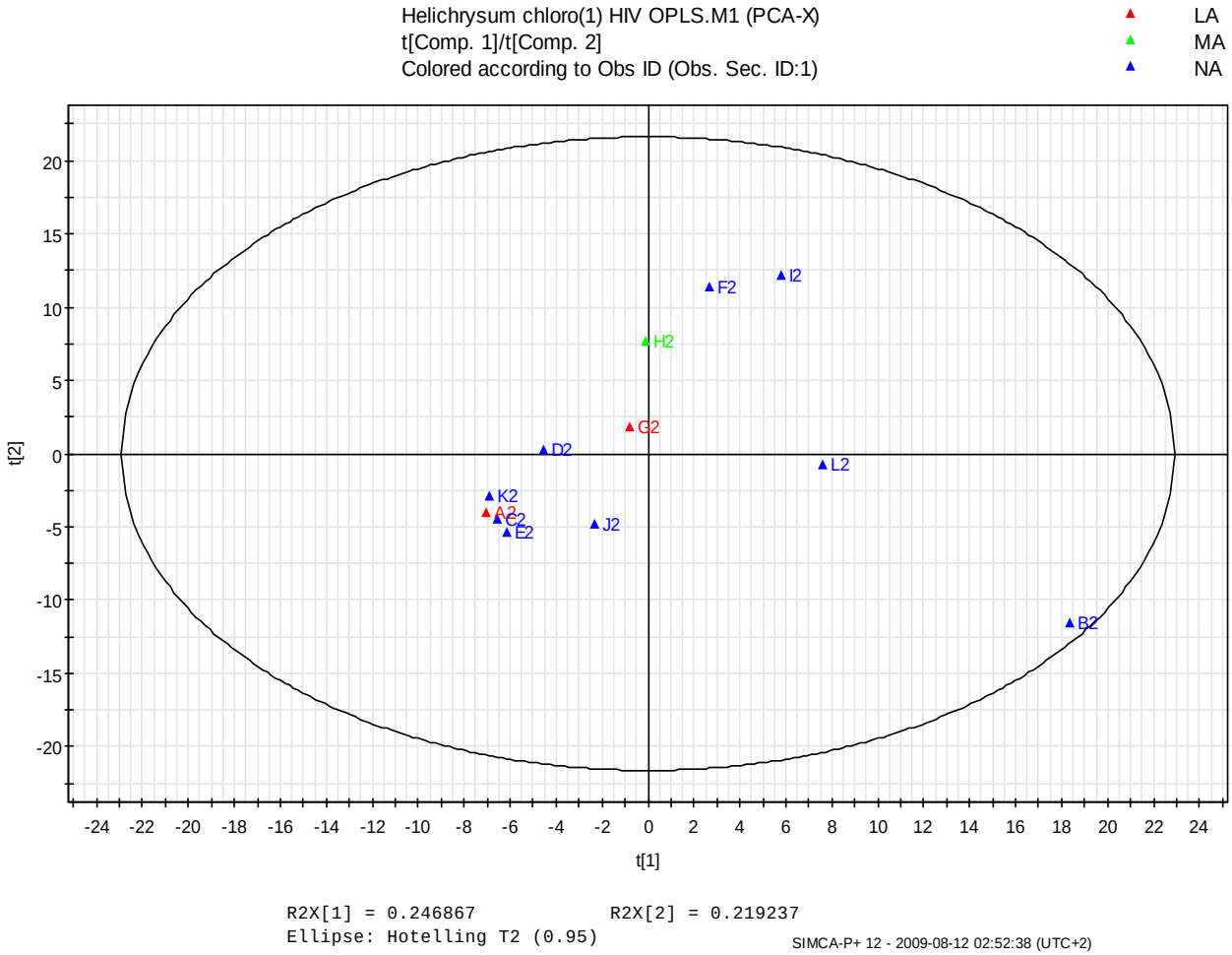

Fig 5.10: PCA scoring plot of the chloroform extracts' activity against HIV-1 RT. *H. panduratum* (A2), *H. rugulosum* (B2), *H. aureonitens* (C2), *H. nudifolium* var. *nudifolium* (D2), *H. appendiculatum* (E2), *H. callicomum* (F2), *H. psilolepis* (G2), *H. herbaceum* (H2), *H. acutatum* (I2), *H. populifolium* (J2), *H. adenocarpum* (K2) and *H. cymosum* subsp. *cymosum* (L2). (MA – medium activity, LA – low activity, NA – no activity)

When analysing into the contribution aspect of these clusters it would be interesting to investigate the reason behind H1 clustering so close to J1 (Fig. 5.11) even with different activity levels. It is also very apparent how close the HA sample is to the NA cluster and not to the two MA samples, indicating that the HA sample has different properties contributing to its activity than those of the MA samples.

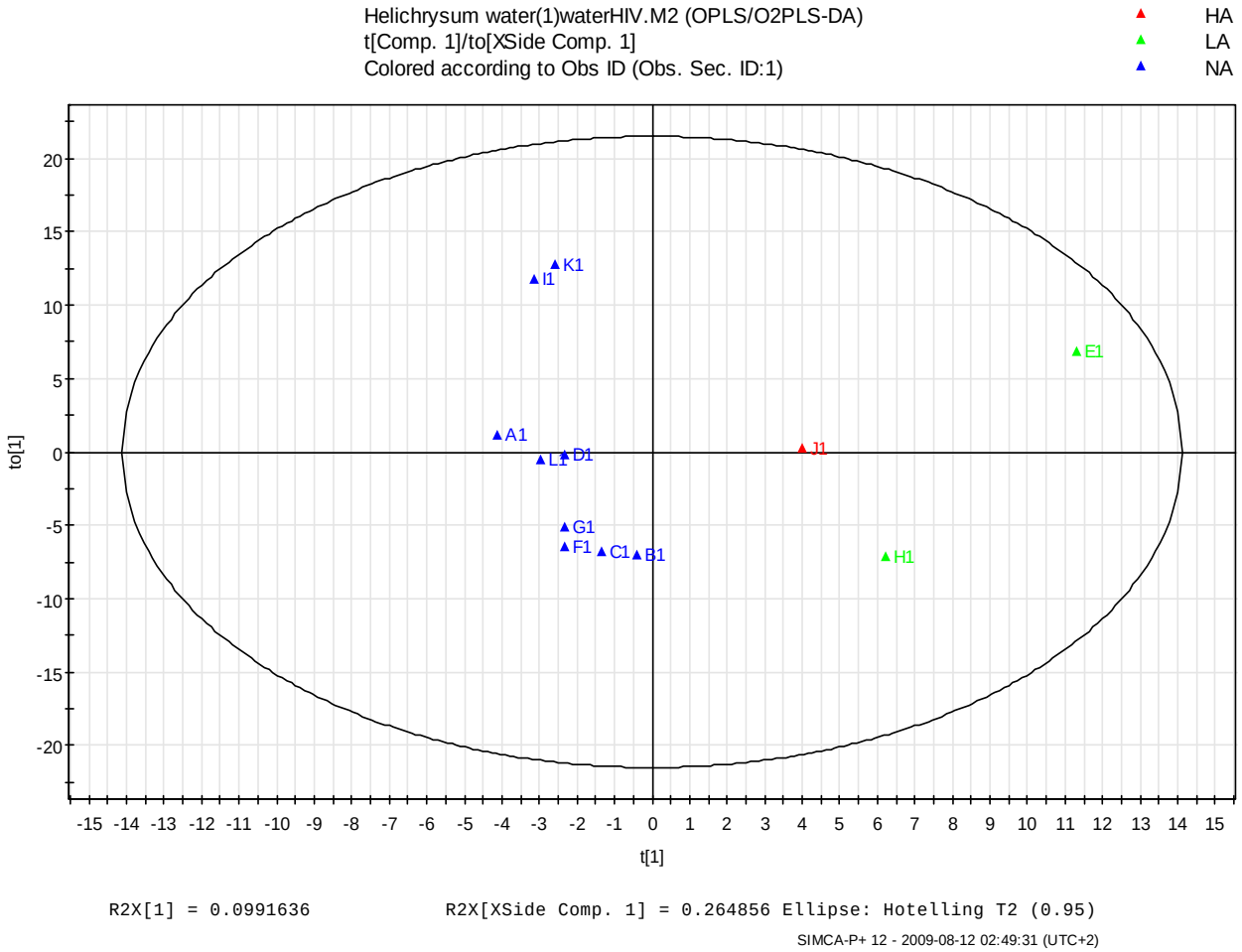

Fig 5.11: OPLS scoring plot of the water-methanol extracts' activity against HIV-1 RT. *H. panduratum* (A1), *H. rugulosum* (B1), *H. aureonitens* (C1), *H. nudifolium* var. *nudifolium* (D1), *H. appendiculatum* (E1), *H. callicomum* (F1), *H. psilolepis* (G1), *H. herbaceum* (H1), *H. acutatum* (I1), *H. populifolium* (J1), *H. adenocarpum* (K1) and *H. cymosum* subsp. *cymosum* (L1). (HA – high activity, LA – low activity, NA – no activity)

The chloroform extracts (Fig 5.12) show a different clustering pattern as compared to that in Fig 5.11. The MA cluster is separated very far from the other two clusters. The LA cluster and NA cluster on the other hand cluster very close to each other indicating very similar characteristics. In the NA cluster, there is a significant variation in the y-axis, this could be indicative of different chemical composition of the different samples.

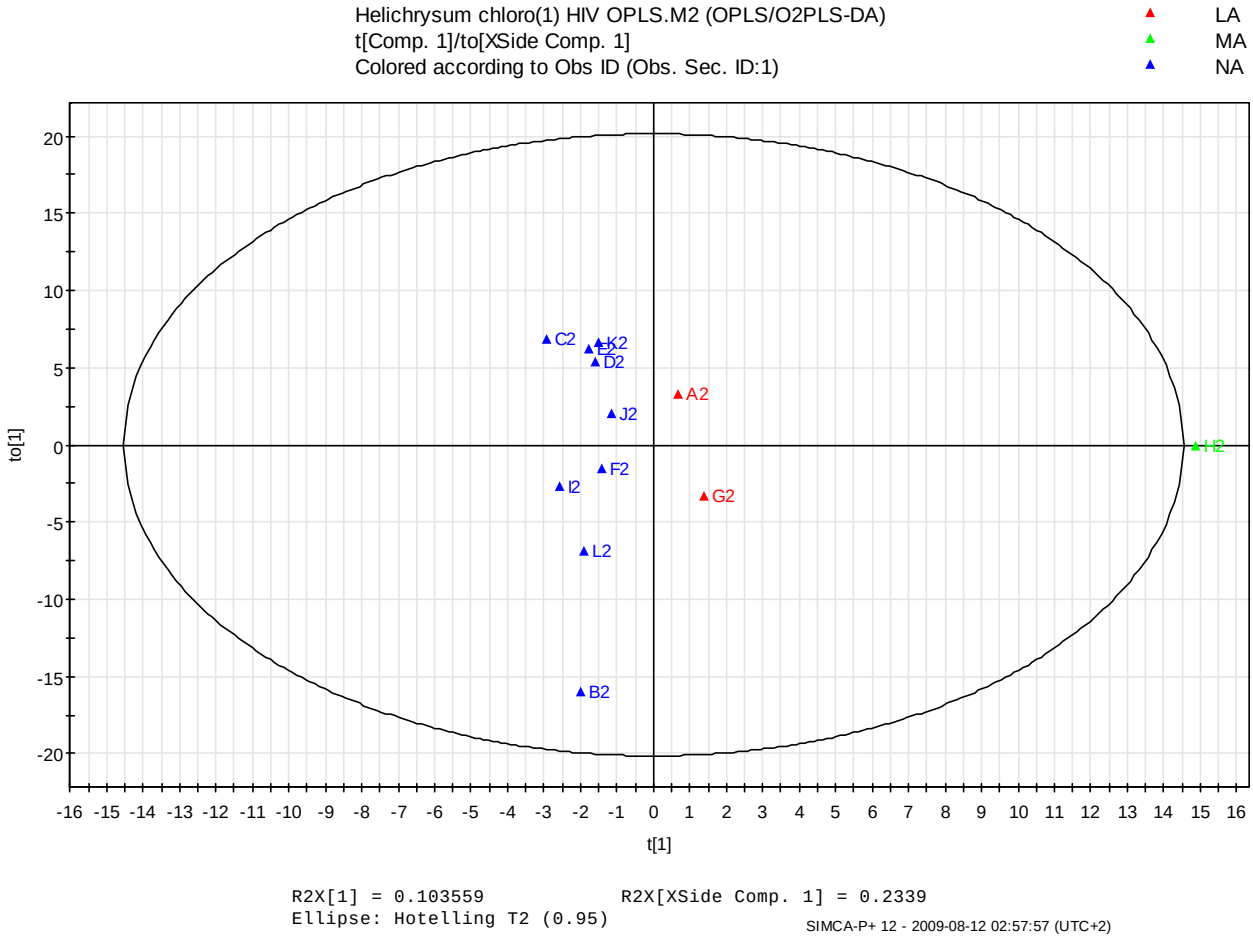

Fig 5.12: OPLS scoring plot of the chloroform extracts' activity against HIV-1 RT. *H. panduratum* (A2), *H. rugulosum* (B2), *H. aureonitens* (C2), *H. nudifolium* var. *nudifolium* (D2), *H. appendiculatum* (E2), *H. callicomum* (F2), *H. psilolepis* (G2), *H. herbaceum* (H2), *H. acutatum* (I2), *H. populifolium* (J2), *H. adenocarpum* (K2) and *H. cymosum* subsp. *cymosum* (L2). (MA – medium activity, LA – low activity, NA – no activity)

5.3.2 Contribution plot analysis

A contribution plot is used to compare different clusters in the above mentioned scoring plots. Where distinct clustering of samples have been observed these contributing factors can be further investigated by drawing up contribution plots. These plots indicate the specific regions that contribute the most to the specific discrimination between the samples. These specific regions will in future be compared to their original NMR spectra (Chapter 8) to identify (if possible) the specific constituent(s) responsibly for the highest activity.

In Fig 5.13 (cytotoxicity effect of the water-methanol extracts' on the vero cells) the buckets/intervals that mainly contribute to the low toxicity are 8.24 ppm – 10.00 ppm, 6.44 ppm – 7.28 pp and 1.84 ppm – 3.04 ppm. The relative high intensity of the region at 8.24 ppm – 10.00 ppm could indicate the importance of the region on the low toxicity nature of the specific samples. The buckets/intervals projecting down (4.32 ppm – 5.12 ppm, 3.16 ppm – 3.64 ppm and 0.00 ppm – 1.72 ppm) would thus represent the regions of the NMR spectra that contribute to the higher toxic nature of the specific samples.

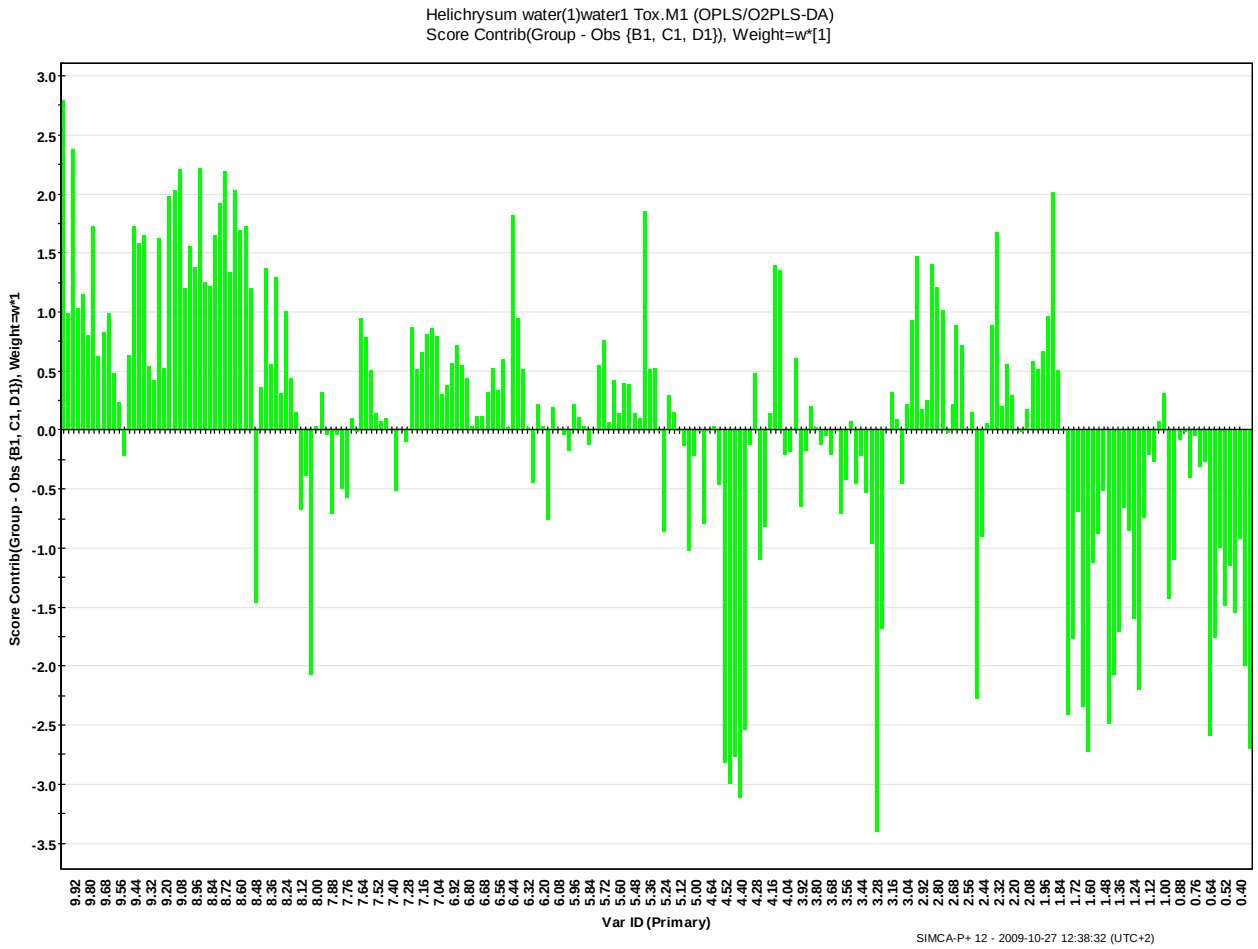

Fig 5.13: Contribution plot of the water-methanol extracts' cytotoxicity on the vero cells. Comparison between the MT cluster and LT cluster. (Bars projecting upwards are the intervals/buckets of the LT cluster). (HT – very high toxicity, MT – medium toxicity, LT – low toxicity)

The contribution plot in Fig. 5.14 indicated the different contributions of the MT cluster and LT cluster in Fig. 5.4 with the chloroform extracts' cytotoxicity effect on the vero

cells. The regions projecting upwards (9.48 ppm – 9.60 ppm, 5.60 ppm – 9.40 ppm and 0.00 ppm – 2.00 ppm) represent the LT cluster and those regions projecting down (9.64 ppm – 10.00 ppm, 5.88 ppm – 9.40 ppm, 4.52 ppm – 5.52 ppm and 2.64 ppm – 4.04 ppm) represent the MT cluster.

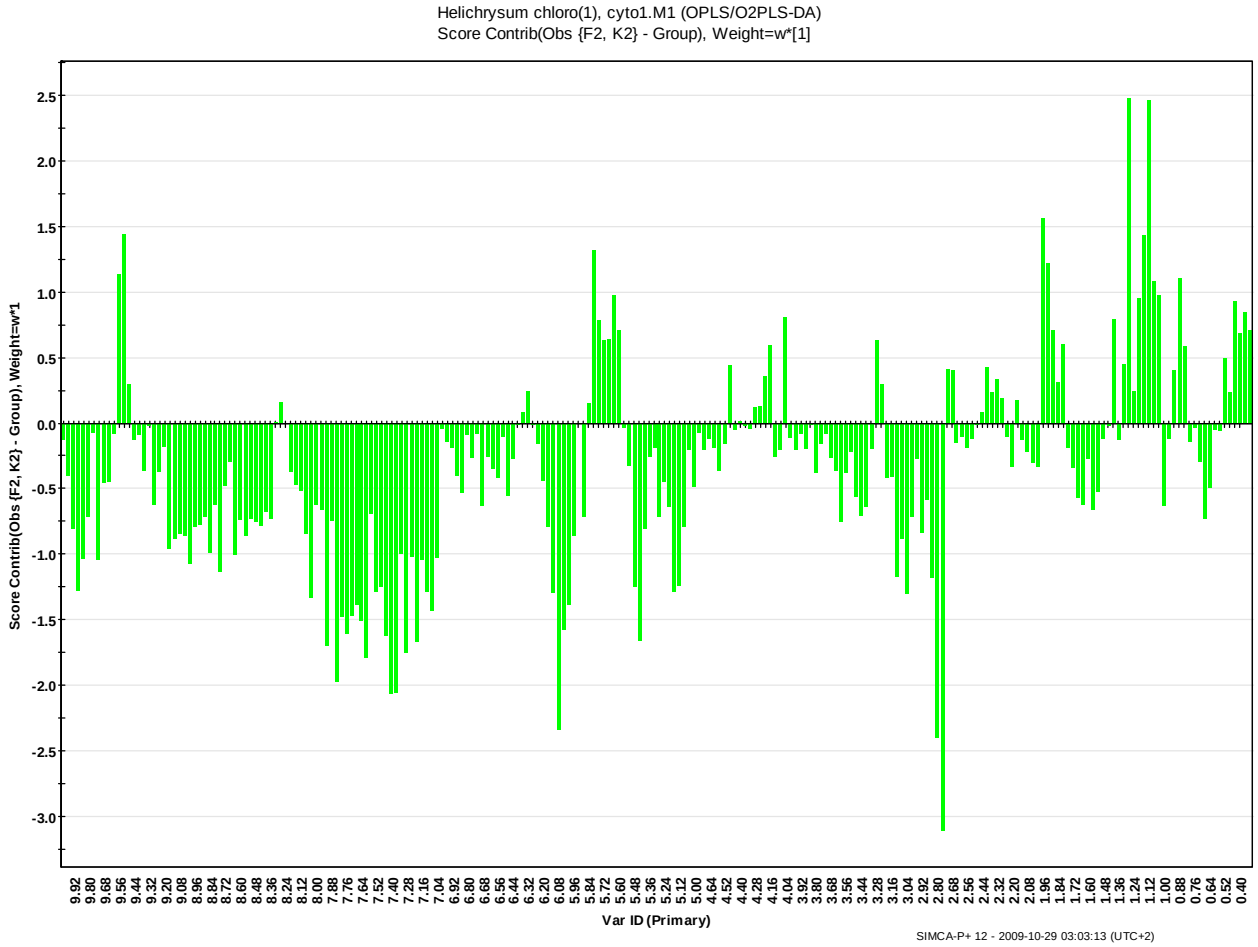

Fig 5.14: Contribution plot of the chloroform extracts' cytotoxicity on the vero cells. Comparison between the LT cluster and MT cluster. (Bars projecting up wards are the intervals/buckets of the LT cluster). (HT – very high toxicity, MT – medium toxicity, LT – low toxicity)

In Fig 5.15 the OPLS scoring plot of the water-methanol extracts activity against HSV (Fig 5.7) is investigated and more specifically the VHA cluster (K1 – *H. adenocarpum* subsp. *adenocarpum*) and the MHA/HA cluster. In this contribution plot the bars projecting upwards represent the buckets/intervals (correlated to the NMR spectra of each sample) that contribute to the K1 being separated from the MHA/HA cluster. The higher the peak of the bucket the more relevant the bucket is and the more it contributes to the position of K1. The bars projecting downwards are representative of the MHA/HA

cluster. These buckets are then also indicative of which buckets are not well represented in the VHA cluster. In Fig 5.15 the buckets from 5.24 ppm to 7.92 ppm, 8.28 ppm to 8.72 ppm and 9.64 ppm to 9.72 ppm are representative of the VHA cluster, which could be indicative of aromatic constituents (phloroglucinols, flavones, flavonoids etc.) being responsible for the discrimination and the activity . The regions from 0.00 ppm to 1.84 ppm (possibly some kind of terpenoids) and 3.12 ppm to 4.28 ppm (probable constituents e.g. polysaccharides) are on the other hand are representative of the MHA/HA cluster.

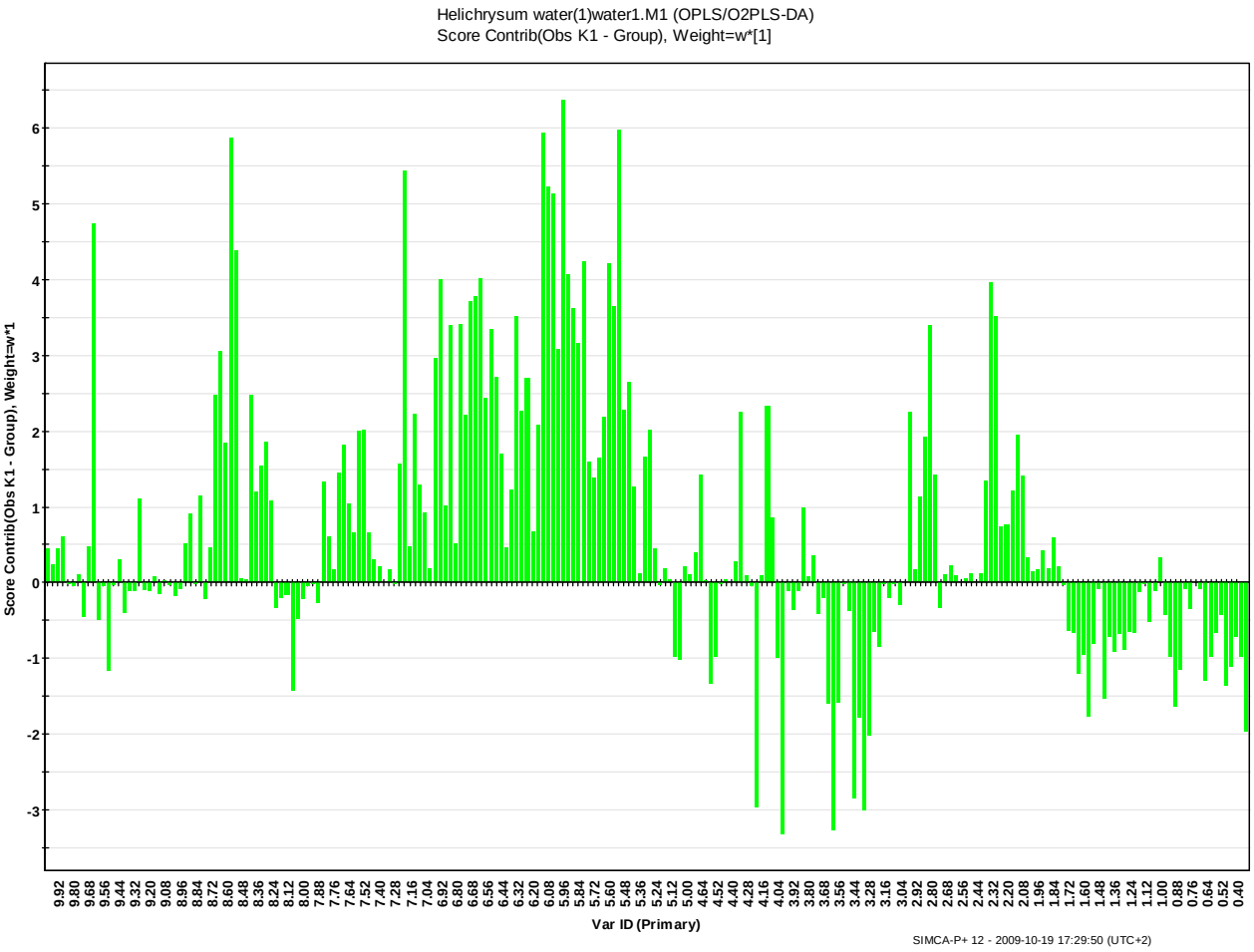

Fig 5.15: Contribution plot of the water-methanol extracts' activity against HSV. Comparison between the MHA/HA cluster and VHA cluster (*H. adenocarpum* subsp. *adenocarpum*). (Bars projecting up wards are the intervals/buckets of the VHA cluster). (VHA – very high activity, HA – high activity, MHA – medium to high activity)

Taking the contribution plot of the chloroform extracts activity against HSV (Fig 5.16) into consideration, the following regions; 9.32 ppm – 9.64 ppm, 5.52 ppm – 5.80 ppm,

4.04 ppm – 4.64 ppm, 1.84 ppm – 2.52 ppm and 0.00 ppm – 1.48 ppm, are mostly representative of VHA/HA cluster. Those contributing to the NA cluster are mostly 9.68 ppm – 10.00 ppm, 7.04 ppm – 9.32 ppm and 2.72 ppm – 3.40 ppm.

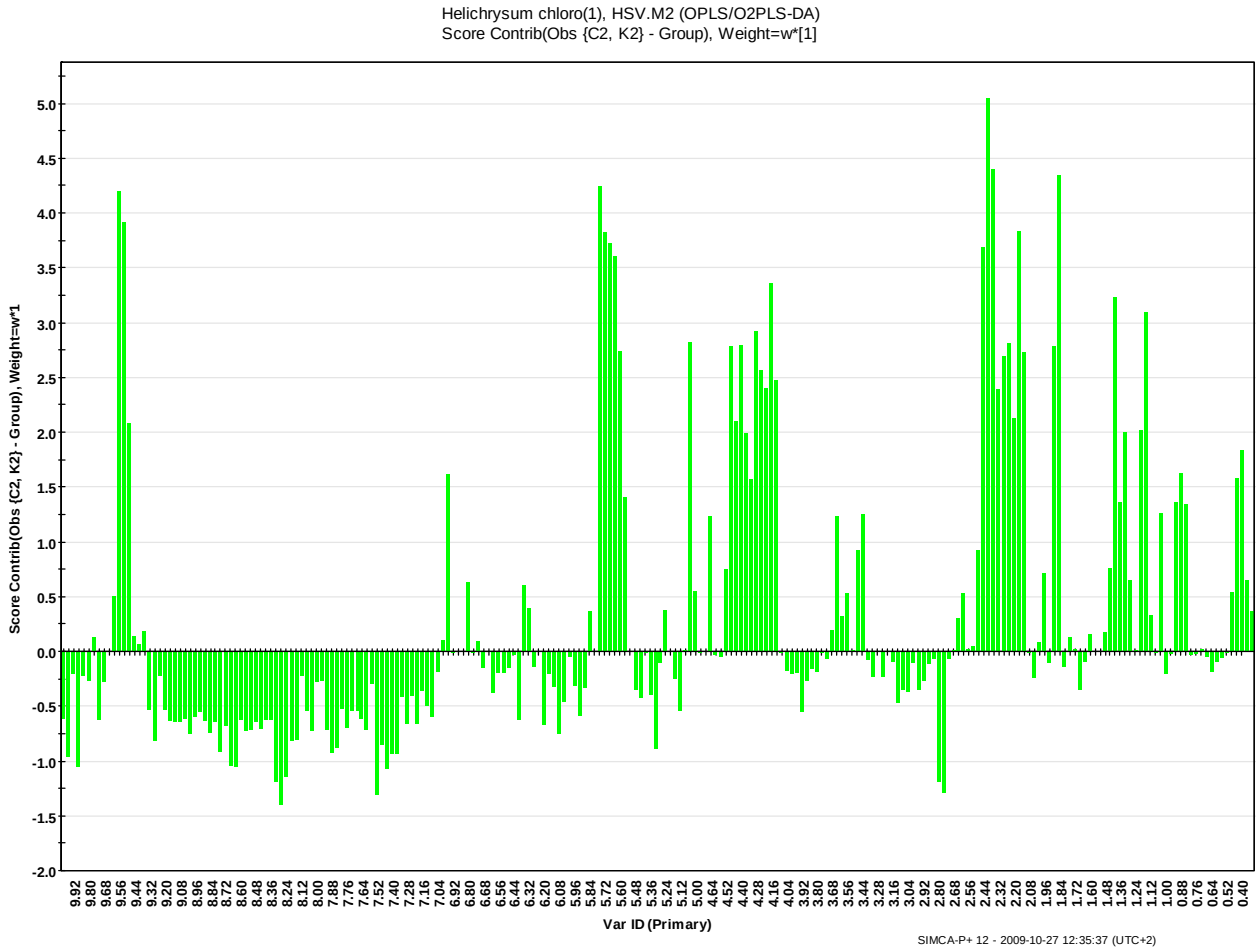

Fig 5.16: Contribution plot of the chloroform extracts' activity against HSV. Comparison between the NA cluster and VHA/HA cluster (*H. adenocarpum* subsp. *adenocarpum* and *H. aureonitens* respectively). (Bars projecting up wards are the intervals/buckets of the VHA/HA cluster). (VHA – very high activity, HA – high activity, NA – no activity)

Fig 5.17 shows the contribution plot of water-methanol extracts with activity against HIV-1 RT. The bars projecting upwards (9.84 ppm – 10.00 ppm, 8.24 ppm – 9.48 ppm, 5.52 ppm – 6.00 ppm, 4.00 ppm – 4.24 ppm and 2.72 ppm – 3.40 ppm) mostly represent the buckets that are contributing to HA (J1) cluster. Those projecting downwards (4.28 ppm – 5.16 ppm, 3.48 ppm – 3.96 ppm and 0.96 ppm – 1.52 ppm) represent NA cluster. There are regions where overlapping occurs but most of the regions contribute as were discussed above.

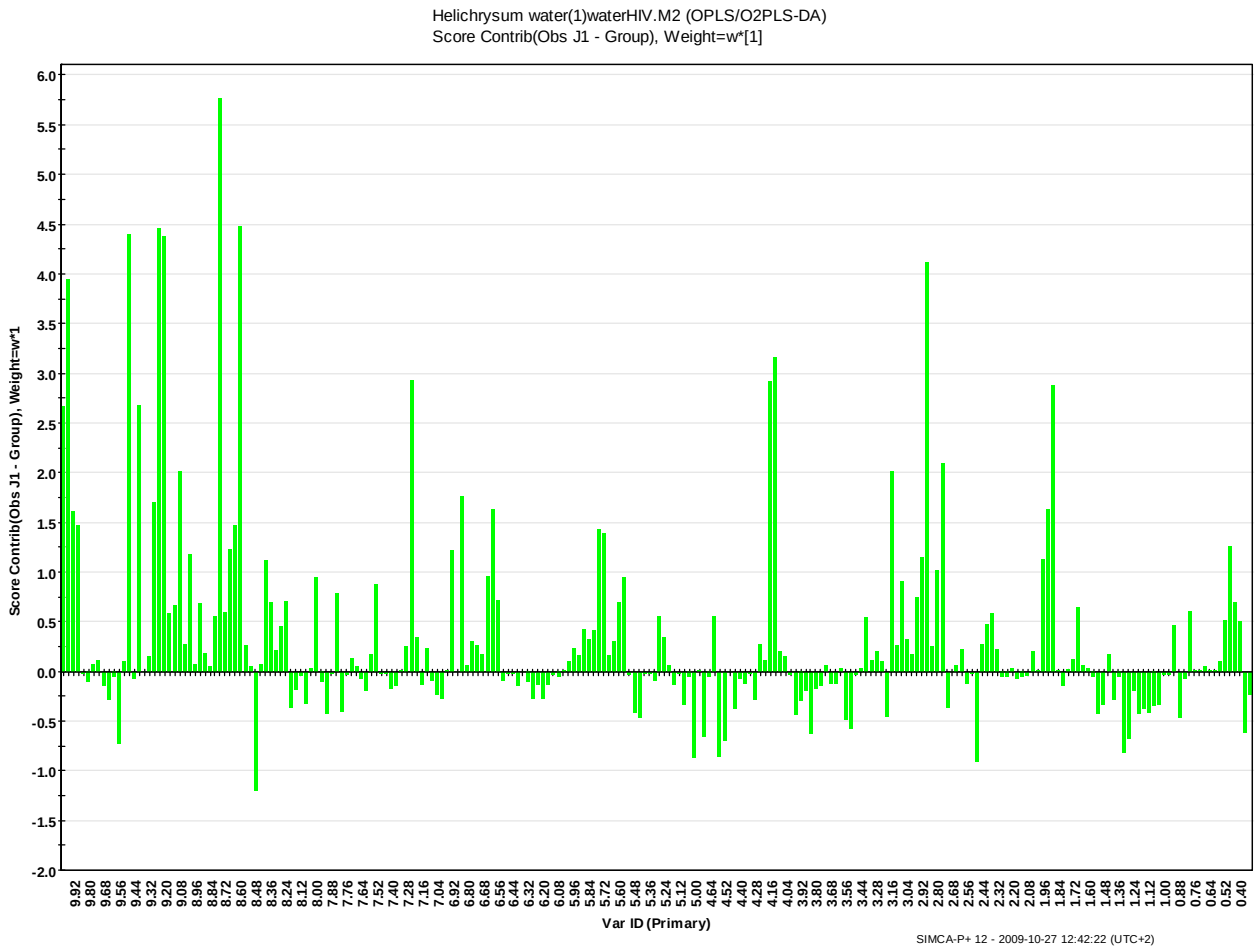

Fig 5.17: Contribution plot of the water-methanol extracts' activity against HIV-1 RT. Comparison between the NA cluster and HA cluster (*H. populifolium*). (Bars projecting up wards are the intervals/buckets of the HA cluster). (HA –high activity, NA – no activity)

The last contribution plot, with the chloroform extracts' activity against HIV-RT (Fig 5.18) has a very clear range of buckets contributing to the discrimination between the two clusters selected (MA – H1 and NA). There are five distinct areas that contribute to the position of H2 (MA) in Fig 5.12. These regions are 7.92 ppm – 8.04 ppm, 7.52 ppm – 7.60 ppm, 6.48 ppm – 6.92 ppm, 6.08 ppm – 6.16 ppm and 3.84 ppm – 4.08 ppm. On the other hand the NA clusters' regions are more broad but not as intense. These include 8.08 ppm – 8.56 ppm, 4.12 ppm – 5.16 ppm and 0.96 ppm – 3.76 ppm.

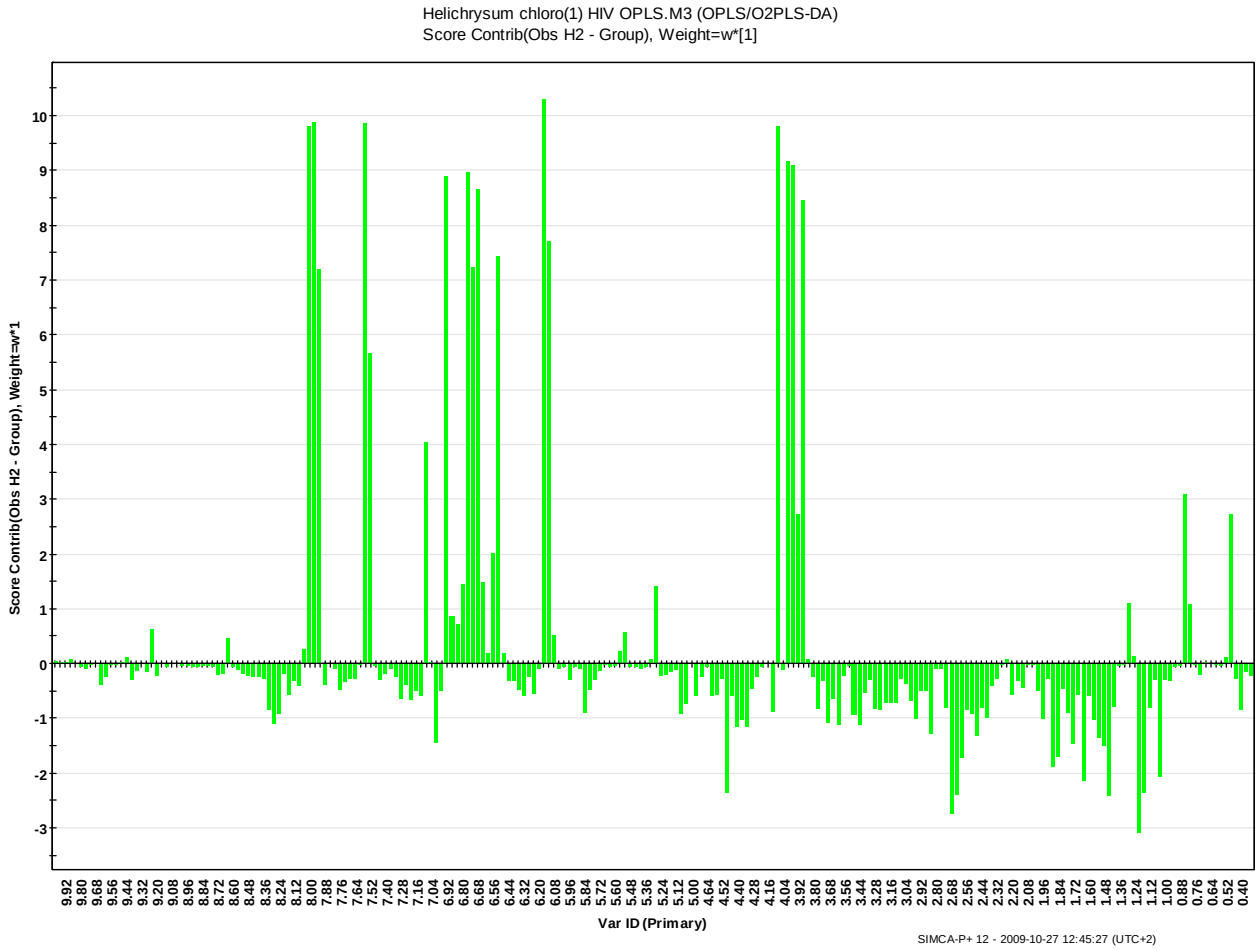

Fig 5.18: Contribution plot of the chloroform extracts' activity against HIV-1 RT. Comparison between the LA cluster and MA cluster (*H. herbaceum*). (Bars projecting up wards are the intervals/buckets of the MA cluster). (MA – medium activity, LA – low activity)

5.4 Discussion and Conclusion

The main aim of this study was to determine if it is possible to use metabolomics as an investigation tool in search of anti-viral properties in the *Helichrysum* species and in future in many other plant species. This method is hypothesized to have the potential to reduce the effort and time spent in search of the active constituents, active fractions or active extracts. In this study the correlation between the activity on HSV and HIV-1 RT and the chemical composition was investigated with the use of multivariate data analysis. From the results obtained a database could be compiled to help in future

searches for activity of the *Helichrysum* species, of which more than 200 South African species have not been investigated for activity on HSV and HIV-1 RT.

Using the PCA in this study did not show any conclusive discrimination between the different plant extract samples. No deductions could be made from this pattern recognition method, due to no separations taking place (Fig 5.1, 5.2, 5.5, 5.6, 5.9 and 5.10).

The use of a supervised method was then used for the analyses, i.e. OPLS. Using the OPLS pattern recognition method, factors like the activity were also incorporated into the dataset. Thus with OPLS, factors like the activity (or no activity) of a specific plant extract is taken into consideration. The OPLS method discriminated very well between the different active plant extracts.

The OPLS scoring plot of the water-methanol extracts with activity against HSV discriminated very well between the very high active *H. adenocarpum* subsp. *adenocarpum* sample and the rest of the samples. There was also some separation between the samples with high activity and the non active samples in the group. From this OPLS scoring plot, two clusters were taken into consideration and a contribution plot was drawn up (Fig 5.15 – water-methanol extracts activity against HSV). The contribution plot indicated regions that are most probably responsible for the difference in activity. The regions from 5.24 ppm to 7.92 ppm in the VHA sample are very prominent and this region is mostly associated with aromatic constituents. Thus it could be proposed that aromatic constituents (e.g. phloroglucinols or flavonoids) in the VHA sample are responsible or are partially responsible for the activity of the *H. adenocarpum* subsp. *adenocarpum* water-methanol extract. Further research will need to be conducted to find the responsible constituents responsible for the differences between the extracts.

The two active chloroform extracts (with activity against HSV), *H. adenocarpum* subsp. *adenocarpum* (VHA) and *H. aureonitens* (HA) separated very well from the NA cluster (Fig 5.8). Once again the contribution plot was drawn up and the regions responsible for the discrimination were highlighted (Fig 5.16). It was shown that mostly regions with

higher chemical shifts contribute to the specific clustering patterns, highlighting the need to investigate the possibility of aromatic compounds being the major contributor to the activity in these *Helichrysum* ssp.

In Fig 5.11 (OPLS scoring plot of HIV-RT water-methanol extract) the OPLS scoring plot discriminates well between HA/LA cluster from the NA clusters, but then there is also a separation made between the E1 (*H. appendiculatum*) and H1 (*H. herbaceum*) both being LA. This separation could possibly be due to different constituents, but these differences do not necessarily contribute to the activity of the E1. The contribution plot (Fig 5.17) was set up to differentiate between the HA/LA (J1 and H1) cluster and the NA cluster. In the contribution plot regions representing sugar compounds and sugar derivatives compounds and aromatics with acidic/aldehyde as functional groups, contributed the most to the discrimination of the HA/LA cluster form the NA cluster.

Lastly looking at the OPLS scoring plot of the chloroform extracts with activity against the HIV-1 RT (Fig 5.12) and the accompanying contribution plot (Fig 5.18) it can be seen that there is a definite correlation between the weights carried by the different clusters and the position or discrimination on the scoring plots. The regions associated with the MA (*H. herbaceum*) cluster (aromatics and polysaccharides) are very prominent and could be further investigated to establish exactly which chemical constituents are responsible for the prominent peaks. The regions on the contribution plot responsible for the NA cluster are broader but not as intense. These include 8.08 ppm – 8.56 ppm, 4.12 ppm – 5.16 ppm and 0.96 ppm – 3.76 ppm. When investigating these plant extracts further, the last three regions should be avoided not to waste time on isolating chemical constituents with no activity.

This study has shown that it is possible to discriminate between different samples (plant extracts) on the basis of their activity and chemical composition. Some results discriminated well whereas others showed little separation, but still the separation between the clusters was observed. It was also noticed that PCA does not discriminate well between the samples; this could be due to small sample size that was included in the study. It is possible to use the data generated from this study and subsequent other (with a large sample size) to set up an initial database for the *Helichrysum* species, that

further investigation on the 244 species in South Africa and the rest of the 600 plus species in the world could be done for the activities. After establishing a database of a large number of *Helichrysum* spp. with repeated analyses on the bioassays it would then be possible to analyze large number of *Helichrysum* spp. samples without the time consuming bioassays.

Using techniques like metabolomics could in future possibly reduce the time spend on tedious bioassays like the HSV cytopathic effect inhibition bioassay (Chapter 3). If the active constituent(s) are identified with metabolomics and NMR, these could then be isolated, purified and identified for possible future drug candidates. Metabolomics is thus a very powerful tool that can help speed up future drug discovery.

5.5 References

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Chapter 6

General Discussion

6.1 General Discussion

In this study the activities of *Helichrysum* species against the herpes simplex virus and HIV RT enzyme of HIV virus was determined and could confirm to a certain extend the traditional use of some of the plants as a skin infection treatment. This could most probably be due to the activity of some of the plants against HSV-1 (see Chapter 3). The plants selected for this specific study ranged from plants with different morphology, different geography and as well as different traditional uses. The results also ranged from high activity to no activity and from high toxicity to low toxicity, thus the need arises to identify the specific constituents responsible for either the activity or the toxicity.

The water-methanol and chloroform extracts of 12 *Helichrysum* species were tested against herpes simplex virus type 1 (HSV-1) (Table 3.1). *Helichrysum adenocarpum* subsp. *adenocarpum* showed the most potent activity for both its water/methanol and chloroform extracts at 25 µg/ml. Three other *Helichrysum* water/methanol extracts also showed good activity. *H. appendiculatum*, *H. callicomum* and *H. populifolium* all showed some activity levels at 50 µg/ml. The chloroform extracts mainly showed toxicity and complete morphology change of the vero cells. Only two of the 12 extracts had measurable results and were only influenced slightly by toxicity. *H. adenocarpum* subsp. *adenocarpum* and *H. aureonitens* had activities at 25 µg/ml and 100 µg/ml respectively (Table 3.2). Problems were encountered with the solubility of the chloroform extracts in the medium. Due to the non-polar nature of these extracts and the polar bio-assay mediums, the complete solubility of the extracts was not possible. Most of the chloroform extracts and especially at higher concentrations precipitated out which resulted in the precipitate settling on the vero cell monolayer at the bottom of the wells, thus it could be speculated that the precipitate, aggregated around the vero cells and possibly "suffocating the cells and thus leading to higher morphological changed of the vero cells.

The 24 extracts were also tested against the human immunodeficiency virus reverse transcriptase (HIV-RT) enzyme. For the water/methanol extracts only *H. populifolium* showed any significant inhibition. It inhibited the HIV-RT by 63.78 % at 200 µg/ml (Fig

4.2). When compared to previous studies (Woradulayapinij et al., 2005), it can only be seen as moderate active at 200 µg/ml. The chloroform extracts only showed low activity against the HIV-RT at 200 µg/ml, with *H. herbaceum* inhibiting the HIV-RT only by 24.81 % (Fig 4.2), this being classified as a low activity extract when as described by Woradulayapinij et al., (2005). It must be taken into account that this is only one enzyme that is part of the HIV and to fully understand the properties of *Helichrysum* plant species against the HIV, extracts must be tested against the whole virus. The same problem was experienced with the HSV-1 bioassay with the solubility of the non-polar extracts on the HIV-RT bioassay, thus bringing the results obtained in this study into question.

The 12 *Helichrysum* species were also tested for their cytotoxicity on the vero cell line. The general trend that was observed was that the polar, water and methanol extracts were less toxic to the cells than the non-polar, chloroform extracts. The active water/methanol plant extracts all showed cytotoxicity results at the IC_{50} level of 200 µg/ml and higher (Table 2.1). Taking the cytotoxicity and the activity data into account the therapeutic indexes (TI) of the water-methanol plant extract of *H. adenocarpum* subsp. *adenocarpum, H. appendiculatum*, *H. callicomum* and *H. populifolium* were calculated as 8.00, 4.35, 4.72 and 2.95 respectively. The chloroform extracts on the other hand were very toxic with *H. callicomum* being the least toxic plant extract having an IC_{50} concentration value of 94.71 µg/ml (Table 2.2). The TI did not show significant values to indicate future use of these plant extracts. It is noteworthy that the cytotoxic results did not necessarily correlate with the observations made with the cytopathic effect (CPE) bio-assay. The CPE showed morphological changes at toxic concentration levels different from those suggested by the cytotoxicity bio-assay. This highlights the fact that *in vitro* bioassays are not always correct and that caution needs to be taken in using these results.

The use of metabolomics in the search of antiviral properties in *Helichrysum* species is a technique that has huge potential in identifying bioactive plants/compounds and to reduce the time spent on tedious bioassays as well as reducing time spend on the long process of bioassay guided fractionation. In this study it was envisaged that the multivariate data

analysis statistical software would differentiate between the plant extracts on the basis of their differences in chemical composition and activity against the viruses. To differentiate between the plant extracts an unsupervised method (PCA) was used to begin with. This was done to observe if discrimination could be made purely on the basis of the chemical composition using the NMR spectral data. Discrimination using the unsupervised method was not good and thus it was decided to use the OPLS method which is a supervised method. With OPLS the activity results are included as Y variables and thus very good separations were obtained.

With the OPLS on the water/methanol extract against HSV, the very high active *H. adenocarpum* subsp. *adenocarpum* (K1) separated very well from the high active cluster and the non-active cluster (Fig 5.5), thus investigation into the reasons behind this were done. The contribution profile indicated various regions on the NMR spectra that could be associated with the separation between the clusters. The NMR region from 5.20 ppm to 8.00 ppm (Fig 5.9) contributed the most to the separation between the clusters. Speculating on the type of compounds that could be contributing to the discrimination between the clusters, it would be safe to say that theses compounds have a high degree of saturation (5.00 ppm – 6.50 ppm) and are also aromatic (6.50 ppm – 8.00 ppm). Typical aromatic compounds would most probably be flavonoids, flavones or phloroglucinols. Chemical classes such as the flavonoids, acylphloroglucinols and diterpenes (not aromatic) from South African *Helichrysum* species exhibit promising antimicrobial activity and plants that contain these compounds seems potential candidates for further study (Lourens et al., 2008).

In considering the chloroform extracts and their activity it can be seen that the very highly active and highly active plant extract groups, *H. adenocarpum* subsp. *adenocarpum* and *H. aureonitens*, separate well from the medium active and non-active clusters (Fig 5.6). The regions around 5.52 ppm – 5.80 ppm (molecules with high levels of saturation = double bonds) and 4.04 ppm – 4.64 ppm (polysaccharides) are the main contributors to the separation between the samples (Fig 5.10).

Discrimination between the clusters was also obtained for the HIV screening data. The major contributing regions are 9.84 ppm – 10.0 ppm and 8.24 ppm – 9.48 ppm (Fig 5.11), which contribute to the separation between the highly active *H. populifolium* (J1) and the non-active cluster (Fig 5.7).

Further investigation into the above mentioned regions that contributed significantly to the separation of the very highly active and high active plant extract cluster from the low and non-active plant extracts needs to be undertaken to establish witch chemical constituents are responsible for the activity of the specific plant extracts.

With this study it was possible to determine that several *Helichrysum* species have anti-viral properties and that it is possible at least to a certain extent to determine the chemical shifts of the active functional groups responsible for the activity in the active plant extracts. This method of investigation has the potential to identify active chemical constituents and ultimately speed up drug discovery.

It is thus necessary to take this study further to determine the extend of the potential when combining the multivariate analysis with that of NMR in search of anti-viral constituents. It is also necessary to investigate additional species of the *Helichrysum* family. The potential of anti-HSV compounds in the *Helichrysum* family is huge and must be explored in greater depth.

6.2 References

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Chapter 7

Acknowledgements

7.1 Acknowledgements

I would like to thank the following people and institutions:

Prof J. J. Marion Meyer for his supervision, support, comments and intellect throughout the course of this research project.

Prof M. B. Taylor for the training and help with the testing of the various plant extracts against HSV.

Dr. F. van der Kooy for his NMR analysis of the plant extract.

Dr. Johan Trigg for his help with the Chemometrics software and understanding of multivariate analysis

Dr. E. Tshikalange for his help with the HIV – RT bioassay testing.

Mrs. Brigitte Binneman for the help with the Vero cell cytotoxicity testing.

Mr. Tony Abbott for his help in the collection and identification of some of the *Helichrysum* species.

Mrs. Minky Welman from SANBI with her help in searching for and identifying most of the *Helichrysum* species.

Mr. Peter Biller for the training and help on the NMR.

Dr. Quenton Kritzinger, Mrs. Rene Swart and students in our group, for their assistance and comments throughout this research project.

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Chapter 8

Appendixes

8.1 NMR spectra of the water/methanol plant extra

8. 1. 1. *Helichrysum acutatum*

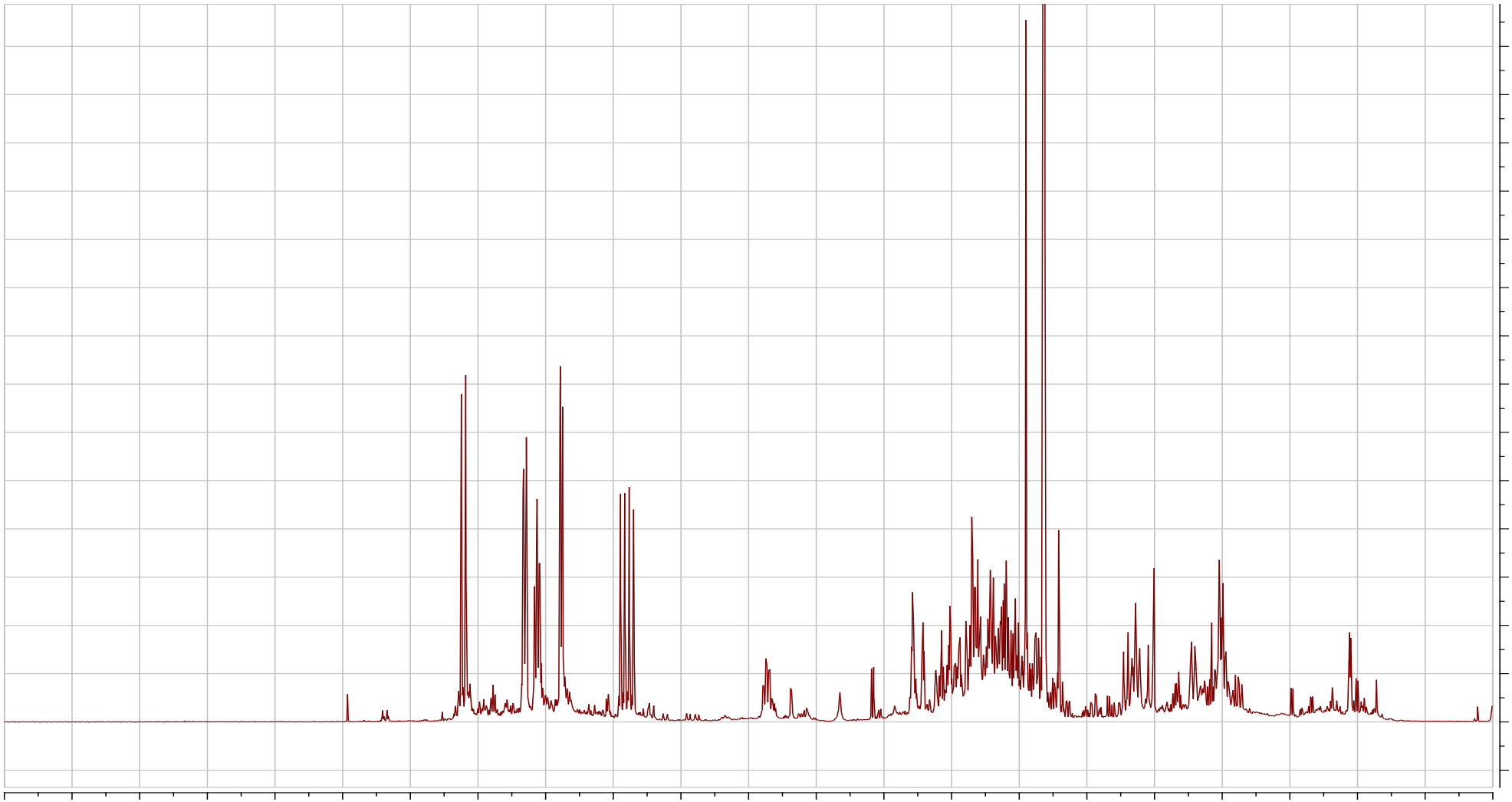

Fig 8. 1: NMR spectrum of the water/methanol plant extract of *H. acutatum* (600 MHz , Leiden University).

8. 1. 2. *Helichrysum adenocarpum* subsp. *adenocarpum*

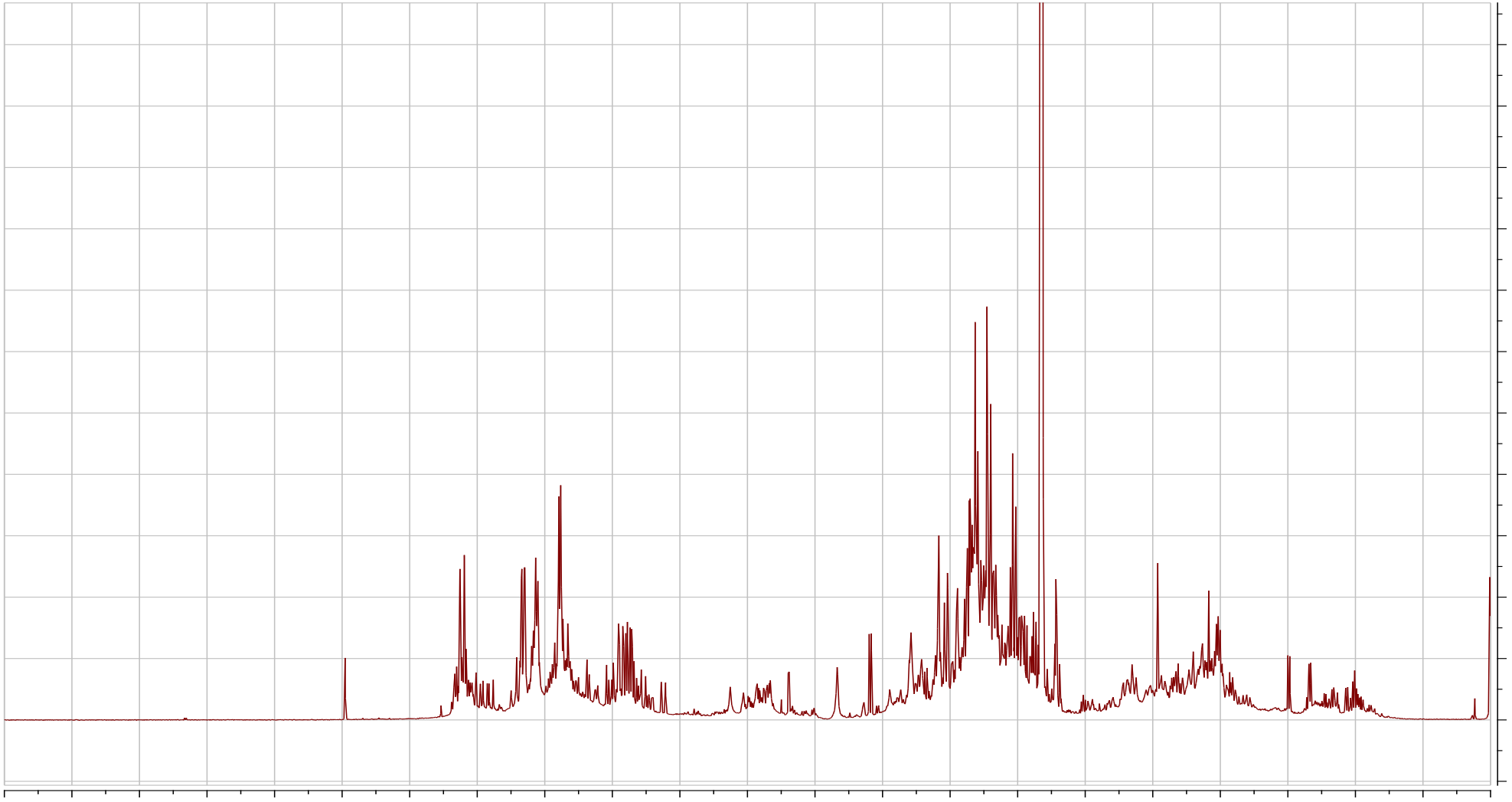

Fig 8. 2: NMR spectrum of the water/methanol plant extract of *H. adenocarpum* subsp. *adenocarpum* (600 MHz , Leiden University).

8. 1. 3. *Helichrysum appendiculatum*

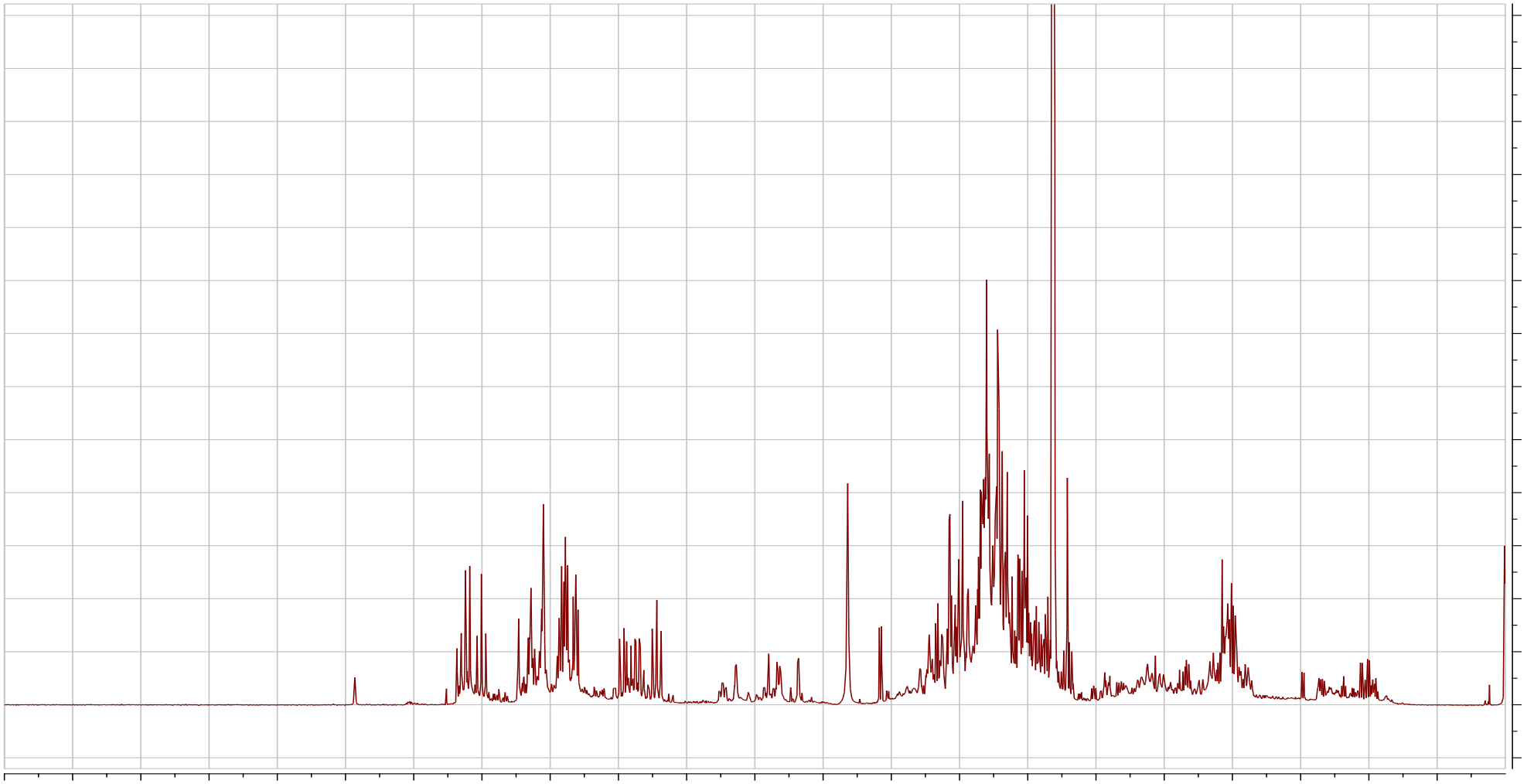

Fig 8. 3: NMR spectrum of the water/methanol plant extract of *H. appendiculatum* (600 MHz , Leiden University).

8. 1. 4. *Helichrysum aureonitens*

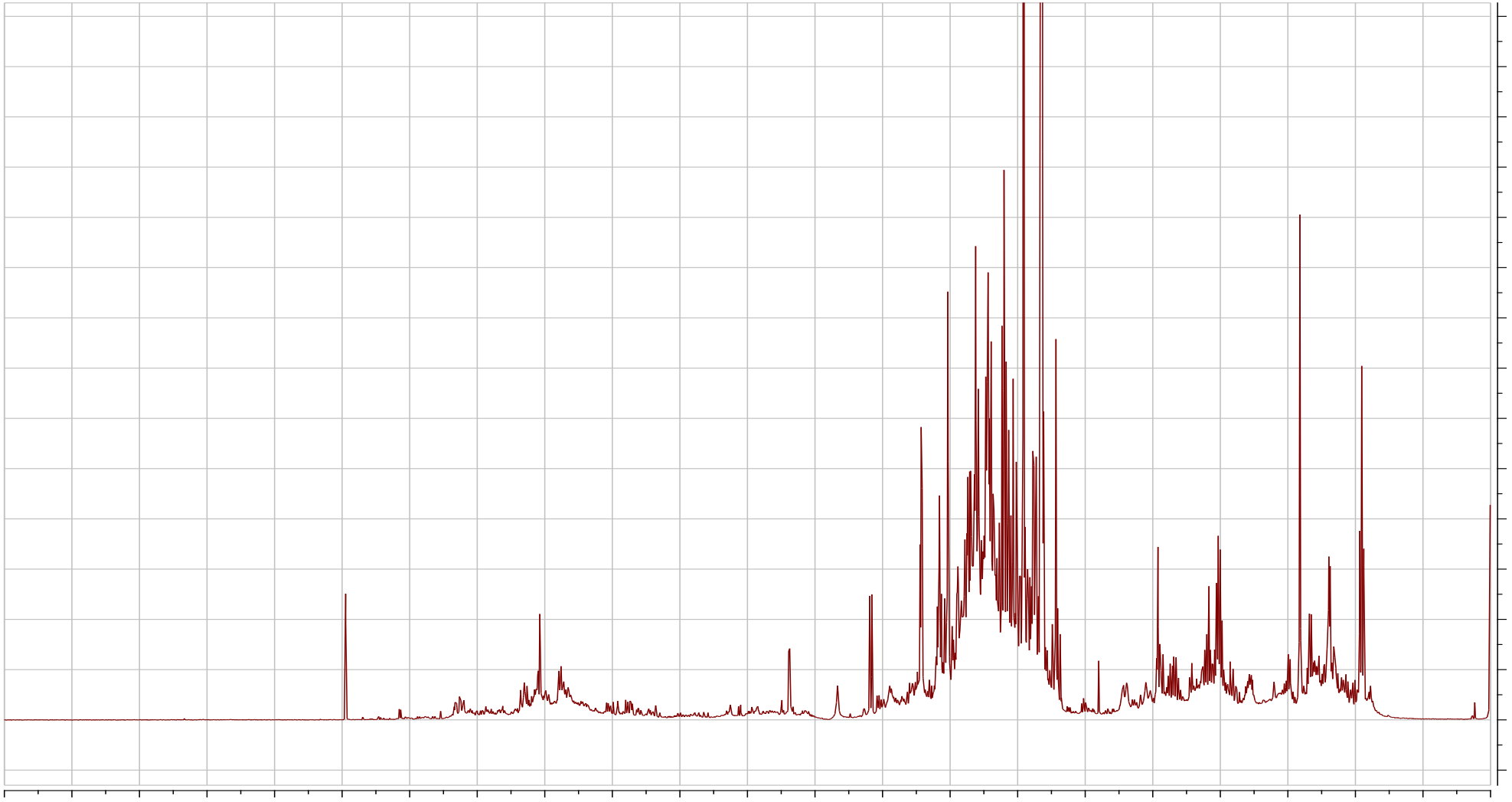

Fig 8. 4: NMR spectrum of the water/methanol plant extract of *H. aureonitens* (600 MHz , Leiden University).

8. 1. 5. *Helichrysum callicomum*

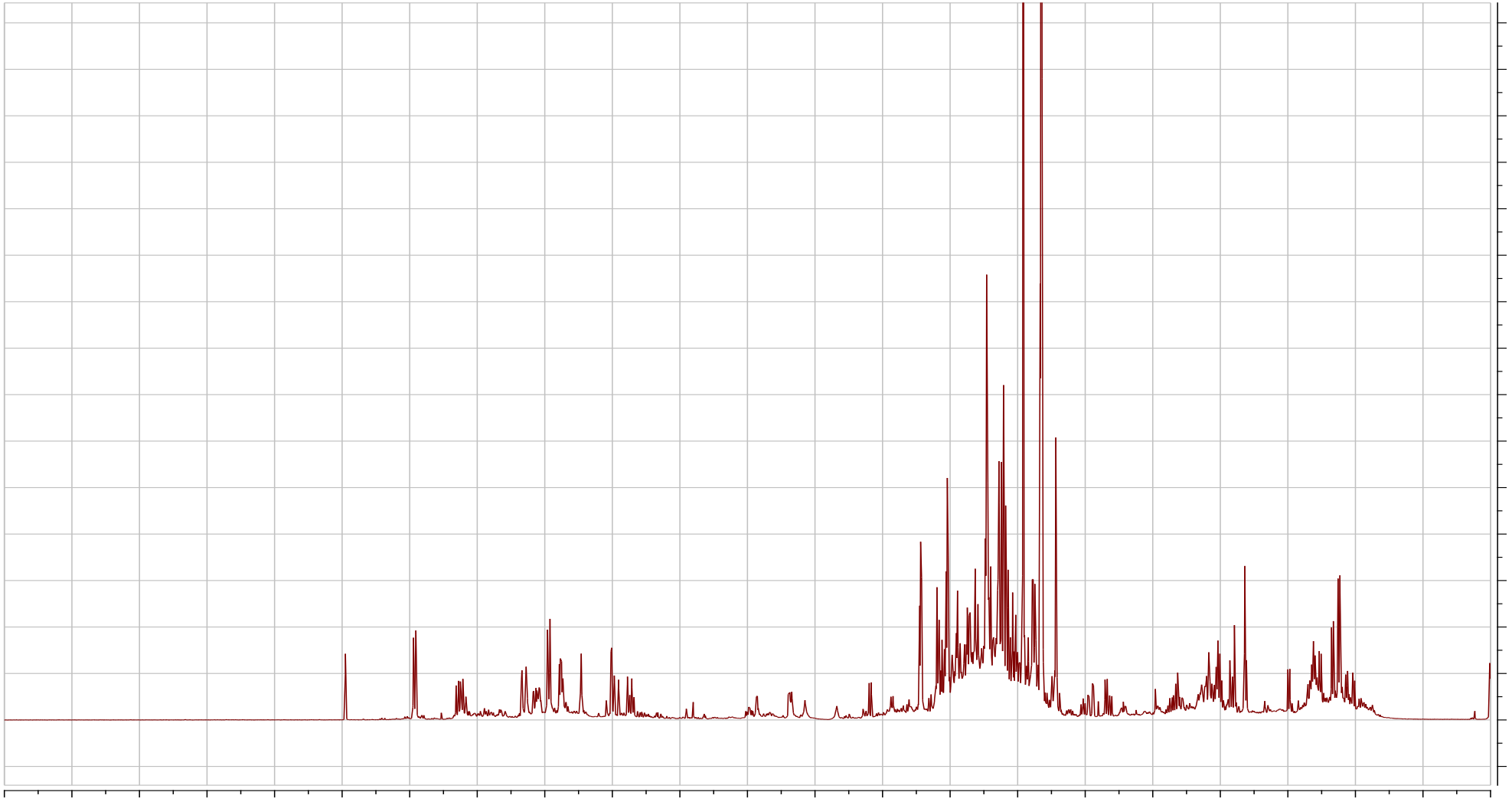

Fig 7. 5: NMR spectrum of the water/methanol plant extract of *H. callicomum* (600 MHz , Leiden University).

8. 1. 6. *Helichrysum cymosum* subsp. *cymosum*

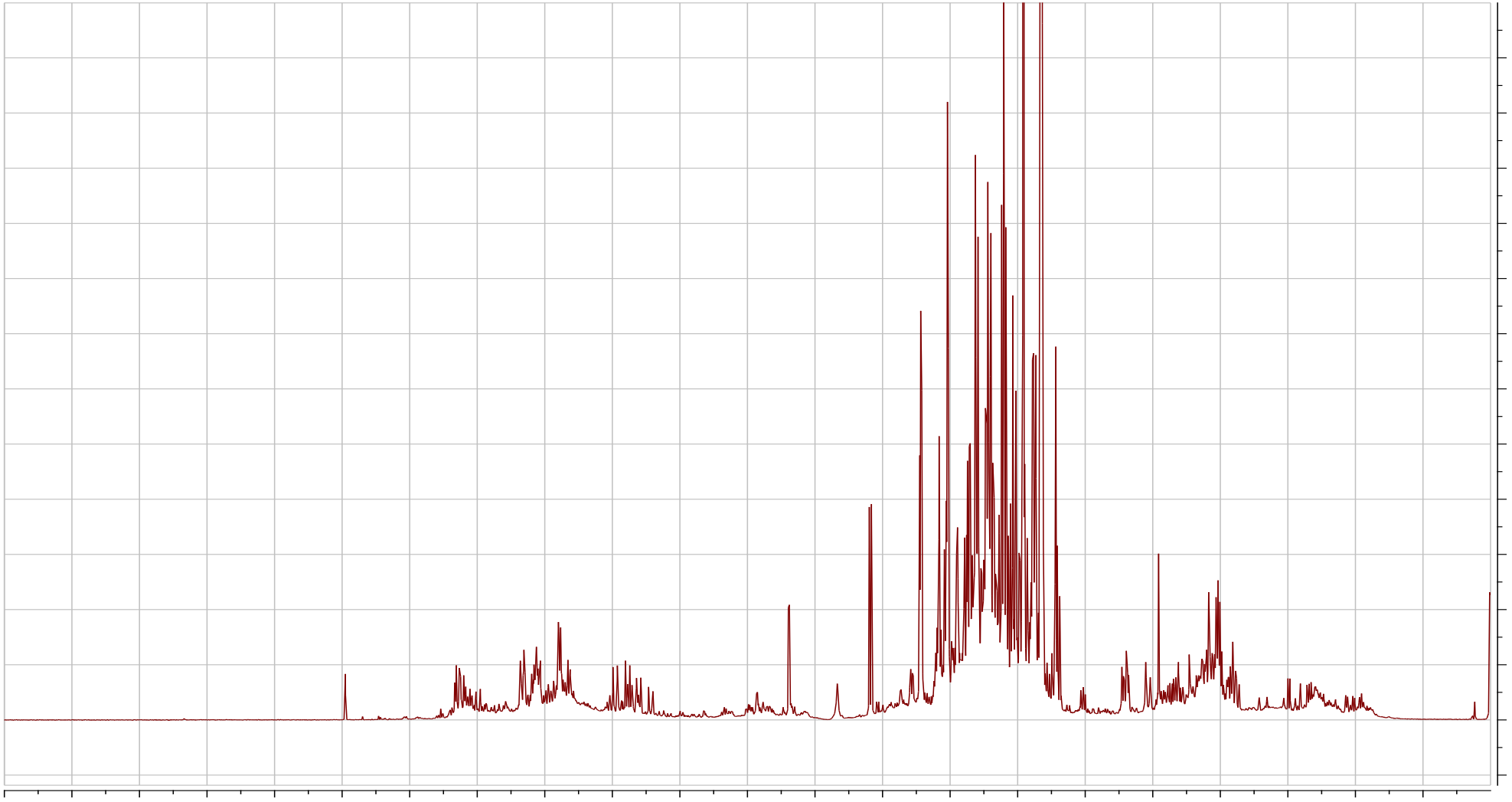

Fig. 8.6: NMR spectrum of the water/methanol plant extract of *H. cymosum* subsp. *cymosum* (600 MHz , Leiden University).

8. 1. 7. *Helichrysum herbaceum*

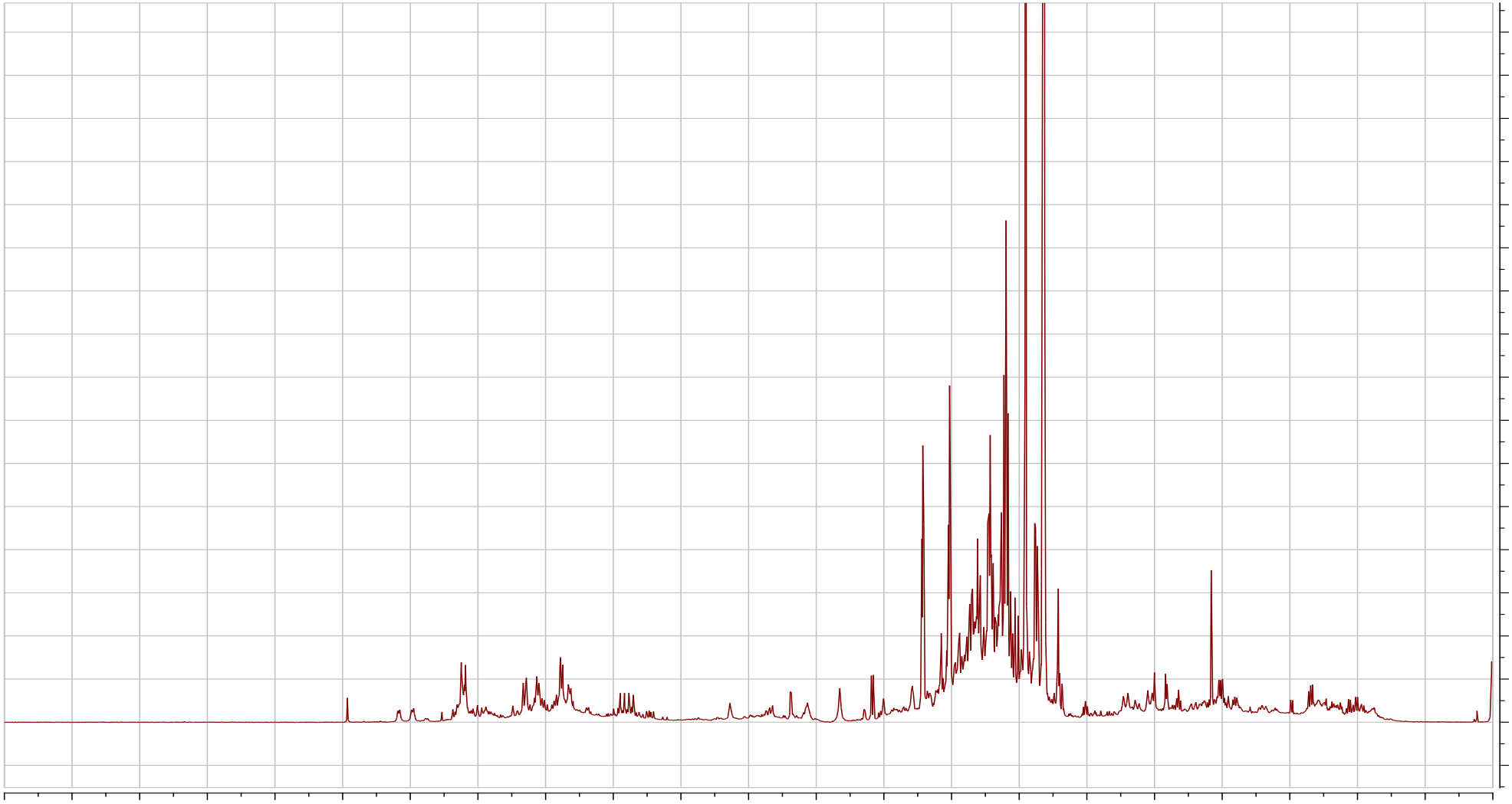

Fig. 8.7: NMR spectrum of the water/methanol plant extract of *H. herbaceum* (600 MHz , Leiden University).

8. 1. 8. *Helichrysum nudifolium* var. *nudifolium*

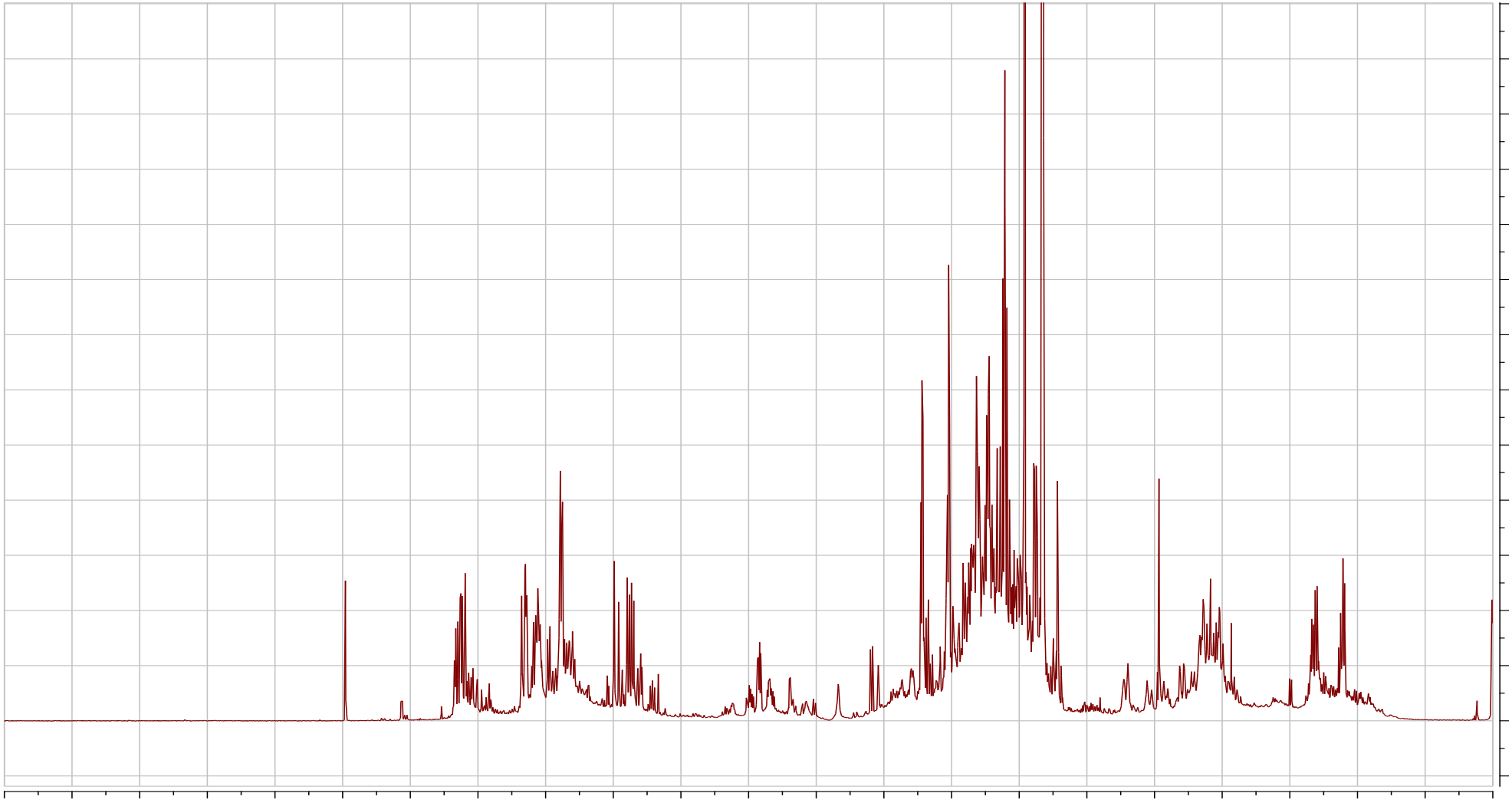

Fig. 8.8: NMR spectrum of the water/methanol plant extract of *H. nudifolium* var. *nudifolium* (600 MHz , Leiden University).

8. 1. 9. *Helichrysum panduratum*

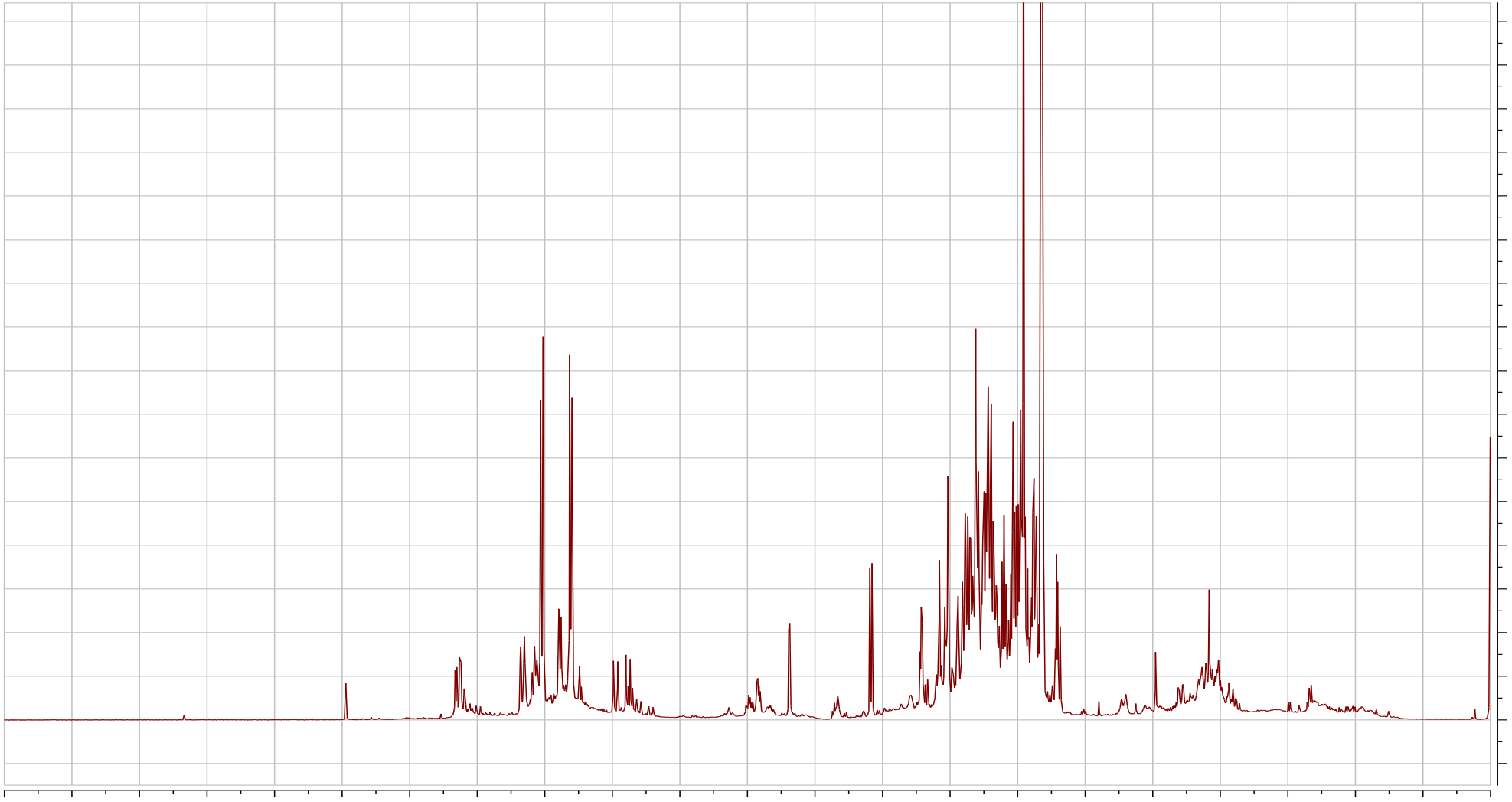

Fig 8.9: NMR spectrum of the water/methanol plant extract of *H. panduratum* (600 MHz , Leiden University).

8. 1. 10. *Helichrysum psilolepis*

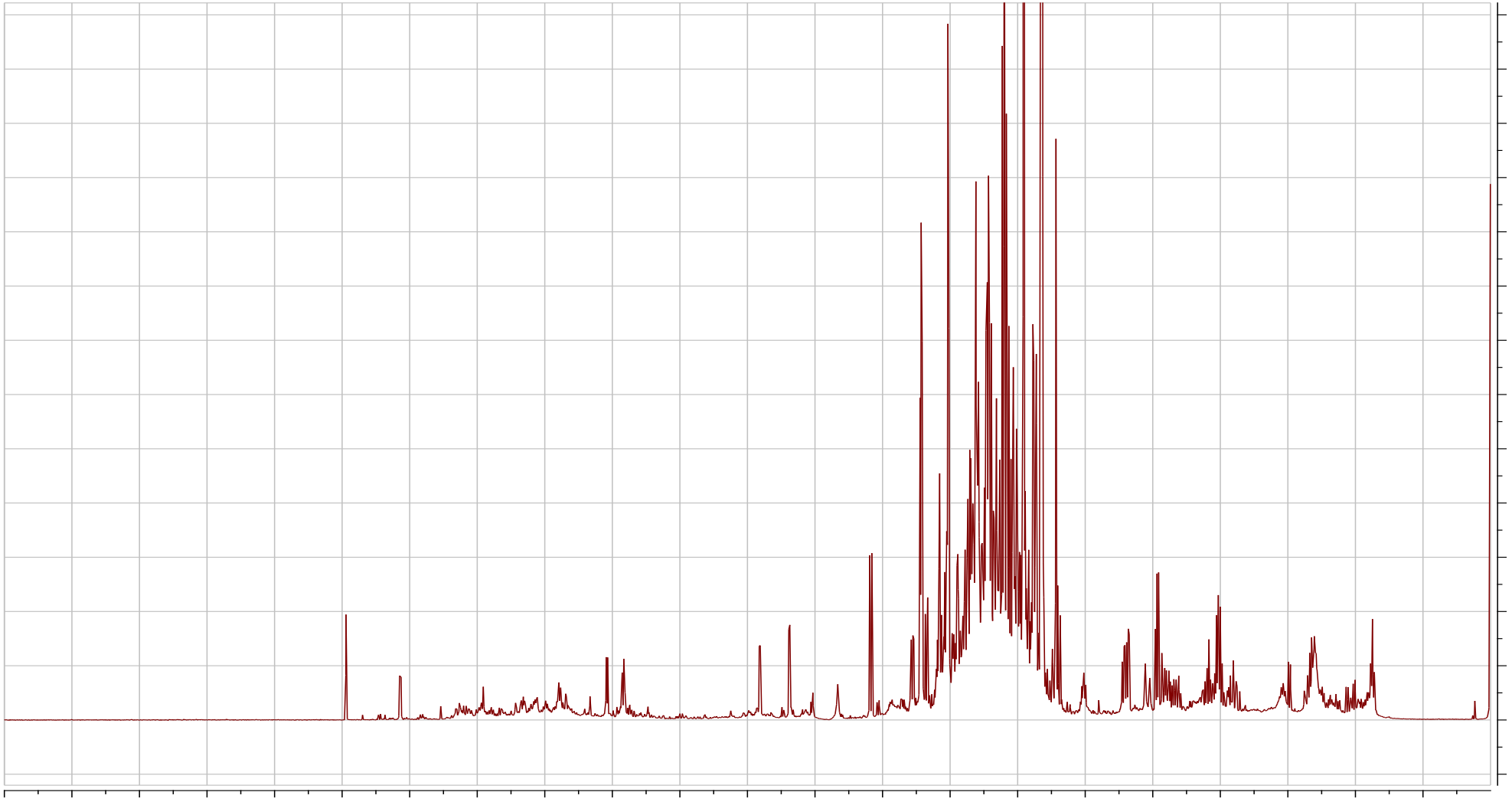

Fig 8.10: NMR spectrum of the water/methanol plant extract of *H. psilolepis* (600 MHz , Leiden University).

8. 1. 11. *Helichrysum populifolium*

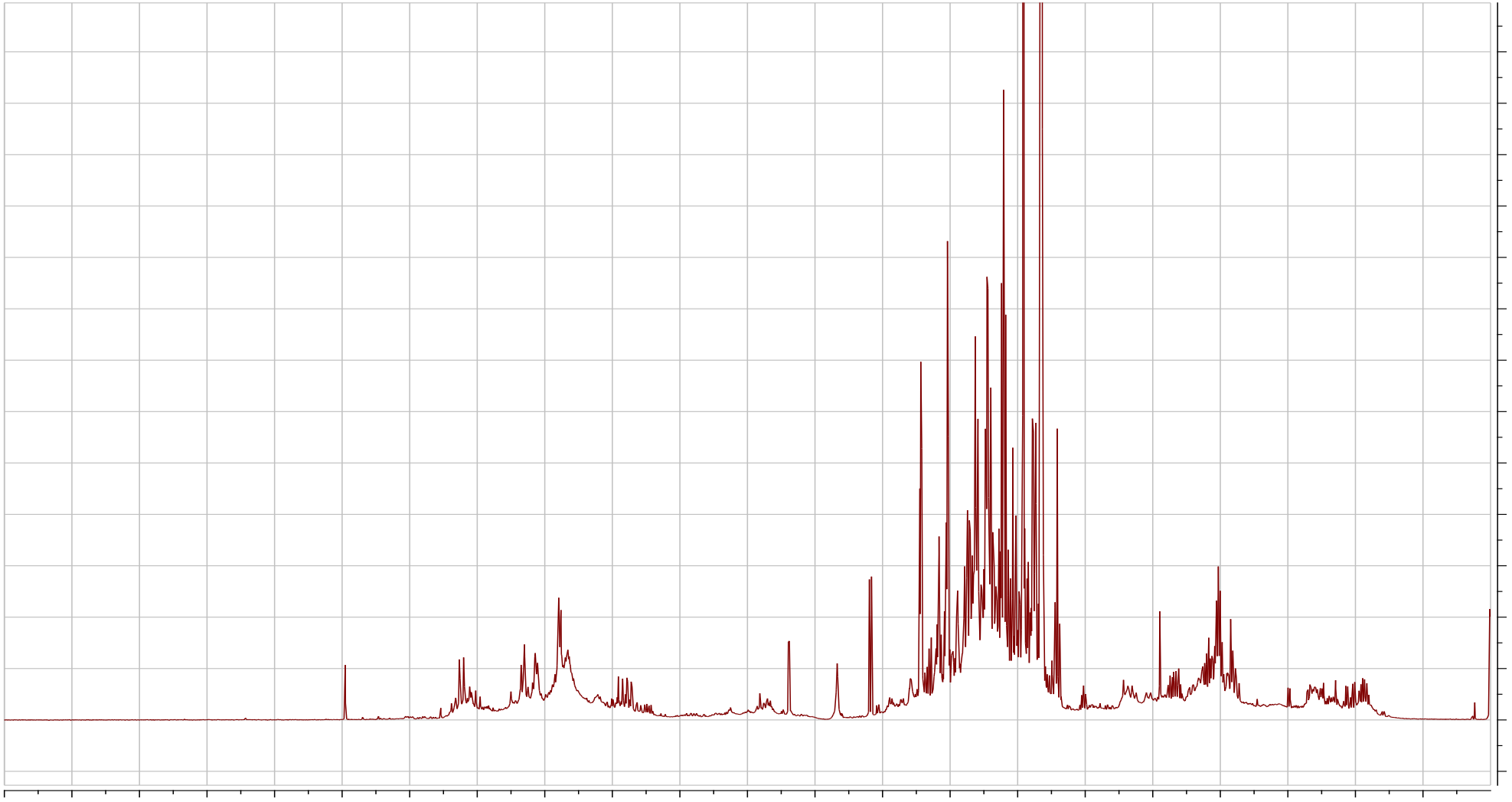

Fig 8.11: NMR spectrum of the water/methanol plant extract of *H. populifolium* (600 MHz , Leiden University).

8. 1. 12. *Helichrysum rugulosum*

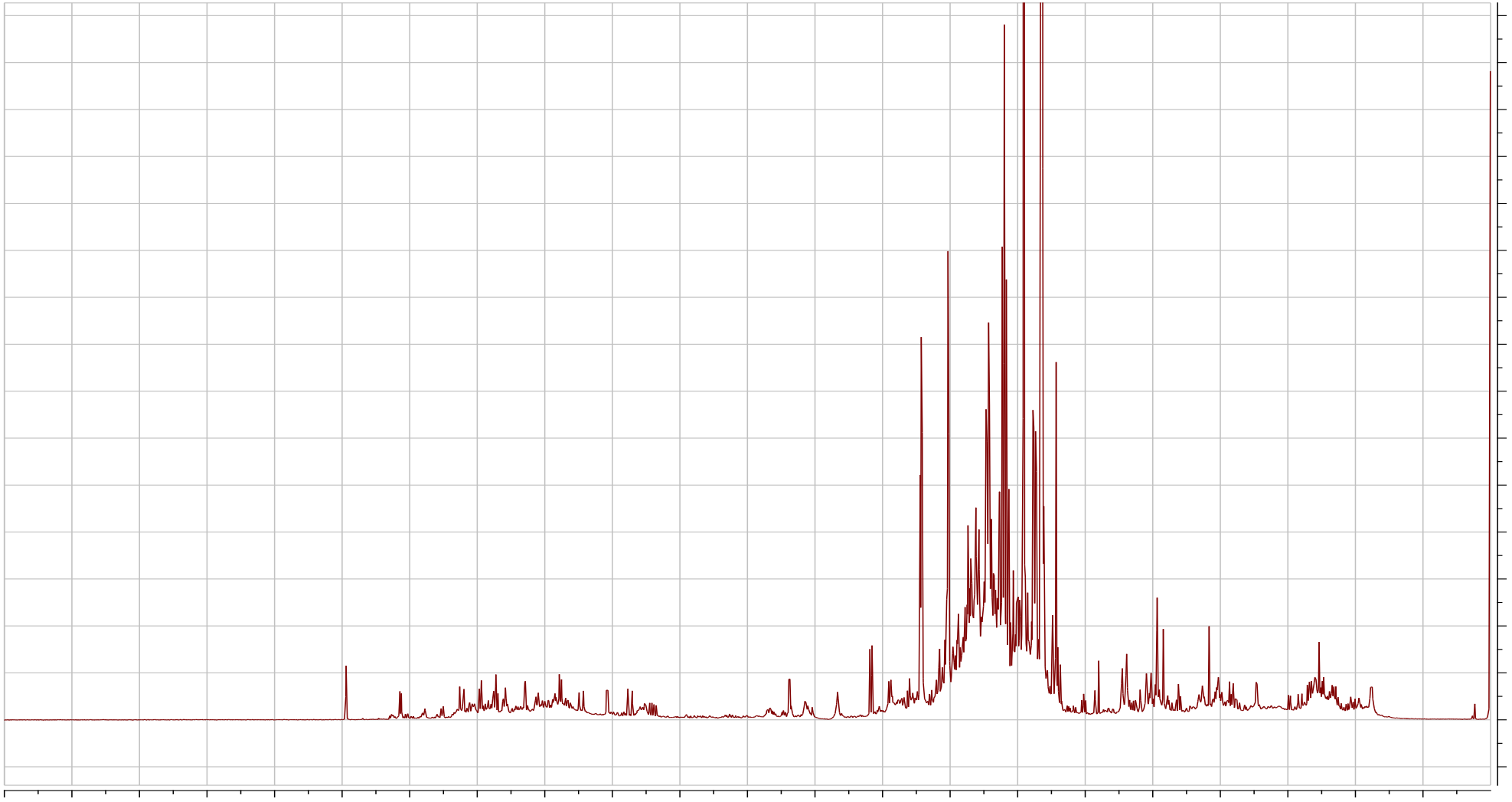

Fig 8. 12: NMR spectrum of the water/methanol plant extract of *H. rugulosum* (600 MHz , Leiden University).

8.2 NMR spectra of the chloroform plant extract

8. 2. 1. *Helichrysum acutatum*

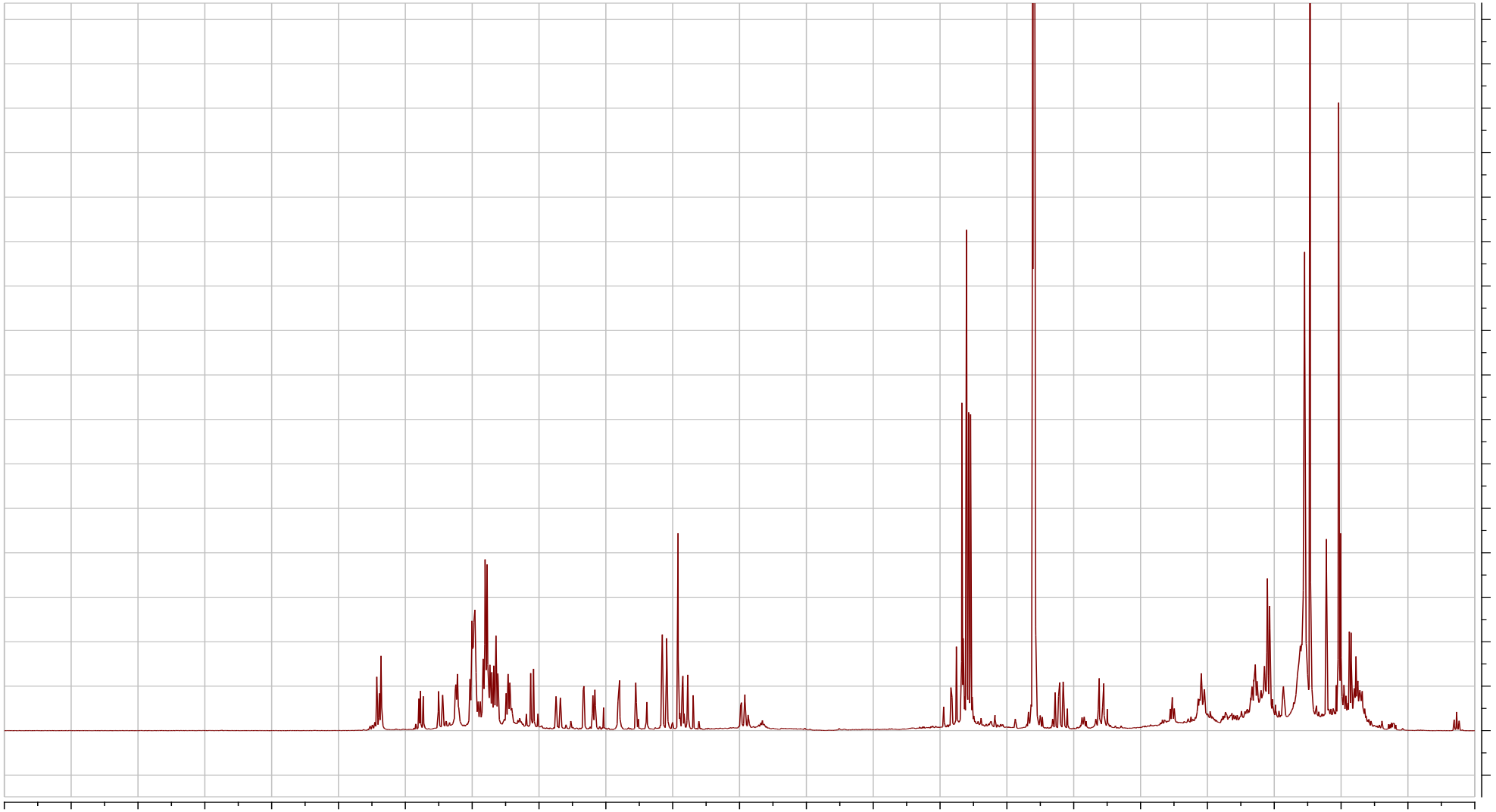

Fig 8. 13: NMR spectrum of the chloroform plant extract of *H. acutatum* (600 MHz , Leiden University).

8. 2. 2. *Helichrysum adenocarpum* subsp. *adenocarpum*

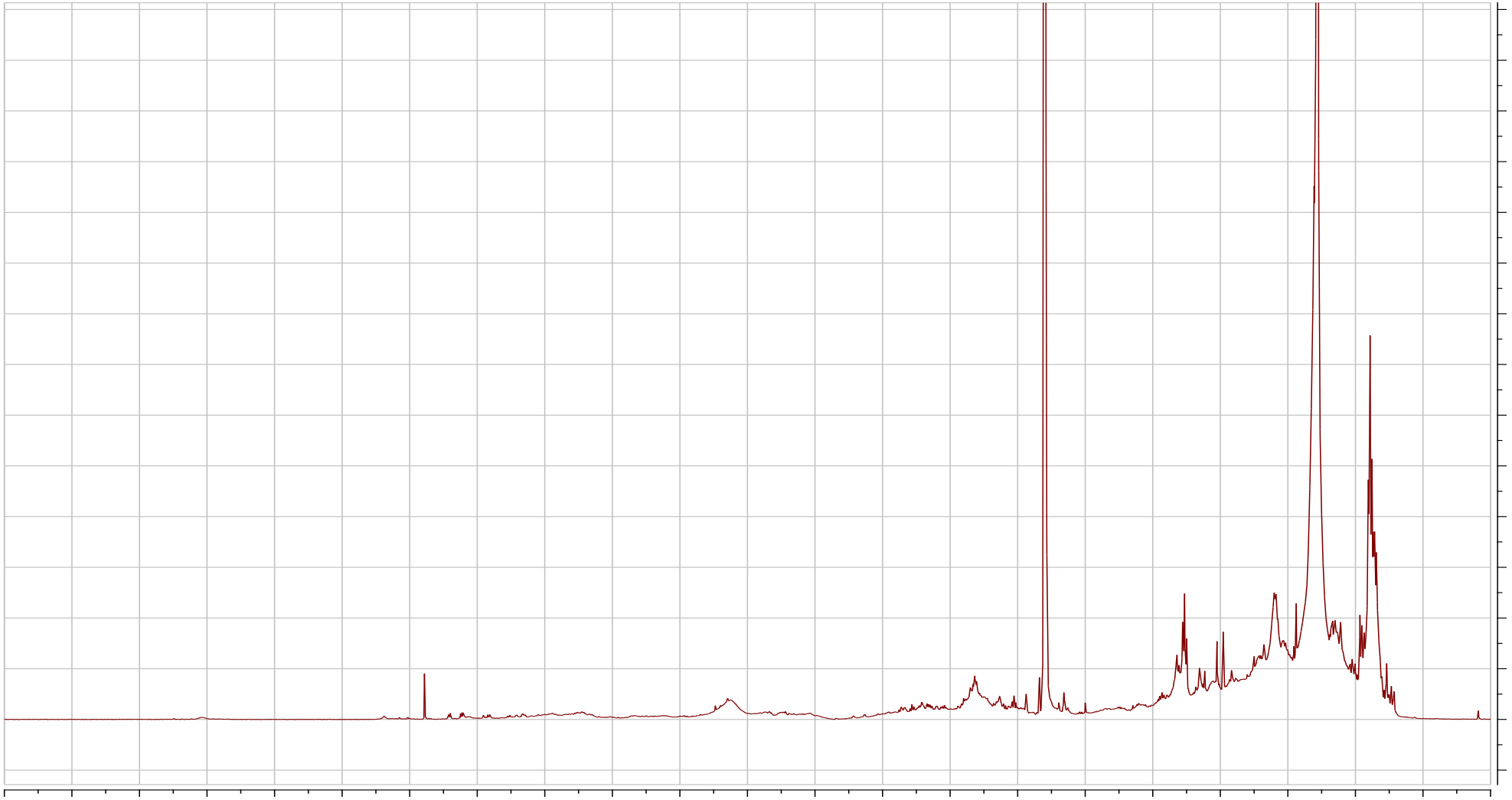

Fig 8. 14: NMR spectrum of the chloroform plant extract of *H. adenocarpum* subsp. *adenocarpum* (600 MHz , Leiden University).

8. 2. 3. *Helichrysum appendiculatum*

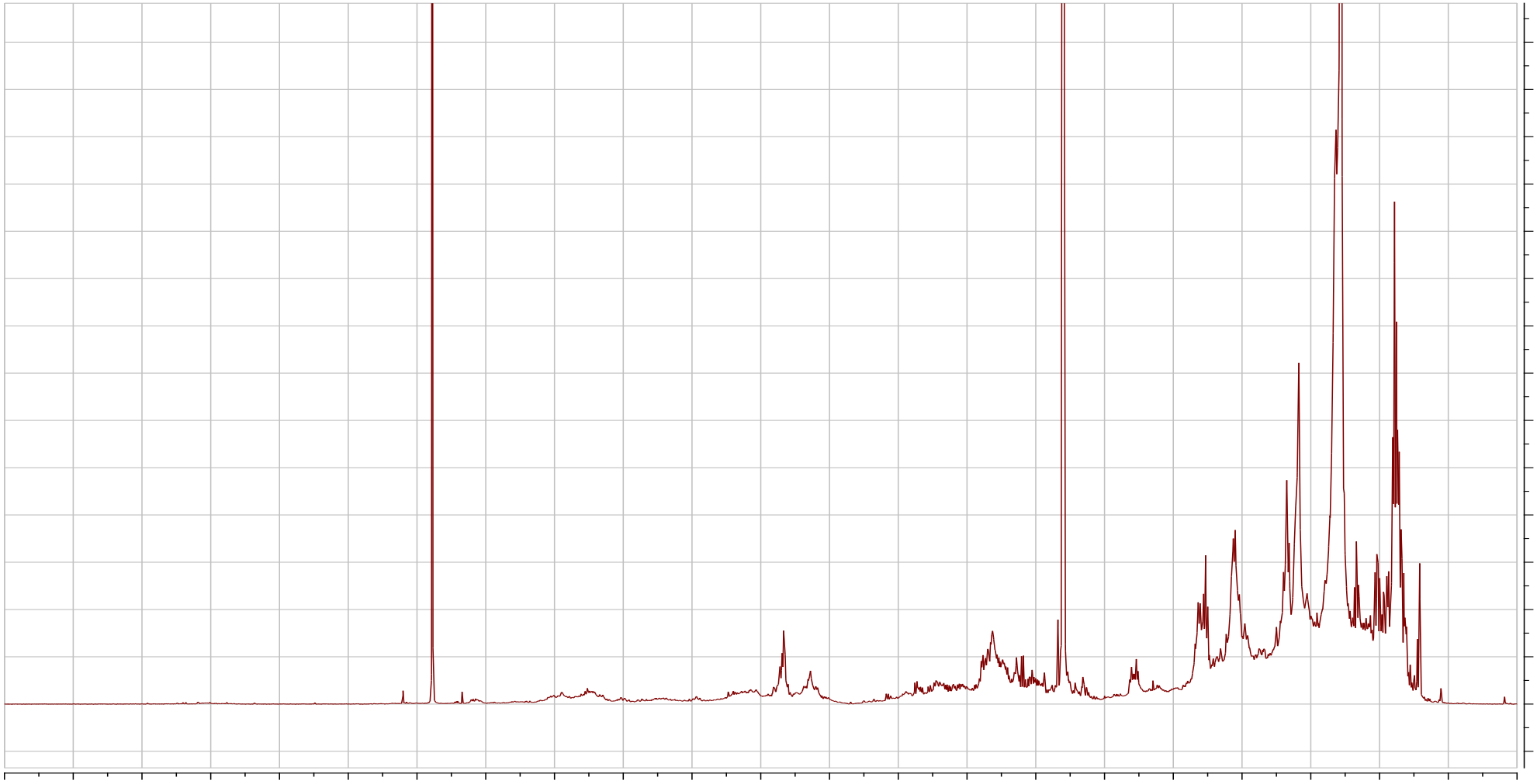

Fig 8. 15: NMR spectrum of the chloroform plant extract of *H. appendiculatum* (600 MHz , Leiden University).

8. 2. 4. *Helichrysum aureonitens*

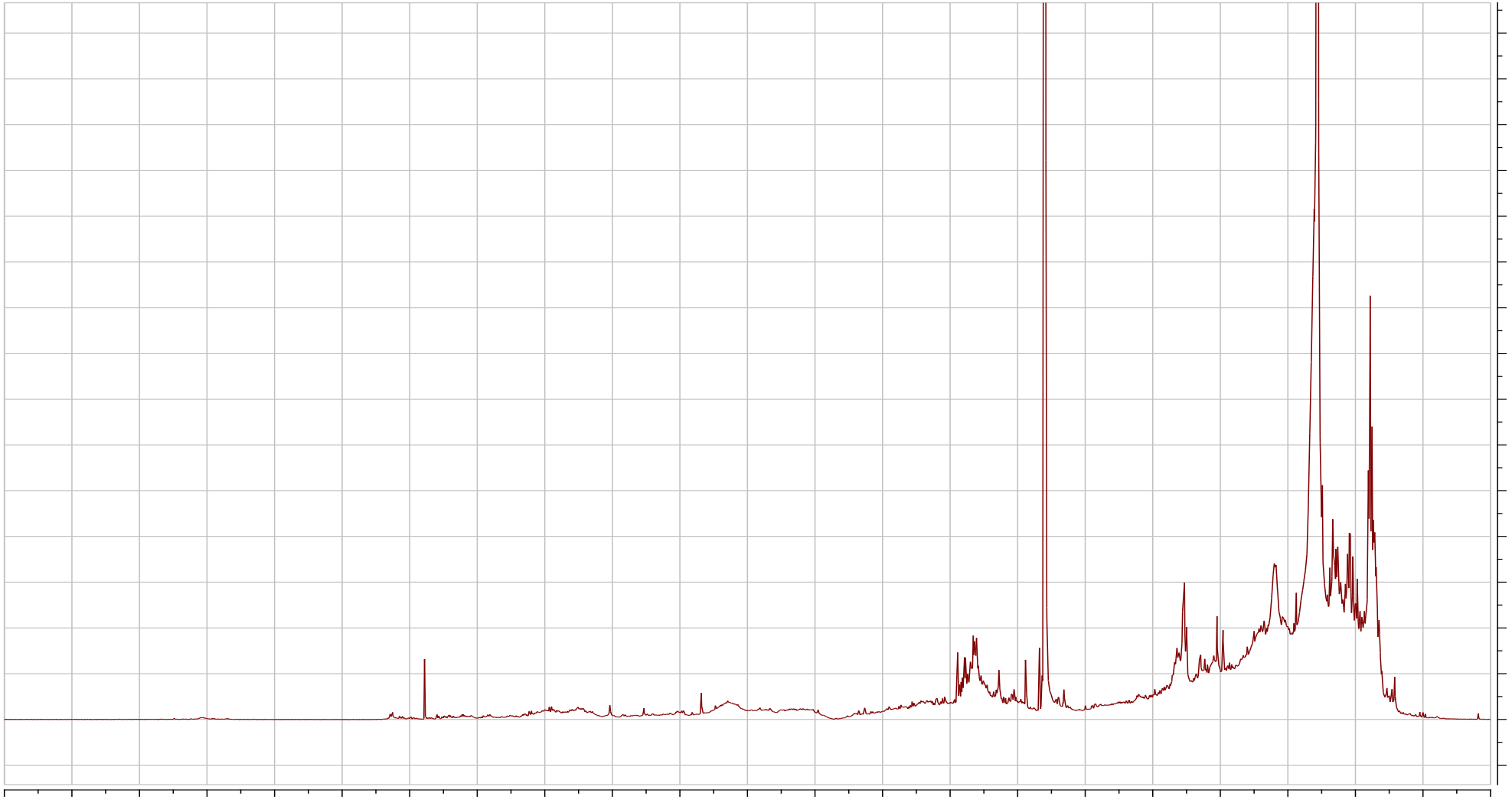

Fig 8. 16: NMR spectrum of the chloroform plant extract of *H. aureonitens* (600 MHz , Leiden University).

8. 2. 5. *Helichrysum callicomum*

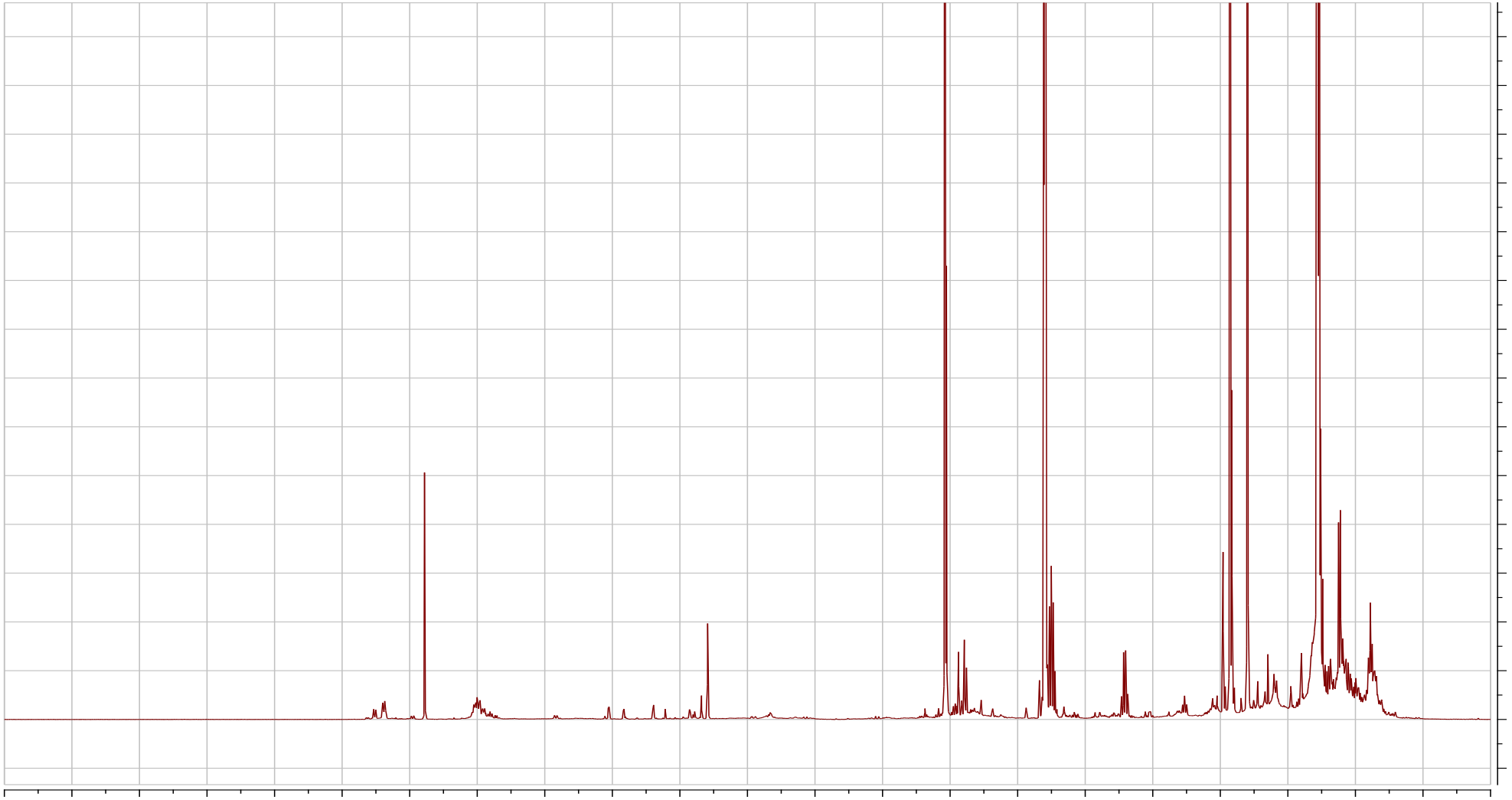

Fig 8. 17: NMR spectrum of the chloroform plant extract of *H. callicomum* (600 MHz , Leiden University).

8. 2. 6. *Helichrysum cymosum* subsp. *cymosum*

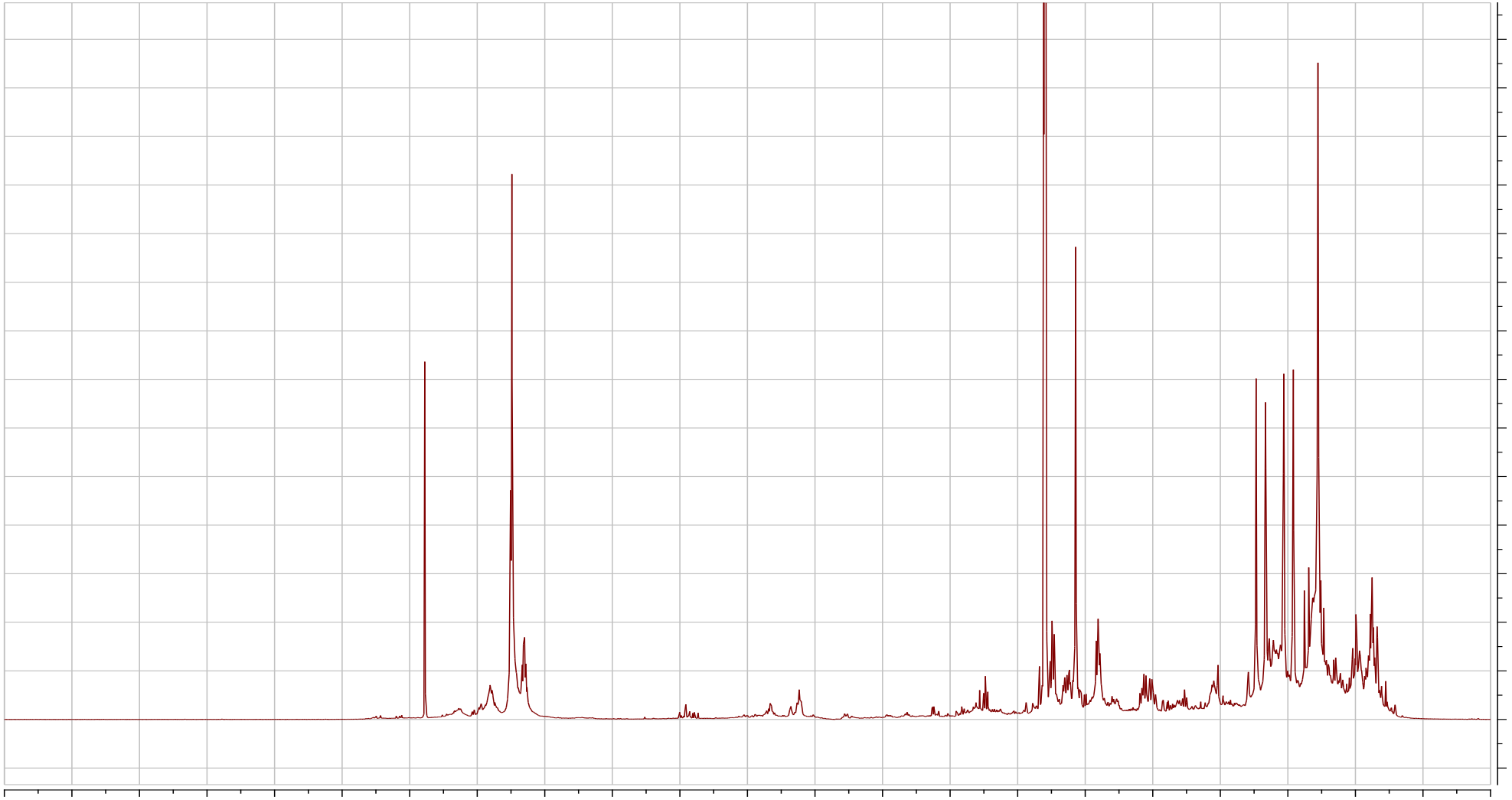

Fig 8. 18: NMR spectrum of the chloroform plant extract of *H. cymosum* subsp*. cymosum* (600 MHz , Leiden University).

8. 2. 7. *Helichrysum herbaceum*

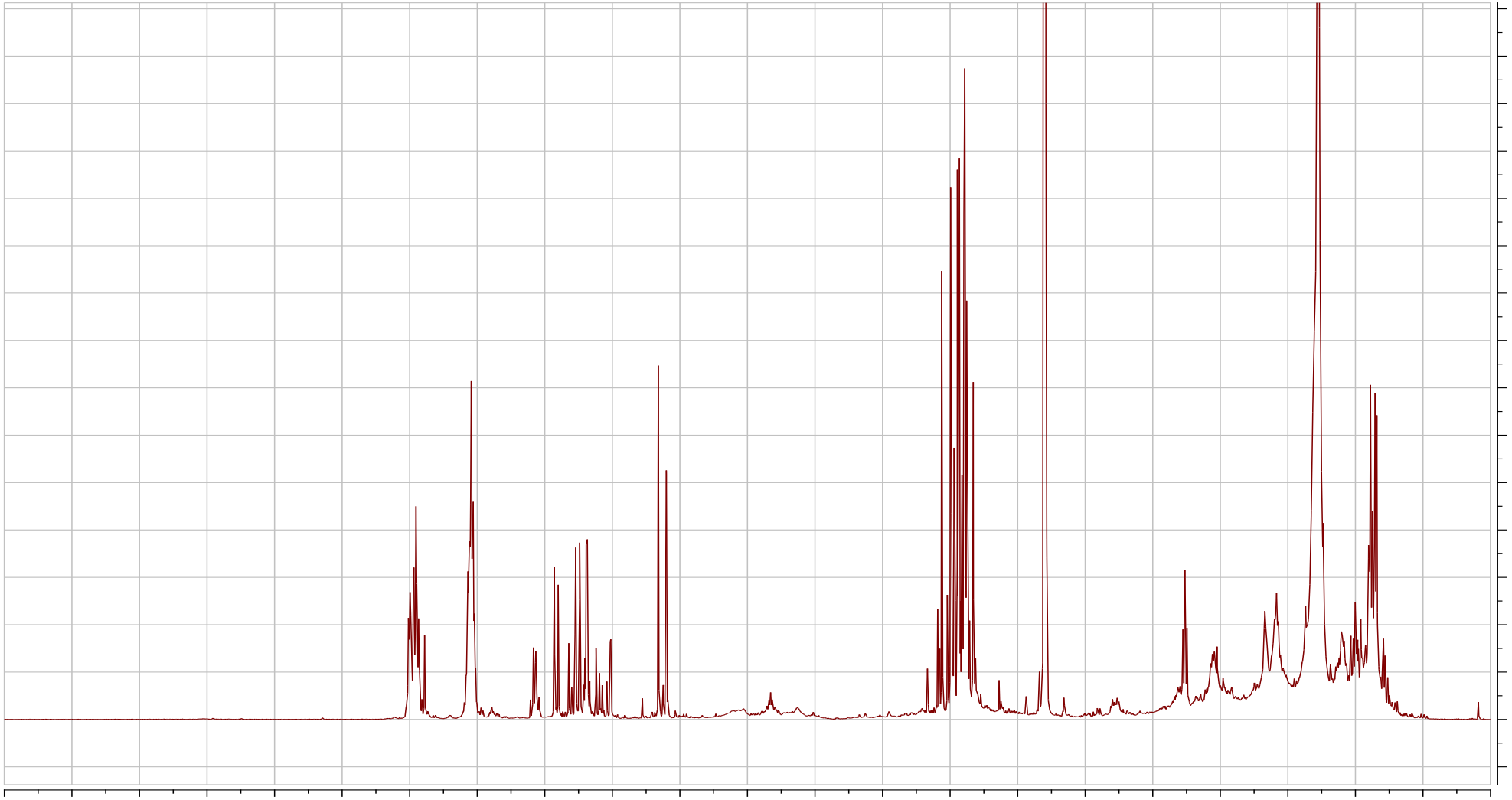

Fig 8. 19: NMR spectrum of the chloroform plant extract of *H. herbaceum* (600 MHz , Leiden University).

8. 2. 8. *Helichrysum nudifolium* var. *nudifolium*

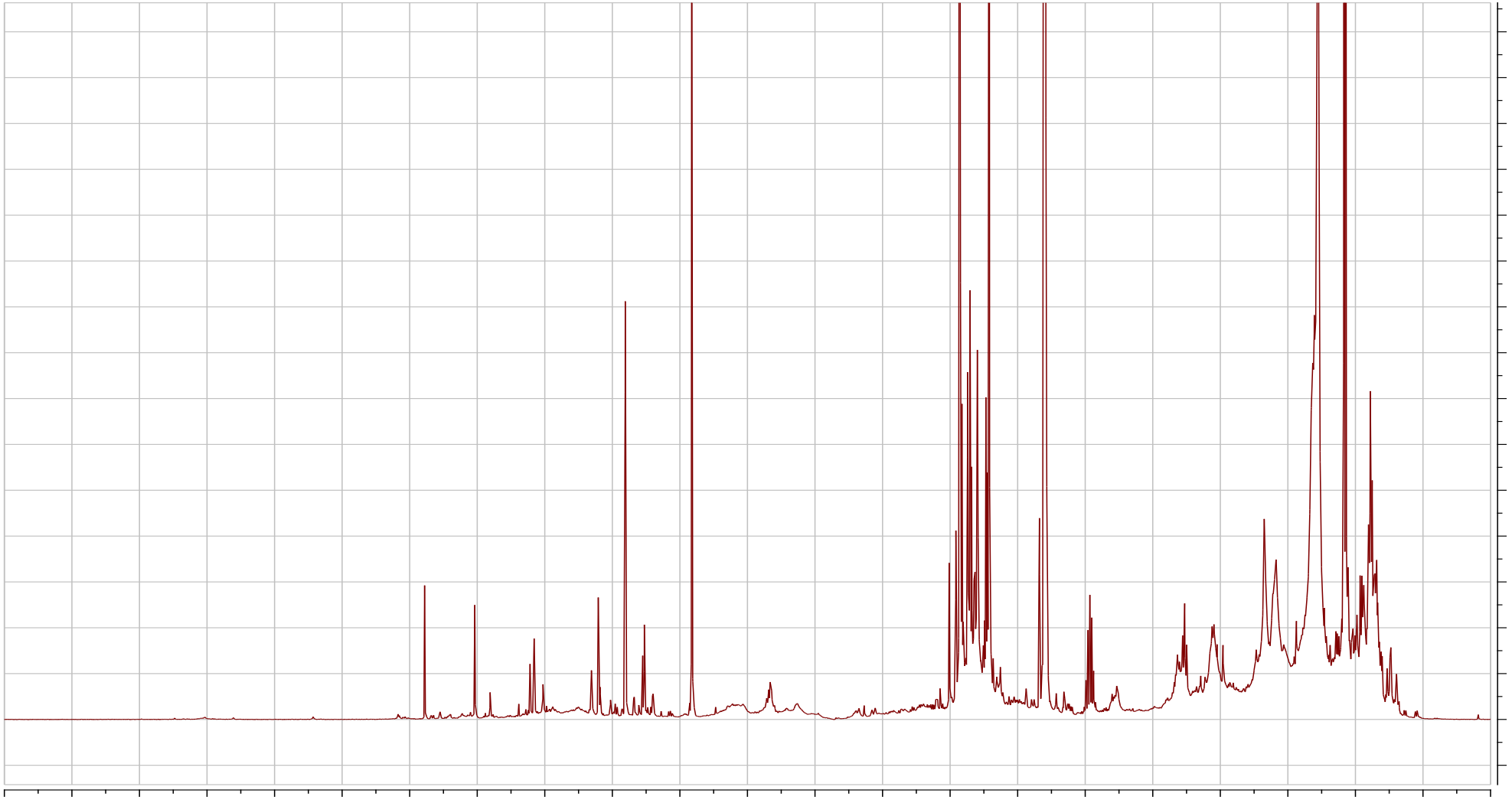

Fig 8. 20: NMR spectrum of the chloroform plant extract of *H. nudifolium* var. *nudifolium* (600 MHz , Leiden University).

8. 2. 9. *Helichrysum panduratum*

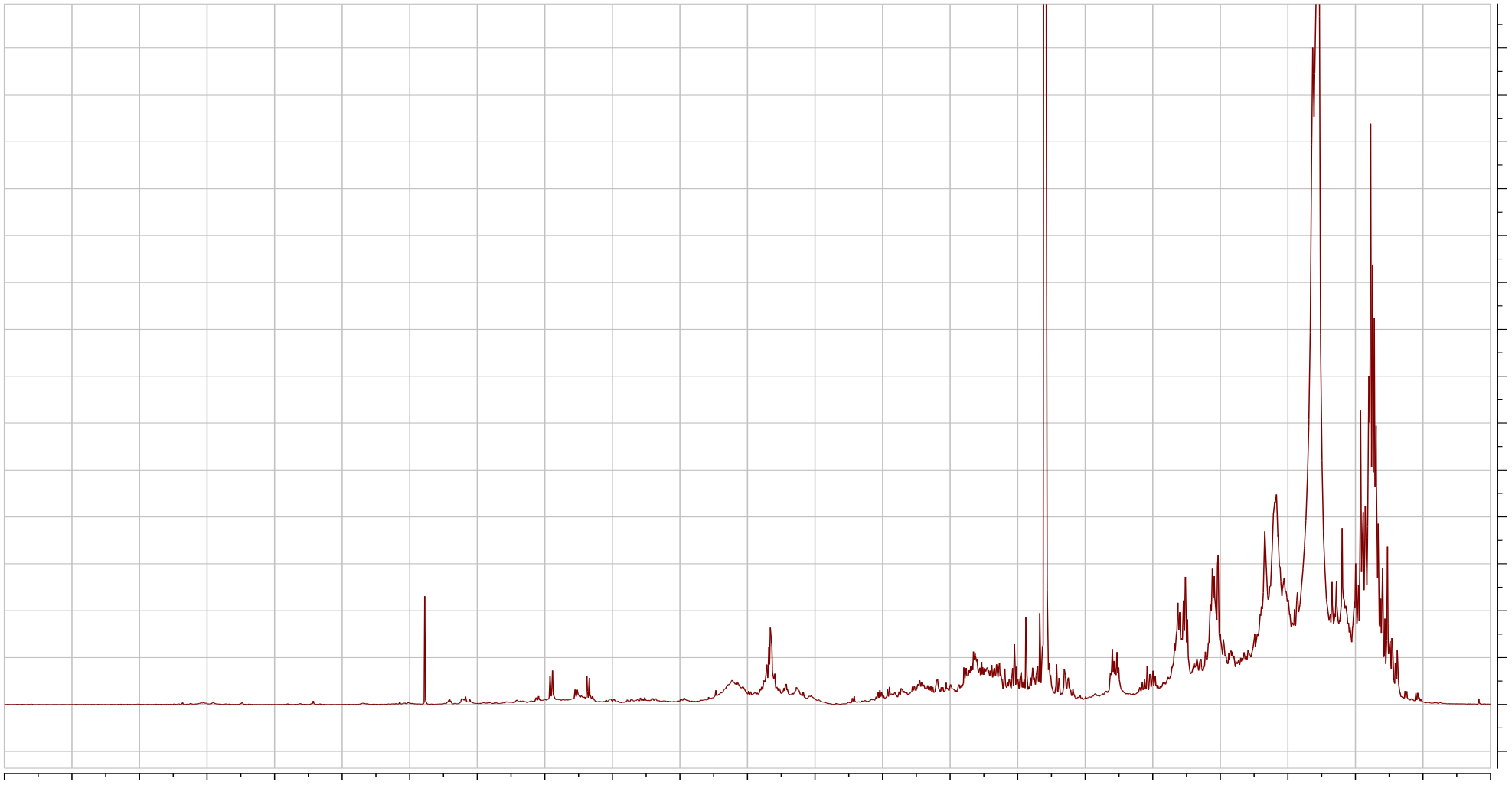

Fig 8. 21: NMR spectrum of the chloroform plant extract of *H. panduratum* (600 MHz , Leiden University).

8. 2. 10. *Helichrysum psilolepis*

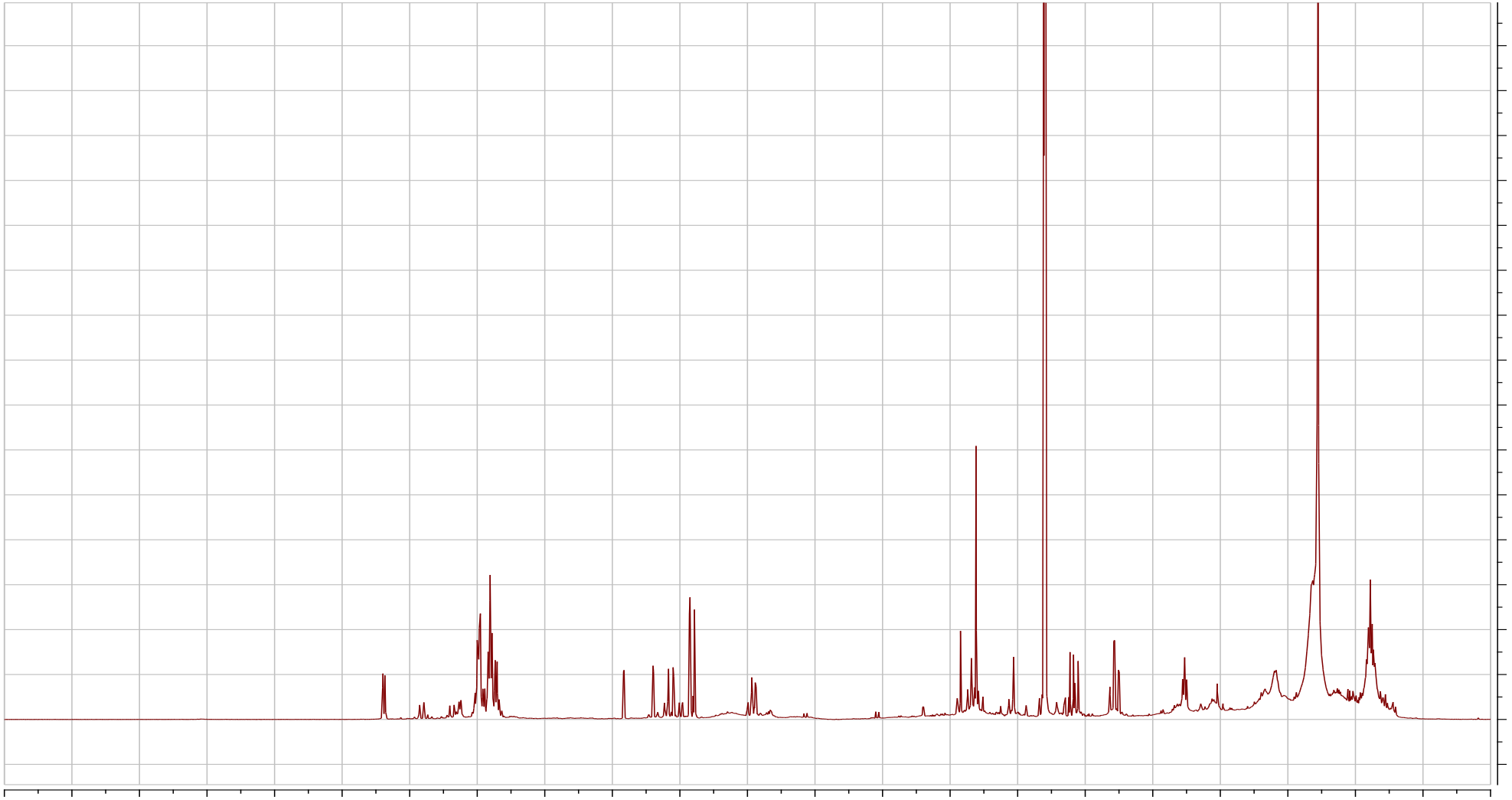

Fig 8. 22: NMR spectrum of the chloroform plant extract of *H. psilolepis* (600 MHz , Leiden University).

8. 2. 11. *Helichrysum populifolium*

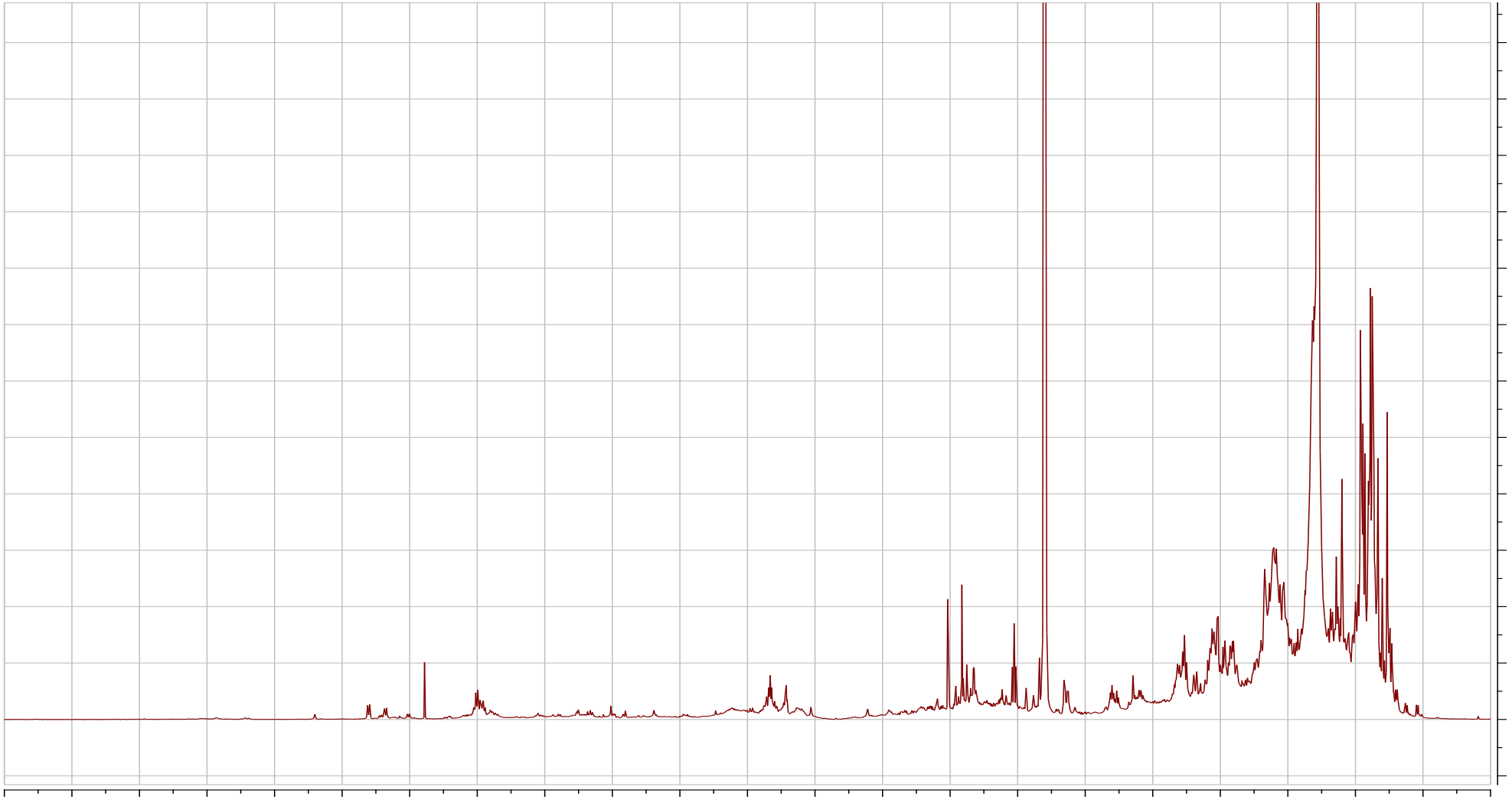

Fig 8. 23: NMR spectrum of the chloroform plant extract of *H. populifolium* (600 MHz , Leiden University).

8. 2. 12. *Helichrysum rugulosum*

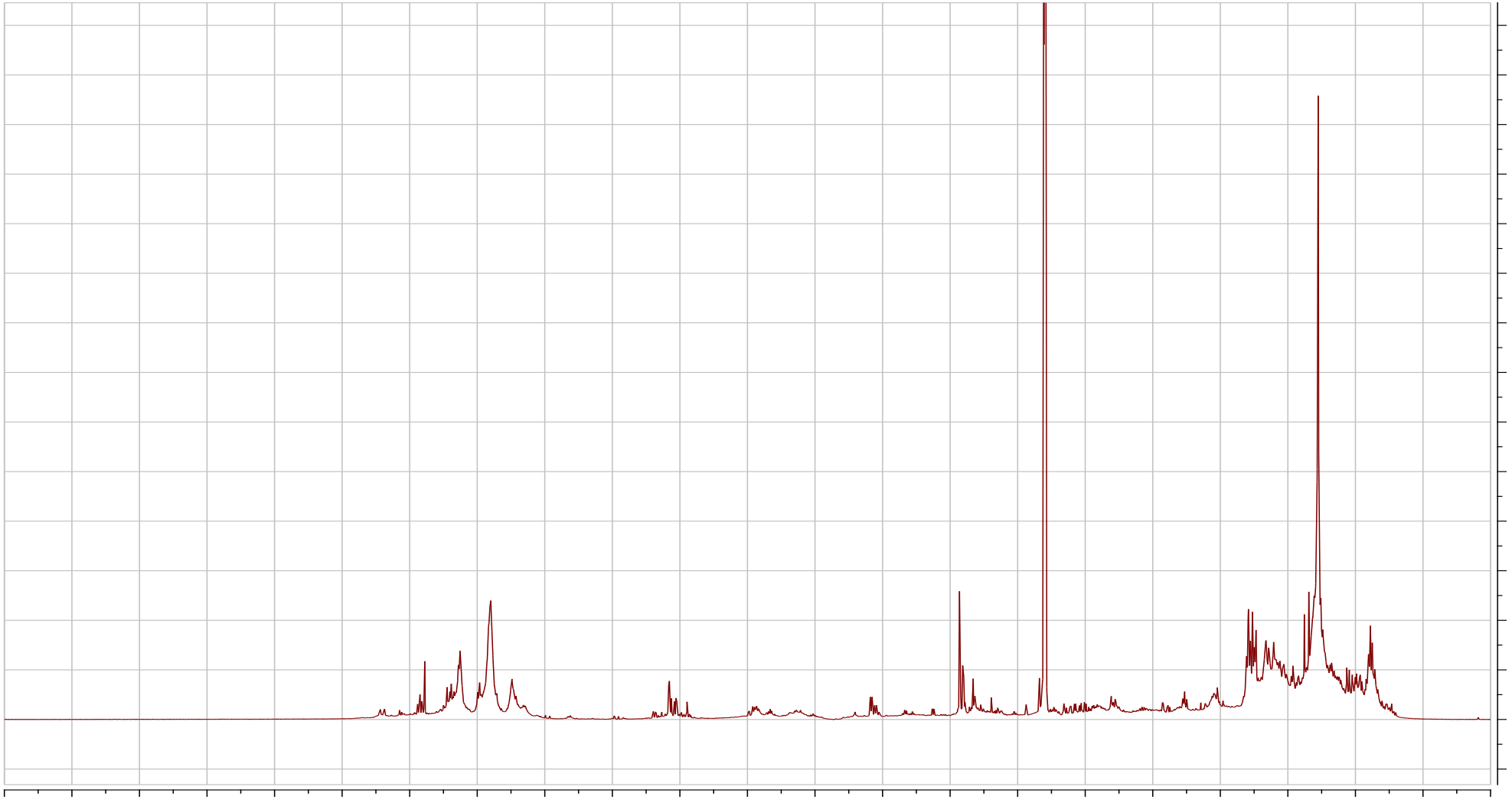

Fig 8. 24: NMR spectrum of the chloroform plant extract of *H. rugulosum* (600 MHz , Leiden University).