Strathclyde Institute of Pharmacy and Biomedical Sciences

Phytochemical and Antimicrobial Studies of Scottish Plants and Fungal Endophytes

by

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A thesis presented in fulfilment of the requirements for the degree of

Doctor of Philosophy

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‘This thesis is the result of the author’s original research. It has been composed by the author and has not been previously submitted for examination which has lead to the award of a degree.’

Signed:

Date:

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List of Abbreviations

AIDS: Acquired Immune Deficiency Syndrome

Ara: Arabinoside

ASE: Accelerated Solvent Extractor

ATCC: American Type Culture Collection

Calcd: calculated

CC: Column Chromatography

CIMS: Chemical Ionisation Mass Spectrometry

COSY: COrrelation SpectroscopY

DEPT: Distortionless Enhancement by Polarisation Transfer

DMF: Dimethylformamide

DMSO: Dimethylsulfoxide

EIMS: Electron Impact Mass Spectrometry

FAB/NOBA: Fast Atom Bombardment/Nitro-benzyl alcohol (matrix)

FC: Flash Chromatography

Gal: Galactoside

Glc: Glucoside

HIV: Human Immunodeficiency Virus

HMBC: Heteronuclear Multiple Bond Correlation

HMQC: Heteronuclear Multiple Quantum Coherence

HREIMS: High Resolution Electron Impact Mass Spectrometry

IC_{50}: Inhibitory Concentration at 50%

IR: Infra-Red

LORA: Low Oxygen-Recovery Assay

MABA: Microplate Alamar Blue Assay

MDR-TB: Multidrug-Resistant Tuberculosis

MIC: Minimum Inhibitory Concentration

MS: Mass Spectrometry

NMR: Nuclear Magnetic Resonance (1D: one dimensional, 2D: two dimensional)

NOE: Nuclear Overhauser Effect

NOESY: Nuclear Overhauser Effect SpectroscopY

NTCC: National Type Culture Collection

NTM: Non-tuberculous Mycobacteria

Rha: Rhamnoside

SI: Selectivity Index

TB: Tuberculosis

TLC: Thin Layer Chromatography

UV: Ultra-Violet

VLC: Vacuum Liquid Chromatography

XDR-TB: extensively drug-resistant tuberculosis

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Abstract

This thesis describes the phytochemical investigation of a selection of Scottish plants and endophytes for the presence of antimicrobial agents.

Extracts (n=321) from 46 plants, 1 lichen and 43 endophyte cultures were tested against *Mycobacterium aurum* and *Mycobacterium tuberculosis*. The results of this screening and a literature survey led to the selection of 8 extracts for further fractionation.

Bioassay-guided fractionation of the *n*-hexane extract of S*kimmia japonica* aerial parts afforded the triterpene taraxerol and three coumarins identified as bergapten and a mixture of meranzin and oxypeucedanin. Unambiguous ^{1}H and ^{13}C NMR assignments for meranzin are reported for the first time.

Bioassay-guided fractionation of the *n*-hexane extract of *Juniperus communis* aerial parts afforded the diterpene *trans*-communic acid. Its activity against *Mycobacterium aurum* is reported for the first time. Bioassay-guided fractionation of the *n*-hexane extract of *Juniperus communis* roots afforded for the first time the sesquiterpene longifolene and the diterpene totarol. This is the first report of the activity of longifolene against *Mycobacterium tuberculosis* and of totarol against *Mycobacterium aurum*. Totarol also showed activity against *Mycobacterium fortuitum* and *Mycobacterium phlei*.

Bioassay-guided fractionations of the ethyl acetate extracts of *Myrica gale* stems and of *Calluna vulgaris* aerial parts afforded the known antitubercular triterpene oleanolic acid. The *n*-hexane extract of *Calluna vulgaris* aerial parts afforded the triterpene epifriedelanol. This is the first report of isolation of epifriedelanol and oleanolic acid from *Calluna vulgaris*.

The *n*-hexane extract of *Myrica gale* stems afforded for the first time the dihydrochalcone myrigalone B and the alkane *n*-nonacosane. The ethyl acetate extract of *Myrica gale* roots afforded the triterpene myricadiol. Myrigalone B was active against rifampicin and isoniazid-resistant *Mycobacterium tuberculosis*.

Last, further antibacterial activity of oleanolic acid and totarol against *Enterococcus faecalis*, *Staphylococcus epidermidis* and *Streptococcus pyogenes* and of toratol against *Bacillus cereus* is reported for the first time.

Chapter 1 Introduction

1.1 Mycobacterial infections and need for novel drugs

1.1.1 Tuberculosis

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*, a rod-shape acid-fast bacterium. Compared to other bacteria, the cell wall of mycobacteria is more lipidic as it contains additional layers of mycolic acid, arabinogalactan and peptidoglycan complex which offer a strong protection against external aggression (Salyers and Whitt 2002).

Tuberculosis is an aerosol-spread disease. By coughing, sneezing or simply speaking, patients suffering from tuberculosis release small droplets containing *Mycobacterium tuberculosis* that remain suspended in the air. An individual will first come in to contact with *Mycobacterium tuberculosis* by being in the same environment as a diseased patient.

Individuals usually get infected after a prolonged contact with the mycobacterium. The diagnosis is made after a positive skin test. This reveals a response of the immune system which in most cases will prevent *Mycobacterium tuberculosis* from growing and spreading. After this primary infection, latent bacilli remain in the host. The infected individual shows no symptoms of TB and is not contagious to others.

Re-infection by contact with another diseased patient, reactivation of latent bacilli, or inability to contain primary infection causes the disease to develop. The patient shows some symptoms: fever, cough, weigh loss and fatigue. The bacilli grow and spread in the lungs causing lesions. In some cases, it can enter the bloodstream and disseminate (Bloom 1994, Salyers and Whitt 2002).

In Europe, the biggest TB outbreak was registered during the 19th century when a quarter of the population was estimated to have been killed by the disease. TB had virtually disappeared from industrialised countries by the mid 20th century thanks to the progress of science and to the improvement of living conditions. Nevertheless, the past two decades have seen a resurgence of tuberculosis. TB is estimated to have caused 1.8 million deaths in 2007 and is currently the leading bacterial killer worldwide (Bloom 1994, WHO 2009).

This resurgence of TB is partly due to the dramatic increase of the immuno-depressed population due to the HIV/AIDS pandemic. Of the 1.8 million deaths caused by TB in 2007, 456,000 occurred in HIV-positive patients. Tuberculosis is the most common opportunistic disease affecting HIV/AIDS patients and case of extra-pulmonary (or disseminated TB) are more frequent in this population (Bloom 1994, Salyers and Whitt 2002, WHO 2009).

The current standard recommended treatment in the United Kingdom is a combination of four first-line antibiotics. The patient is given fixed doses of rifampicin and isoniazid daily for six months. In addition during the first two months the patient also receives pyrazinamide and ethambutol or streptomycin (National Collaborating Centre for Chronic Conditions 2006).

TB treatments are long and therefore met with poor patient compliance. As a result, in the current TB outbreak, isoniazid or rifampicin-resistant *Mycobacterium tuberculosis* strains are widespread. The World Health Organisation is recommending the implementation of Directly Observed Therapy to tackle this issue as resistant strains are more difficult to treat and require the use of expensive drugs that are less efficient and more toxic (Cole et al. 2005, WHO/IUATLD 1997).

Among the 9.27 million cases of TB, there is an estimated 0.5 million cases of multidrug-resistant (MDR) TB. Of the 27 countries that account for 85% of the MDR-TB cases, 15 are in the European region. Moreover, 55 countries have reported at least one case of extensively drug-resistant (XDR) TB. As a consequence, the WHO with its proposed Stop TB strategy has made drug-resistance one of the six main issues to target the TB burden (WHO 2009).

1.1.2 Non Tuberculous Mycobacterial infections

Non-tuberculous mycobacteria (NTM) are usually found in soils and natural or treated water sources. Diseases are usually caused following exposure to NTM in the environment (Griffith et al. 2007).

The epidemiology and pathogenesis of NTM is not well documented. General interest in NTM has only arisen in the past 20 years due to co-infection with NTM diseases in HIV/AIDS patients. It has been subsequently observed that the occurrence of lung diseases caused by NTM is increasing in the non AIDS-population (Jarzembowski and Young 2008).

The 125 species of NTM identified so far are traditionally divided into four groups (Runyon classes) based on their growing rate and their pigment production. This classification is still used despite the availability of genetic classification (Jarzembowski and Young 2008).

- Class I includes slow growing NTM that produce pigments only in the presence of light such as *M. kansasii* and *M. marinum*
- Class II includes slow growing NTM that produce pigments whether or not exposed to light such as *M. scrofulaceum, M. gordonae* and *M. szulgai*

- Class III includes slow growing NTM that produce no pigments such as *M. avium-intracellulare, M. xenopi* and *M. terrae*
- Class IV includes rapidly growing NTM that produce no pigments such as *M. fortuitum, M. peregrinum, M abscessus* and *M. chelonae*

Among these 125 NTM species, 60 are known or thought to be pathogenic, causing a range of diseases from benign skin infections to life-threatening disseminated infections. In the majority of cases, standard TB treatments cannot be applied successfully since NTM species usually show less sensitivity than *Mycobacterium tuberculosis* to first-line anti-TB antibiotics *in vitro*. As a result, treatment of NTM infections is often long and difficult (Griffith et al. 2007, Jarzembowski and Young 2008).

1.1.3 The need for novel antimycobacterial agents

Both TB and NTM infections represent a major health burden. Treatments usually require a long course of multidrug therapy. The high incidence of drug-resistant TB due to interrupted treatments calls for the use of expensive and more toxic drugs and no specific drugs are available to treat NTM infections. In this context, novel antimycobacterial agents are needed to shorten treatment courses, target drug-resistance, and specifically tackle NTM infections (Cole et al. 2005, Jarzembowski and Young 2008).

1.2 Antimycobacterial natural products

Streptomycin (isolated from *Streptomyces griseus*) and capreomycin (isolated from *Streptomyces capreolus*) are two examples of anti-TB drugs of natural origin (Copp 2003). Other natural sources, such as marine natural products, have been investigated for the presence of antimycobacterial activity and

several comprehensive reviews have reported the presence of antimycobacterial natural products (Cantrell et al. 2001, El Sayed et al. 2000, Newton et al. 2000, Okunade et al. 2004).

Terpenoids from terrestrial plants, which are known to have developed complex terpenoids biosynthesis, are the most reported antimycobacterial natural products isolated so far (Cantrell et al. 2001, Copp 2003)

It should be noted that many studies so far have reported on the antimycobacterial activity of crude extracts (Newton et al. 2000). With the help of recent development in chromatography and the availability of rapid *in vitro* and *in vivo* bioassays, it can be anticipated that many other novel antimycobacterial agents will be discovered in the future (Pauli et al. 2005).

1.2.1 Potential of Scottish plants.

In Scotland, several plants have been reported for their traditional use in the treatment of TB and other respiratory diseases and to alleviate symptoms such as cough and chest pains (Table 1).

Another reason for looking at Scottish plants is that mycobacteria thrive in soils which are wet, acidic and covered with dense vegetation primarily composed of mosses, ericaceous plants and conifers (Kazda 2000). Such an environment is typical of the heaths and boglands of Scotland where many ericaceous and conifer plants grow well (Lindsay 1995, Scottish National Heritage 1995). Given the tendency of such plants to develop in this unique ecosystem despite the mycobacterial challenge in the soil it can be anticipated that they are likely to exhibit some antimycobacterial activity and contain novel antimycobacterial compounds.

Table 1. Herbal remedies traditionally used in Scotland to treat TB symptoms.

Name	Mode of administration	Reference(s)
Alexanders (*Smyrmium olusatrum*)	Eaten in salads, as a herb or in broth with *Ligusticum scoticum*	(Allen and Hatfield 2004, Darwin 1996)
Bog-bean (*Menyanthes trifoliate*)	Fresh juice or tea from leaves, stem or roots (internal use)	(Allen and Hatfield 2004, Darwin 1996)
Common nettle (*Urtica dioica*)	Tea (internal use)	(Allen and Hatfield 2004)
Common sorrel(*Rumex acetosa*)	Leaves (internal use)	(Allen and Hatfield 2004)
Elecampane (*Inula helenium*)	Roots decoction (internal use)	(Allen and Hatfield 2004)
Ground ivy (*Glechoma hederacea*)	whole plant decoction or infusion (internal use)	(Darwin 1996)
Heathers (*Calluna vulgaris, Erica cinerea, Erica tetralix*)	Infusion of aerial parts (internal use)	(Allen and Hatfield 2004, Darwin 1996)

Table 1. (continued) Herbal remedies traditionally used in Scotland to TB symptoms.

Name	Mode of administration	Reference(s)
Honeysuckle (*Lonicera periclymenum*)	Patients passed through a wreath of *Lonicera periclymenum* Infusion of flowers drunk against coughs	(Allen and Hatfield 2004, Darwin 1996)
Horsetail *(Equisetum* species)	Crushed fresh stems (internal use)	(Darwin 1996)
Ivy (*Hedera helix*)	Leaves, flowers and berries (internal use)	(Darwin 1996)
Maidenhair spleenwort (*Asplenium trichomanes*)	Ale brewed from *Phyllitis scolopendrium* and *Asplenium tricomanes* (internal use)	(Darwin 1996)
Mugwort (*Artemisia vulgaris*)	Internal use	(Allen and Hatfield 2004, Darwin 1996)
Mullein (*Verbascum thapsus*)	Internal use	(Darwin 1996)
Oak (*Quercus petraea*)	Tannins in oak bark prevents TB symptoms	(Darwin 1996)

Table 1. (continued) Herbal remedies traditionally used in Scotland to treat TB symptoms.

Name	Mode of administration	Reference(s)
Scots lovage (*Ligusticum scoticum*)	Eaten raw in salad, boiled as vegetable or in broth with *Smyrmium olusatrum*	(Allen and Hatfield 2004, Darwin 1996)
Thrift (*Armeria maritime*)	Decoction of roots in milk (internal use)	(Allen and Hatfield 2004)
Thyme *(Thymus* species)	Tea (internal use)	(Allen and Hatfield 2004)
Tree lungwort (*Lobaria pulmonaria*)	Infusion (internal use)	(Allen and Hatfield 2004)
Watercress *(Rorippa* species)	Internal use	(Darwin 1996)
Wild rose *(Rosa* species)	Flowers and hips (internal use)	(Darwin 1996)
Woodruff (*Galium odoratum*)	Tea from leaves (internal use)	(Darwin 1996)
Yarrow (*Achillea millefolium*)	Decoction in milk with quartz (internal use)	(Darwin 1996)

1.2.2 Potential of fungal endophytes

Endophytes (mostly bacteria and fungi) are microorganisms symptomlessly colonising internal living plant tissues (Tan and Zou 2001). The precise nature of the plant-endophyte relationship is unknown. Sometimes described as a latent pathogenesis and other times as a mutualistic symbiosis, the host-endophyte interaction is thought to have evolved through time to become highly specific and influence biosynthetic pathways in both host and endophytes (Strobel 2003, Tan and Zou 2001). It can be anticipated that such interactions might generate novel chemical entities. Endophytes are therefore receiving increasing attention as potential sources of bioactives metabolites (Ramasamy et al. 2009, Strobel and Daisy 2003, Strobel 2003, Tan and Zou 2001).

Endophytes are found in virtually all plants and some endophytes are known to improve their hosts tolerance to environmental stress (Tan and Zou 2001). Thus, it can be anticipated that the endophytes associated with Scottish plants are likely to exhibit some antimycobacterial activity.

1.3 *Skimmia japonica* Thunb.

Table 2. Botanical classification of *Skimmia japonica*

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Sapindales
Family	Rutaceae
Genus	*Skimmia*, Thunb.
Species	*Japonica*

1.3.1 Description

Skimmia are evergreen shrubs native to Japan, most of them sexually distinct or dioecious. They have long been cultivated for the fragrance of their leaves, flowers and fruits. *Skimmia japonica* is 1.2 to 1.5m high with bright green elliptic leaves (8-12 cm). Small white flowers grow as dense clusters. If both male and female plants are grown together, the female flowers are later replaced by bright red berry-like fruits (Bailey 1949-1977, Genders 1977-1994). *Skimmia* species are not fastidious but prefer peaty, slightly acidic soils. Therefore, although *Skimmia japonica* is native of Japan, this species is a common ornamental plant in Scotland (Haworth-Booth 1970).

Figure 1. Foliage of *Skimmia japonica*.

1.3.2 Traditional uses

Tonic, restorative and carminative properties are traditionally attributed to the stems and leaves of *Skimmia japonica*. A decoction of the stem is given to treat rheumatic pains and a tincture is used to treat atrophy of the muscles. The plant is also known to be poisonous and requires cautious use (Perry and Metzger 1980).

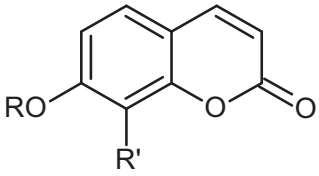
1.3.3 Previous phytochemical work

Previously phytochemical work on *Skimmia japonica* mainly afforded coumarins. Compounds isolated from *Skimmia japonica* to date are compiled in Tables 3 to 5 and Figures 2 to 4.

Table 3. Coumarins previously isolated from *Skimmia japonica*

Compound	Reference(s)
Auraptenol **(1)**	(Reisch and Achenbach 1991)
Isomeranzin **(2)**	(Atkinson et al. 1974, Reisch and Achenback 1991)
Meranzin **(3)**	(Reisch and Achenbach 1991)
Meranzin hydrate **(4)**	(Atkinson et al. 1974, Reisch and Achenback 1991)
Osthenol **(5)**	(Reisch and Achenback 1991)
Osthol **(6)**	(Nakatani et al. 1991, Reisch and Achenback 1991)
Scopoletin **(7)**	(Reisch and Achenbach 1989, Reisch and Achenbach 1991)
Seselin **(8)**	(Späth and Neufeld 1938)
Umbelliferone **(9)**	(Atkinson et al. 1974, Reisch and Achenbach 1989, Reisch and Achenback 1991)

Figure 2. Coumarins previously isolated from *Skimmia japonica*



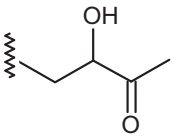
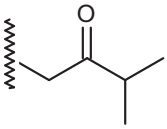
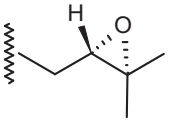
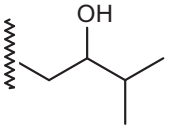
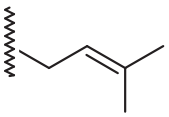
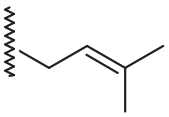
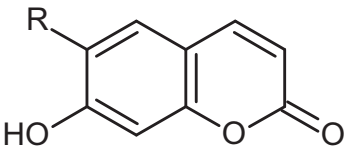
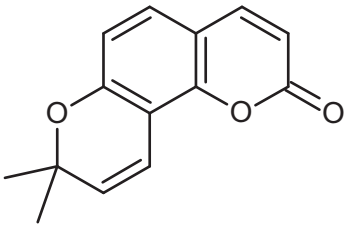
Compound	R	R’
(1)	Me	
(2)	Me	
(3)	Me	
(4)	Me	
(5)	H	
(6)	Me	

Figure 2 (continued). Coumarins previously isolated from *Skimmia japonica*



Compound	R
(7)	OMe
(9)	H

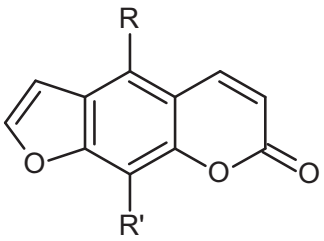


(8)

Table 4. Furanocoumarins previously isolated from *Skimmia japonica*

Compound	Reference(s)
(+)-heraclenin **(10)**	(Reisch and Achenbach 1989)
(+)-saxalin **(11)**	(Reisch and Achenbach 1989, Reisch and Achenbach 1991, Reisch and Achenbach 1992a)
Arnocoumarin **(12)**	(Reisch and Achenbach 1989)
Bergapten **(13)**	(Reisch and Achenbach 1989, Reisch and Achenbach 1991)
Heraclenol **(14)**	(Reisch and Achenback 1991)
Imperatorin **(15)**	(Nakatani et al. 1991, Reisch and Achenbach 1989, Reisch and Achenbach 1992a, Reisch and Achenback 1991)
Isoimperatorin **(16)**	(Atkinson et al. 1974, Nakatani et al. 1991, Reisch and Achenbach 1989, Reisch and Achenbach 1992a, Reisch and Achenback 1991)
Isopimpinellin **(17)**	(Reisch and Achenbach 1989)
Oxypeucedanin **(18)**	(Atkinson et al. 1974, Reisch and Achenbach 1989, Reisch and Achenbach 1992a, Reisch and Achenback 1991)
Oxypeucedanin hydrate **(19)**	(Atkinson et al. 1974, Reisch and Achenbach 1989, Reisch and Achenbach 1992a, Reisch and Achenback 1991)
Oxypeucedanin methanolate **(20)**	(Atkinson et al. 1974)
Phellopterin **(21)**	(Nakatani et al. 1991, Reisch and Achenbach 1992a, Reisch and Achenback 1991)
Xanthotoxin **(22)**	(Reisch and Achenbach 1989, Reisch and Achenback 1991)

Figure 3. Furanocoumarins previously isolated from *Skimmia japonica*



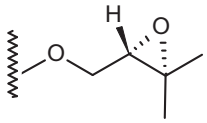
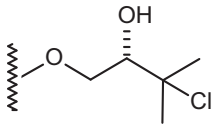
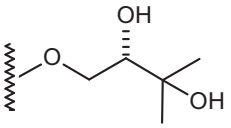
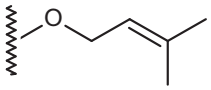
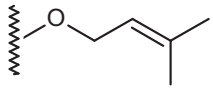
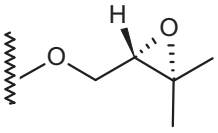
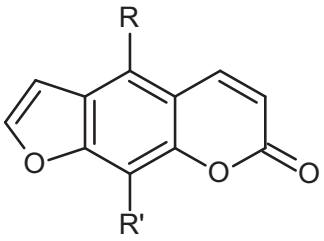
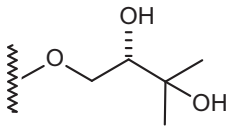
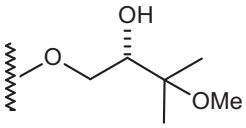
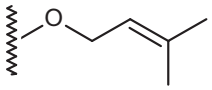
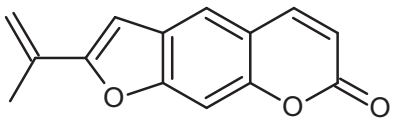
Compound	R	R’
(10)	H	
(11)		H
(13)	OMe	H
(14)	H	
(15)	H	
(16)		H
(17)	OMe	OMe
(18)		H

Figure 3 (continued). Furanocoumarins previously isolated from *Skimmia japonica*



Compound	R	R’
(19)		H
(20)		H
(21)	OMe	
(22)	H	OMe

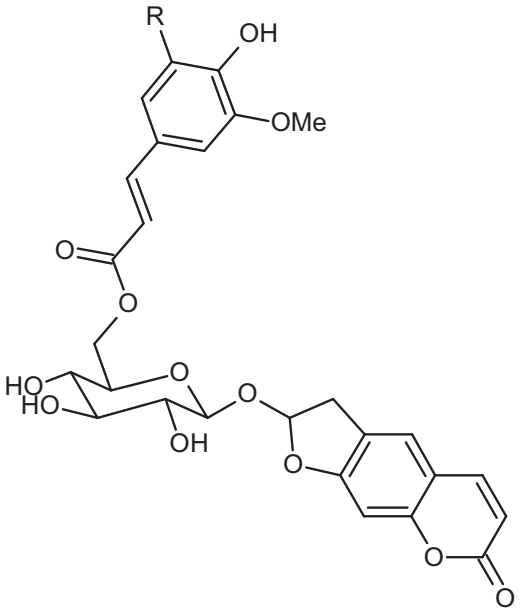


(12)

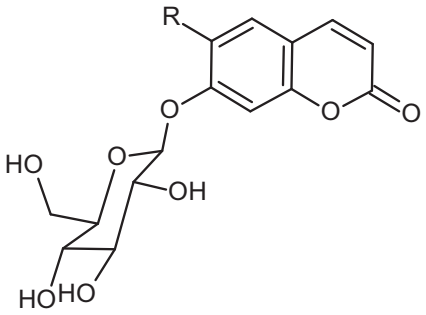
Table 5. Miscellaneous compounds previously isolated from *Skimmia japonica*

Compound	Reference(s)
Coumarin glucosides	
Scopolin **(23)**	(Reisch and Achenbach 1992b)
Skimmin **(24)**	(Reisch and Achenbach 1992b)
Furanocoumarin glucosides **(25, 26**)	(Reisch and Achenbach 1992b)
Alkaloids	
(+)-platydesminium salt **(27)**	(Boyd and Grundon 1967)
Dictamnine **(28)**	(Boyd and Grundon 1967, Reisch and Achenbach 1992a)
Skimmianine **(29)**	(Boyd and Grundon 1967, Takeda 1941b, Tomita and Ishii 1958)
Triterpenoids	
Friedoolea*n*-14-e*n*-3β-ol	(Atkinson et al. 1974, Takeda 1941b)
(taraxerol or skimmiol) **(30)**	(Nakatani et al. 1991, Reisch and Achenbach 1991)
Skimiarepin A **(31)** and B **(32)**	(Ochi et al. 1988)
Skimmione (taraxerone) **(33)**	(Nakatani et al. 1991, Reisch and Achenback 1991, Takeda 1941b)
Phytosterols	
β-sitosterol **(34)**	(Reisch and Achenback 1991)

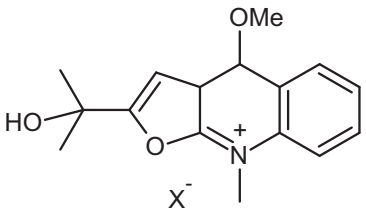
Figure 4. Coumarins glucosides and alkaloids previously isolated from *Skimmia japonica*



Compound	R
(25)	H
(26)	OH



Compound	R
(23)	OMe
(24)	H



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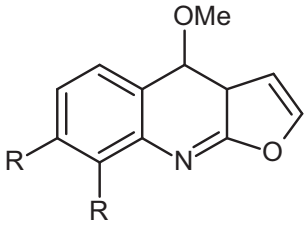
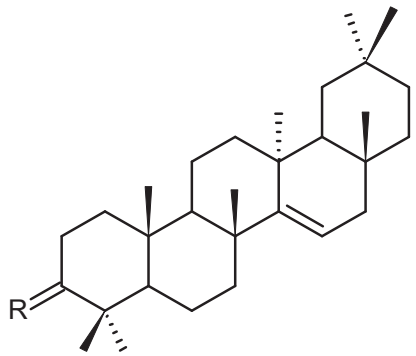
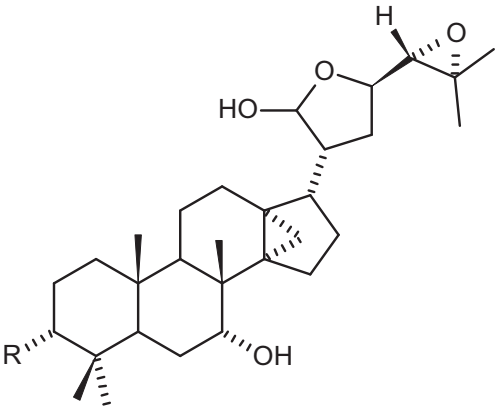


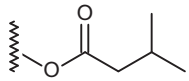
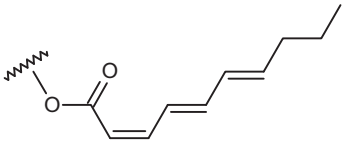
Compound	R
(28)	H
(29)	OMe

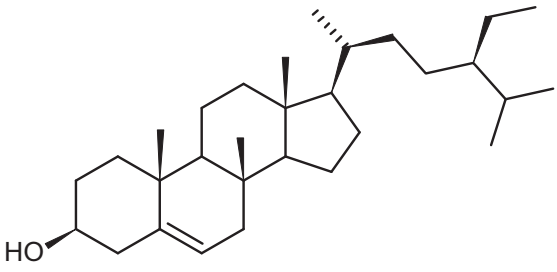
Figure 4 (continued). Triterpenoids and phytosterols previously isolated from *Skimmia japonica*



Compound	R
(30)	β-OH, α-H
(33)	O



Compound	R
(31)	
(32)	



(34)

1.3.4 Previous biological work

Limited biological work has been reported for *Skimmia japonica* extracts or isolates. Skimmianine from *Skimmia japonica* was found to potentiate the effect of adrenaline in cats when administered at a concentration of 50-100mg/kg. It also sensitised spinal reflexes to stimuli, relaxed intestinal muscles and increased the tonus of striated muscles, showing effects similar to ephedrine (Berezhinskaya and Trutneva 1963). Furanocoumarins from the petroleum ether extract of *Skimmia japonica* inhibited the growth of *Gleosporium limetticola* and *Botrytis cinerea* (two fungal plant parasites) at concentrations ≤650ppm (Martin et al. 1966). Several polysaccharides isolated from the leaves from *Skimmia japonica* leaves disclosed inhibitory activity against Ehrlich carcinoma solid tumours in mice (at a dose of 15mg/kg) as well as anti-complementory activity (Hashi 1991).

1.4 *Juniperus communis* L.

Table 6. Botanical classification of *Juniperus communis*

Kingdom	Plantae	Plants
Subkingdom	Tracheobionta	Vascular plants
Superdivision	Spermatophyta	Seed plants
Division	Coniferophyta	Conifers
Class	Pinopsida	
Order	Pinales	
Family	Cupressaceae	Cypress family
Genus	*Juniperus* L.	Juniper
Species	*Juniperus communis* L.	Common Juniper

1.4.1 Description

Juniper is an odorous low evergreen shrub that is widely found in Scotland. Its red-brown bark is flakous and fibrous. Its leaves are short (1.5-2.5 cm) and needle-like, 3 at a whorl and crowded on the twig (Figure 5). Though a conifer, Juniper does not bear wooden cones but black-blue, berry-like fleshy cones. Juniper can tolerate wide ranges of temperatures and soil pH and is, as a consequence, quite widespread in the temperate and cold zones of the Northern hemisphere (Bentham 1924, Hooker 1937).

Figure 5. *Juniperus communis*

1.4.2 Traditional uses

Most of the medicinal properties of *Juniperus communis* have been attributed to its berries which were traditionally used to heal wounds, swellings, pains, fevers, rheumatism, bites and headaches (Newton et al. 2002, Tunón et al. 1995). A decoction of the branches with berries was said to relieve kidney infection (Ritch-Krc 1996). The berries, when eaten raw, were claimed to be beneficial in cases of rheumatism whereas the oil obtained following alcoholic extraction had a reputation as a diuretic. They are also said to stimulate the appetite (Foster 1999) and are used as a seasoning agent. The berries have been reported in the Lothians for purging, stomach ailments, epilepsy and purification and also as a general medicine in the Highlands (Darwin 1996).

Juniper and its volatile oils were also used to treat conditions of the kidneys and bladder and as a carminative in cases of indigestion and flatulence. Juniper has been reported for the treatment of cystitis, flatulence, colic, rheumatism and pain in the joints or muscles (Newall 1996). It has also been reported as a traditional cure for respiratory illnesses (Johnson 2006) and symptoms of tuberculosis (Jimenez-Arellanes et al. 2003, McCutcheon et al. 1997).

1.4.3 Previous phytochemical work

Numerous phytochemical studies have been performed on *Juniperus communis*. An up to date list of the compounds previously isolated from *Juniperus communis* is presented in Tables 7 to 10 and Figures 6 to 9.

Several reports on the GC analysis of *Juniperus communis* fruit and leaf essential oils indicated that these contain mainly mono- and sesquiterpenes (Adams 1998, Angioni et al. 2003, Butkiene et al. 2004, Butkiene et al. 2006, Butkiene et al. 2007, Duke 1995, Gonny et al. 2006, Guerra Hernandes 1988).

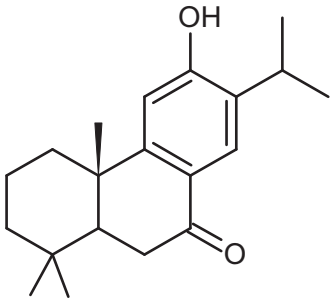
Table 7. Terpenoids previously isolated from *Juniperus communis*.

Compound	Reference(s)
Sugiol **(35)**	(Bredenberg and Gripenberg 1954)
Communic acid **(36)**	(Arya 1962a, Arya et al. 1961, De Pascual Teresa 1973)
1,4-dimethylcyclohex-3-enylmethyl ketone **(37)**	(Thomas 1973a)
Junione **(38)**	(Thomas 1973b)
Imbricatalic acid **(39)**	(De Pascual Teresa 1973)
Imbricatolic acid **(40)**	(De Pascual Teresa 1973)
Isopimaric acid **(41)**	(De Pascual Teresa 1973)
Myrceocommunic acid **(42)**	(De Pascual Teresa 1973)
Sandaracopimaric acid **(43)**	(De Pascual Teresa 1973)
Torulosic acid **(44)**	(De Pascual Teresa 1973)
Junicedral **(45)**	(De Pascual Teresa 1977a)
β-elemen-7-α-ol **(46)**	(De Pascual Teresa 1977b)
5-(4,5-dihydroxy-3-methylenepentyl)-1,4-dimethyl-6-methylene-decahydronaphtalene-1-carboxylate **(47)**	(De Pascual Teresa et al. 1980)

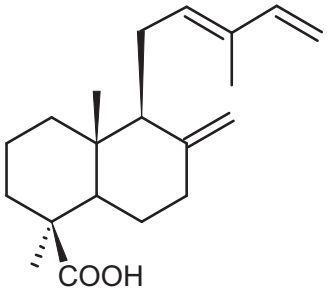
Table 7 (continued). Terpenoids previously isolated from *Juniperus communis*.

Compound	Reference(s)
1,4,7-trimethyl-9-oxo-7-vinyl-tetradecahydrophenanthrene-1-carboxylate **(48)**	(De Pascual Teresa et al. 1980)
1,4,7-trimethyl-9-hydroxy-7-vinyl-tetradecahydrophenanthrene-1-carboxylate **(49)**	(De Pascual Teresa et al. 1980)
15-*O*-palmitoylisocupressic acid **(50)**	(San Feliciano et al. 1991)
3α,15-dihydroxy-labda-8(17), 13E-diene **(51)**	(Kagawa et al. 1993)
3α-acetoxyisocupressic acid **(52)**	(Kagawa et al. 1993)
3α-hydroxy-12, 13E-biformene **(53)**	(Kagawa et al. 1993)
3α-hydroxy-labda-8(17),13E-die*n*-15-oic acid **(54)**	(Kagawa et al. 1993)
3α-hydroxymanool **(55)**	(Kagawa et al. 1993)
3α-hydroxy-labda-8(17),12E,14-trie*n*-19-oic acid **(56)**	(Kagawa et al. 1993)
Isocupressic acid **(57)**	(Kagawa et al. 1993)
Manool **(58)**	(Kagawa et al. 1993)

Figure 6. Terpenoids previously isolated from *Juniperus communis*.



(35)



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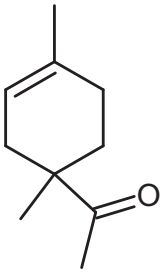

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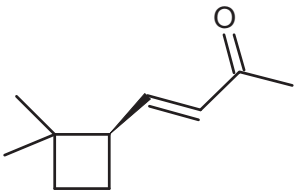
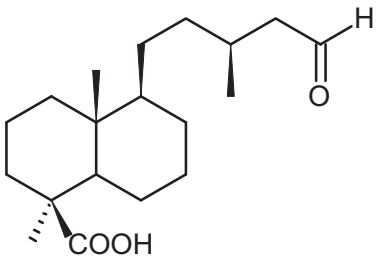


(38)



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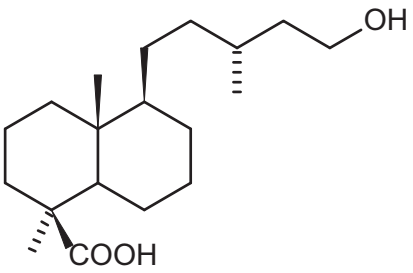
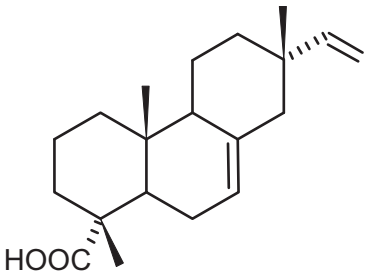


(40)

Figure 6 (continued). Terpenoids previously isolated from *Juniperus communis*.



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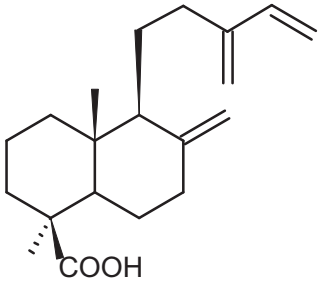
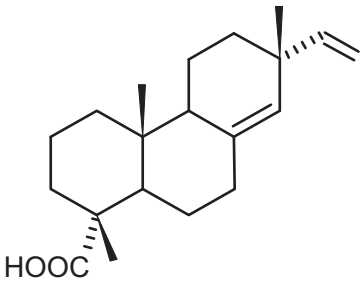
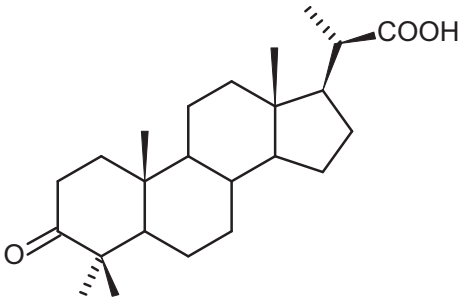


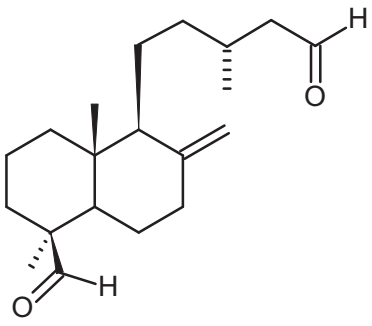
(42)



(43)



(44)



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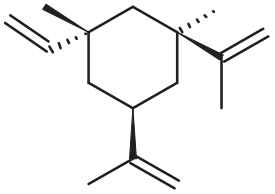
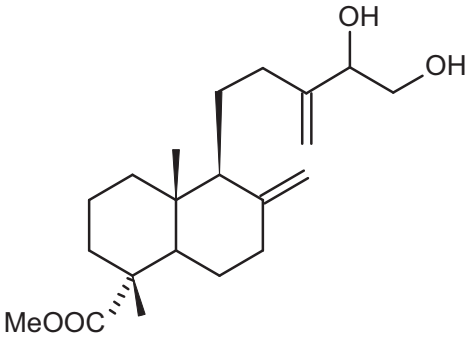


(46)

Figure 6 (continued). Terpenoids previously isolated from *Juniperus communis*.



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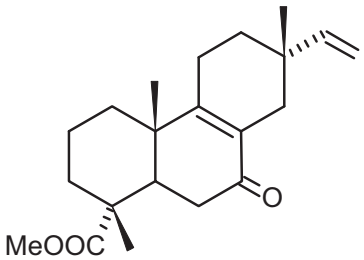

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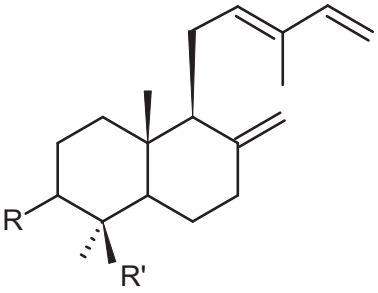
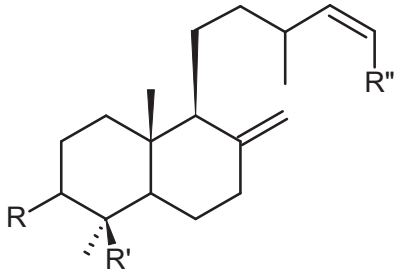
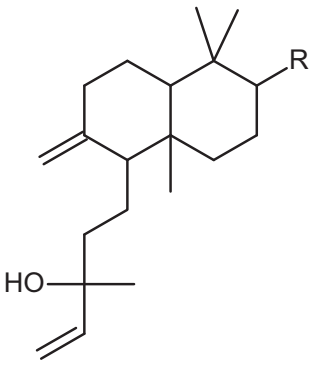


Compound	R	R’
(49)	α-OH	COOH
(53)	α-OH	Me

Figure 6 (continued). Terpenoids previously isolated from *Juniperus communis*.



Compound	R	R’	R”
(50)	H	COOH	CH_2-O-$C_{16}H_{34}$
(51)	α-OH	Me	CH_2OH
(52)	OAc	COOH	CH_2OH
(54)	α-OH	Me	COOH
(57)	H	COOH	CH_2OH

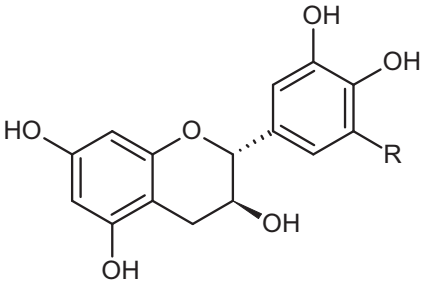


Compound	R
(55)	OH
(58)	H

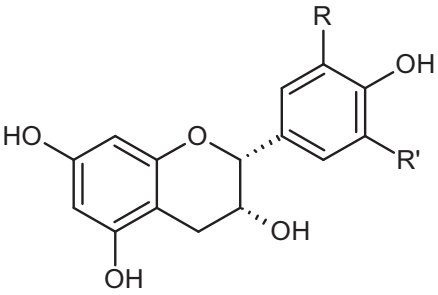
Table 8. Flavonoids previously isolated from *Juniperus communis*.

Compound	Reference(s)
(+)-catechin **(59)**	(Friedrich and Engelshowe 1978, Lamer-Zarawska 1977)
Nepetin **(60)**	(Lamer-Zarawska 1977)
Quercetin **(61)**	(Lamer-Zarawska 1977)
(-)-epiafzelechin **(62)**	(Friedrich and Engelshowe 1978)
(-)-epicatechin **(63)**	(Friedrich and Engelshowe 1978, Iida et al. 2007)
(+)-afzelechin **(64)**	(Friedrich and Engelshowe 1978)
(+)-epigallocatechin **(65)**	(Friedrich and Engelshowe 1978)
(+)-gallocatechin **(66)**	(Friedrich and Engelshowe 1978)
Apigenin **(67)**	(Hiermann 1996)
Hinokiflavone **(68)**	(Ilyas and Ilyas 1990; Hiermann 1996)
Luteolin **(69)**	(Hiermann 1996)
Podocarpusflavone A **(70)**	(Hiermann 1996)
Robustaflavone **(71)**	(Hiermann 1996)
(-)-catechin or catechol) **(72)**	(Iida et al. 2007)

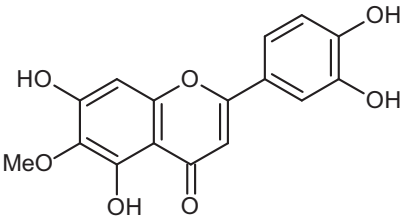
Figure 7. Flavonoids previously isolated from *Juniperus communis*.



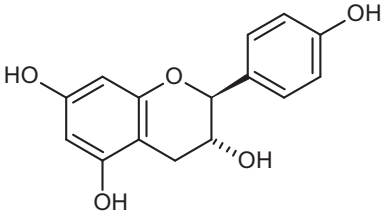
Compound	R
(59)	H
(66)	OH



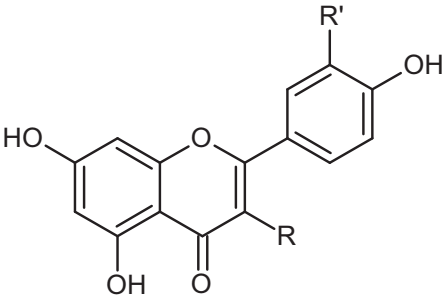
Compound	R	R’
(62)	H	H
(63)	OH	H
(65)	OH	OH



(60)

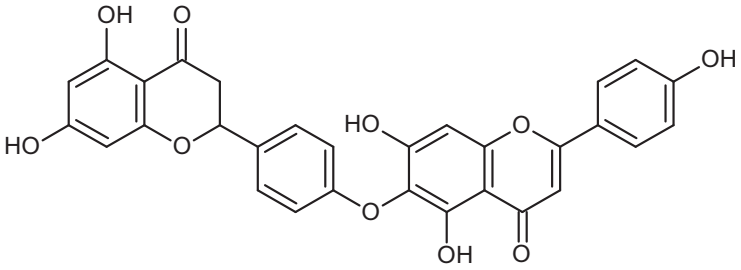


(64)

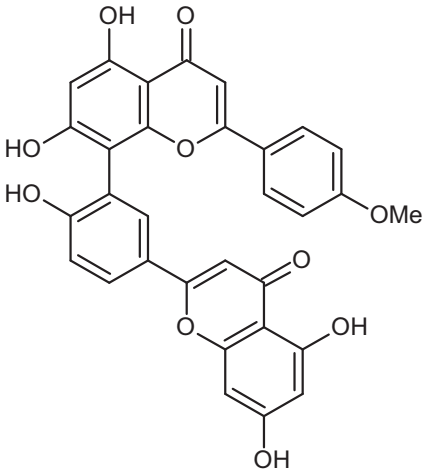


Compound	R	R’
(61)	OH	OH
(67)	H	H
(69)	H	OH

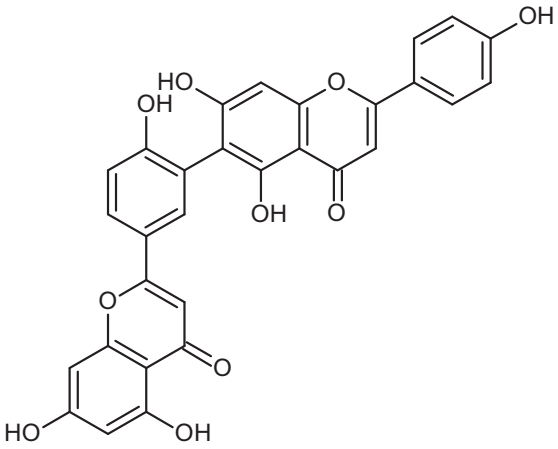
Figure 7 (continued). Flavonoids previously isolated from *Juniperus communis*.



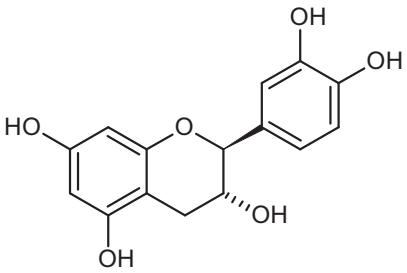
(68)



(70)



(71)



(72)

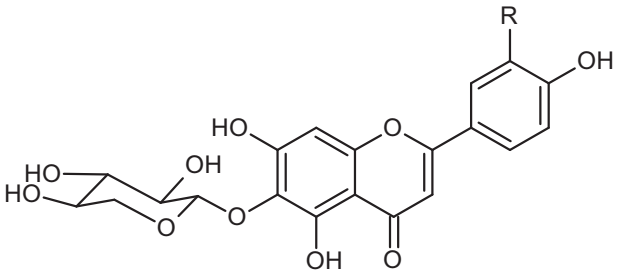
Table 9. Flavonoid glycosides previously isolated from *Juniperus communis*.

Isolated compound	Reference
3’,4’,5,6,7-pentahydroxyflavone 6-β-D-xyloside **(73)**	(Lamer-Zarawska 1977; Lamer-Zarawska 1980)
4’,5,6,7-tetrahydroxyflavone 6-β-D-xyloside **(74)**	(Lamer-Zarawska 1977; Lamer-Zarawska 1980)
Isoquercitrin **(75)**	(Iida et al. 2007, Kowalska 1980, Lamer-Zarawska 1977, Lamer-Zarawska 1980)
Nepitrin **(76)**	(Lamer-Zarawska 1977)
7-hydroxy-4’,5 6-trimethoxyflavone 7-*O*-glucoside **(77)**	(Lamer-Zarawska 1980)
Apigenin 7-*O*-glucoside **(78)**	(Lamer-Zarawska 1980)
Hypolactin 7-*O*-glucoside **(79)**	(Lamer-Zarawska 1980)
Quercetin 3-*O*-arabinosylglucoside 7-*O*-glucoside **(80)**	(Lamer-Zarawska 1980)
Rutin or rutoside **(81)**	(Lamer-Zarawska 1980; Kowalska 1980)
Scutellarein 7-*O*-glucoside **(82)**	(Lamer-Zarawska 1980)
Kaempferol-3-*O*-α-L-rhamnoside **(83)**	(Hiermann 1996)
Kaempferol-3-*O*- β-D-glucoside **(84)**	(Hiermann 1996)
Luteoli*n*-7-*O*-β-D-glucoside **(85)**	(Hiermann 1996)

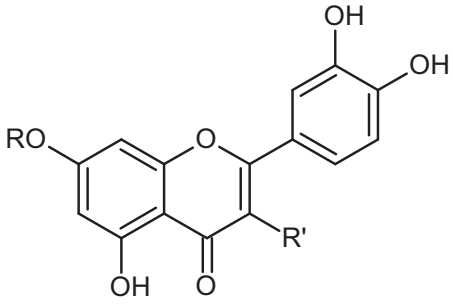
Table 9 (continued). Flavonoid glycosides previously isolated from *Juniperus communis*.

Isolated compound	Reference
Quercitrin **(86)**	(Hiermann 1996)
(*M*)-**(87)** and (*P*)-**(88)**cupressuflavone 4'-*O*-glucosides	(Inatomi et al. 2005)
3'-methoxy-5,7,8,4',5'-pentahydroxyflavone-7-*O*-β-D-xylopyranoside **(89)**	(Iida et al. 2007)
(+)-afzelechin 7-*O*-β-D-xylopyranoside **(90)**	(Iida et al. 2007)
(+)-3'-*O*-methylcatechi*n*-5-*O*-β-D-glucopyranoside **(91)**	(Iida et al. 2007)
isoscutellari*n*-7-*O*-β-D-glucopyranoside **(92)**	(Iida et al. 2007)
apigeni*n*-4'-*O*-β-D-glucopyranoside **(93)**	(Iida et al. 2007)
(+)-4'-*O*-methylcatechi*n*-5-*O*-β-D-glucopyranoside **(94)**	(Iida et al. 2007)
(+)-3'-*O*-methylepicatechi*n*-7-*O*-β-D-glucopyranoside **(95)**	(Iida et al. 2007)
luteoliflava*n*-5-*O*-β-D-glucopyranoside **(96)**	(Iida et al. 2007)
phlorizin **(97)**	(Iida et al. 2007)

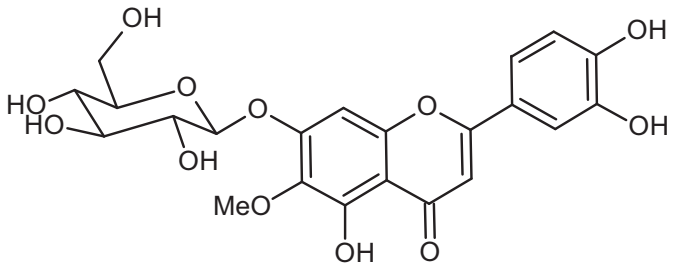
Figure 8. Flavonoid glycosides previously isolated from *Juniperus communis.*



Compound	R
(73)	OH
(74)	H

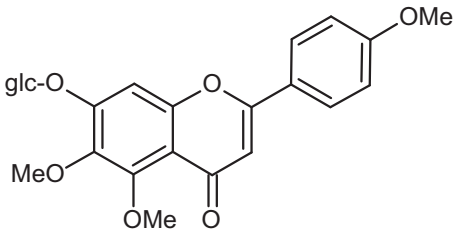


Compound	R	R’
(75)	H	*O*-rha
(80)	glc	*O*-glc-ara
(81)	H	*O*-glc-rha
(85)	glc	H
(86)	H	*O*-glc

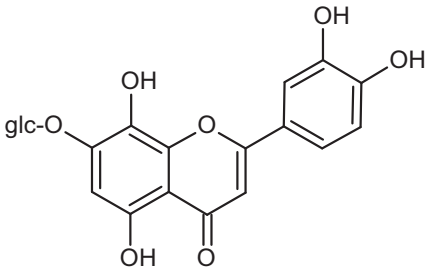


(76)

Figure 8 (continued). Flavonoid glycosides previously isolated from *Juniperus communis*.



(77)



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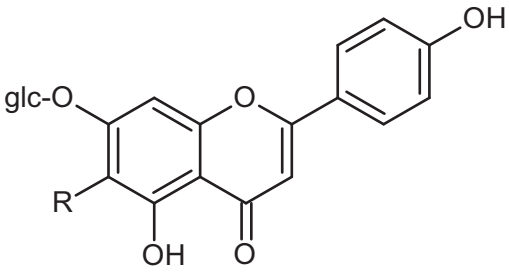
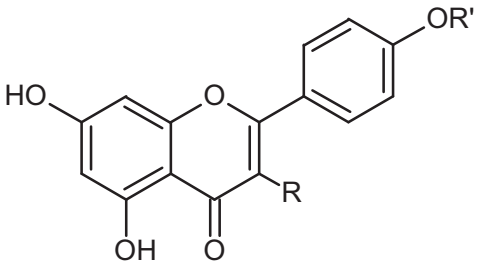
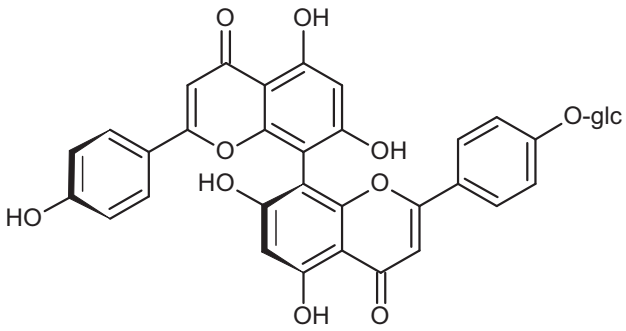


Compound	R
(78)	H
(82)	OH

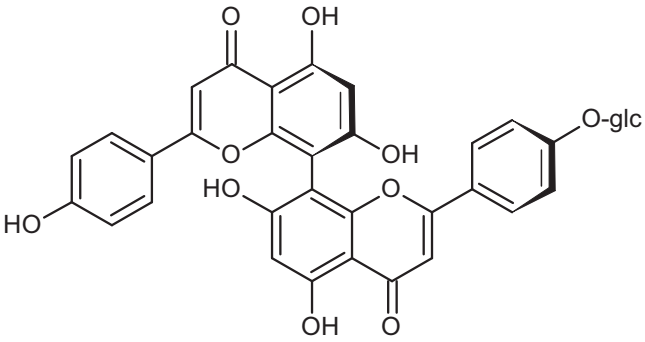


Compound	R	R’
(83)	*O*-rha	H
(84)	*O*-glc	H
(93)	H	*O*-glc

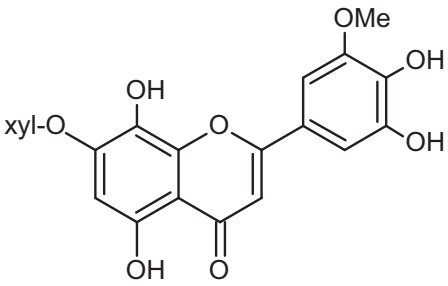
Figure 8 (continued). Flavonoid glycosides previously isolated from *Juniperus communis*.



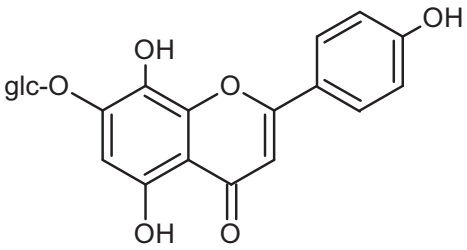
(87)



(88)

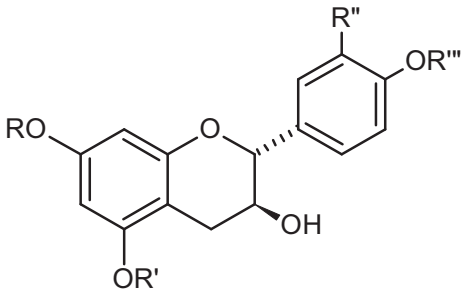


(89)

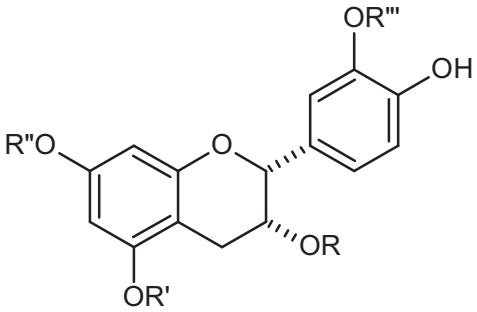


(92)

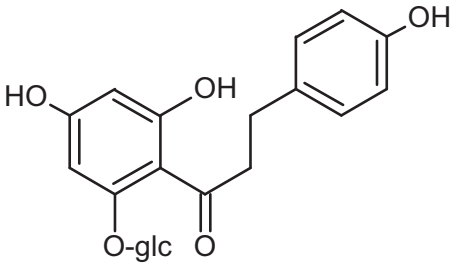
Figure 8 (continued). Flavonoid glycosides previously isolated from *Juniperus communis*.



Compound	R	R’	R”	R”’
(90)	xyl	H	H	H
(91)	H	glc	OMe	H
(94)	H	glc	OH	Me



Compound	R	R’	R”	R”’
(95)	OH	H	glc	Me
(96)	H	glc	H	H

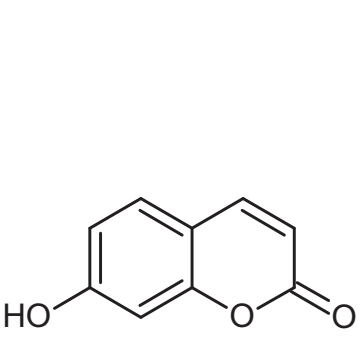


(97)

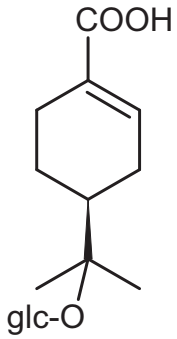
Table 10. Miscellaneous compounds previously isolated from *Juniperus communis*.

Isolated compound	Reference
Umbelliferone **(98)**	(Kowalska 1980)
Junipercomnoside A **(99)** and B **(100)**	(Nakanishi et al. 2004)
(-)-oleuropeic acid 8-*O*-β-D-glucopyranoside **(101)**	(Nakanishi 2005)

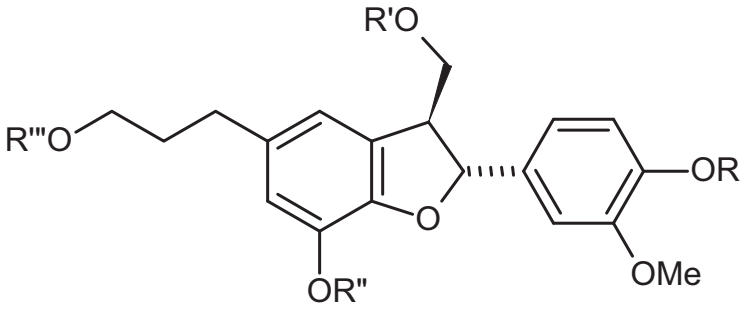
Figure 9. Miscellaneous compounds previously isolated from *Juniperus communis*.



(98)



(101)



Compound	R	R’	R”	R”’
(99)	xyl	H	H	H
(100)	H	rha	Me	glc

1.4.4 Previous biological work

Juniperus communis extracts and isolates have been investigated for various biological properties including anticancer (Bayazit 2004), anti-inflammatory (Tunón et al. 1995), antioxidant (Elmastaş et al. 2006), antiviral (Markhanen et al. 1981, Simons et al. 1963), diuretic (Vollmer and Giebel 1938) and hypoglycaemic activity (Sanches de Medina et al. 1994). They also showed inhibition of lipase (Kim and Kang 2005) and of platelet aggregation (Kagawa et al. 1993).

One study reported that the essential oil of *Juniperus communis* leaf and of the ripe and unripe berries did not inhibit the growth of *Candida albicans*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Angioni et al. 2003). Strong antifungal activity against *Candida* species and dermatophytes was reported for the essential oil prepared from the berries (Pepeljnjak et al. 2005).

The methanol extract of *Juniperus communis* berries showed no antimycobacterial acticity (MIC>500µg/mL) against *Mycobacterium aurum* and *Mycobacterium smegmatis* when tested using a broth microdilution assay (Newton et al. 2002). The methanol extract of the whole plant showed good inhibition of *Mycobacterium tuberculosis* growth in a disc diffusion assay (McCutcheon et al. 1997) and similar activity is reported using tube dilution assay and broth dilution assay (Gautam et al. 2007). The *n*-hexane and methanol extracts from the leaf showed good activity against *Mycobacterium tuberculosis* H_{37}Rv and its isoniazid-resistant and ethambutol-resistant variants (MIC values of 100µg/mL) when using a microplate Alamar blue assay (Jimenez-Arellanes et al. 2003) .

1.5 *Calluna vulgaris* L.

Table 11. Botanical classification of *Calluna vulgaris*

Kingdom	Plantae	Plants
Subkingdom	Tracheobionta	Vascular plants
Superdivision	Spermatophyta	Seed plants
Division	Magnoliophyta	Flowering plants
Class	Magnoliopsida	
Order	Ericales	
Family	Ericaceae	Heath family
Genus	*Calluna*	Heather
Species	*Calluna vulgaris* L.	

1.5.1 Description

Calluna vulgaris, also known as ‘ling’, ‘Scotch heather’ or ‘true heather’, is an evergreen low bushy shrub widely found in Scotland (Figure 10). The leaves are very short (about 3mm) and densely crowded on the twig. The flowers are also very short, bell-shaped and range from white to purple in colour depending on the cultivar.

Calluna vulgaris grows best on humus rich acidic soils in full sun and is the dominant plant species of Scottish boglands and woodlands. (Bentham 1924, Darwin 1996, Horwood 1919)

Figure 10. *Calluna vulgaris* in heathland.

1.5.2 Traditional uses

Calluna vulgaris is reported to have been traditionally used as antiseptic, cholagogue, nerve tonic, lung tonic, diaphoretic, diuretic, expectorant, anti-rheumatic and for the treatment of gout. An infusion prepared from heather tops was traditionally drunk in Scotland to treat cough and consumption (Allen and Hatfield 2004, Atkinson et al. 1974, Darwin 1996, Kumarasamy et al. 2002).

1.5.3 Previous phytochemical work

Previous phytochemical work on *Calluna vulgaris* mainly yielded some flavonoid glycosides. Compounds isolated to date are compiled in Tables 12 to 13 and Figures 11 to 12.

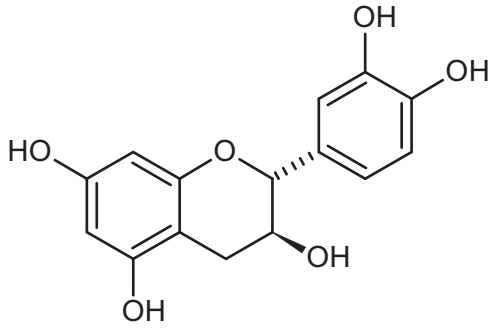
Table 12. Flavonoids and flavonoid glycosides previously isolated from *Calluna vulgaris.*

Compound	Reference(s)
(+)-catechin **(102)**	(Jalal et al. 1982)
2’’-acetylcallunin **(103)**	(Allais et al. 1995)
3-desoxycallunin **(104)**	(Allais et al. 1995)
Apigenin-7-(2-acetyl)glucuronic acid methyl ester **(105)**	(Allais et al. 1991)
Callunin **(106)**	(Jalal et al. 1982, Simon et al. 1993a)
Cyanidin-3-glucoside **(107)**	(Santamour and Lucente 1967)
Delphinidin **(108)**	(Santamour and Lucente 1967)
Dihydroherbacetin **(109)**	(Shelyuto et al. 1977)
Dihydroherbacetin-8-β-D-glucoside **(110)**	(Lamer-Zarawska et al. 1986)
Herbacetin-8-gentiobioside **(111)**	(Olechnowicz-Stepien et al. 1978)
Herbacetin-8-β-D-glucoside **(112)**	(Olechnowicz-Stepien et al. 1978, Shelyuto et al. 1977)
Kaempferol-3-[2’’’,3’’’,4’’’-triacetylarabinosyl(1→6)glucoside] **(113)**	(Simon et al. 1993b)
Kaempferol-3[2’’’,3’’’,5’’’-triacetylarabinosyl(1→6)glucoside] **(114)**	(Allais et al. 1991)
Quercetin-3-[2’’’,3’’’,4’’’-triacetyl-α-L-arabinosyl(1→6)-β-D-glucoside] **(115)**	(Simon et al. 1993a)
Quercetin-3-[2’’’,3’’’,5’’’-triacetylarabinosyl(1→6)-β-D-glucoside] **(116)**	(Simon et al. 1993b)

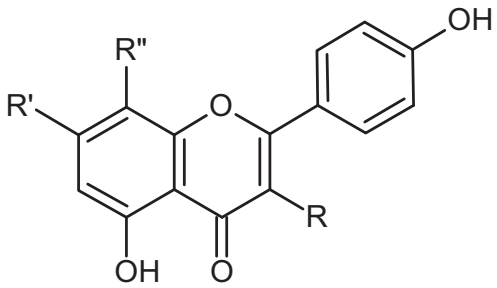
Table 12 (continued). Flavonoids and flavonoid glycosides previously isolated from *Calluna vulgaris*.

Compound	Reference(s)
Quercetin-3-galactoside **(117)**	(Jalal et al. 1982, Olechnowicz-Stepien et al. 1978)
Quercetin-3-glucoside **(118)**	(Jalal et al. 1982)
Quercetin-3-α-L-arabinoside (avicularin) **(119)**	(Simon et al. 1993a, Zozulya et al. 1974)
Taxifolin-3-β-D-glucoside **(120)**	(Olechnowicz-Stepien et al. 1978)

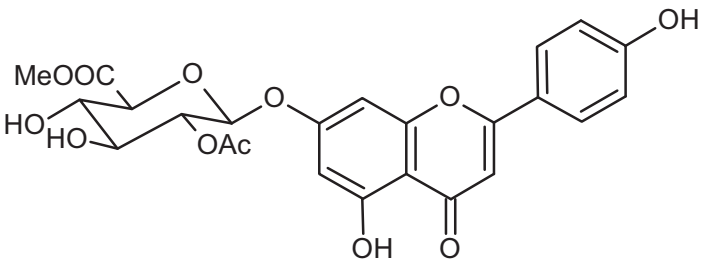
Figure 11. Flavonoids and flavonoid glycosides previously isolated from *Calluna vulgaris.*



(102)

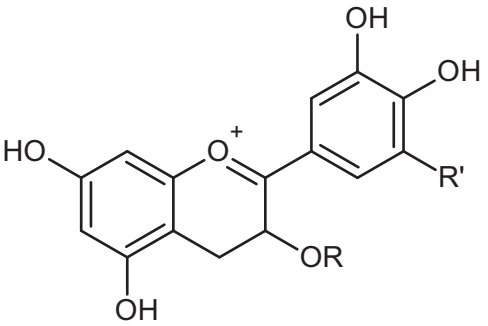


Compound	R	R’	R”
(103)	OH	glc	2”-acetyl-glc
(104)	H	OH	*O*-glc
(106)	OH	OH	glc

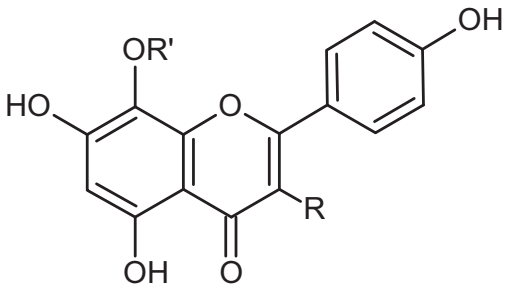


(105)

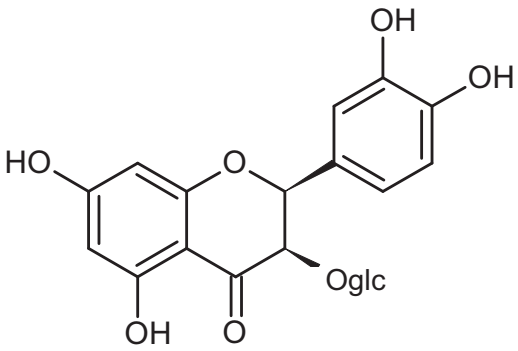
Figure 11 (continued). Flavonoids and flavonoid glycosides previously isolated from *Calluna vulgaris*



Compound	R	R’
(107)	Glc	H
(108)	H	OH

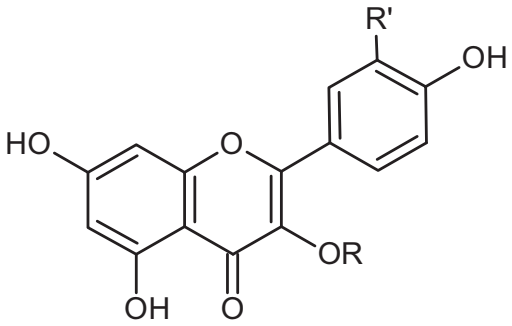


Compound	R	R’
(109)	H	H
(110)	H	Glc
(111)	OH	glc-β(1→6)-glc
(112)	OH	Glc



(120)

Figure 11 (continued). Flavonoids and flavonoid glycosides previously isolated from *Calluna vulgaris*

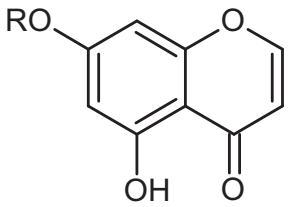


Compound	R	R’
(113)	[2’’’,3’’’,4’’’-triacetylarabinosyl(1→6)glucoside]	H
(114)	[2’’’,3’’’,5’’’-triacetylarabinosyl(1→6)glucoside]	H
(115)	[2’’’,3’’’,4’’’-triacetylarabinosyl(1→6)glucoside]	OH
(116)	[2’’’,3’’’,5’’’-triacetylarabinosyl(1→6)glucoside]	OH
(117)	gal	OH
(118)	glc	OH
(119)	ara	OH

Table 13. Miscellaneous compounds previously isolated from *Calluna vulgaris*

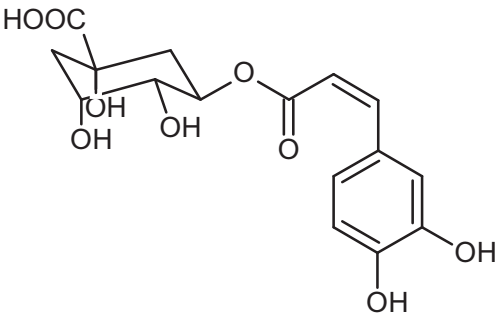
Isolated compound	Reference (s)
5,7-dihydroxychromone **(121)**	(Simon et al. 1994)
5,7-dihydroxychromone 7-glucoside **(122)**	(Simon et al. 1994)
Chlorogenic acid **(123)**	(Jalal et al. 1982)
Ursolic acid **(124)**	(Najid et al. 1992, Simon et al. 1992)
Procyanidin D1 **(125)**	(Jalal et al. 1982)

Figure 12. Miscellaneous compounds previously isolated from *Calluna vulgaris*



Compound	R
(121)	H
(122)	glc

Figure 12 (continued). Miscellaneous compounds previously isolated from *Calluna vulgaris*



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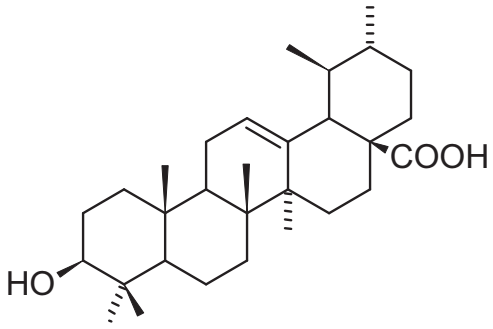

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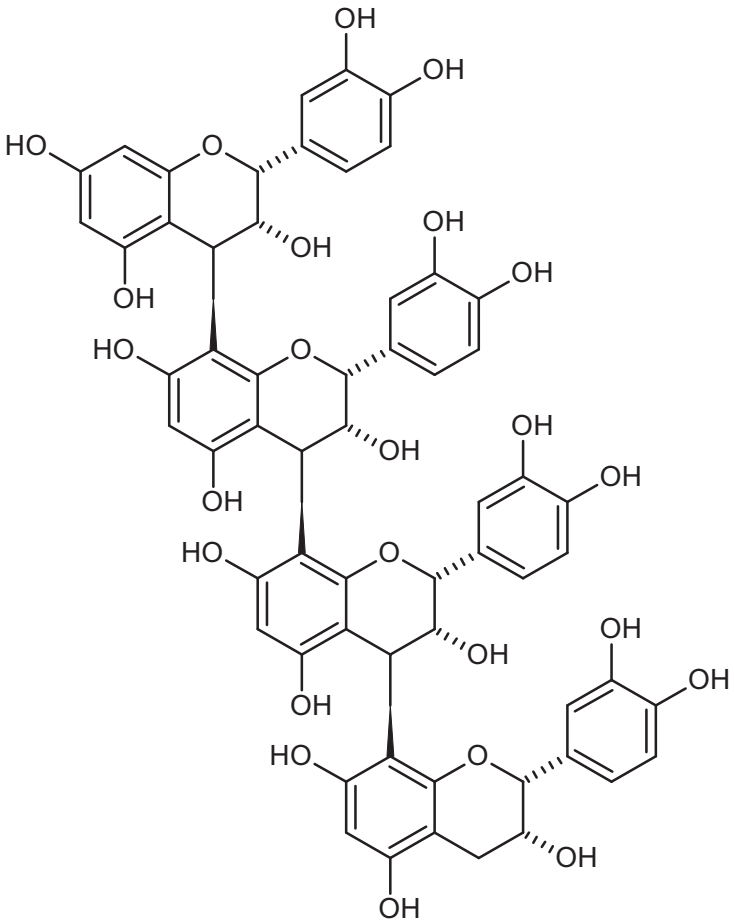


(125)

1.5.4 Previous biological work

A crude extract prepared from *Calluna vulgaris* aerial parts reduced arterial pressure, had hypothermal effect and facilitated blood coagulation (Zozulya et al. 1974).

Ursolic acid from *Calluna vulgaris* was found to inhibit lipoxygenase and cyclooxygenase enzyme, thus partly explaining the anti-inflammatory activity of calluna. Ursolic acid (at a concentration of 1µM) also blocked arachidonate metabolism in mouse peritoneal macrophages, human platelets and differenciated HL60 leukemic cells (Najid et al. 1992). Further studies disclosed that ursolic acid from *Calluna vulgaris* inhibited DNA synthesis of HL60 cells in a dose-dependent manner (Simon et al. 1992).

The ethyl acetate extract of *Calluna vulgaris* aerial parts showed anti-inflammatory activity in a carrageenan-induced hind paw edema assay. This activity was attributed to kaempferol-3-*O*-β-D-galactoside isolated through biosassay-guided fractionation (Orhan et al. 2007).

The methanol extract of *Calluna vulgaris* seeds showed antibacterial activity (MICs of 100µg/mL against *Staphylococcus aureus* and *Staphylococcus hominis* (Kumarasamy et al. 2002).

The ethanol and diethyl ether extracts of *Calluna vulgaris* aerial parts showed no antimicrobial activity. The aqueous extract, however, showed good activity against *Staphylococcus aureus* and *S. epidermidis*, *Candida albicans*, and *Cryptococcus neoformans.* It also showed moderate activity against *Escherichia coli*, *Pseudonomas aerugonisa* and *Proteus vulgaris* (Braghilori et al. 1996).

1.6 *Myrica gale* L.

Table 14. Botanical classification of *Myrica gale*

Kingdom	Plantae	Plants
Subkingdom	Tracheobionta	Vascular plants
Superdivision	Spermatophyta	Seed plants
Division	Magnoliophyta	Flowering plants
Class	Magnoliopsida	
Subclass	Hamamelididae	
Order	Myricales	
Family	Myricaceae	Bayberry family
Genus	*Myrica* L.	Sweetgale
Species	*Myrica gale* L	

1.6.1 Description

Myrica gale, also known as sweetgale or bog myrtle, is an aromatic shrub that is native to Scotland (Figure 13). The leaves are dark green, long (3-8cm) and sweet-scented. The flowers grow in short catkins (under 2cm). The fruits are small waxy ovoid drupes. *Myrica gale* grows best on peaty acidic soil and is a common species of Scottish boglands (Genders 1977-1994, Horwood 1919).

Figure 13. *Myrica gale*

1.6.2 Traditional uses

Leaves and fruits of *Myrica gale* are used for their aromatic properties in cooking and brewing. An infusion from the leaves and the essential oil were traditionally used for their stomachic, astringent, emmenagogue, abortifient and insect repellent properties (Duke 1995, Genders 1977-1994).

1.6.3 Previous phytochemical work

Several studies have investigated the chemical composition of essential oils obtained from *Myrica gale* fruits and leaves by GC analysis. The main constituents in the essential of the dried leaves by steam distillation were myrcene (16.2%), selin-11-e*n*-4-ol (14.6%) and limonene (10.8%) (Lawrence and Weaver 1974). The main constituents in the essential oil of the air-dried fruits oil obtained by supercritical fluid extraction were 1,8-cineole (25.7%) and α-pinene (20.6%) (Sokolova et al. 2005). The main constituents in the essential oil of the fresh leaves obtained by hydrodistillation were selin-11-e*n*-4-ol (11.5%) myrcene (11.2%) and β-caryophyllene (8.3%) (Sylvestre et al. 2006).

Previous phytochemical investigation of *Myrica gale* mainly afforded flavonoids. All compounds isolated from *Myrica gale* to date are compiled in Tables 15 to 17 and Figures 14 to 16.

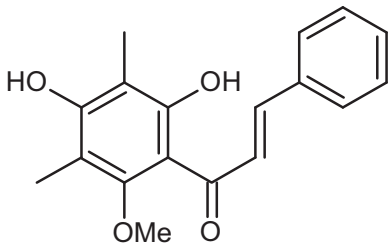
Table 15. Flavonoids previously isolated from *Myrica gale*.

Compound	Origin	Reference(s)
2’,4’’-dihydroxy-6’-methoxy-3’,5’-dimethylchalcone **(126)**	Acetone extract of leaves	(Malterud et al. 1977)
2’,6’-dihydroxy-4’-methoxy-3’,5’-dimethyldihydrochalcone or myricagalone B **(127)**	Fruits extract Diethyl-ether extract of fruits	(Anthonsen et al. 1971, Uyar et al. 1978)
2’-hydroxy-4’,6’-dimethoxy-3’-methyldihydrochalcone **(128)**	Ether extract of fruits	(Malterud et al. 1977)
Angoletin **(129)**	Diethyl-ether extract of fruits	(Malterud 1992)
Catechin (Figure 7, **59**)	Aerial parts extract	(Santos and Waterman 2000)
Epicatechin (Figure 7, **63**)	Aerial parts extract	(Santos and Waterman 2000)
Epigallocatechin (Figure 7, **65**)	Aerial parts extract	(Santos and Waterman 2000)
Epigallocatechin-3-*O*-gallate **(130)**	Aerial parts extract	(Santos and Waterman 2000)
Gallocatechin (63) (Figure 7, **66**)	Aerial parts extract	(Santos and Waterman 2000)
Kaempferol-3-(2,3-diacetoxy-4-p-coumaroyl)-rhamnoside **(131)**	Methanol extract of leaves	(Carlton et al. 1990)

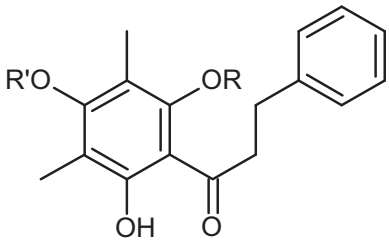
Table 15 (continued). Flavonoids previously isolated from *Myrica gale*.

Compound	Origin	Reference(s)
Myrcetin 3,3’-di--D-galactoside **(132)**	Leaves extract	(Bodalski and Rzadkowska-Bodalska 1969)
Myrcetin 3-D-galactoside **(133)**	Leaves extract	(Bodalski and Rzadkowska-Bodalska 1969)
Myrcetin 3-*O*-(6’’-galloyl)-β-D-galactopyranoside **(134)**	Methanol extract of stems	(Nagai et al. 1995)
Myricagalone E **(135)**	Diethyl-ether extract of fruits	(Malterud 1992)
Myricagalone G **(136)**	Diethyl-ether extract of fruits	(Malterud 1992)
Myricagalone H **(137)**	Diethyl-ether extract of fruits	(Malterud 1992)
Quercetin (Figure 7, **61**)	Leaves extract	(Bodalski and Rzadkowska-Bodalska 1969)
Quercetin 3-*O*-galactoside (Figure 11, **117**)	Leaves extract	(Bodalski and Rzadkowska-Bodalska 1969)
Quercetin 3-*O*-glucoside (Figure 11, **118**)	Leaves extract	(Bodalski and Rzadkowska-Bodalska 1969)

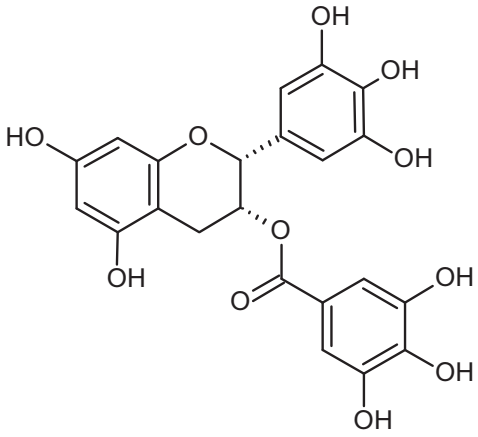
Figure 14. Flavonoids previously isolated from *Myrica gale*.



(126)

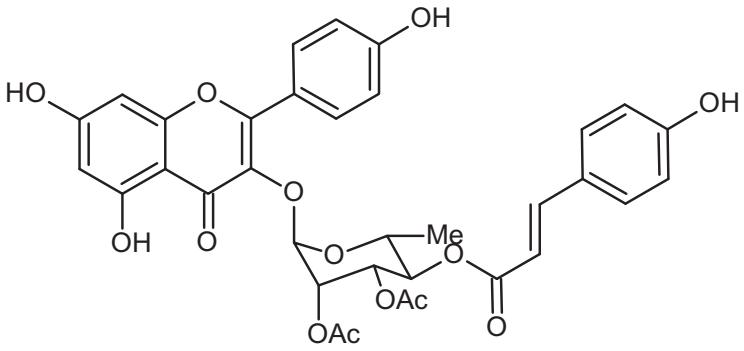


Compound	R	R’
(127)	H	Me
(128)	Me	H
(129)	Me	Me



(130)

Figure 14 (continued). Flavonoids previously isolated from *Myrica gale*



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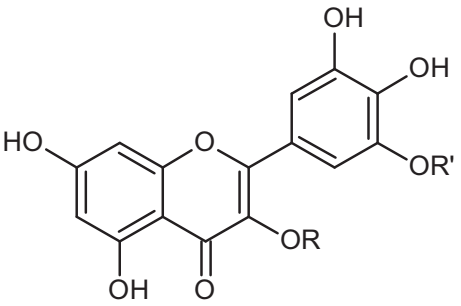
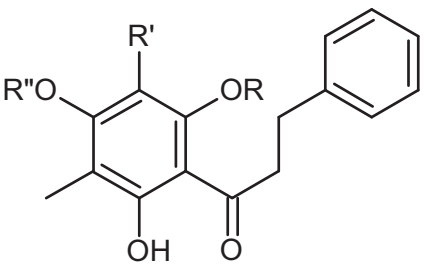


Compound	R	R’
(132)	gal	gal
(133)	gal	H
(134)	6”-galloyl-gal	H

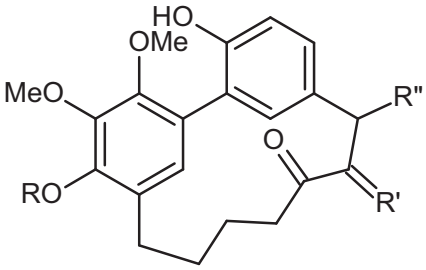


Compound	R	R’	R”
(135)	Me	Me	Me
(136)	H	H	Me
(137)	Me	H	H

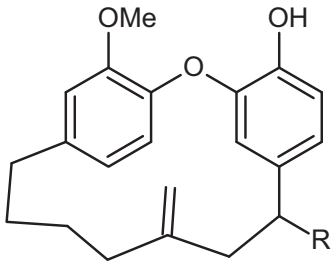
Table 16. Diarylheptanoids previously isolated from *Myrica gale*.

Compounds	Origin	Reference(s)
12-dehydroporson **(138)**	Methanol extract of stems	(Nagai et al. 1995)
12-hydroxymyricanone **(139)**	Methanol extract of stems	(Nagai et al. 1995)
Galeon **(140)**	Ethyl acetate extract of stems	(Malterud et al. 1976, Morihara et al. 1997)
Hydroxygaleon **(141)**	Ethyl acetate extract of stems	(Malterud et al. 1976)
Myricanone **(142)**	Methanol extract of stems	(Malterud 1981, Nagai et al. 1995)
Myricatomentoside I **(143)**	Methanol extract of branches	(Morihara et al. 1997)
Myricatomentoside II **(144)**	Methanol extract of branches variety	(Morihara et al. 1997)
Porson **(145)**	-	(Anthonsen et al. 1975, Nagai et al. 1995)
Porson (revised structure) **(146)**	Methanol extract of stems	(Nagai et al. 1995)

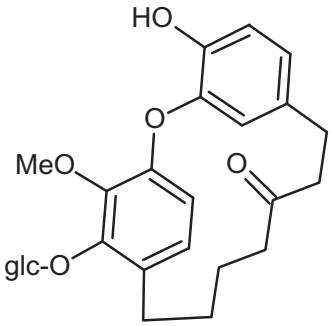
Figure 15. Diarylheptanoids previously isolated from *Myrica gale*



Compound	R	R’	R”
(138)	Me	O	H
(139)	H	H, OH	H
(142)	H	H, H	H
(145)	Me	H, H	OH
(146)	Me	H, OH	H



Compound	R
(140)	H
(141)	OH



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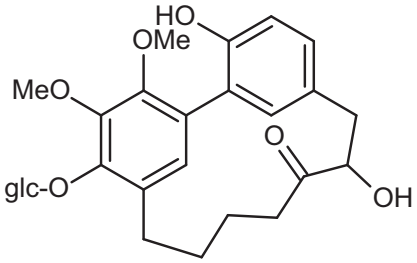
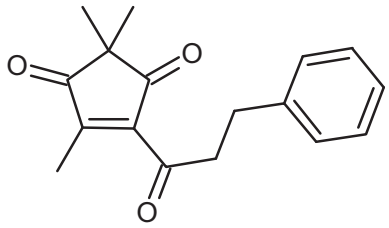


(144)

Table 17. Miscellaneous compounds previously isolated from *Myrica gale*.

Compounds	Origin	Reference(s)
2,2,5-trimethyl-a(3-phenyl-propionyl)-cyclopent-4-ene-1,3-dione **(147)**	Fruits extract	(Anthonsen et al. 1971)
4,4,6-trimethyl-2-(3-phenylpropionyl)-cyclohexane-1,3,5-trione	Fruits extract	(Anthonsen et al. 1971, Uyar et al. 1978)
or myricagalone A **(148)**	Diethyl-ether extract of fruits	(Malterud 1992)
Gallic acid **(149)**	Methanol extract of stems	(Nagai et al. 1995)
Gallocatechin-(4α-8)-epicatechin **(150)**	Aerial parts extract	(Santos and Waterman 2000)
Gallocatechin-(4α-8)-epigallocatechin **(151)**	Aerial parts extract	(Santos and Waterman 2000)
Gallocatechin-(4α-8)-gallocatechin-(4α-8)-gallocatechin **(152)**	Aerial parts extract	(Santos and Waterman 2000)
Myricardiol or myricadiol **(153)**	Acetone extract of bark	(Ryabinin and Matyukhina 1959)
Oleanolic acid **(154)**		(Borovkov and Belova 1962)
Ursolic acid (Figure 12, **124**)		(Borovkov and Belova 1962)

Figure 16. Miscellaneous compounds previously isolated from *Myrica gale*



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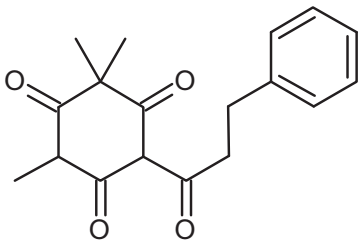

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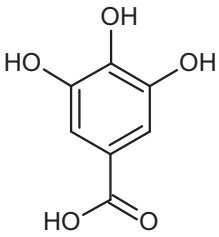

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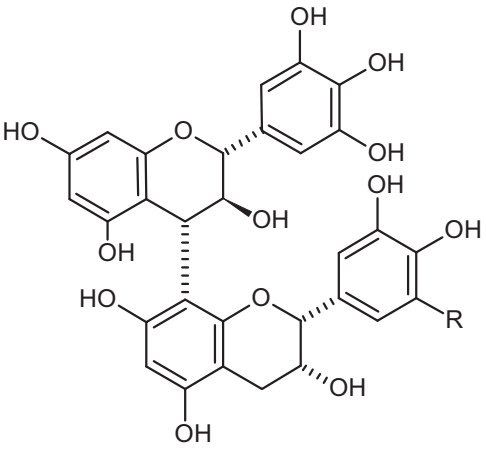
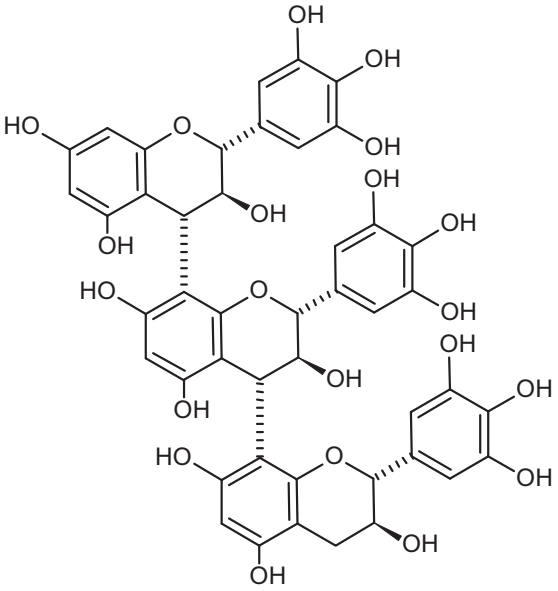


Compound	R
(150)	H
(151)	OH



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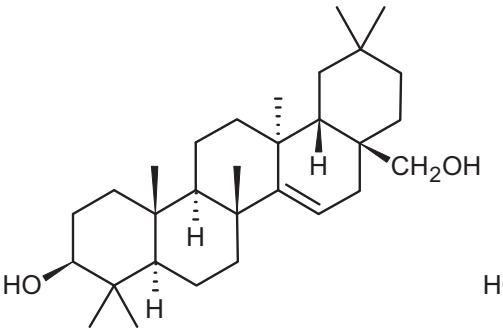
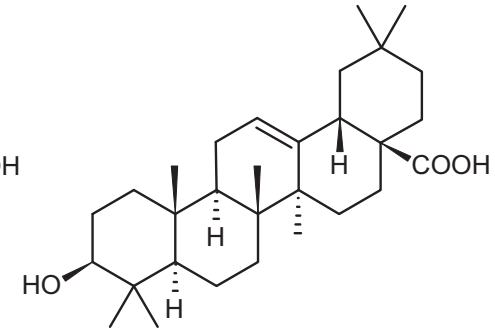


(153)



(154)

1.6.4 Previous biological work

There are very few reports on biological activity of *Myrica gale* extracts or isolates. Most studies carried out so far have focussed on investigating the activity of *Myrica gale* essential oil.

Myrigalone B, isolated from the fruit exudate of *Myrica gale,* was found to be a potent antioxidant (Mathiesen et al. 1996, Mathiesen et al. 1995). It also showed bactericidal activity (Malterud and Faegri 1982). Myrigalone A was reported to inhibit growth of Gram-positive bacteria and *Aspergillus fumigatus* (Malterud and Faegri 1982).

The oil distilled from *Myrica gale* leaves and a 10% dilution of this oil both demonstrated antifungal activity against *Trichophyton interdigitale* (Stuart 1998).

The essential oil obtained following hydrodistillation of *Myrica gale* leaves showed promising anti-cancer activity against human lung carcinoma cell line A-549 and human colon adenocarcinoma cell line DLD-1 with IC_{50} values of 88±1µg/mL in both case (Sylvestre et al. 2006).

The *n*-hexane, ethyl acetate and methanol extracts of *Myrica gale* leaves as well as its essential oil showed weak tick-repellent activity on *Ixodes ricinus* (Jaenson et al. 2005). The oil of *Myrica gale* is also efficient against the biting midges *Culicoides impunctatus* and is used in commercialised midges-repellent products (Simpson et al. 1996).

1.7 Aims and objectives

As there is currently a need for new anti-TB drugs and since Scottish natural sources have been largely unexplored so far, the primary aim of this work was to isolate and identify antimycobacterial products from a selection of plants, lichen and fungal endophytes collected in Scotland.

In addition, the secondary goal of the project was to purify and identify other secondary metabolites from the selected plants. Isolation of structural analogues of the active compounds for comparison and of other classes of compound is a way of assessing the validity and the limitations of the bioassay-guided fractionation method.

The third goal was to established whether some of the isolated compounds, from both active and inactive fractions, exhibited broader antibacterial activy

Thus the objectives of the work were to:

- Perform a primary screening of extracts against *Mycobacterium aurum* and *Mycobacterium tuberculosis* with a view to select promising extracts for further fractionation work.
- Perform bioassay-guided fractionation of selected extracts using various chromatographic techniques to isolate the compound(s) responsible for the antimycobacterial activity.
- Isolate compounds from fractions deemed inactive
- Elucidate the structure of isolated compounds using spectroscopic techniques such as NMR, IR, UV and MS.

- Screen identified compounds for activity against drug-susceptible, drug-resistant and non replicating strains of *Mycobacterium tuberculosis* as well as against non-tuberculous mycobacteria.

- Perform cytotoxicity studies to establish the selectivity of isolated compounds towards mycobacteria.

- Perform further screening of selected isolated compounds against a panel of Gram-positive and Gram-negative bacteria to establish whether the isolated compounds had further antibiotic activity.

Chapter 2: MATERIALS AND METHODS

Chapter 2 Materials and Methods

2.1 General

2.1.1 Solvents and chemicals

Analytical grade ethyl acetate and methanol were obtained from Fisher Scientific UK. Analytical grade *n*-butanol, chloroform and dimethylformamide (DMF) were obtained from VWR UK. Analytical grade dimethylsulfoxide (DMSO) and ethanol were obtained from Sigma-Aldrich UK. HPLC grade *n*-hexane was obtained from Rathburn Chemicals Ltd UK. Ethanol, ethyl acetate and methanol were distilled before use.

Bergapten, longifolene, oleanolic acid and totarol and oleanolic acid were obtained from Sigma-Aldrich UK. Oxypeucedanin was obtained from PhytoLab GmbH, Germany.

2.1.2 Plant material

Plants were either purchased from a local nursery (Alba Trees Plc, Gladsmuir, UK) or collected in the wild. Details of collection sites of plants and lichen are presented in the appendices (Table 1). Voucher specimens were deposited at the herbarium of the Natural Product Research Laboratories, Strathclyde Institute of Pharmacy and Biomedical Sciences.

The plant material was dried at room temperature over several days then ground to a fine powder using an all basicS2® analytical mill (Ika® Werke GmbH & Co. KG, Germany) purchased from VWR, UK. A Fritsch® cutting mill equipped with three rotating knives and a fine sieve was used to grind larger quantities.

2.1.3 Isolation and culture of endophytes

Isolation and culture were performed by Mr Kevin INGLEBY. (Centre for Ecology and Hydrology, Penicuik, UK)

Where possible, ectomycorrhizal (ECM) fungal endophytes were isolated from clean tissue transferred aseptically from the fruiting bodies to agar plates. If clean tissue was not available, collections of spores were made so that isolates could be obtained by germinating spores. ECM isolates were also obtained from plant ECM roots. All ericoid mycorrhizal (ERM) fungal endophytes were isolated from plant ERM roots. Details of collections sites are presented in the appendices (Table 1). ECM and ERM root isolates were made by first selecting and cleaning roots under a dissecting microscope. Root fragments (5-10 mm) containing ECM or ERM were washed under tap water and retained on a flame-sterilised tea strainer. Fragments were sonicated (2 x 15s) and gently shaken in 1% Tween 80 (1min), then rinsed in sterile distilled water. This was followed by additional shaking in 30% H_2O_2 for ca. 15s (ERM) or 30s to 2min (ECM), and a final rinse (3 times) in sterile distilled water. Root fragments were blotted on sterile filter paper in a Petri dish. Roots containing ERM were aseptically cut into 1mm fragments whilst branched roots with ECM were cut into single elements. Some roots with ECM were dissected to expose the hyphae penetrating between root cells (i.e. the Hartig net). Root pieces were then transferred aseptically and spread out on agar plates. Several agar media were prepared to identify the optimal nutritional requirements for the growth of each fungal strain. Agar media used included modified Melin Norkrans (MMN) containing (L^{-1}): KH_2PO_4 (0.5g), $MgSO_4$ $7H_2O$ (0.15g), $CaCl_2$ (50mg), NaCl (25mg), thiamine (100µg), $(NH_4)_2HPO_4$ (0.25g), $FeCl_3$ (1% solution) (1.2mL), agar (10g), supplemented with glucose (10g/L) or malt extract (3g/L); Potato dextrose and Malt extract agar (Oxoid, UK).

Media were made at full or half strength formulation. Streptomycin and penicillin G (both 30mg/L) (Sigma-Aldrich, UK), with or without the fungicidal agent benomyl (2mg/L) (Sigma-Aldrich, UK), were added to the media in order to selectively isolate endophytes from root samples. Plates were incubated at 15-20°C and checked daily for contamination or the emergence of the target fungi. Once cultures were obtained, sub-cultures were performed as necessary on the required nutrient agar to purify isolates. ECM fungal isolates were identified from their ECM morphology (Agerer et al. 1996-2008, Ingleby et al. 1990) and by comparing the growth of the cultures with that of known strains from fungal fruiting bodies. ERM fungal isolates and other fungal endophytes were identified by comparing morphological characterisation with published descriptions and taxonomic keys.

Endophytes cultures were provided as flasks containing a single species mycelium growing in aqueous broth. Cultures were filtered using N301 Bio PrepNylon™ cloth (Biodesign Inc. NY). Mycelia were rinsed with distilled water and air dried. Aqueous broths and mycelia were stored at 5°C before extraction.

Details of collection sites of endophytes and extraction yields are presented in the appendices (Table 2). Voucher specimens of fruiting bodies and freeze-dried stocks of endophytes are kept in the Herbarium of the Natural Product Research Laboratories, Strathclyde Institute of Pharmacy and Biomedical Sciences.

2.2 Extraction

Various methods of extractions with solvent were used. All extracts obtained were dried using a Büchi R-205 rotary evaporator and stored at -20°C before testing. Extraction yields are presented in the appendices (Tables 1 and 2).

2.2.1 Soxhlet extraction

Juniperus communis and *Abies fraseri* aerial parts were extracted successively with 3L of *n*-hexane, ethyl acetate and methanol each for 25h using a Soxhlet apparatus. The extracts obtained were then filtered using qualitative filter paper (Whatman® #1), dried and stored at -20°C.

2.2.2 Accelarated Solvent Extraction

For all other plants and for the lichen species, the dried powdered material was extracted successively with *n*-hexane, ethyl acetate and methanol in an Accelerated Solvent Extractor (ASE 100®, Dionex) using pressurised liquid extraction. Four static cycles with a static time of 8min and a volume flush of 60% were run at a fixed oven temperature of 100°C. Extracts obtained were then dried and stored at -20°C.

2.2.3 Maceration and sonication

Fungal endophyte mycelia were sonicated in 25mL of distilled ethanol at 45°C for 5 min. The process was repeated thrice and the extracts were pooled.

2.2.4 Liquid-Liquid partition

Fungal endophyte aqueous broths were successively partitioned between *n*-hexane, chloroform and *n*-butanol. The volume of solvent used for each partition was equal to the volume of broth (ca. 150mL). The process was repeated thrice with each solvent and the extracts were pooled, dried and stored.

2.3 Chromatographic techniques

2.3.1 Thin Layer Chromatography

Thin Layer Chromatography (TLC) was performed on aluminium supported silica gel 60 F254 TLC plates (Merck®).

Binary solvent systems *n*-hexane/chloroform, *n*-hexane/ethyl acetate, chloroform/methanol, ethyl acetate/methanol were used as mobile phases. A few drops of acetic acid were occasionally added to the methanol-containing mobile phases to prevent spot 'tailing'.

Compounds were first visualised under UV light at 254nm. Plates were then spayed with either anisaldehyde-H_2SO_4 reagent which allow visualisation of most compounds or with Dragendorff's reagent which allow a more specific visualisation of nitrogenous compounds.

2.3.2 Vacuum Liquid Chromatography

Vacuum Liquid Chromatography (VLC) was performed as in a vacuum fritted Büchner funnel as previously described (Targett et al. 1979). A compressed layer (ca. 5cm) of silica gel 60H (Merck®) was used as the stationary phase.

Samples were dissolved in an appropriate solvent, adsorbed on a small amount of silica gel 60 (Merck®) and dried to achieve a free flowing powder. The powder was loaded and packed as a uniform thin layer on top of the compressed silica layer and covered with cotton wool.

Elution was started with *n*-hexane and followed with ethyl acetate and *n*-hexane mixtures of increasing polarity and finally with mixtures of ethyl acetate and methanol (up to 20% methanol in ethyl acetate).

2.3.3 Open Column Chromatography

2.3.3.1 Size exclusion chromatography

Lipophilic Sephadex® LH20100 (Sigma-Aldrich®, UK) was soaked in a solution of 5% *n*-hexane in chloroform for several hours. The slurry was then poured and packed in a glass chromatography column of appropriate size.

Samples were dissolved in a small volume of solution of 5% *n*-hexane in chloroform. The concentrated sample solution was loaded at the top of the column.

Elution started with the same solution of 5% *n*-hexane in chloroform. If needed elution was continued with 100% chloroform followed by 5% methanol in chloroform. Sephadex® was then washed several times with methanol and kept dried for further use.

2.3.3.2 Silica gel column

Open column chromatography (CC) was performed on silica gel 60 (Merck®) suspended in a starting solvent system chosen after TLC analysis of the sample to be fractionated. The slurry was then poured and packed in a glass chromatography column of appropriate size. Samples were adsorbed on a small amount of silica gel 60 and loaded at the top of the column. The columns were eluted with solvent systems of increasing polarity.

2.3.4 Flash Chromatography

Flash chromatography (FC) was performed on a FlashMaster™ Personal apparatus (Jones Chromatography).

Samples were adsorbed on bulk Isolute® sorbent HM-N (International Sorbent Technology Ltd) and loaded onto an Isolute® Flash SiII cartridge (International Sorbent Technology Ltd). Columns were eluted under pressure with solvent systems of increasing polarity chosen by prior TLC analysis of the sample to be fractionated.

2.4 Structure elucidation

2.4.1 NMR spectroscopy

1D and 2D NMR spectra either recorded on a Brucker Advance DMX spectrometer operating at 600MHz (^{1}H) and 150MHz (^{13}C) or on a JEOL JNM-LA400 FT spectrometer operating at 400MHz (^{1}H) and 100MHz (^{13}C). All spectral data obtained were processed with MestReNova software (version 5.2.5-4119).

2.4.2 Mass spectrometry

Mass spectrometry experiments were recorded by Mr James Tweedie (University of Glasgow Chemistry department) on a JEOL JMS-700 high resolution mass spectrometer.

2.4.3 Infrared spectroscopy

Infrared sprectroscopy experiments were run on an ATI Mattson Genesis Series FTIR^{TM} spectrophotometer. Spectra were processed with Winfirst software (version 1.05) and functional groups were identified based on the literature (Hesse et al. 1997).

2.4.4 Ultraviolet spectroscopy

Ultraviolet sprectroscopy experiments were run on a Unicam UV300 UV-Visible spectrophotometer. Spectra were processed using Vision32 software (version 1.05).

2.4.5 Optical rotation

Optical rotations were measured by Mr James Tweedie (University of Glasgow Chemistry department) at 21°C using a 10mm cell on an Autopol V, automatic polarimeter (Rudolph Research Analytical, USA)

2.5 Antimicrobial assays

2.5.1 Screening against *Mycobacterium tuberculosis*

The activity against *Mycobacterium tuberculosis* H_{37}Rv (ATCC 27294), H_{37}Rv-rifampicin-resistant (ATCC 35838), H_{37}Rv-isoniazid-resistant (ATCC 35822), H_{37}Rv-streptomycin-resistant (ATCC 35820) and a moxifloxacin-resistant isolate (generated from H_{37}Rv at the University of Illinois at Chicago with an aspartic acid to asparagine mutation in codon 94) was assessed by Ms. Baojie WAN (ITR, Chicago, USA) according to a method based on the microplate Alamar Blue assay (MABA) (Collins and Franzblau 1997). Stock solutions of extracts were prepared in DMSO and added, at a concentration of 100 μg/mL, to two wells of a microplate containing Middlebrook 7H12 broth (Falzari et al. 2005). One well was inoculated with broth containing $2x10^4$CFU *M. tuberculosis* H_{37}Rv. The second well received only media in order to assess background fluorescence.

Isoniazid, rifampicin, moxifloxacin and PA-824 (a nitroimidazopyran-derived experimental antitubercular drug candidate) were included as antibiotic controls. Additional controls consisted of growth control (bacteria+DMSO) and sterility control (media only). For MICs, two-fold serial dilutions were performed in 7H12 media. Each microplate was incubated for 5 days at 37 °C in a 5% CO_2 atmosphere in a sealed plastic bag. After 5 days of incubation at 37°C, one control growth was developed with a mixture of Alamar blue solution (20μL) (Trek Diagnostics, Westlake Ohio, USA) and sterile 10% Tween 80 (12μL). The plates were re-incubated at 37°C for 24h.

After this, if the well turned pink, the dye mixture was placed into all wells and the plates were re-incubated for an additional 24h. The mean fluorescence units (FU) of media-only wells were subtracted from all other wells. Each sample was assayed in duplicate. Results were expressed in terms of percentage of inhibition defined as follows:

$$\%\ \text{inhibition} = 1 - \left(\frac{\text{test well FU}}{\text{mean FU of triplicate bacteria} - \text{only wells}} \times 100\right)$$

The MIC was defined as the lowest concentration effecting a reduction in fluorescence of ≥90% relative to bacteria only controls. Each sample was assayed in duplicate.

The potential of samples to target the subpopulation of *M. tuberculosis* in the non-replicating persistent (NRP) state, was assessed using a low oxygen recovery assay (LORA) (Cho et al. 2007). This employed *M. tuberculosis* H_{37}Rv (pFCA-luxAB) which synthesises luciferase when actively growing. The strain was cultured in 300mL of Dubos Tween-albumin broth in a BioStatQ fermentor with a head space ratio of 0.5 and agitated at 120rpm with no detectable perturbation of the medium surface.

The dissolved oxygen concentration (DOC) was monitored with an Ingold oxygen sensor probe. Cells were harvested when the OD at 570nm indicated achievement of the desired growth phase (i.e. late non-replicating persistence). Aliquots of bacterial culture (50mL) were centrifuged at 2,700g for 30min, washed once with phosphate buffered saline (PBS), suspended in PBS (1mL), and stored at -80°C. Prior to use, cultures were thawed, diluted in Middlebrook 7H12 broth and sonicated for 15s. Cultures were diluted to obtain an OD at 570nm of 0.03-0.05 and 2000-5000 relative light units (RLU) per 100μL (i.e. $5x10^5$ to $2x10^6$ CFU/mL). Two-fold serial dilutions of compounds were prepared in 100μL in black 96 well plates, and 100μL of the cell suspension was subsequently added. The microplate cultures were placed under anaerobic conditions (oxygen<0.16%) using an Anoxomat Model WS-8080, three cycles of evacuation and filling with a mixture of 10% H_2, 5% CO_2, balance N_2. Plates were incubated at 37°C for 7 days and then transferred to an ambient gaseous condition (5% CO_2-enriched air) incubator for a 24h 'recovery'. On day 7 and 8, 100μL of culture were transferred to white opaque 96-well plates. A 10% solution of *n*-decanal in ethanol was freshly diluted 10-fold in PBS and 100μL added to each well with an auto-injector. Luminescence was measured in a Victor^2 multi-label reader (1s reading time). Samples reducing viability under these non-growth conditions led to a decreased luciferase signal following aerobic recovery. Pure compounds were tested at 50 and 10 μg/mL and MICs were determined.

2.5.2 Screening against non-tuberculous mycobacteria

The activity against *Mycobacterium aurum* A+ (CIP 10482, Pasteur Institute, Paris, France), *Mycobacterium fortuitum* (ATCC 6841) *Mycobacterium phlei* (ATCC 11758) and *Mycobacterium smegmatis* (ATCC 14468) was evaluated using a modification of a broth microdilution procedure previously reported (Seidel and Taylor 2004).

Samples were dissolved in organic solvents (dimethylformamide, dimethylsulfoxide, ethanol or methanol) and diluted in Sensititre® Cation-Adjusted Mueller-Hinton Broth with TES (Trek Diagnostics Systems® Ltd, UK). A dye solution of thiazolyl blue tetrazolium bromide (Sigma-Aldrich®, UK) in distilled methanol was prepared at a concentration of 5mg/mL.

Mycobacteria were subcultured onto a fresh slope of Columbia agar (Oxoid®, UK) supplemented with 5% defibrinated horse blood (Oxoid®, UK) and incubated at 37°C for 3 days. The slopes were then rinsed with normal saline and the bacterial suspensions were transferred to clean sterile universals. To obtain the desired inoculum, bacterial suspensions were mixed vigorously in normal saline to disrupt visible clumps, and left to settle for 5 min. Supernatants were diluted in normal saline to match the turbidity of a McFarland 0.5 standard using a biowave CO8000 cell density meter (Biochrom WPA®, UK). An aliquot (50µL) was then transferred to cation-adjusted Mueller–Hinton broth (10 mL) (Sensititre®, Trek-Diagnostics Systems, UK).

The assays were performed in flat-bottomed 96-well plate. Each well first received 100µL broth. The first sample well received an additional 100µL of sample. Two fold serial dilutions were performed by moving 100µL of this mixture to the next well and discarding the remaining 100µL.

Each sample containing well then received 100µL of bacterial inoculum at a concentration of 5.10^5CFU/mL. Isoniazid, ethambutol and rifampicin (Sigma-Aldrich, UK) were used as positive controls. Sterile control (broth only), growth control (broth and inoculum only), solvent control (blank sample) were also included. The plates were incubated at 37°C for 3 days (*Mycobacterium fortuitum* and *Mycobacterium smegmatis*) or 5 days (*Mycobacterium aurum* and *Mycobacterium phlei*). After incubation, 20µL of the dye solution was added to each well and the plates were further incubated for an hour. In the presence of bacterial growth, the initially yellow dye turns dark blue/black permitting determination of the minimal inhibitory concentration (MIC) as the lowest concentration at which no growth was observed. Each sample was assayed on duplicate on two different days.

2.5.3 Antibacterial assay

The activity against *Bacillus cereus* (ATCC 9634), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC12228) and *Streptococcus pyogenes* (ATCC 19615) was evaluated by Dr Jamil A. SHILPI (SIPBS) using a broth microdilution assay following a method previously reported (Seidel et al. 2008).

Briefly, bacteria from freshly grown cultures were transferred in sterile tryptic soy broth by a sterile loop and incubated at 37°C sufficient bacterial growth could be visualised. The bacterial suspension were then diluted in normal saline to match the turbidity of a McFarland standard 0.5 using a biowave CO8000 cell density meter (Biochrom WPA®, UK). The absorbance of the bacterial suspensions was recorded against a blank saline solution and the concentration was adjusted to get an absorbance within 0.1 to 0.15.

An aliquot (100μL) was then transferred to cation-adjusted Mueller–Hinton broth (10mL) (Sensititre®, Trek-Diagnostics Systems, UK) giving 1×10^6 CFU/mL bacterial inoculum.

The assays were performed in flat-bottomed 96-well plates. The plates were prepared as previously described for screening against non-tuberculous mycobacteria. Ciprofloxacin was used as positive control. Plates were incubated at 37°C for 16-18h. After incubation, 20μL of the dye solution was added to each well and the plates were further incubated for 20min (1h for *Enterococcus faecalis* and *Streptococcus pyogenes*). In the presence of bacterial growth, the initially yellow dye turns dark blue/black permitting determination of the minimal inhibitory concentration (MIC) as the lowest concentration at which no growth was observed. Each sample was assayed on duplicate on two different days.

2.6 Cytotoxicity assay

Cytotoxicity against mammalian Vero cells (ATCC CCL-81) was assessed at the ITR using a cell proliferation assay previously described (Falzari et al. 2005). Results were expressed as the concentration effecting 50% inhibition of the growth of Vero cells (IC_{50}). Selectivity indices were calculated for each compound by dividing the IC_{50} value with the MIC value.

Chapter 3: RESULTS AND DISCUSSION

Chapter 3 Results and discussion

3.1 Preliminary screening of crude extracts for antimycobacterial activity.

The results of the evaluation of the activity of crude plant, lichen and endophyte extracts against *Mycobacterium aurum* are reported in Tables 18 and 19. MICs of active extract (MIC≤64µg/mL) are in red and bold. MICs of extracts selected for further bioassay-guided fractionation monitored by the activity against *Mycobacterium aurum* are highlighted in yellow.

Plant (n=208), lichen (n=3) and endophyte (n=13) extracts were screened against *M. aurum*. Among these extracts, 11 plant extracts, the 3 lichen extracts and 2 endophyte extracts disclosed MICs ≤64µg/mL.

The most active plant extract was the *n*-hexane extract of *Juniperus communis* roots (MIC of 4µg/mL). Out of the 11 most active plant extracts, 8 were *n*-hexane extracts. In addition, for one plant out of three, the *n*-hexane extract was more active then the ethyl acetate extract. A specificity of mycobacteria is their very lipidic cell wall (Salyers and Whitt 2002). Hexane extracts mainly contain non polar or low polarity compounds. Such compounds are more likely to easily penetrate or disrupt the mycobacterial cell wall.

Furthermore, out of the 11 most active plant extracts, 7 were roots extracts. The production of antimycobacterial compounds by the roots may be a response to the mycobacterial stress in the soil where these plants are growing.

The *n*-hexane and ethyl acetate extracts of *Cladonia arbuscula*, the only lichen species investigated, had an MIC of 4µg/mL. The methanol extract of *Cladonia arbuscula* showed an MIC of 32µg/mL unlike methanol extracts from plants which showed MICs ≥512µg/mL. The antimycobacterial activity of *Cladonia arbuscula* extracts against *Mycobacterium aurum* is reported here for the first time. Usnic acid (Figure 17) isolated from *Cladonia arbuscula* diethyl ether extract have previously been identified as an antimycobacterial agent (MIC = 32µg/mL against *M. aurum* in a BACTEC assay) (Ingólfsdóttir et al. 1998). Synthesis of dibenzofurans derivatived from usnic acid led to the discovery of a new class of antitubercular agent (Prado et al. 2006).

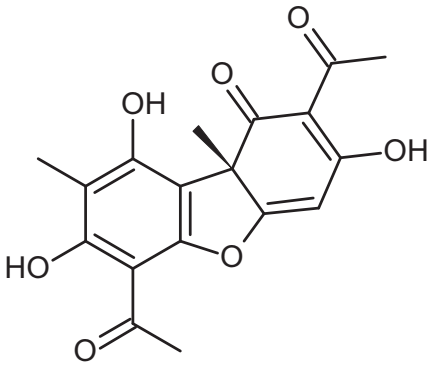


Figure 17. Structure of (+)-usnic acid

Among the endophyte extracts screened, the most active were ethanolic extracts from the mycelium of an ericoid endophyte species isolated from *Vaccinium myrtillus* (MIC of 8µg/mL) and of another ericoid endophyte species isolated from *Calluna vulgaris* (MIC of 32µg/mL).

Table 18. Activity of plant and lichen extracts against *Mycobacterium aurum* (MICs in µg/mL).

Plant	H	E	M
Abies alba aerial parts	256	>512	>512
Abies alba roots	512	>512	>512
Abies fraseri aerial parts	512	512	>512
Abies fraseri roots	256	256	>512
Abies grandis aerial parts	512	512	>512
Abies grandis roots	256	>512	>512
Abies nobilis aerial parts	256	128	>512
Abies nobilis roots	256	512	>512
Anemone nemorosa whole flowering plant	>512	>512	>512
Betula pendula aerial parts	256	256	>512
Betula pendula roots	256	>512	>512
Calluna vulgaris aerial parts	>512	>512	>512
Cornus alba aerial parts	512	>512	512
Cornus alba roots	>512	>512	512
Crataegus monogyna aerial parts	>512	>512	>512
Crataegus monogyna roots	128	>512	>512
Empetrum nigrum whole plant	>512	256	>512
Fagus sylvatica aerial parts	>512	>512	>512
Fagus sylvatica roots	256	128	>512
Fraxinus excelsior stems	**64**	>512	>512

H : *n*-hexane extract E : ethyl acetate extract M : methanol extract

Table 18 (continued). Activity of plant and lichen extracts against *Mycobacterium aurum* (MICs in µg/mL).

Plant	H	E	M
Fraxinus excelsior roots	**32**	>512	>512
Ilex aquifolium aerial parts	>512	>512	>512
Ilex aquifolium roots	>512	>512	>512
Juniperus communis aerial parts	**64**	512	512
Juniperus communis roots	**4**	**32**	256
Knautia arvensis whole plant	>512	>512	>512
Larix leptoleptis aerial parts	512	512	>512
Larix leptoleptis roots	32	512	>512
Lonicera periclymenum aerial parts	>512	>512	>512
Malus sylvestris aerial parts	512	>512	>512

Plant	H	E	M
Malus sylvestris roots	>512	>512	>512
Myrica gale stems	128	128	>512
Myrica gale roots	**64**	128	>512
Phragmites australis whole plant	256	512	>512
Picea abies aerial parts	**64**	512	>512
Picea abies roots	128	>512	>512
Pinus mugo aerial parts	**64**	256	>512
Pinus mugo roots	128	256	>512
Pinus nigra maritima aerial parts	128	512	>512
Pinus nigra maritima roots	128	512	ND

H : *n*-hexane extract E : ethyl acetate extract M : methanol extract

Table 18 (continued). Activity of plant and lichen extracts against *Mycobacterium aurum* (MICs in µg/mL).

Plant	H	E	M
Pinus nigra nigra aerial parts	128	256	>512
Pinus nigra nigra roots	512	512	>512
Pinus sylvestris Scotica aerial parts	256	>512	>512
Pinus sylvestris Scotica roots	128	512	>512
Polygonum persicaria whole flowering plant	512	512	>512
Populus alba aerial parts	128	>512	>512
Populus alba roots	512	>512	>512
Populus nigra stems	512	512	>512
Populus nigra roots	>512	>512	>512
Populus tremula aerial parts	512	>512	>512
Populus tremula roots	512	>512	>512
Prunella vulgaris whole plant	256	>512	>512
Pulmonaria officinalis flowering aerial parts	256	>512	>512
Quercus petraea aerial parts	>512	>512	>512
Quercus petraea roots	>512	>512	>512
Rumex obtusifolius aerial parts	512	>512	>512
Rumex obtusifolius roots	>512	**64**	512
Salix caprea aerial parts	512	>512	>512
Salix caprea roots	>512	128	>512
Salix cinerea aerial parts	512	>512	>512

H : *n*-hexane extract E : ethyl acetate extract M : methanol extract

Table 18 (continued). Activity of plant and lichen extracts against *Mycobacterium aurum* (MICs in µg/mL).

Plant	H	E	M
Salix cinerea roots	>512	>512	>512
Skimmia japonica aerial parts	512	512	>512
Sorbus aucuparia roots	512	>512	>512
Sphagnum whole plant	512	>512	>512
Taxus baccata aerial parts	512	>512	>512
Taxus baccata root	256	512	>512
Thuja plicata aerial parts	>512	>512	>512

Plant/Lichen	H	E	M
Thuja plicata roots	256	**32**	512
Tsuga heterophylla aerial parts	512	>512	>512
Tsuga heterophylla roots	512	>512	>512
Vaccinium myrtillus whole plant	>512	>512	>512
Viburnum opulus aerial parts	>512	>512	>512
Viburnum opulus roots	>512	>512	>512
Cladonia arbuscula whole plant	**4**	**4**	**32**

H : *n*-hexane extract E : ethyl acetate extract M : methanol extract

Antibiotic controls: Isoniazid (MIC 0.125 µg/mL), Rifampicin (1 µg/mL)

Table 19. Activity of ethanol extracts of endophytes (mycelia) against *Mycobacterium aurum*.

Endophyte	MIC (µg/mL)
Xerocomus badius #1	>512
Xerocomus badius #2	256
Chalciporus piperatus	128
Cenococcum species	512
Rhizopogon species #1	>512
Ericoid root endophyte #1	>512
Ericoid root endophyte #2	512
Ericoid root endophyte #3	>512
Ericoid root endophyte #4	**32**
Ericoid root endophyte #5	>512
Ericoid root endophyte #6	>512
Ericoid root endophyte #7	**8**
Ericoid root endophyte #8	>512
Isoniazid	0.125
Rifampicin	1

Results of the evaluation of activity of plant, lichen and endophyte extracts against *Mycobacterium tuberculosis* $H_{37}Rv$ (expressed in percentage of inhibition at an extract concentration of 100µg/mL) are reported in Tables 20 and 21. Active extracts (≥ 90% growth inhibition) are in red and bold. Extracts selected for further bioassay-guided fractionation monitored by the activity against *Mycobacterium tuberculosis* are further highlighted in yellow.

Plant (n=209) lichen (n=3) and endophyte (n=110) extracts were screened against *M. tuberculosis*. Five plant extracts, two lichen extracts and nine endophyte extracts showed 90% or more growth inhibition at 100µg/mL.

The most active plant extracts with a percentage of inhibition of 97% were the ethyl acetate extracts of *Calluna vulgaris* aerial parts and *Myrica gale* roots. Out of the five most active plant extracts, two were *n*-hexane extracts and three were ethyl acetate extracts. Based on this observation, no conclusion can be drawn regarding the correlation between activity and extract polarity. However for almost three plants out of four, the *n*-hexane extract was more active than the ethyl acetate extract. The hypothesis of non-polar extracts being more active because their compounds are more likely to easily penetrate or disrupt the mycobacterial cell wall remains valid.

The higher activity of root extracts compared to aerial part extracts observed against *M. aurum* was not observed against *M. tuberculosis*.

The *n*-hexane and ethyl acetate extracts of *Cladonia arbuscula* showed 96 and 99% growth inhibition, respectively. Althought, usnic acid from *Cladonia* extract has previously been found to be active against *M. aurum*, *M. tuberculosis* var. *homini* and var. *bovis* (Ingólfsdóttir 2002, Ingólfsdóttir et al. 1998), this is the first report of the antimycobacterial activity of crude extracts from *C. arbuscula*.

Among the endophyte extracts screened, the most active were ethanol extracts of *Chalciporus piperatus* and *Xerocomus Badius* (96% inhibition). They were both cultured from fruiting bodies collected on *Pinus sylvestris* growing in two different collection sites. The nine endophyte extracts with percentage of inhibition over 90% were all ethanolic extracts prepared from mycelia. No other specific activity pattern was observed for the endophyte extracts.

On completion of this preliminary screening, poor correlation was observed between the sensitivity profiles of *M. aurum* and *M. tuberculosis* when using a broth microdilution assay contrarily to the results reported when using a BACTEC assay (Chung et al. 1995). The MABA allows rapid screening against *M. tuberculosis* (Collins and Franzblau 1997, Franzblau et al. 1998). Based on the results reported above, *M. aurum* is not a good surrogate for *M. tuberculosis*. However testing against *M. aurum* and other non-tuberculous mycobacteria can lead to the discovery of some antimycobacterial compounds overlooked when screened only against *M. tuberculosis*.

A literature search on the traditional uses of plants and lichens identified *Abies grandis, Betula pendula, Calluna vulgaris, Cladonia arbuscula, Crataegus monogyna, Empetrum nigrum, Ilex aquifolium, Juniperus communis, Knautia arvensis, Lonicera periclymenum, Phragmites australis, Pinus sylvestris, Prunella vulgaris, Pulmonaria officinalis, Thuja plicata* and *Tsuga heterophylla* as species reputed to be effective in the treatment of tuberculosis (Allen and Hatfield 2004, Atkinson 2003, Flora Celtica Database 2009, Ingólfsdóttir et al. 1998, McCutcheon et al. 1997, UK CropNet Medicinal Plants of Native America Database 2009). Whilst the antitubercular activity of *B. pendula, E. nigrum* and *J. communis* has already been reported (Jimenez-Arellanes et al. 2003, McCutcheon et al. 1997, Newton et al. 2002), this is the first report of the activity of *C. vulgaris*, *C. arbuscula, K. arvensis* and *P. vulgaris* against MTB.

The activity of these plants in the *in vitro* assay justifies to some extent their use as traditional anti-TB remedies. On the other hand, *A. grandis, C. monogyna, I. aquifolium, L. periclymenum, P. australis, P. sylvestris, P. officinalis, T. plicata and T. heterophylla* failed to exhibit any activity against *M. tuberculosis* in our assay. It is possible that for these plants the anti-TB effect is mediated through immunostimulation rather than direct inhibition of mycobacterial growth.

Table 20. Activity of plant and lichen extracts against *Mycobacterium tuberculosis* (% of growth inhibition at 100µg/mL)

Plant	H	E	M
Abies alba aerial parts	69	65	3
Abies alba roots	39	51	1
Abies fraserii aerial parts	52	60	15
Abies fraserii roots	59	35	2
Abies grandis aerial parts	41	36	2
Abies grandis roots	33	33	10
Abies nobilis aerial parts	74	58	35
Abies nobilis roots	70	68	36
Anemone nemorosa whole flowering plant	43	42	19
Betula pendula aerial parts	78	78	50
Betula pendula roots	69	73	48
Calluna vulgaris aerial parts	79	**97**	22
Cornus alba aerial parts	56	53	22
Cornus alba roots	55	40	18
Crataegus monogyna aerial parts	55	14	8
Crataegus monogyna roots	65	47	35
Empetrum nigrum whole plant	**95**	37	23
Fagus sylvatica aerial parts	57	30	6
Fagus sylvatica roots	69	51	39
Fraxinus excelsior stems	68	48	38

H : *n*-hexane extract E : ethyl acetate extract M : methanol extract

Table 20 (continued). Activity of plant and lichen extracts against *M. tuberculosis* (% of growth inhibition at 100µg/mL)

Plant	H	E	M
Fraxinus excelsior roots	88	63	52
Ilex aquifolium aerial parts	39	53	7
Ilex aquifolium roots	60	68	39
Juniperus communis aerial parts	64	53	45
Juniperus communis roots	**93**	62	51
Knautia arvensis whole plant	47	77	22
Larix leptoleptis aerial parts	76	36	1
Larix leptoleptis roots	27	35	17
Lonicera periclymenum aerial parts	62	23	13
Malus sylvestris aerial parts	42	7	7
Malus sylvestris roots	50	5	6
Myrica gale stems	89	**96**	16
Myrica gale roots	52	**97**	17
Phragmites australis whole plant	28	24	5
Picea abies aerial parts	43	24	8
Picea abies roots	77	56	44
Pinus mugo aerial parts	41	46	10
Pinus mugo roots	48	44	12
Pinus nigra maritima aerial parts	28	16	13
Pinus nigra maritima roots	37	25	0

H : *n*-hexane extract E : ethyl acetate extract M : methanol extract

Table 20 (continued). Activity of plant and lichen extracts against *M. tuberculosis (*% of growth inhibition at 100µg/mL)

Plant	H	E	M
Pinus nigra nigra aerial parts	38	37	0
Pinus nigra nigra roots	33	33	5
Pinus sylvestris Scotica aerial parts	28	34	13
Pinus sylvestris Scotica roots	32	23	6
Polygonum persicaria whole flowering plant	67	69	62
Populus alba aerial parts	26	22	13
Populus alba roots	37	22	20
Populus nigra stems	41	8	11
Populus nigra roots	48	21	12

Plant	H	E	M
Populus tremula aerial parts	33	18	8
Populus tremula roots	43	36	18
Prunella vulgaris whole plant	75	56	38
Pulmonaria officinalis flowering aerial parts	38	31	11
Quercus petraea aerial parts	32	31	8
Quercus petraea roots	76	66	39
Rumex obtusifolius aerial parts	68	41	13
Rumex obtusifolius roots	57	55	47
Salix caprea aerial parts	81	25	18

H : *n*-hexane extract E : ethyl acetate extract M : methanol extract

Table 20 (continued). Activity of plant and lichen extracts against *M. tuberculosis (*% of growth inhibition at 100µg/mL)

Plant	H	E	M
Salix caprea roots	85	39	13
Salix cinerea aerial parts	42	15	3
Salix cinerea roots	44	27	2
Skimmia japonica aerial parts	63	36	19
Sorbus aucuparia stems	45	33	3
Sphagnum whole plant	80	34	29
Taxus baccata aerial parts	71	43	37
Taxus baccata root	35	23	10
Thuja plicata aerial parts	46	27	18

Plant	H	E	M
Tsuga heterophylla aerial parts	51	40	1
Tsuga heterophylla roots	45	28	2
Vaccinium myrtillus whole plant	64	26	17
Viburnum opulus aerial parts	35	25	4
Viburnum opulus roots	71	35	5

Lichen	H	E	M
Cladonia arbuscula whole	**96**	**99**	59

H : *n*-hexane extract E : ethyl acetate extract M : methanol extract

Antibiotic controls: Isoniazid (99% growth inhibition), Rifampicin (99% growth inhibition)

Table 21. Activity of endophyte extracts against *Mycobacterium tuberculosis* (% of growth inhibition at 100μg/mL)

Endophyte	H	C	B	E
Amanita muscaria #1	56	59	27	85
Amanita muscaria #2	ND	ND	12	78
Xerocomus badius #2	ND	72	51	**96**
Xerocomus badius #1	ND	59	38	**93**
Xerocomus edulis	ND	46	ND	28
Cortinarius semisanguineus	ND	ND	ND	27
Gyromitra esculenta	ND	ND	20	57
Lactarius deliciosus	ND	36	12	14
Leccinum holopus	21	56	14	13
Paxillus involutus	51	48	7	66
Suillus bovinus	50	35	20	24
Suillus luteus #3	ND	64	19	33
Suillus luteus #1	ND	37	14	**90**
Suillus luteus #2	ND	40.3	33.0	42.7
Chalciporus piperatus	ND	75	47	**96**

H : *n*-hexane extract C : chloroform extract

B : butanol extract E : ethanol extract

Table 21 (continued). Activity of endophyte extracts against *M. tuberculosis* (% of growth inhibition at 100µg/mL)

Endophyte	H	C	B	E
Suillus variegatus	ND	72	39	70
Tricholoma imbricatum	66	26	57	20
Cenococcum species	ND	74	16	15
Rhizopogon species #3	ND	39	49	29
Rhizopogon species #1	ND	ND	39	37
Rhizopogon species #2	ND	44	57	50
Russula species	ND	10	8	32
Tomentellopsis species	29	38	13	ND
Ericoid root endophyte #2	ND	45	10	**91**
Ericoid root endophyte #3	ND	52	36	**91**
Ericoid root endophyte #5	ND	ND	ND	**91**
Ericoid root endophyte #6	ND	ND	30	**92**
Ericoid root endophyte #7	46	35	12	28
Ericoid root endophyte #8	ND	42	33	**95**
Ericoid root endophyte #9	38	51	25	11
Ericoid root endophyte #10	ND	42	20	69

H : *n*-hexane extract C : chloroform extract

B : butanol extract E : ethanol extract

Table 21 (continued). Activity of endophyte extracts against *M. tuberculosis (*% of growth inhibition at 100µg/mL)

Endophyte	H	C	B	E
Ericoid root endophyte #11	ND	ND	19	76
Ericoid root endophyte #12	ND	ND	24	22
Ericoid root endophyte #13	57	66	36	1
Ericoid root endophyte #14	38	40	16	33
Ericoid root endophyte #15	42	23	11	32
Ericoid root endophyte #16	ND	44	21	21

H : *n*-hexane extract C : chloroform extract

B : butanol extract E : ethanol extract

Antibiotic controls: Isoniazid (99% growth inhibition)

Rifampicin (99% growth inhibition)

3.2 Selection of material for bioassay-guided fractionation studies

On the basis of i) the above results obtained following the preliminary screening of plant, lichen and endophyte extracts against *M. aurum* and *M. tuberculosis*, ii) previously reported work and of iii) the availability of material in hand, the following extracts were selected for further bioassay-guided fractionation:

- *n*-hexane extract of *Skimmia japonica* aerial parts
- *n*-hexane extract of *Juniperus communis* aerials parts
- *n*-hexane extract of *Juniperus communis* roots
- ethyl acetate extract of *Calluna vulgaris* aerial parts
- *n*-hexane extract of *Myrica gale* stems
- ethyl acetate extract of *Myrica gale* stems
- *n*-hexane extract of *Myrica gale* roots
- ethyl acetate extract of *Myrica gale* roots

Fractionation schemes of the selected extracts are presented in the appendices (Figures 1 to 10).

The aim of the bioassay-guided fractionation process is to isolate the compounds responsible for the activity observed in crude extracts.

This method proved successful for most extracts and the activity was concentrated after each fractionation steps. However in some case the process proved difficult.

For example, bioassay-guided fractionation became quite challenging if the active compound was present within a fraction available in too little amount to allow successful fractionation. Moreover there is no guarantee that the active compound(s) isolated will present novel or interesting chemical scaffolds or structures. In this instance, some studies report the use of proton NMR (Kalaitzis 2004) to monitor novel or unusual signals or advise on the use of dereplication techniques (Houghton and Raman 1998) to eliminate common groups of compounds known to be active in the assay chosen.

3.2.1 Fractionation of *Skimmia japonica*

Bioassay-guided fractionation of the *n*-hexane extract of *Skimmia japonica* was monitored by its activity against *M. tuberculosis*. This led to the identification of **(SJ2)** and **(SJ3)** (Appendices, Figure 4). Although the initial crude extract was only moderately active (63% growth inhibition at 100µg/mL), good activity was observed after the first fractionation step (Appendices, Figure 1).

The fractionation became more challenging thereafter resulting in some loss of activity as well as the isolation of insufficient amount of material for further fractionation and/or failure to separate substances obtained in active mixtures.

As part of the phytochemical investigation of fractions deemed inactive, **(SJ1)** was readily isolated from both 69H9 and 69H10 upon precipitation during the size exclusion chromatography fractionation step (Appendices, Figures 2 and 3). The characterisation of **(SJ1)** and of inactive compounds from other extracts is reported in chapter 3, section 3.3

3.2.2 Fractionation of *Juniperus communis*

The fractionation of the *n*-hexane extract of *Juniperus communis* aerial parts was monitored by its activity against *Mycobacterium aurum*. This led to the isolation of **(JC3)** (Appendices, Figure 6)

The fractionation of the *n*-hexane extract of *Juniperus communis* roots was monitored by its activity against *Mycobacterium tuberculosis*. This led to the isolation of **(JC1)** and **(JC2)** (Appendices, Figure 5).

In both cases, greater activity was observed for a few fractions after each fractionation step and activity of the extract could be attributed to pure compounds.

3.2.3 Fractionation of *Calluna vulgaris*

The fractionation of the ethyl acetate extract of *Calluna vulgaris* aerial parts was monitored by its activity against *Mycobacterium tuberculosis*. This led to the isolation of **(CV1)** (Appendices, Figure 7).

Greater activity was observed for a few fractions after each fractionation step and the activity of the extract could be attributed to subfractions 309E6C **(CV1)** and 309E7E.

NMR data revealed 309E7E was a mixture of **(CV1)** with a compound of closely related structure and the subfraction was not further investigated. Phytochemical investigation of the *n*-hexane extract led to the isolation of **(CV2)**.

3.2.4 Fractionation of *Myrica gale*

The fractionation of the ethyl acetate extract of *Myrica gale* aerial parts was monitored by the activity against *Mycobacterium tuberculosis*. The bioassay-guided fractionation afforded two active fractions: 299E3E and 299E6F (Appendices, Figure 9). The ^{1}H NMR spectrum of fraction 299E6F was similar to the one obtained for a commercial sample of oleanolic acid and this fraction was not further investigated. The ^{1}H NMR spectrum of fraction 299E3E revealed the presence of a mixture of compounds which could not be purified due to the insufficient amount of material obtained.

The bioassay-guided fractionation of the *n*-hexane extract of *Myrica gale* aerial parts afforded 299H6E as the most active fraction. As for 299E3E, the ^{1}H NMR spectrum revealed the presence of a mixture of compounds. Further separation was unsuccesful, the insufficient amount of material obtained did not permit the characterisation of the compounds separated.

Phytochemical investigation of the *n*-hexane extract of the aerial parts afforded **(MG1)** and **(MG2)** whilst phytochemical investigation of the ethyl acetate extract of the roots afforded **(MG3)** (Appendices, Figures 8 and 10).

3.3 Characterisation of isolated compounds

3.3.1 Characterisation of **(JC1)** as (+)-longifolene

(JC1) was isolated from the *n*-hexane extract of *Juniperus communis* roots as a pale yellow oil. On TLC, it showed a single faint spot under UV light (λ=254nm). After spraying with anisaldehyde-sulfuric acid reagent and heating, the spot took a brown/orange colour.

On the ESI+ MS spectrum a fragment with low relative intensity was observed at *m/z* 205.2 that could account for a pseudo molecular ion $[M+H]^+$. The main fragment was observed at m/*z* 83.

On the IR spectrum, peaks were observed at 3064 (=C-H stretching), 2953 and 2868cm^{-1} (–CH_3 stretching). The spectrum indicated no heteroatom.

The ^1H NMR spectrum (Figure 18) showed two singlets at δ4.49 and 4.74ppm attributable to an exo-methylene group. Three sharp singlets, each integrating for three protons at δ0.89, 0.94 and 0.99ppm indicated the presence of three tertiary methyl groups.

The ^{13}C and Dept135 NMR spectra (Figure 18 and 19) disclosed fifteen carbons identified as three methyls, six methylenes including an exo-methylene at δ99.0ppm, three methines and three quaternary carbons. These data suggested a molecular formula of $C_{15}H_{24}$ indicating four degrees of unsaturation. With the presence of an exomethylene confirmed, this suggested **(JC1)** had a tricyclic structure.

The HMBC spectrum (Figure 22) revealed the methyls groups at δ0.89ppm (Me-13) and 0.94ppm (Me-14) were gem-dimethyls. They correlated to each other and to a quaternary center at δ33.6ppm. They also showed ^3J correlations to carbons at δ36.4 (C-4) and 62.1ppm (C-2). The remaining methyl protons at δ0.99ppm (Me-12) correlated to carbons at δ45.1 (C-1), 43.4 (C-6) and 168.1ppm (C-8) indicating these carbons were directly attached to the quaternary carbon bearing Me-12.

The protons at δ1.39ppm (H-2) showed ^3J correlations with six carbons at δ25.5, 29.7, 30.5, 30.6, 44.0 47.9 and 168.1ppm indicating this position was a ring junction.

The proton at δ2.07ppm (H-1) correlated with carbons at δ29.7 (C-10), 43.4 (C-C-6), 47.9 (C-9) and 168.01ppm (C-8). Key HMBC correlations are schematically presented in Figure 23.

The relative position of the methyls at δ0.89 (Me-13) and 0.94ppm (Me-14) was established based on NOESY correlations observed between the methyl at δ0.89ppm (Me-13) and the proton at δ2.61ppm (H-9) and between the methyl at δ0.94ppm (Me-14) and the proton at δ2.07ppm (H-1). Selected NOE correlations are presented in Figure 24.

The above data led to the identification of **(JC1)** as longifolene. The ^{1}H and ^{13}C NMR data showed good agreement with previous reports (Lei and Fallis 2002).

Longifolene has previously been isolated from the bark of *Juniperus communis* (Arya 1962b). It has also been detected in the roots essential oil but not in the essential oils from the wood, berries or needles (Butkiene et al. 2004, Butkiene et al. 2006, Butkiene et al. 2007, Gonny et al. 2006). This is the first report of its isolation from *Juniperus communis* roots.

Table 22. ^{1}H (600MHz) and ^{13}C (150MHz) NMR data for **(JC1)** in $CDCl_3$.

Position	^{13}C	^{1}H
1	45.1	2.07(d, 3.5)
2	62.1	1.39(br s)
3	33.6	
4	36.4	1.02(m)/1.67*
5	21.1	1.39*
		1.59*
6	43.4	1.62*
		1.70*
7	44.0	
8	168.1	
9	47.9	2.61(d, 4.8)
10	29.7	1.15(m)
		1.71*
11	25.5	1.39*
		1.67*
12	30.1	0.99(s)
13	30.6	0.89(s)
14	30.5	0.94(s)
15a	99.0	4.49(br s)
15b		4.74(br s)

* overlapping signals

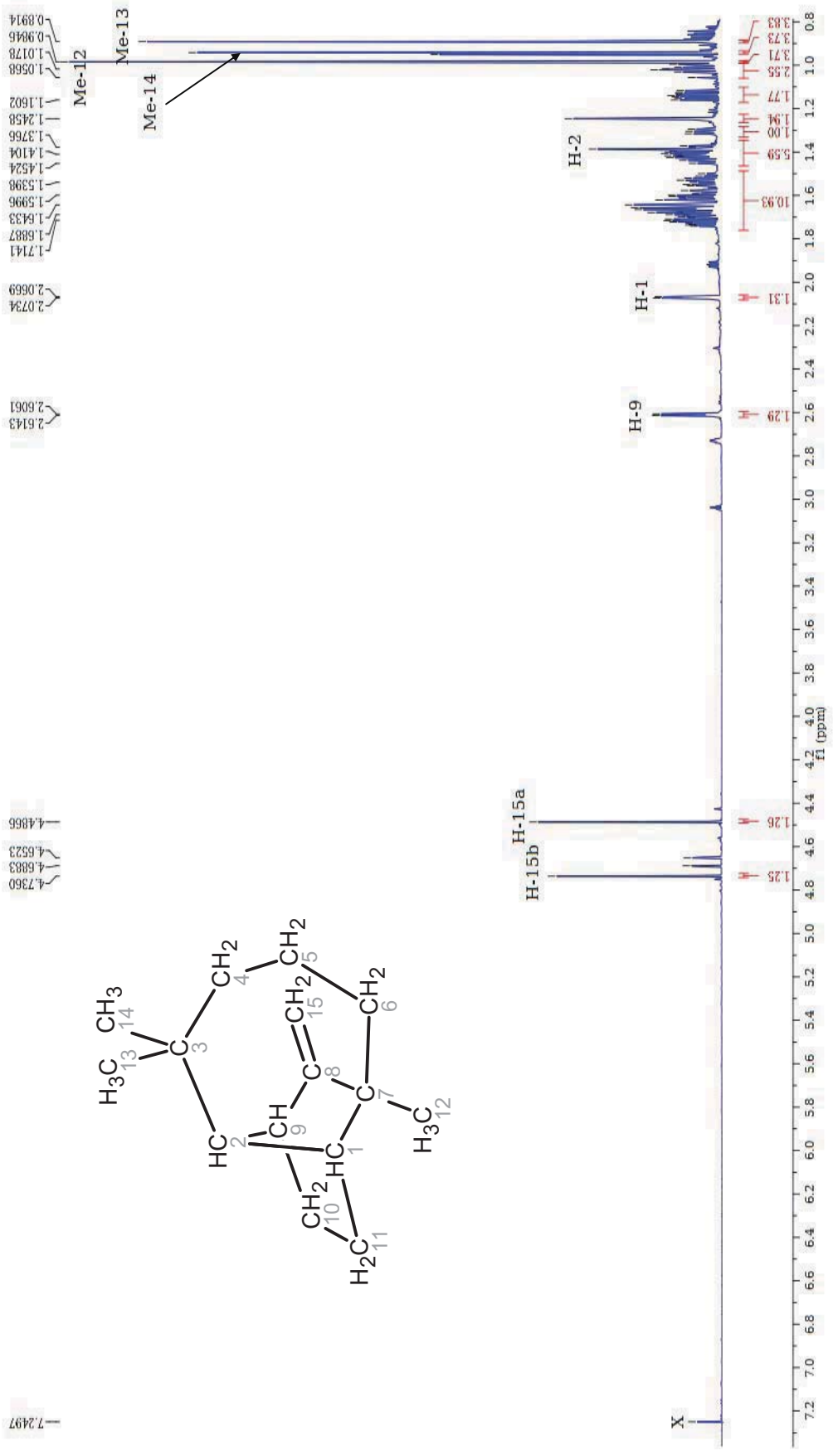


Figure 18. ^{1}H NMR spectrum of **(JC1)**, 600MHz, $CDCl_3$ (X).

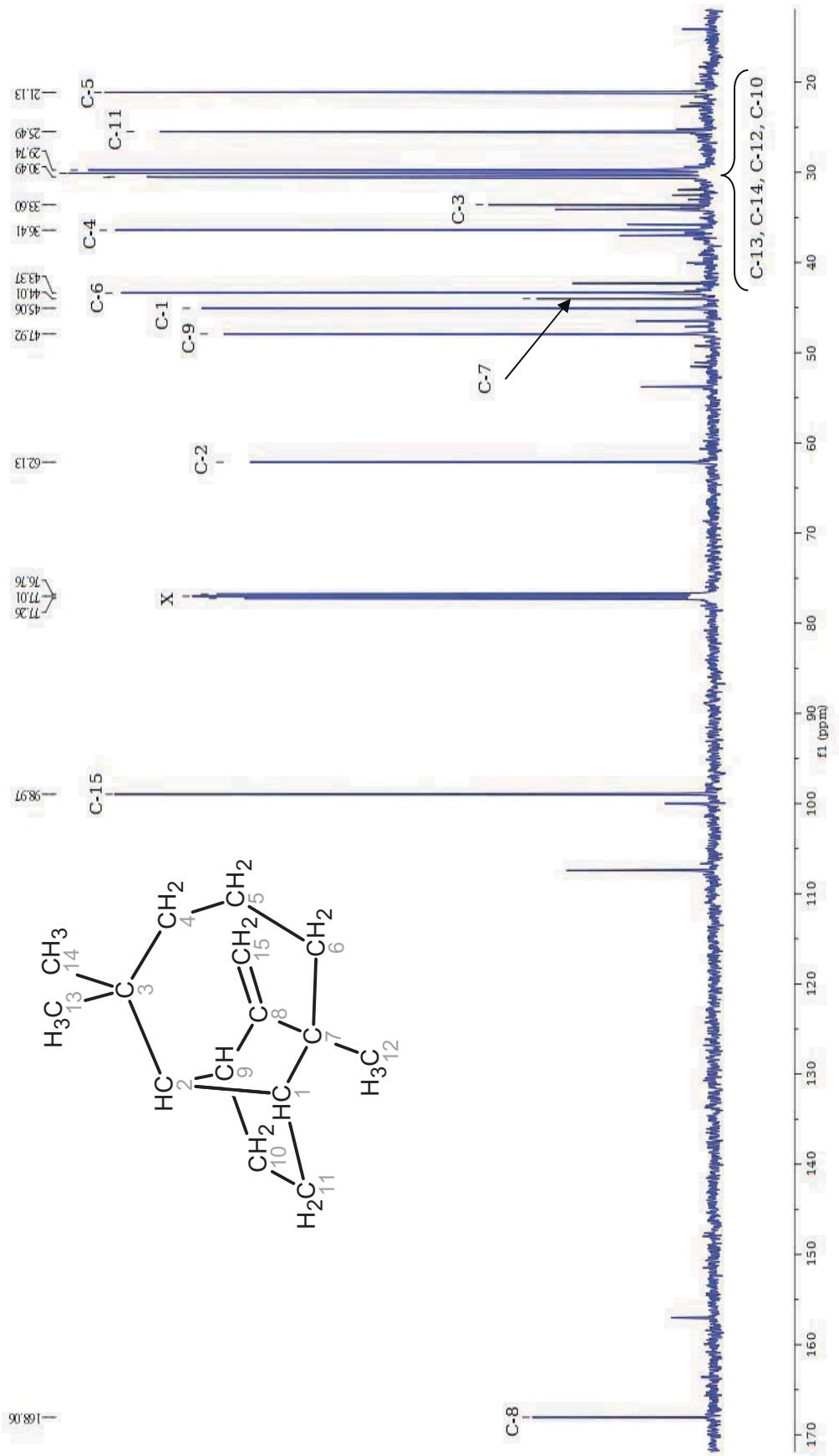


Figure 19. ^{13}C NMR spectrum of **(JC1)**, 150MHz, $CDCl_3$ (X).

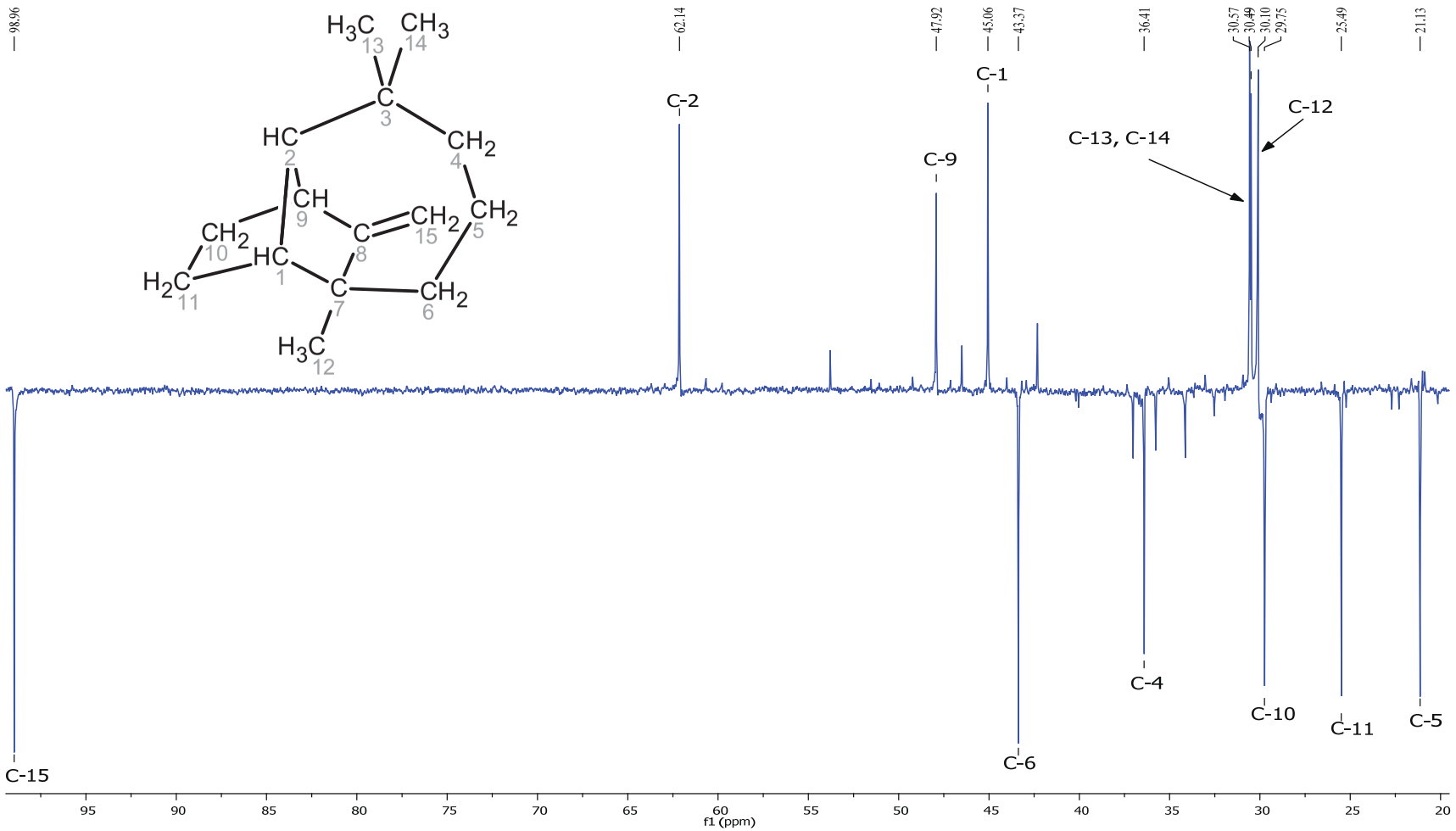


Figure 20. DEPT 135 NMR spectrum of **(JC1)**, 150MHz, $CDCl_3$ (X).

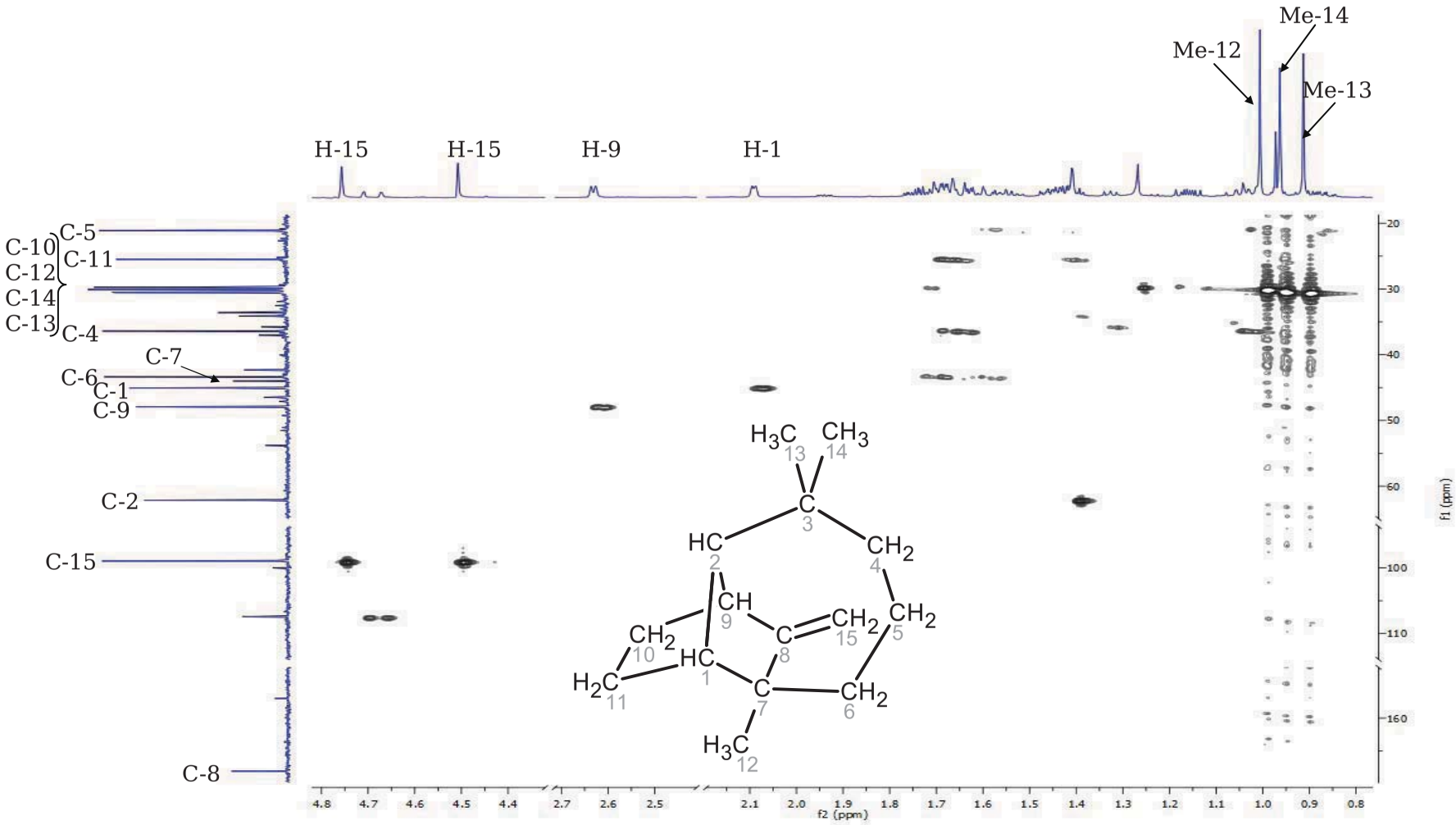


Figure 21. HMQC spectrum of **(JC1)**, 600MHz, $CDCl_3$ (X).

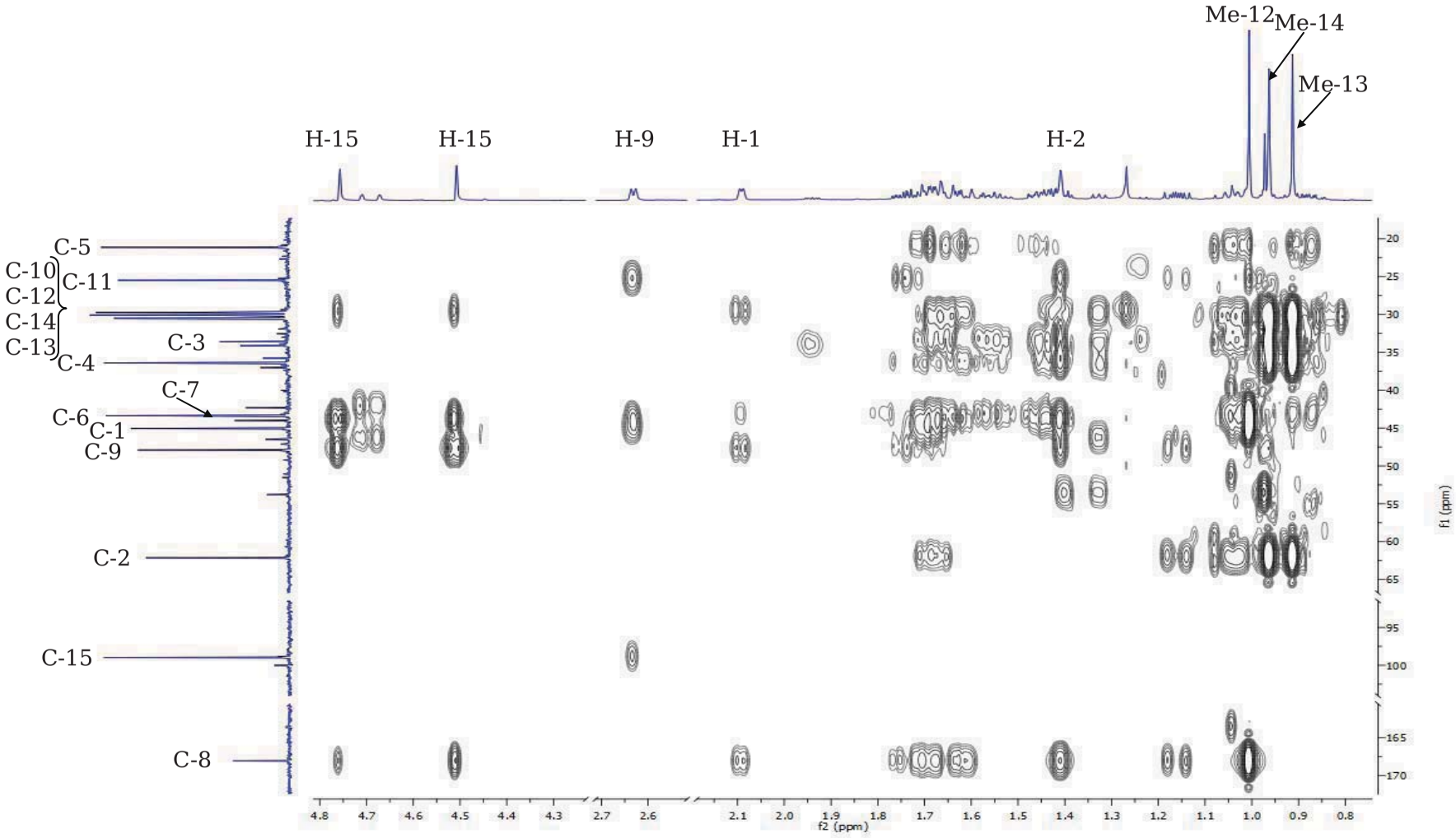


Figure 22. HMBC spectrum of **(JC1)**, 600MHz, $CDCl_3$ (X)

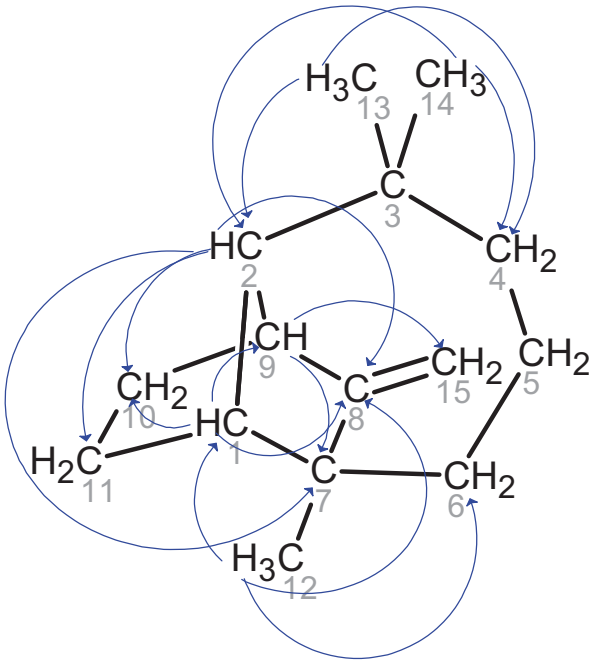


Figure 23. Selected HMBC correlations of **(JC1)**.

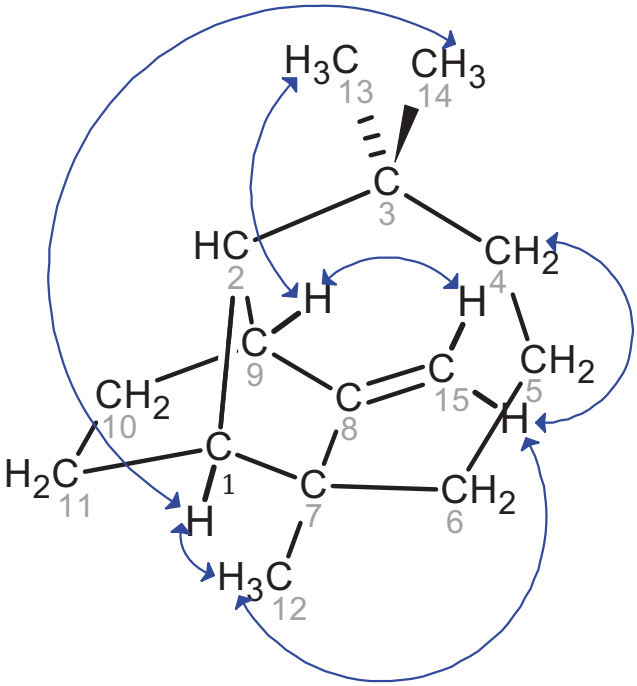


Figure 24. Selected NOE correlations of **(JC1)**

3.3.2 Characterisation of **(JC2)** as (+)-totarol

(JC2) was isolated from the *n*-hexane extract of *Juniperus communis* roots as white crystals. On TLC, it showed a single, strongly absorbing, spot under UV light (λ=254nm). After spraying with anisaldehyde-sulfuric acid reagent and heating, the spot took a brown/orange colour.

EIMS showed a molecular ion $[M]^+$ at *m/z* 286.3 compatible with the molecular formula $C_{20}H_{30}O$. This molecular formula indicated 6 double bond equivalents.

The ^1H NMR spectrum (Figure 25) indicated two aromatic protons at δ7.02ppm (d, *J*=8.4Hz) and δ6.52ppm (d, *J*=8.4Hz). These splitting patterns and coupling constant are typical of two ortho-coupled protons on a substituted benzene ring. A singlet at δ4.53ppm suggested the presence of a phenolic substituent. Three sharp singlets, integrating for three protons each, indicated three tertiary methyl groups at δ0.94, 0.97 and 1.20 ppm. Finally, a proton appearing as a septet at δ3.31ppm and the two doublets, integrating for three protons each, at δ1.36 and 1.38ppm suggested an isopropyl group.

On the COSY spectrum (Figure 26), the signal at 3.31ppm (1H, septet, *J*=7.04Hz), only showed correlation to the two secondary methyls at δ1.36ppm (Me, d, *J*=7.04Hz) and δ1.38ppm (Me, d, *J*=7.04Hz) confirming the presence of the isopropyl group (red circle on Figure 26).

Moreover, the presence of the CH_2-CH_2-CH fragment could be observed through correlations of the geminal protons at δ1.67 and 1.93ppm with a single proton at δ1.29ppm and another pair of the geminal protons at δ2.77 and 2.97ppm (purple circles on Figure 26).

The ^{13}C NMR spectrum (Figure 27) confirmed the presence of twenty carbons including six in the aromatic region (δ130-160ppm). The HMQC spectrum (Figure 28) disclosed that fourteen carbons were directly attached to protons as five methylenes, five methyls and four methines whilst six carbons were identified as quaternary centres.

The HMBC spectrum (Figure 29) indicated that the two methyls at δ0.94 (Me-19) and 0.97ppm (Me-19) were geminal. This was established since they correlated respectively to the carbons at δ33.22 (C-18) and 21.55ppm (C-19) as well as both correlating to the carbons at δ33.22 (C-4), 41.53 (C-3) and 49.52ppm (C-5). In addition, the proton at δ1.29ppm (H-5) showed ^{2}J and ^{3}J correlations to C-1, C-3, C-4, C-6, C-7, C-9, C-10, C-18, C-19 and C-20 indicating its position at a ring junction.

Further analysis of HMBC correlations in the aromatic region allowed to clearly determine the relative positions of the substituents on that ring. Thus, the phenolic proton at δ4.53ppm showed ^{3}J correlations to the carbons at δ114.25 (C-12) and 130.95ppm (C-14). The aromatic proton at δ6.52ppm (H-12) showed ^{3}J correlations to the carbons at δ130.95 (C-14) and 143.15ppm (C-9). The other aromatic proton at δ7.02ppm (C-11) showed ^{3}J correlations to the carbons at δ37.65 (C-10), 133.96 (C-8) and 151.92pm (C-13). Lastly, the isopropyl proton at δ3.31ppm (H-15) also showed ^{3}J correlations to the carbon at δ151.92ppm.

The coupling constant values for the proton at δ1.29ppm (H-5, 12.6Hz, 2.0Hz) indicated this proton was in an axial position. Moreover, NOESY correlations were observed between this proton at δ1.29ppm (H-5) and the methyl at δ0.97ppm (Me-18) and also between the methyls groups at δ0.94 (Me-19) and 1.20ppm (Me-20). These correlations indicated a *trans*-diaxial configuration of the substituents at the A/B ring junction.

The above data led to the identification of **(JC2)** as (+)-totarol. All NMR data were in good agreement with previous reports (Hanari et al. 2003, Marcos et al. 2003). Totarol has previously been isolated from *Juniperus procera* (Muhammad et al. 1995) and other plants within the Cupressaceae family (Constantine et al. 2001, Iwamoto et al. 2001, Sharp et al. 2001). Although its detection in juniper berry-based spirit and the *n*-hexane extract of ripe juniper berries has recently been reported (Vichi et al. 2008), this is the first report of its isolation from *Juniperus communis* roots.

Table 23. ^{1}H (400MHz) and ^{13}C (100MHz) NMR data, COSY and NOESY correlations for **(JC2)** in $CDCl_3$.

Position	^{13}C	^{1}H	COSY	NOESY
1α	39.6	1.36*	1β, 2α, 2β, 20	1β, 11, 12
1β		2.25 (d, 12.3)	1α, 2α, 2β, 3β	1α, 11, 20
2α	19.5	1.76*	1α, 1β, 2β, 3α, 3β	2β, 19, 20
2β		1.62*	1α, 1β, 2α, 3α, 3β	2α
3α	41.5	1.24 (dd, 13.6, 3.6)	2α, 2β,3β, 18, 19	3β
3β		1.49 (dm, 13.2)	1α, 1β, 2α, 2β, 3β, 18	3α, 19
4	33.2*			
5	49.5	1.29 (dd, 12.6, 2.0)	6α, 6 β	18
6α	19.3	1.93 (dd, 13.4, 7.9)	5, 6β, 7α, 7β	6β, 18
6β		1.67*	5, 6α, 7α, 7β	6α, 18
7α	28.7	2.77 (ddd, 17.2, 11.4, 7.9)	6α, 6β, 7β	7β, 15
7β		2.97 (dd, 15.8, 6.4)	6α, 6β, 7α	7α, 15
8	134.0			
9	143.2			
10	37.7			
11	123.0	7.02 (d, 8.4)	12	1β, 12, 20
12	114.3	6.52 (d, 8.4)	11	11, **OH**
13	151.9			
14	131.0			
15	27.1	3.31 (septet, 7.0)	16, 17	7α, 7β, 16, 17
16	20.3*	1.36 (d, 7.0) †	15	15
17	20.3*	1.38 (d, 7.0) †	15	15
18	33.2*	0.97 (s)	3α, 3β, 19	5, 6α
19	21.6	0.94 (s)	3α, 18	3β
20	25.2	1.20 (s)		1β, 11
OH		4.53 (s)		12

* overlapping signals † interchangeable signals

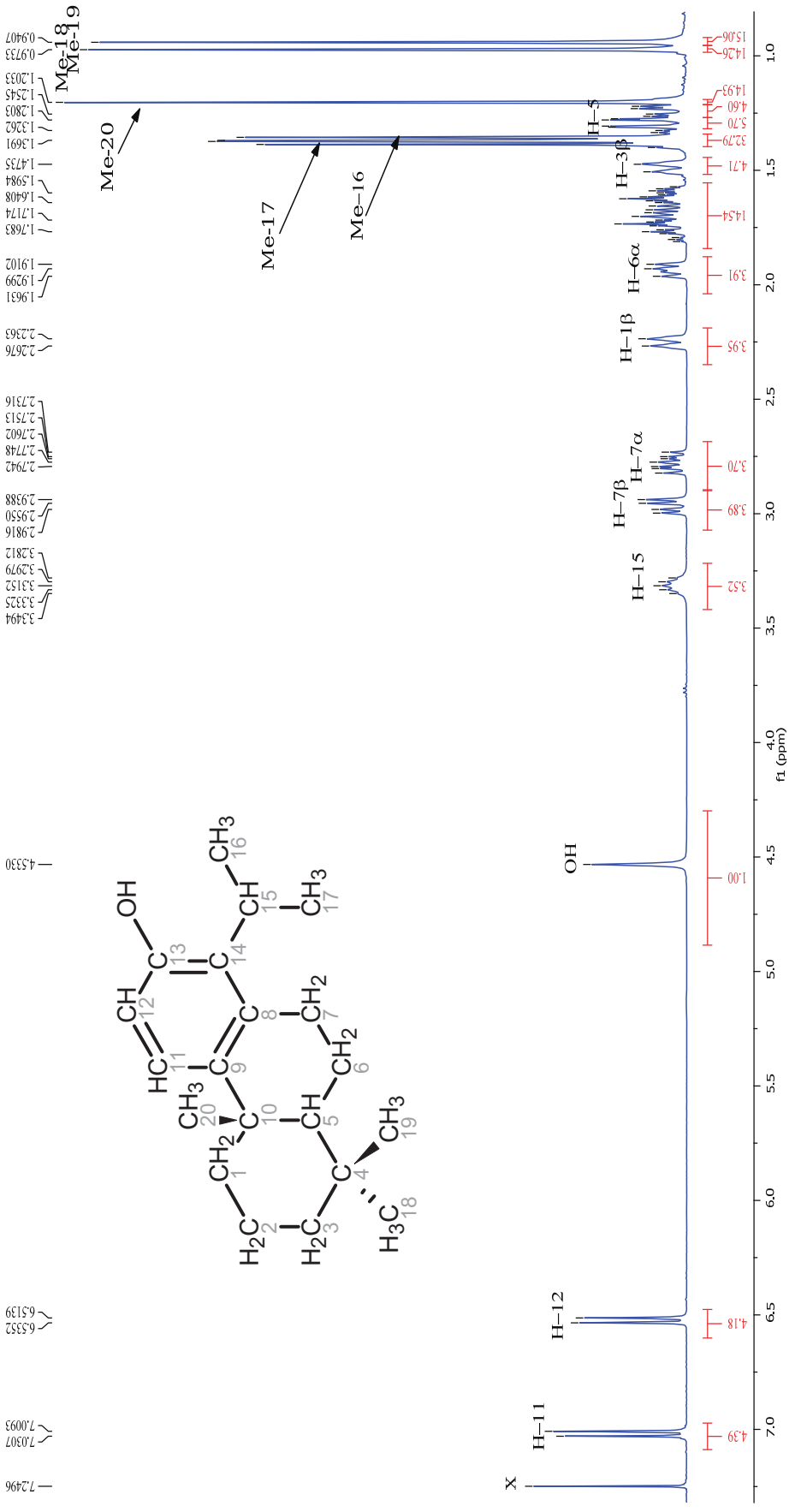


Figure 25. ^{1}H NMR spectrum of **(JC2)**, 400MHz, $CDCl_3$ (X)

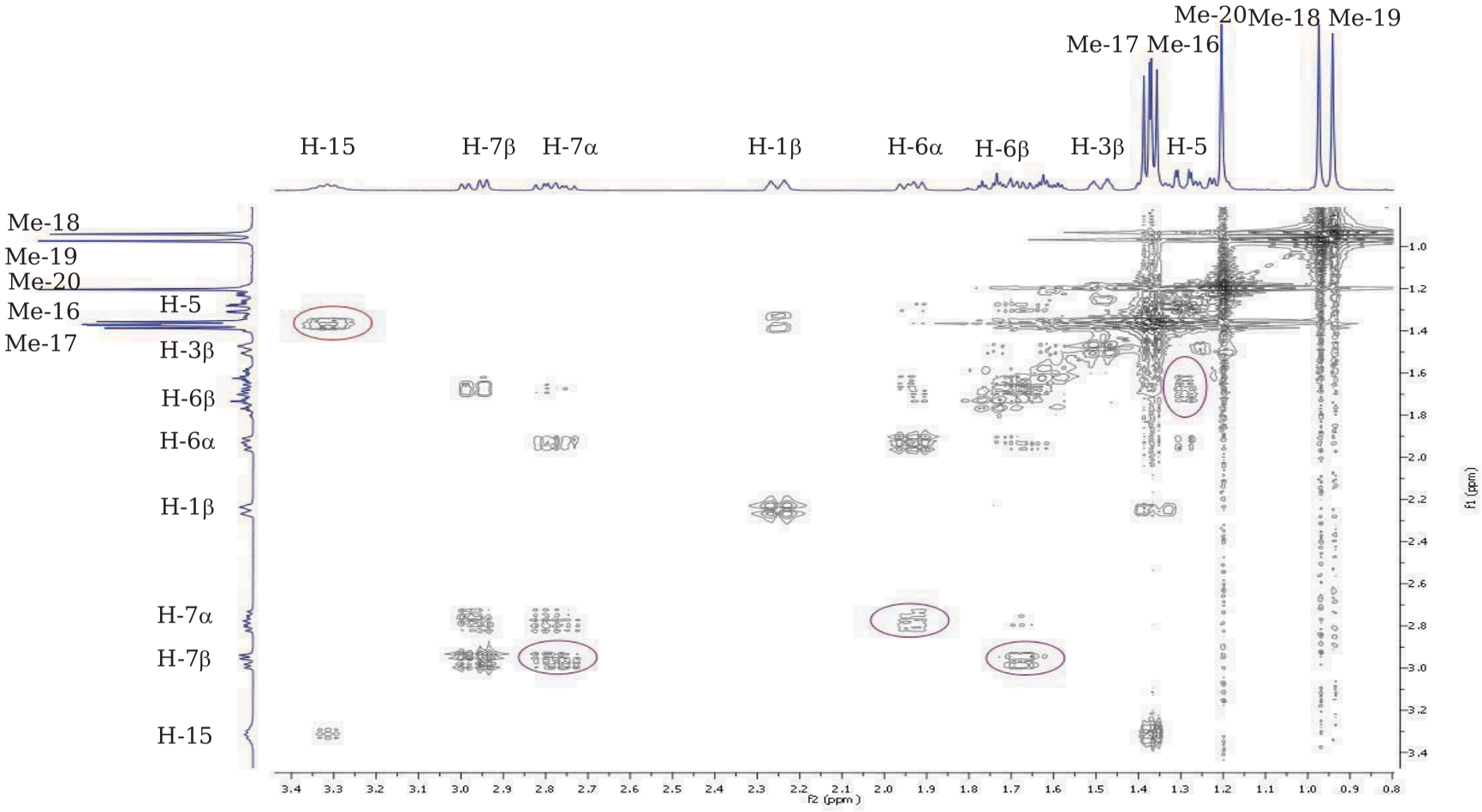


Figure 26. COSY spectrum of **(JC2)**, 400MHz, $CDCl_3$ (X).

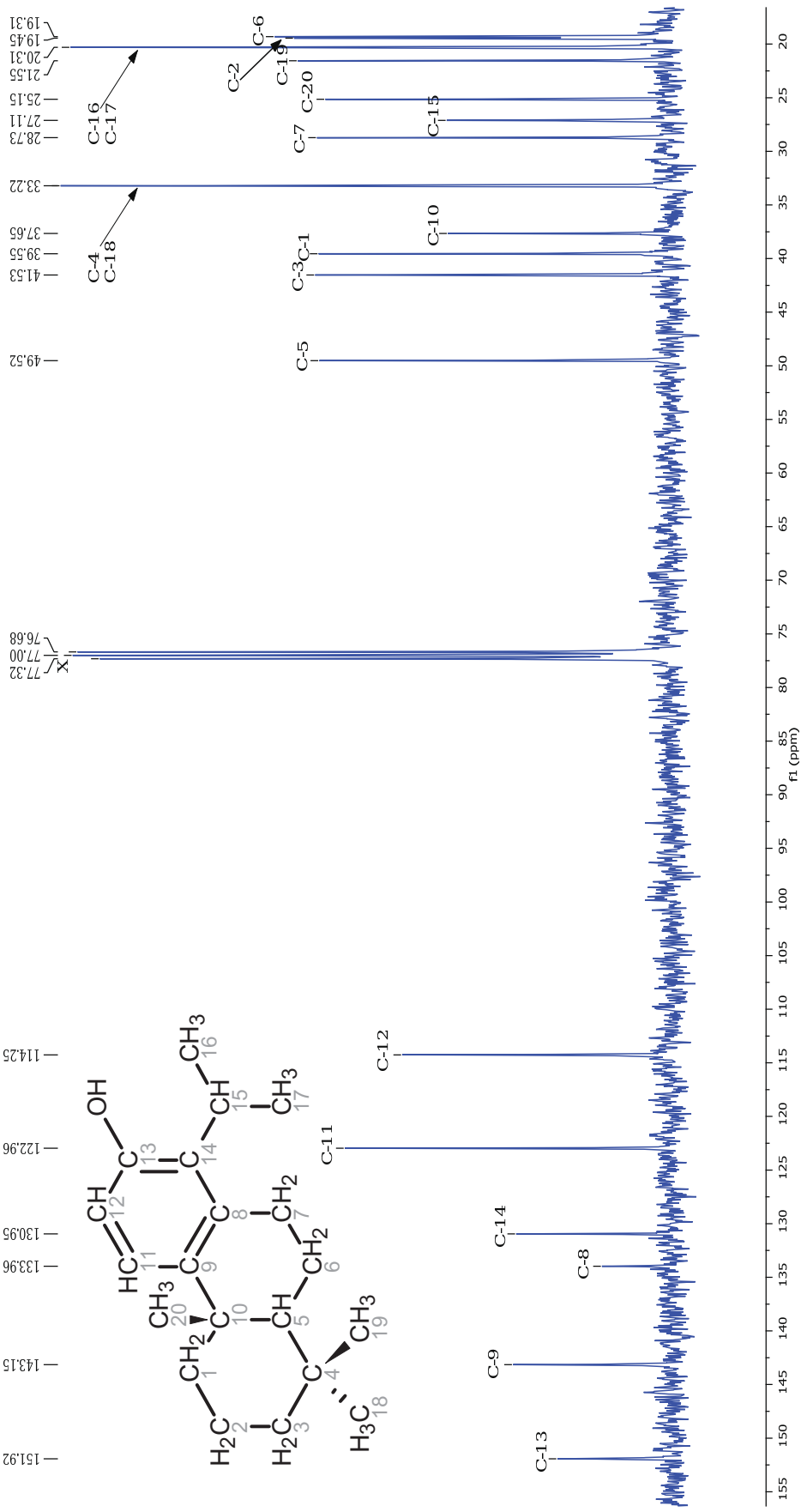


Figure 27. ^{13}C NMR spectrum of **(JC2)**, 100MHz, $CDCl_3$ (X).

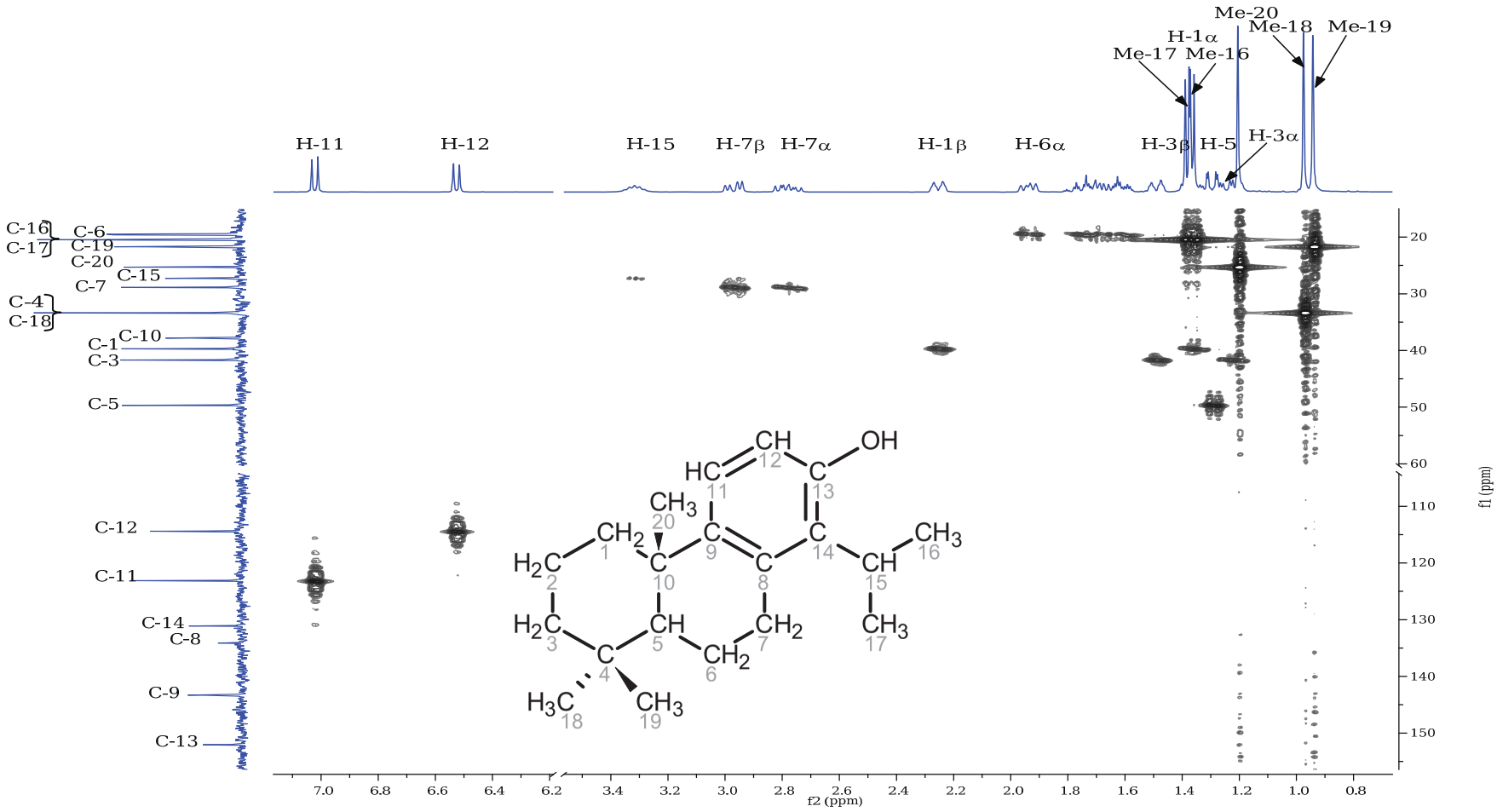


Figure 28. HMQC spectrum of **(JC2)**, 400MHz, $CDCl_3$ (X).

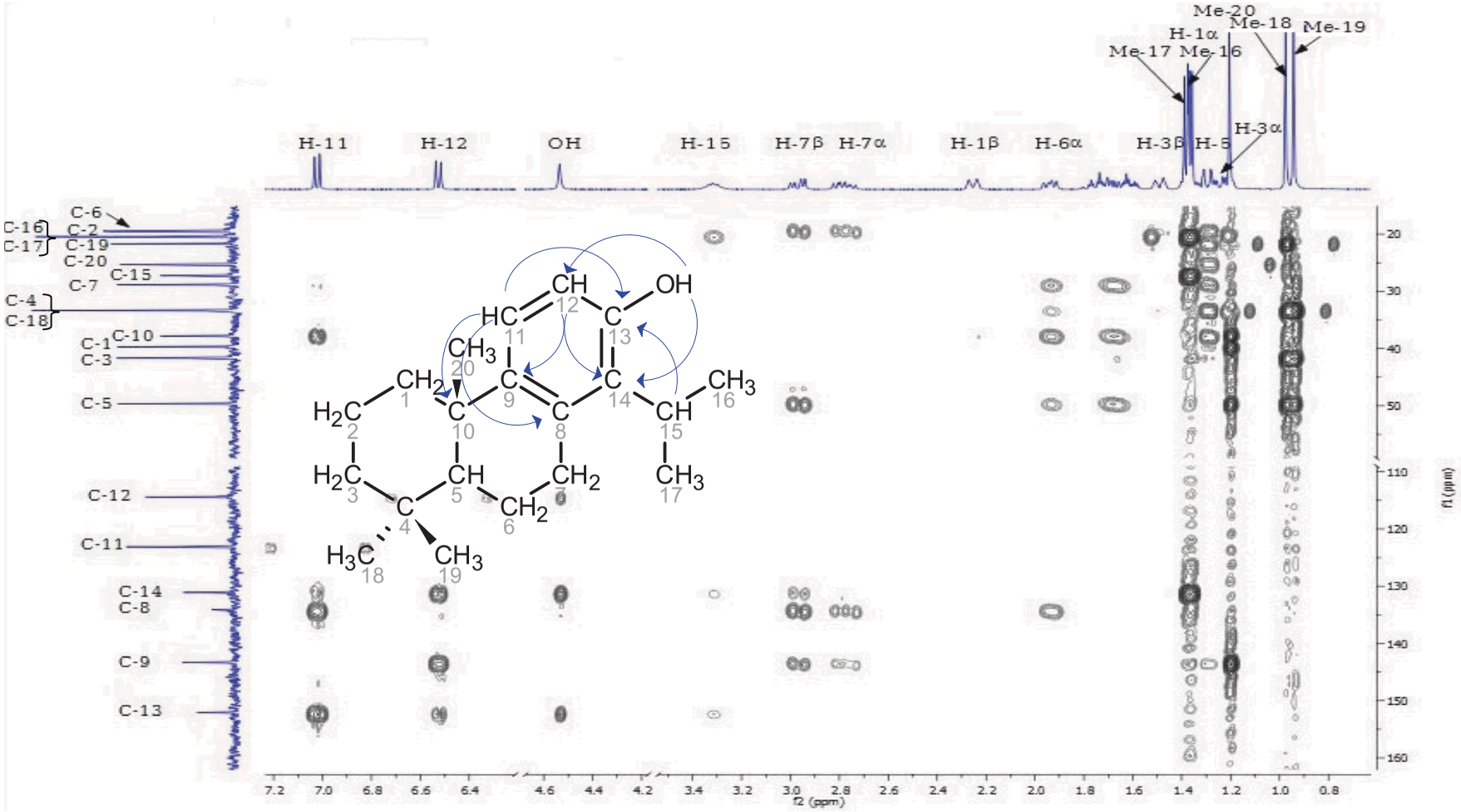


Figure 29. HMBC spectrum of **(JC2)** and selected HMBC correlations in the aromatic region, 400MHz, $CDCl_3$ (X).

3.3.3 Characterisation of **(JC3)** as *trans*-communic acid

(JC3) was isolated as a white amorphous solid from the *n*-hexane extract of *Juniperus communis* aerial parts. No spot was detected under UV light (λ=254nm) on the TLC plate. After spraying with anisaldehyde-sulfuric acid reagent and heating, a single pink/purple spot appeared suggesting the presence of a terpenoid.

HRCIMS disclosed a molecular ion $[M+H]^+$ at *m/z* 303.3 compatible with the molecular formula $C_{20}H_{30}O_2$ and indicating six degrees of unsaturation.

The ^1H NMR spectrum (Figure 30) showed two broad singlets at δ4.45 and 4.83ppm attributable to an exo-methylene group and two doublets at δ4.87 and 5.04ppm attributable to a second exo-methylene group. The spectrum also disclosed two other olefinic protons; a triplet at δ5.40ppm and a doublet of doublet at 6.32ppm. Three sharp singlets at δ0.64, 1.24 and 1.74ppm, each integrating for three protons, indicated the presence of three tertiary methyl groups.

On the COSY NMR spectrum (Figure 31) correlations were observed between the two exo-methylene singlets at δ4.45 and 4.83ppm (red circles on Figure 31). These two protons also showed long range correlation to a signal at δ1.74 ppm. The two doublets of the second exo-methylene group at δ4.87 and 5.04ppm correlated to one another (purple circles on Figure 31). They both correlated to a signal at δ6.32ppm as well. The triplet at δ5.40ppm showed correlation with two protons at 2.13 and 2.39ppm and also long range correlation with the methyl group at 1.74ppm (narrow cross peak) (orange circles on Figure 31).

The ^{13}C NMR spectrum (Figure 32) confirmed the presence of twenty carbons including two signals between 100 and 110ppm typical for exo-methylene groups and a signal at δ184.3ppm typical for a carboxylic acid group. Six signals between 100 and 150ppm suggested three alkene bounds.

On the HMBC spectrum (Figure 34), the exo-methylene protons at δ4.87 and 5.04ppm (H-15b and H-15a) showed ^{3}J correlation with the carbon at δ133.4ppm (C-13). The methyl at δ1.74ppm (Me-16) showed ^{3}J correlations to the carbons at δ133.9 (C-12) and 141.6ppm (C-14). The proton at δ5.40ppm (H-12) correlated with the carbon at δ56.4ppm (C-9). The latter was identified as the attachement point of the side chain to the main structure. Key HMBC correlations are schematically presented in Figure 34.

The NOESY spectrum did not allow concluding on the stereochemistry of the A/B ring junction. However comparison of the above spectroscopic data with previous reports (Fang et al. 1989, Muhammad et al. 1995, Smith et al. 2007) led to the unambiguous identification of **(JC3)** as *trans*-communic acid. The assignements proposed by Smith et al. for position 1 and 3 were revised.

Trans-communic acid has previously been isolated from *Juniperus communis* berries (Arya et al. 1961). This is the first report of its isolation from the aerial parts (leaves and stems).

Table 24. ^{1}H (400MHz) and ^{13}C (100MHz) spectral data of (JC3) in $CDCl_3$.

Position	13C	1H
1	39.2	1.09*
		1.89*
2	19.9	1.54*
3	37.9	1.09*
		2.13*
4	44.2	
5	56.2	1.34
6	25.8	1.89*
7	38.5	1.89*
		2.39*
8	147.9	
9	56.4	1.74*
10	40.3	
11	23.3	2.13*
		2.39*
12	133.9	5.40(t, 6.6)
13	133.4	
14	141.6	6.32(dd, 17.4, 10.8)
15	109.9	4.87(d, 10.6)
		5.04(d, 17.5)
16	11.8	1.74(s)
17	107.7	4.45(br s)
		4.83(br s)
18	29.0	1.24(s)
19	184.3	
20	12.9	0.64(s)

* overlapping signals

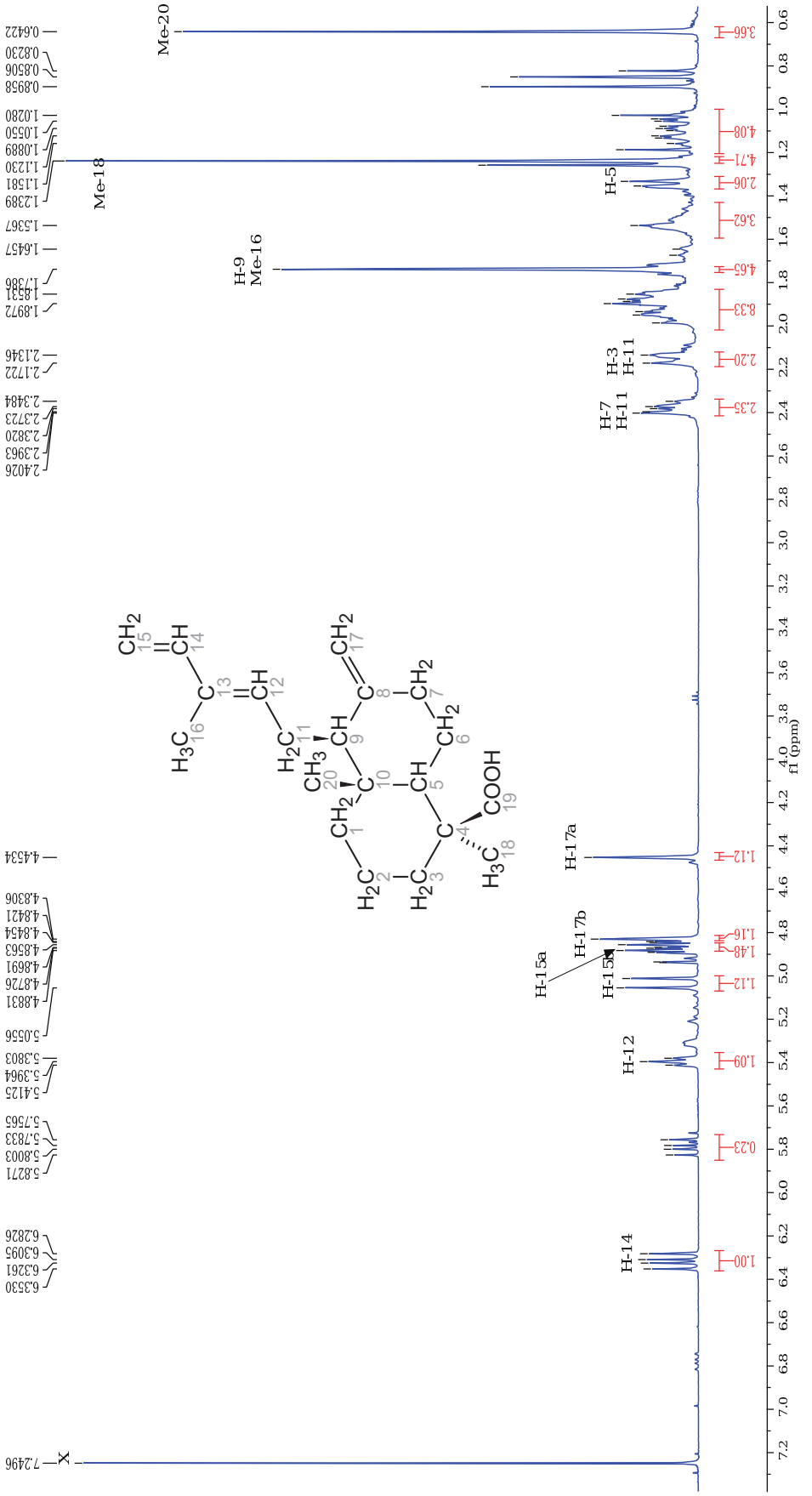


Figure 30. ^{1}H NMR spectrum of **(JC3)**, 400MHz, $CDCl_3$ (X)

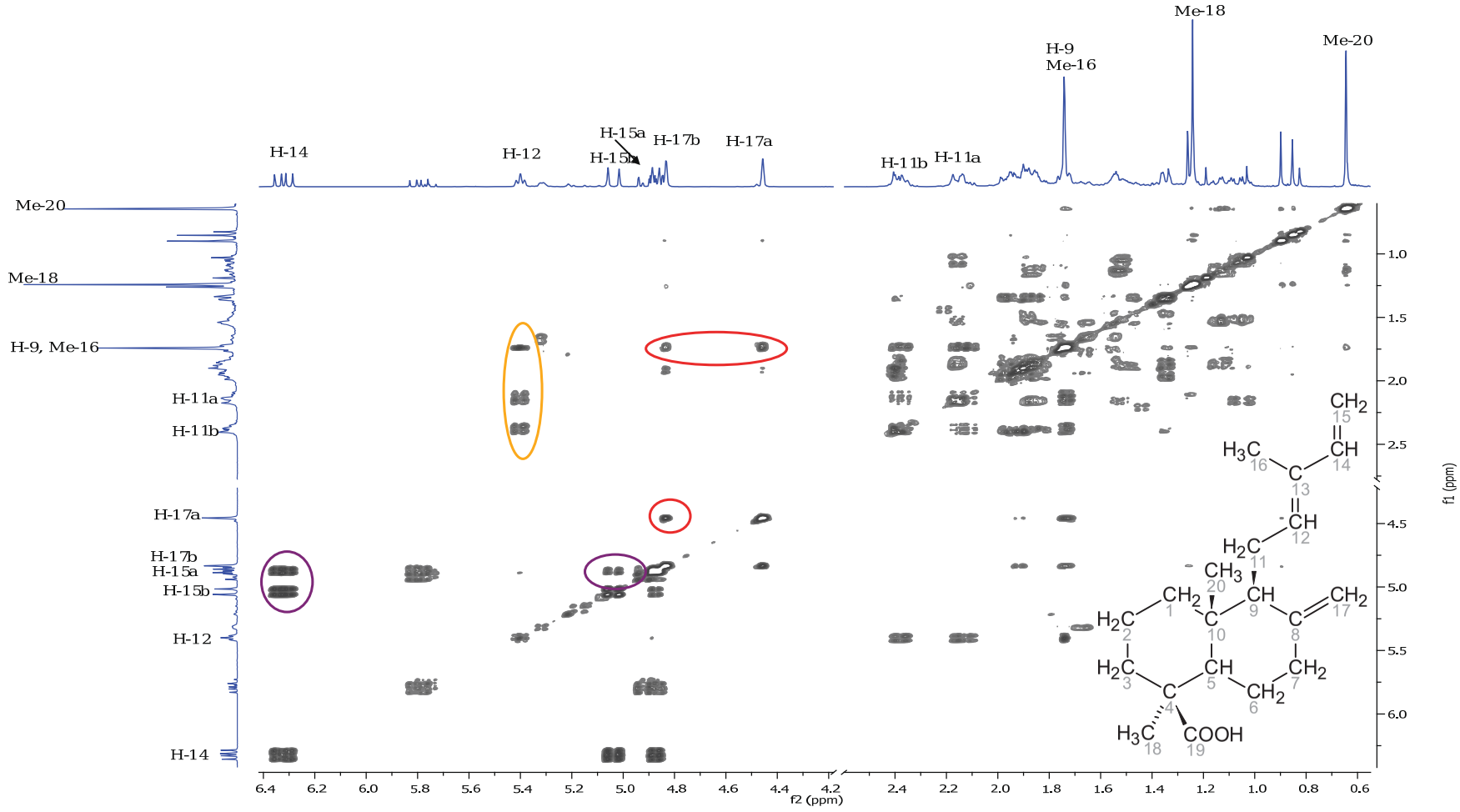


Figure 31. COSY NMR spectrum of **(JC3)**, 400MHz, $CDCl_3$ (X)

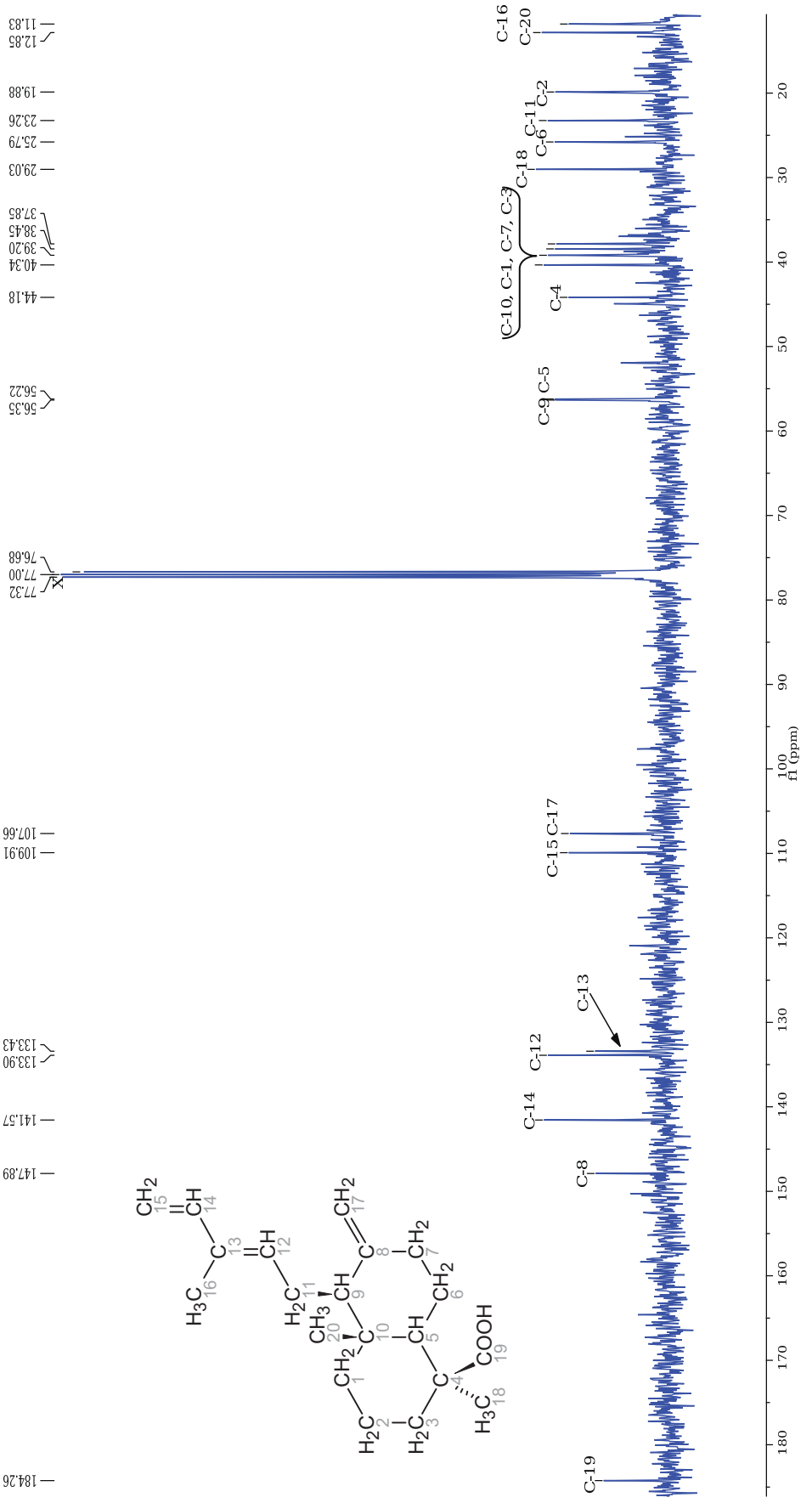


Figure 32. ^{13}C NMR spectrum of **(JC3)** 100MHz, $CDCl_3$ (X)

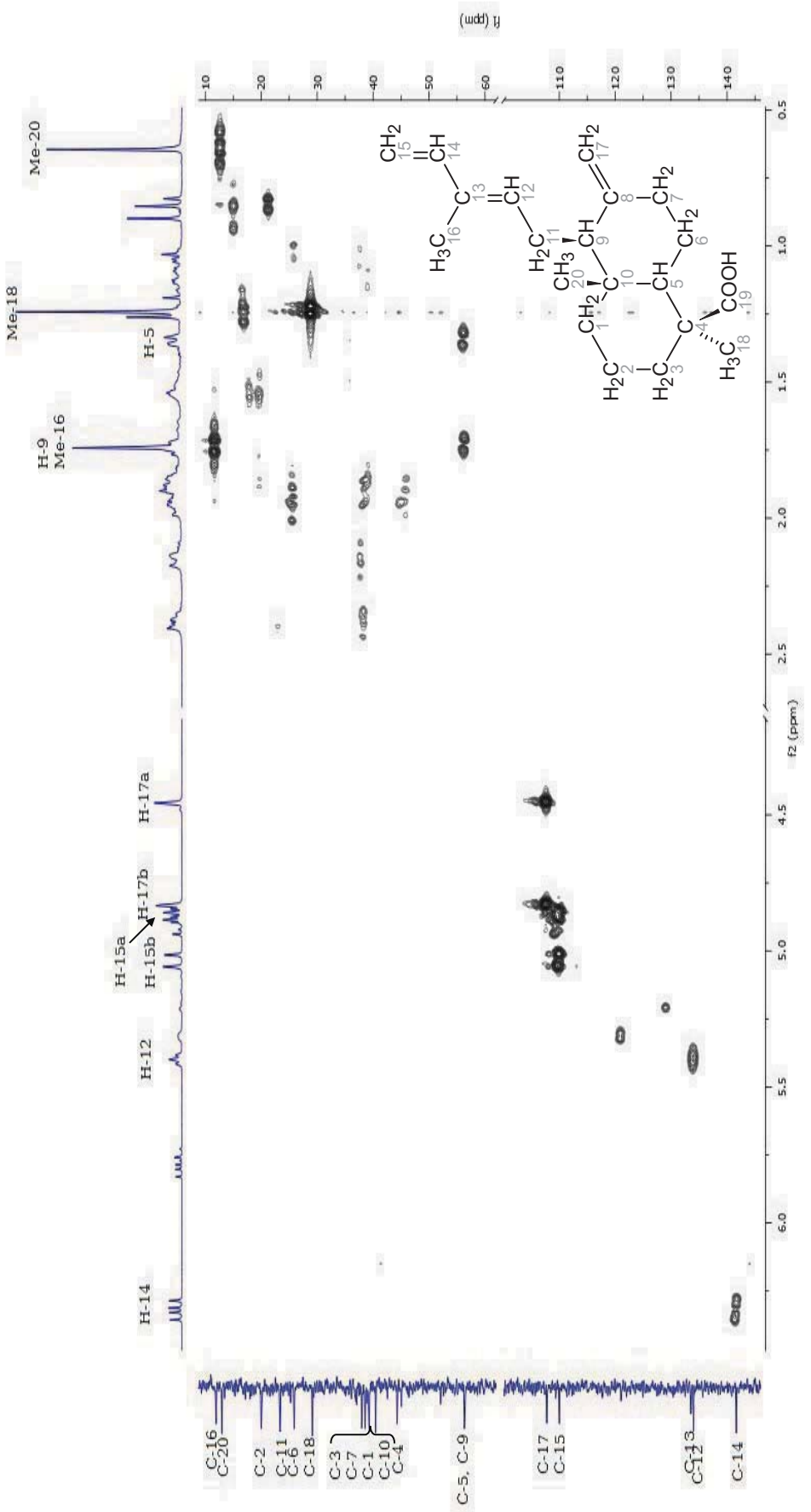


Figure 33. HMQC spectrum of **(JC3)**, 400MHz, $CDCl_3$ (X)

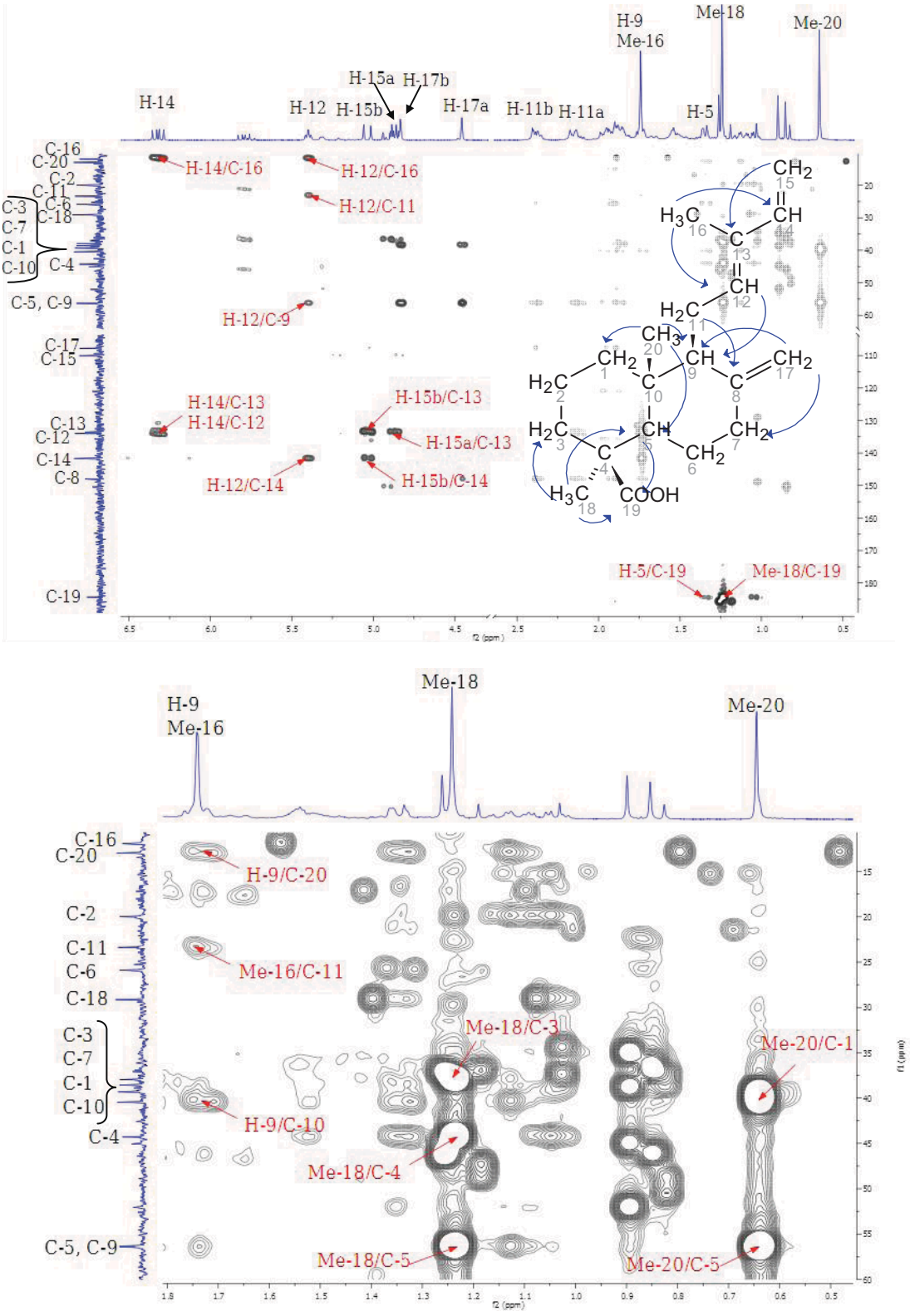


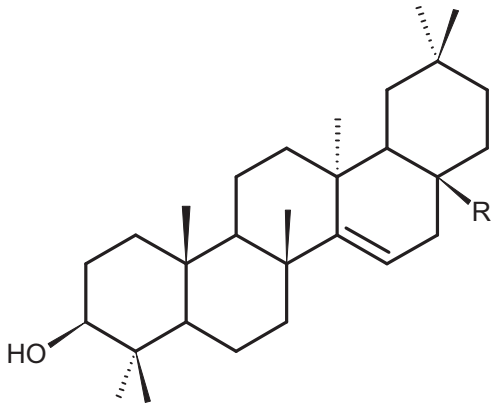
Figure 34. HMBC spectrum of **(JC3)** and selected HMBC correlations, 400MHz, $CDCl_3$ (X)

3.3.4 Characterisation of triterpenes

Four pentacyclic triterpenoids **(SJ1)**, **(CV1)**, **(CV2)** and **(MG3)** were isolated in this study. On TLC, these compounds were characterised under UV light (λ=254nm) by faint or non visible spots turning pink to purple after treatment with anisaldehyde-sulfuric acid spraying reagent.

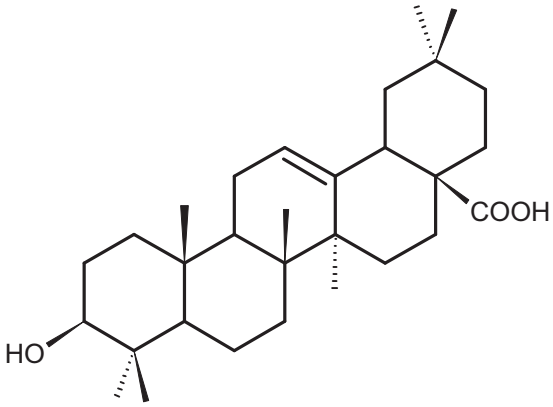
On the ^{1}H NMR spectra, common features included an aliphatic region with many overlapping signals and 7 or 8 methyl groups clearly distinguishable as sharp singlet or doublet, and an oxymethine between δ3 and 4ppm. Three of these isolated triterpenes **(SJ1)**, **(CV1)** and **(MG3)** also disclosed an olefinic proton between 5 and 6ppm

On the ^{13}C NMR spectra, **(SJ1)**, **(CV1)** and **(MG3)** showed two carbons in the regions of δ115-125ppm and δ150-160ppm indicating the presence of a double bond in the triterpene skeleton. All four triterpenes showed an oxygen bearing carbon appearing between δ70 and 80ppm.

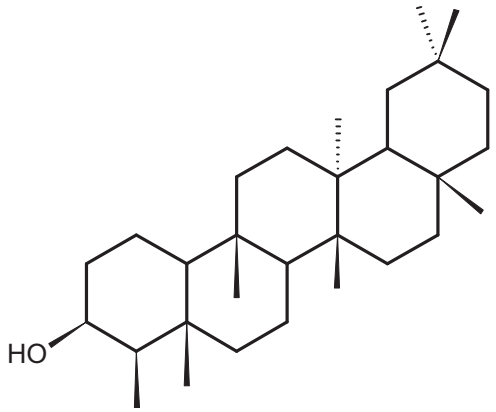


(SJ1) R=Me

(MG3) R=CH_2OH



(CV1)



(CV2)

Figure 35. Structure of isolated triterpenes.

Table 25. Selected ^{1}H (400MHz) NMR spectral data of isolated triterpenes.

Position	δ(ppm)			
	(SJ1)[a]	**(MG3)**[b]	**(CV1)**[b]	**(CV2)**[a]
3	3.18(m)	3.45(dd, 10.1, 6.0)	3.45(dd, 10.2, 5.8)	3.72(br s)
5	0.76(dd*)	0.82(dd, 11.9, 2.2)	0.84(m)	_
9	1.42*	1.46*	1.70(t, 8.8)	_
12			5.50(t, 3.2)	ND
15	5.52(dd, 8.2, 3.2)	5.64(dd, 8.2, 3.0)	ND	ND
18	ND	0.72(dd, 13.7, 3.5)	3.31(dd, 13.8, 4.0)	1.52*
23	0.97(s)	1.24(s)	1.25(s)	0.92(d, 6.7)
24	0.79(s)	1.07(s)	1.03(s)	0.95(s)
25	0.91(s)	0.95(s)	0.90(s)	0.84(s)
26	1.08(s)	1.09(s)	1.03(s)	0.98(s)
27	0.89(s)	1.10(s)	1.30(s)	0.99(s)
28	0.81(s)	3.48(d, 10.2) 3.63(d, 10.7)	_	1.16(s)
29	0.94(s)	1.01(s)	0.99(s)	0.93(s)
30	0.89(s)	1.05(s)	1.02(s)	0.98(s)

[a] in $CDCl_3$ [b] in C_5D_5N * overlapping signals ND: not determined

Table 26. ^{13}C (100MHz) NMR spectral data of isolated triterpenes.

Position	δ(ppm)			
	(SJ1)[a]	**(MG3)**[b]	**(CV1)**[b]	**(CV2)**[a]
1	37.7†	38.6	39.3	15.8
2	27.1	28.4	28.4	35.2
3	79.1	78.5	78.4	72.8
4	38.7	39.6	39.7	49.2
5	55.5	56.3	56.2	37.8
6	18.8	19.5	19.2	41.7
7	41.3	42.0	33.6	17.5
8	38.9	39.7	40.1	53.2
9	49.2	49.9	48.5	37.1
10	37.5	38.6	37.7	61.3
11	17.5	18.2	24.2†	35.5
12	33.1	34.2	122.9	30.6
13	38.0	38.1	145.2	38.4
14	158.0	158.9	42.5	39.7
15	116.9	117.2	28.7	32.8
16	37.7†	31.6	24.1†	36.1
17	35.8	41.5	46.8	30.0
18	48.7	45.9	42.3	42.8
19	36.6	36.7	47.0	35.3
20	28.8	29.2	31.3	28.2
21	33.7	33.6	34.6	32.3
22	35.1	28.7	33.6	39.3
23	28.0	29.2	29.1	11.6
24	15.4†	16.8	16.9	16.4
25	15.4†	16.0	15.9	18.2
26	25.9	26.6	17.8	20.1
27	21.3	22.3	26.5	18.6
28	29.8	64.9	180.5	32.1
29	33.3	34.1	33.5	35.0
30	29.9	30.4	24.0	31.8

[a] in $CDCl_3$ [b] in C_5D_5N †interchangeable signals

3.3.4.1 Characterisation of **(SJ1)** as taraxerol

(SJ1) was isolated as colourless crystals from the *n*-hexane extract of *Skimmia japonica* aerial parts. HREIMS indicated a molecular ion $[M]^+$ at *m/z* 426.4 corresponding to the molecular formula $C_{30}H_{50}O$ (6 double bond equivalents). The IR spectrum disclosed a hydroxy group (ν_{max}=3482 cm^{-1}).

The ^1H NMR spectrum (Figure 36) showed an olefinic proton at δ5.52ppm (dd, 8.2Hz, 3.2Hz), a multiplet at δ3.18ppm suggesting the presence of an oxymethine and 8 tertiary methyl groups appearing as singlets at δ0.79, 0.81, 0.89, 0.89, 0.91, 0.94, 0.97 and 1.08ppm.

On the COSY spectrum, correlations were observed between the olefinic proton at 5.52ppm and two protons at 1.60 and 1.90ppm. This indicated a CH_2 adjacent to the double bond as suggested by the splitting pattern of the olefinic proton.

The ^{13}C NMR spectra (Figure 37) confirmed the presence of 30 carbons. Noticeable signals included two carbons at δ116.9 and 158.0ppm and an oxymethine at δ79.1ppm.

The HMBC spectrum (Figure 38) showed two geminal methyls at δ0.97 (Me-23) and 0.79ppm (Me-24) respectively correlating with carbons at δ15.4 (C-24) and 28.0ppm (C-23) as well as correlating with three other carbons at δ38.7 (C-4), 55.5 (C-5), 79.0ppm (C-3). The methyl group at δ0.91ppm (Me-25) also showed a ^3J correlation to the carbon at δ55.5ppm (C-5).

Similarly, the non-geminal methyl groups at δ0.91 (Me-25) and 1.08ppm (Me-26) both correlated with the carbon at δ49.2ppm (C-9) while another pair of non-geminal methyl groups at δ0.81 (Me-28) and 0.891ppm (Me-27) both correlated with the carbon at δ48.7ppm (C-18). Me-27 also correlated to the carbon at δ158.0ppm (C-14).

The NOESY spectrum showed a correlation between the proton at δ3.18ppm (H-3) and the methyl group at δ0.97ppm (Me-23) indicating they were on the same side of the ring.

Comparison of these data with previous reports (Chien et al. 2004) allowed characterisation of **(SJ1)** as friedoolean-14-en-3-β-ol or taraxerol.

Taraxerol has previously been reported from the methanol and the ethanol extract of *Skimmia japonica* aerial parts (Atkinson et al. 1974, Nakatani et al. 1991, Reisch and Achenbach 1991, Takeda 1941a).

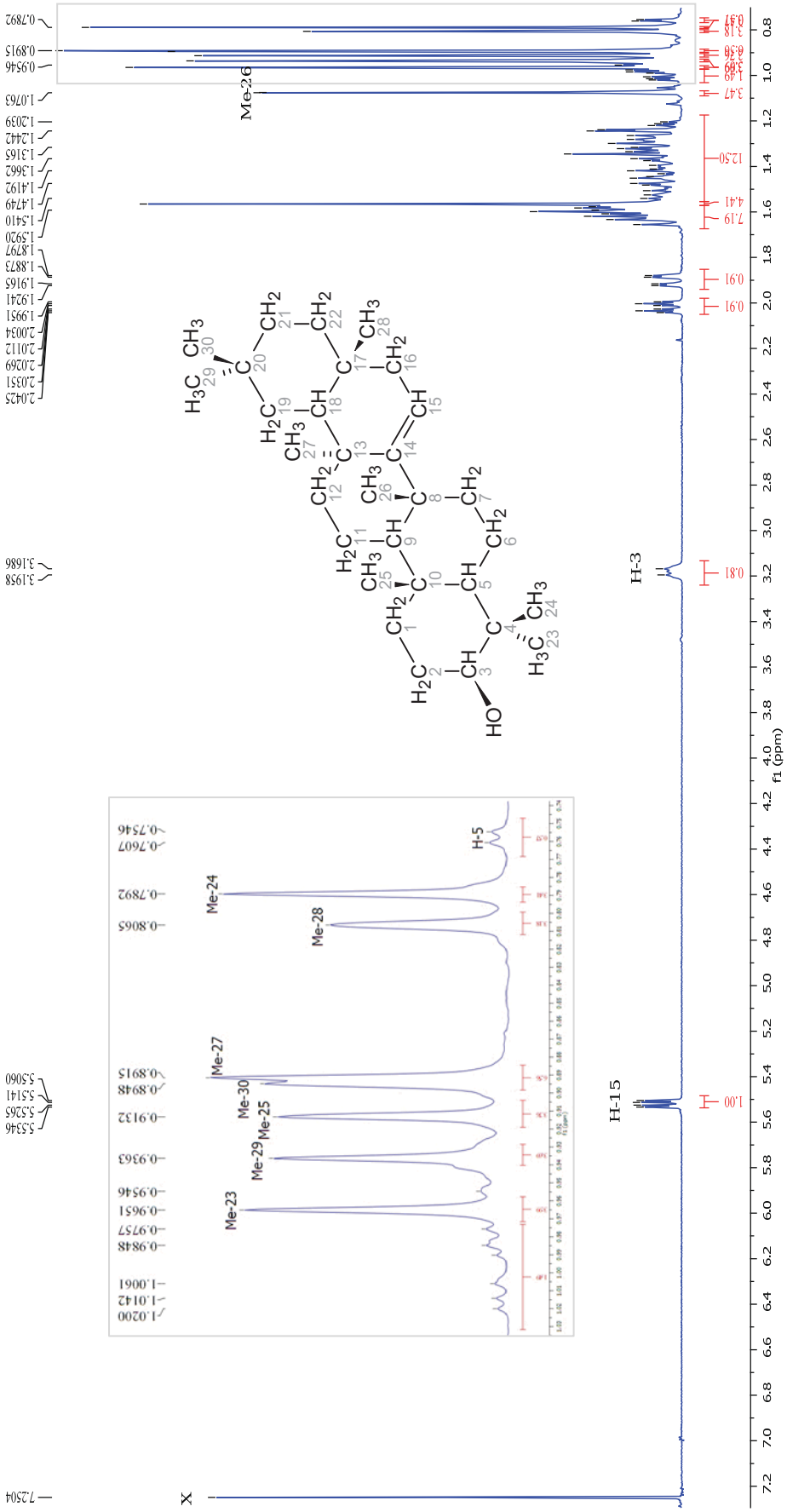


Figure 36. ^{1}H NMR spectrum of **(SJ1)**, 400MHz, $CDCl_3$ (X)

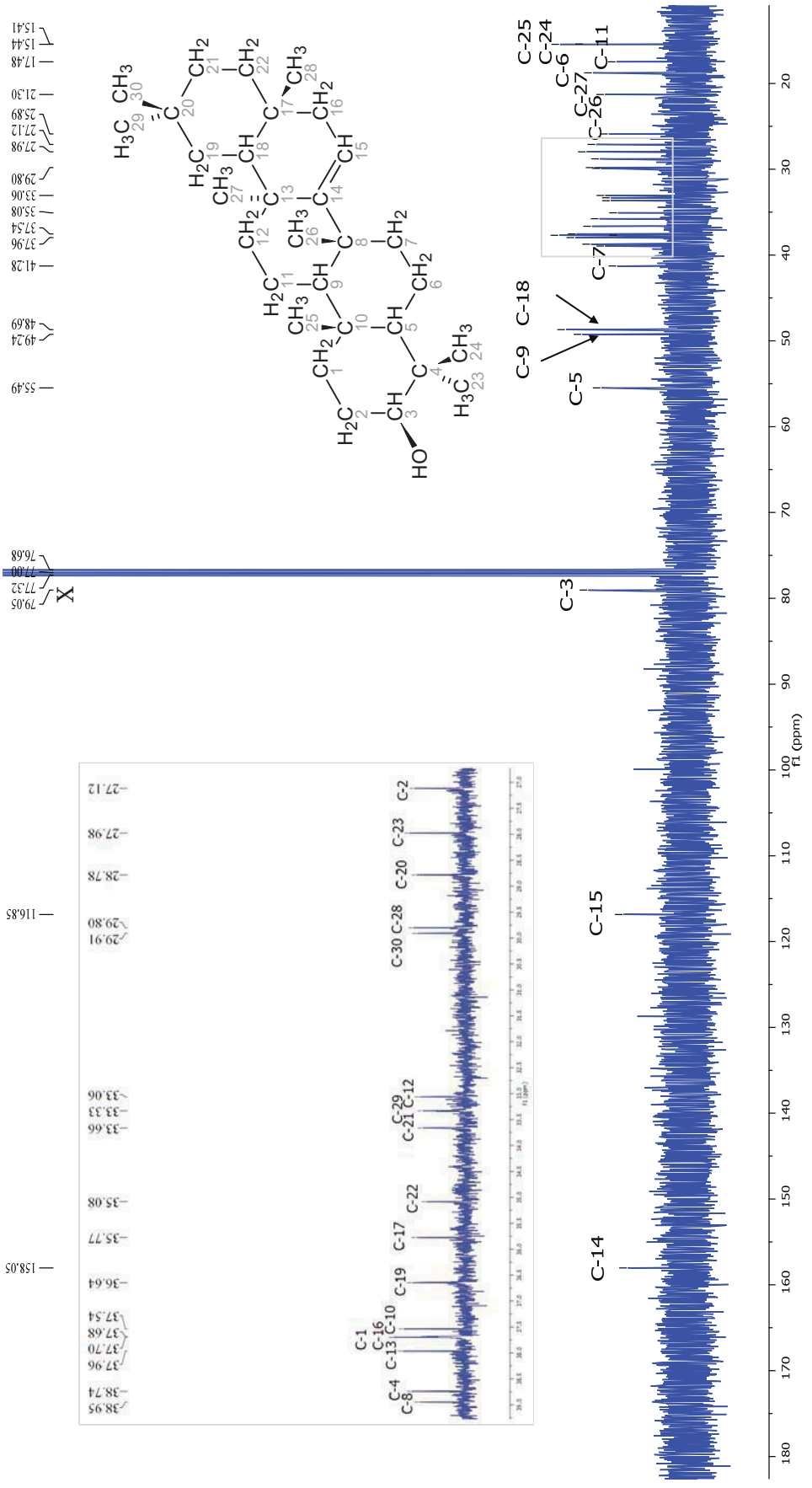


Figure 37 ^{13}C NMR spectrum of **(SJ1)**, 100MHz, $CDCl_3$ (X)

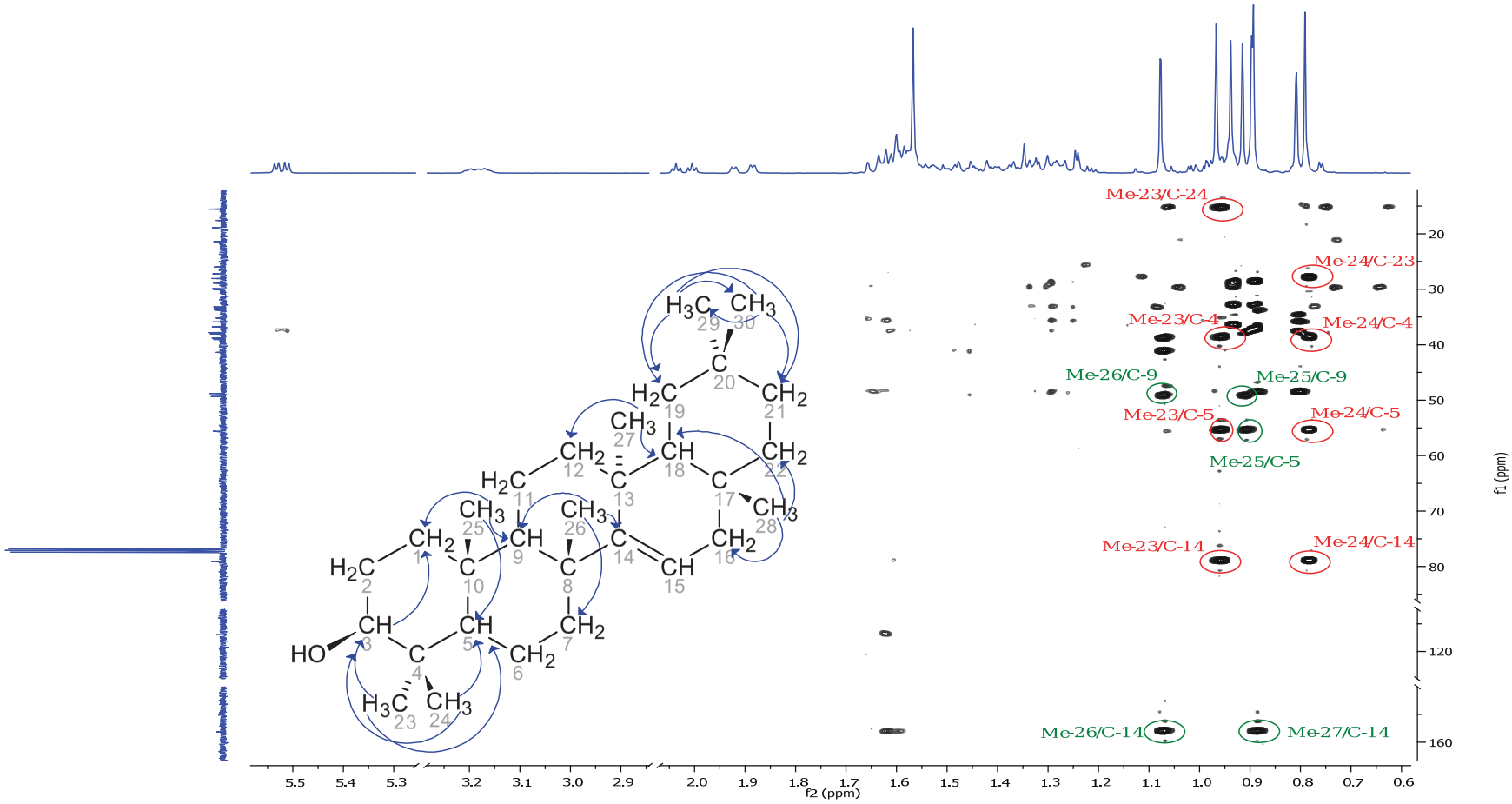


Figure 38. HMBC spectrum of **(SJ1)** and selected HMBC correlations, 400MHz, $CDCl_3$ (X)

3.3.4.2 Characterisation of **(MG3)** as myricadiol

(MG3) was isolated as a white flaky solid from the *n*-hexane extract of *Myrica gale* roots.

Accurate FABMS showed a pseudo-molecular ion $[M+H]^+$ of *m/z* 443.3885 corresponding to the molecular formula $C_{30}H_{51}O_2$ indicating a molecular formula of $C_{30}H_{50}O_2$ (6 double bond equivalents) for **(MG3)**.

The IR spectrum was almost identical to the one obtained for **(SJ1)**. It only differed in the intensity of the peak corresponding to the O-H stretch (ν_{max}=3369cm^{-1}) suggesting the presence of an additional hydroxyl group.

The NMR spectra disclosed many features previously observed for **(SJ1)** and was therefore characterised as a taraxerane-type triterpene bearing a tertiary alcohol, a double bond in $\Delta_{14,\,15}$ and 7 tertiary methyls among which two pairs of geminal methyl groups.

The main difference observed in comparison with **(SJ1)** was the replacement of one methyl group with one oxymethylene group (δ^H3.48 and 3.63ppm, δ^C64.9ppm). On the HMBC spectrum (Figure 41), the proton at δ3.48ppm showed ^3J correlation with the carbon at δ28.7ppm (C-22) and ^2J correlations with the carbon at δ41.5ppm (C-17). In addition, the proton at δ3.63ppm showed ^3J correlation with the carbon at δ31.6ppm (C-16). This indicated the oxymethylene was at position 28.

The proton at 3.45ppm (H-3) appeared as a doublet of doublet. The coupling constant values, 10.1 and 6.0Hz, respectively indicated axial-axial and axial-equatorial coupling with the protons at position 2. This indicated H-3 was in an axial position.

On the NOESY spectrum (Figure 42) correlation was oberserved between the proton at 3.45ppm (H-3) and the proton at δ0.82ppm (H-5), indicating H-5 was also in an axial position. Correlation was also observed between the proton at δ0.82ppm (H-5) and the proton at δ1.46ppm (H-9) indicating H-9 was in an axial position as well. The proton at δ0.82ppm (H-5) also showed correlation with the methyl group at δ1.24ppm (Me-23) indicating this methyl group was on the same side of ring A as H-5 that is to say in an equatorial position. This implied Me-24 was in an axial position. Finally the NOESY correlation observed between the methyls groups at 0.94 (Me-25) and 1.07ppm (Me-24) allowed to conclude on a *trans*-diaxial configuration for ring junctions A/B and B/C.

Further NOESY correlations were observed between the proton at δ0.71ppm (H-18) and the oxymethylene group. These observations allowed to conclude on a *cis* D/E ring junction.

These data led to the identification of **(MG3)** as 14-taraxeren-3,28-diol or myricadiol. Previous ^{13}C NMR assignements (Merfort et al. 1992, Sakurai et al. 1987) were recorded in deuterated chloroform. In this study, **(MG3)** was poorly soluble in chloroform.

Full NMR data for myricadiol are thus reported in deuterated pyridine for the first time.

Myricadiol was previously isolated from the acetone extract of *Myrica gale* stem bark (Ryabinin and Matyukhina 1959). This is the first report of its presence in the roots.

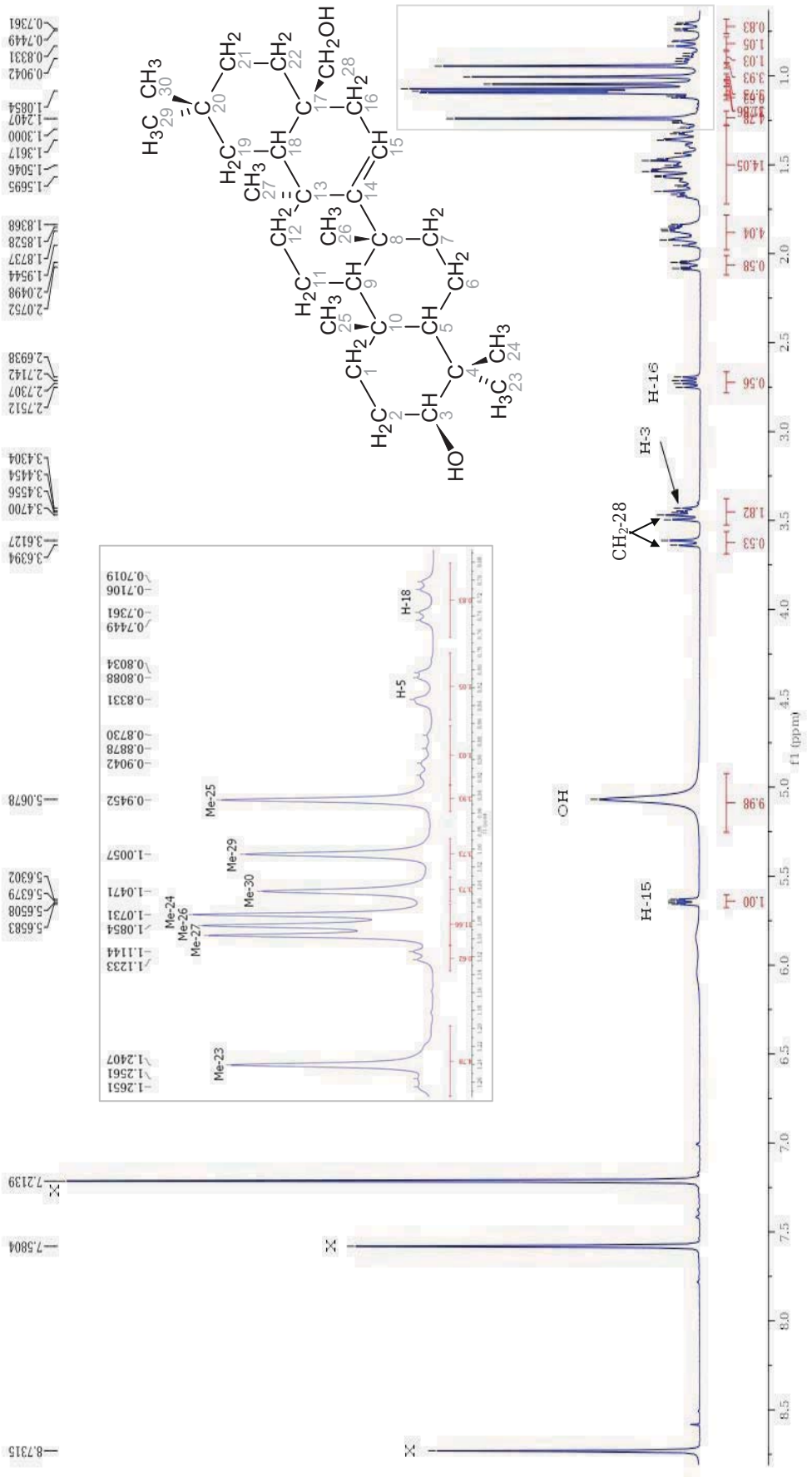


Figure 39. ^{1}H NMR spectrum of **(MG3)**, 400MHz, C_5D_5N (X)

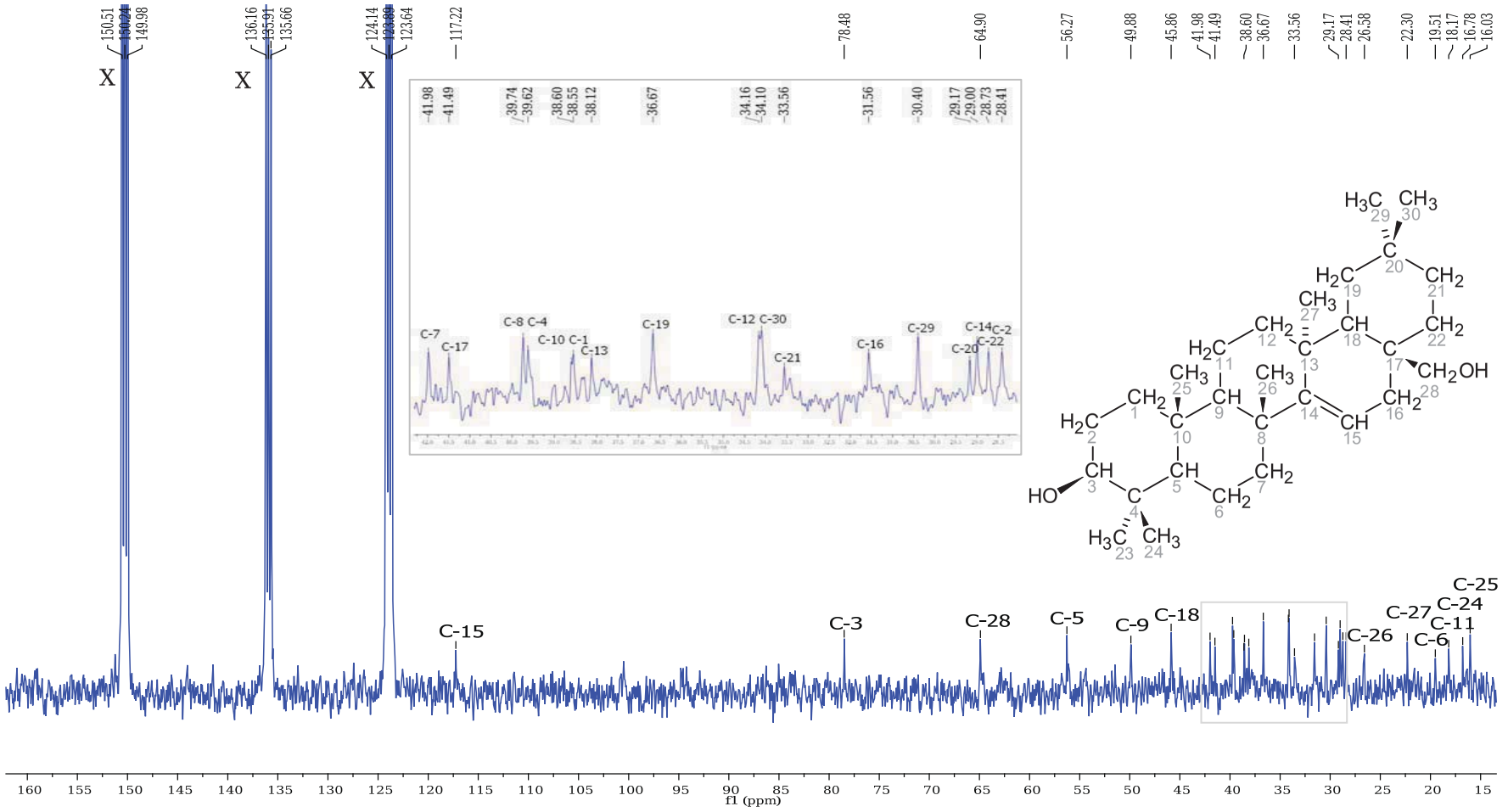


Figure 40. ^{13}C NMR spectrum of **(MG3)**, 100MHz, C_5D_5N (X)

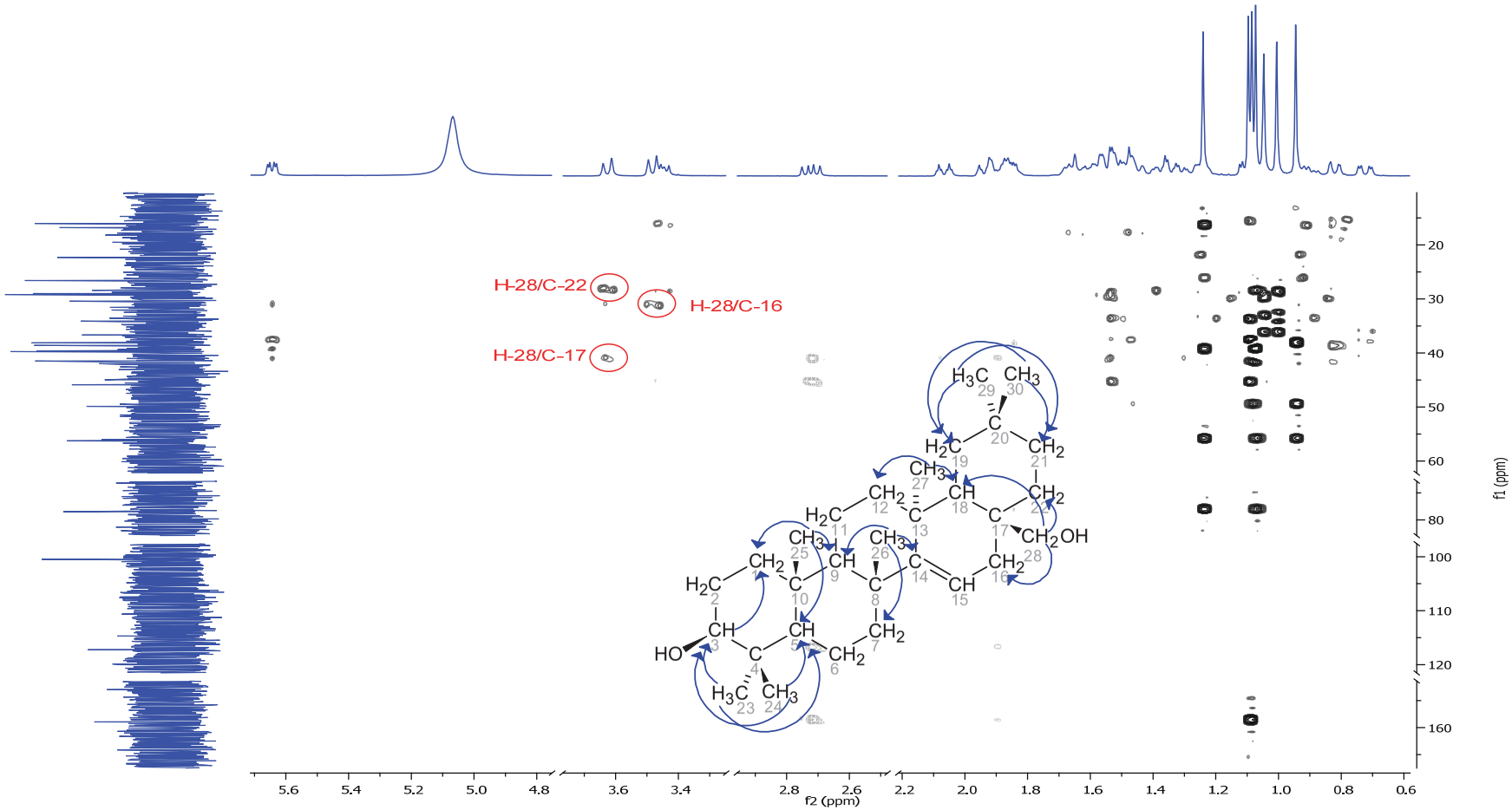


Figure 41. HMBC spectrum (400MHz, C_5D_5N (X)) and selected HMBC correlations of **(MG3)**

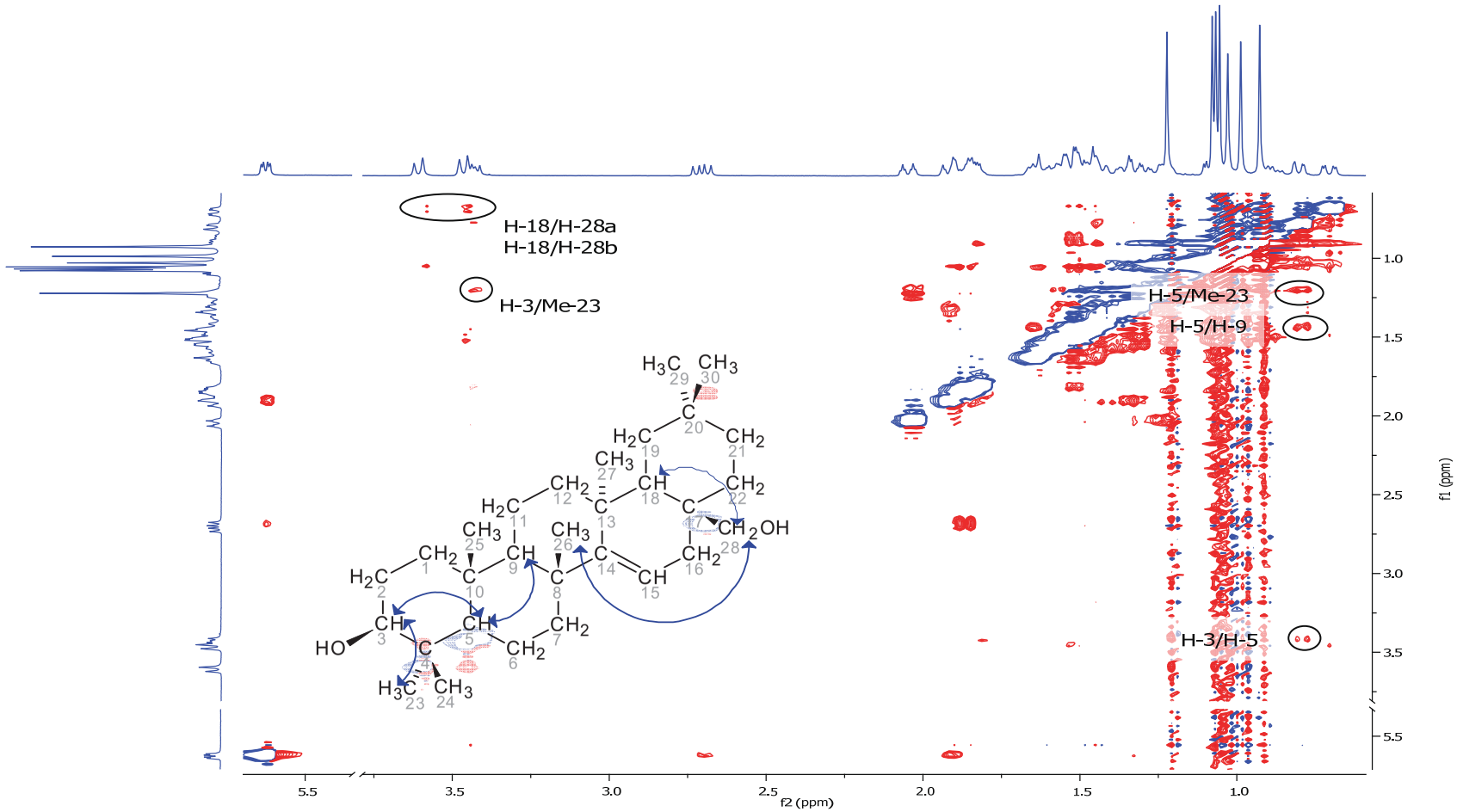


Figure 42. NOESY spectrum (400MHz, C_5D_5N (X)) and key NOE correlations of **(MG3)**

3.3.4.3 Characterisation of **(CV1)** and as oleanolic acid

(CV1) was isolated as a white crystalline powder from the ethyl acetate extract of *Calluna vulgaris* aerial parts. HREIMS indicated a molecular ion $[M]^+$ at *m/z* 456.3602 corresponding to the molecular formula $C_{30}H_{48}O_3$ (7 double bond equivalents).

The ^1H and ^{13}C NMR spectra (Figures 43 and 44) disclosed many or the features previously observed for **(SJ1)** and **(MG3)**. Therefore **(CV1)** was characterised as a pentacyclic triterpene bearing a tertiary alcohol, a alkene bond, one olefinic proton and 7 tertiary methyls among which two pairs of geminal methyls. In addition, the ^{13}C NMR spectrum indicated a carboxylic acid group at δ180.5ppm and the proton spectrum disclosed a second deshielded methine proton at 3.31ppm.

(CV1) was unambiguously characterised as oleanolic acid by comparison of the NMR data obtained with those of an authentic commercial sample. NMR data were in good agreement with previous reports (Seebacher et al. 2003).

To the best of our knowledge, this is the first report of the isolation of oleanolic acid from *Calluna vulgaris*.

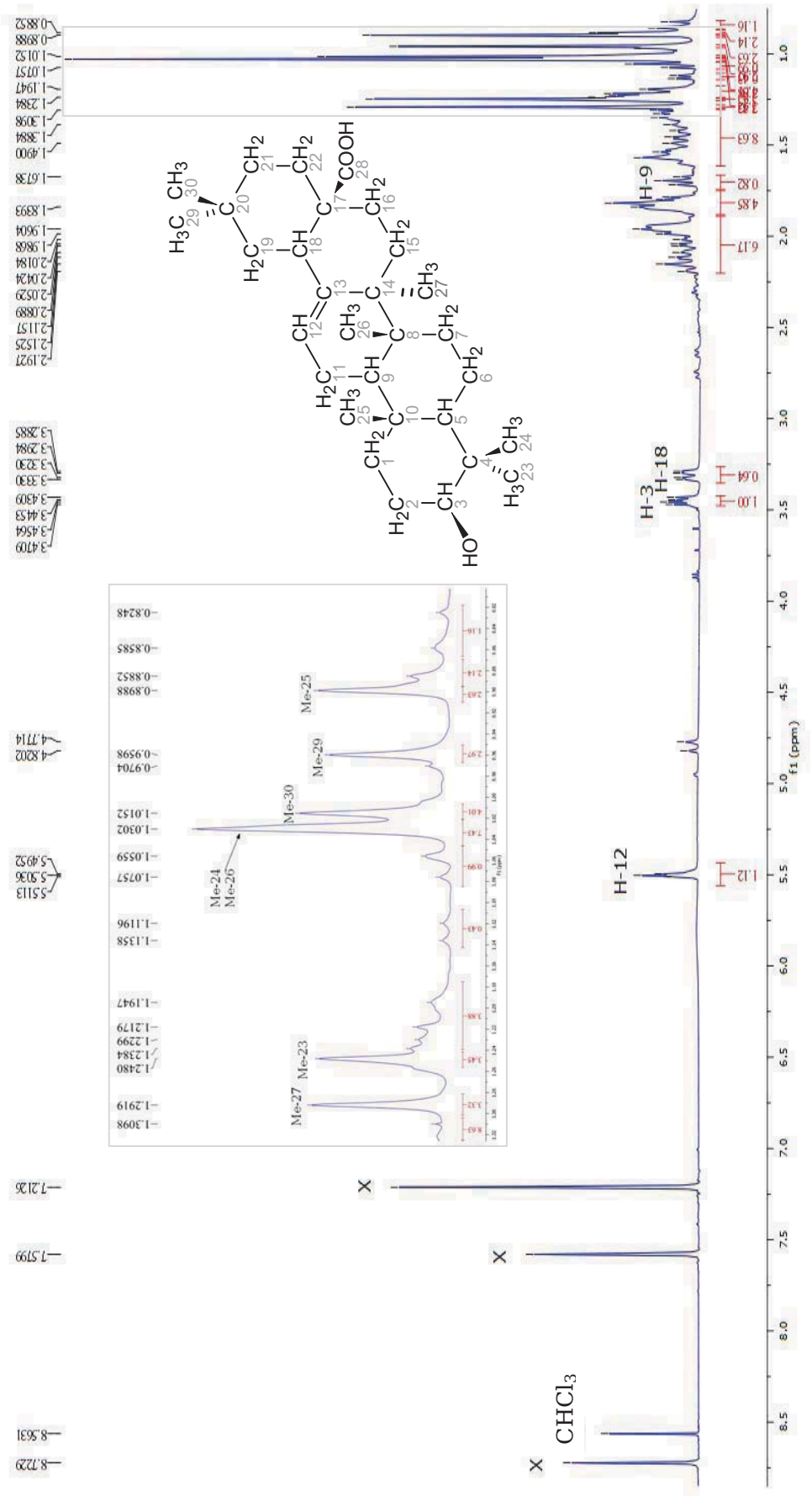


Figure 43. ^{1}H NMR spectrum of **(CV1)**, 400MHz, C_5D_5N (X)

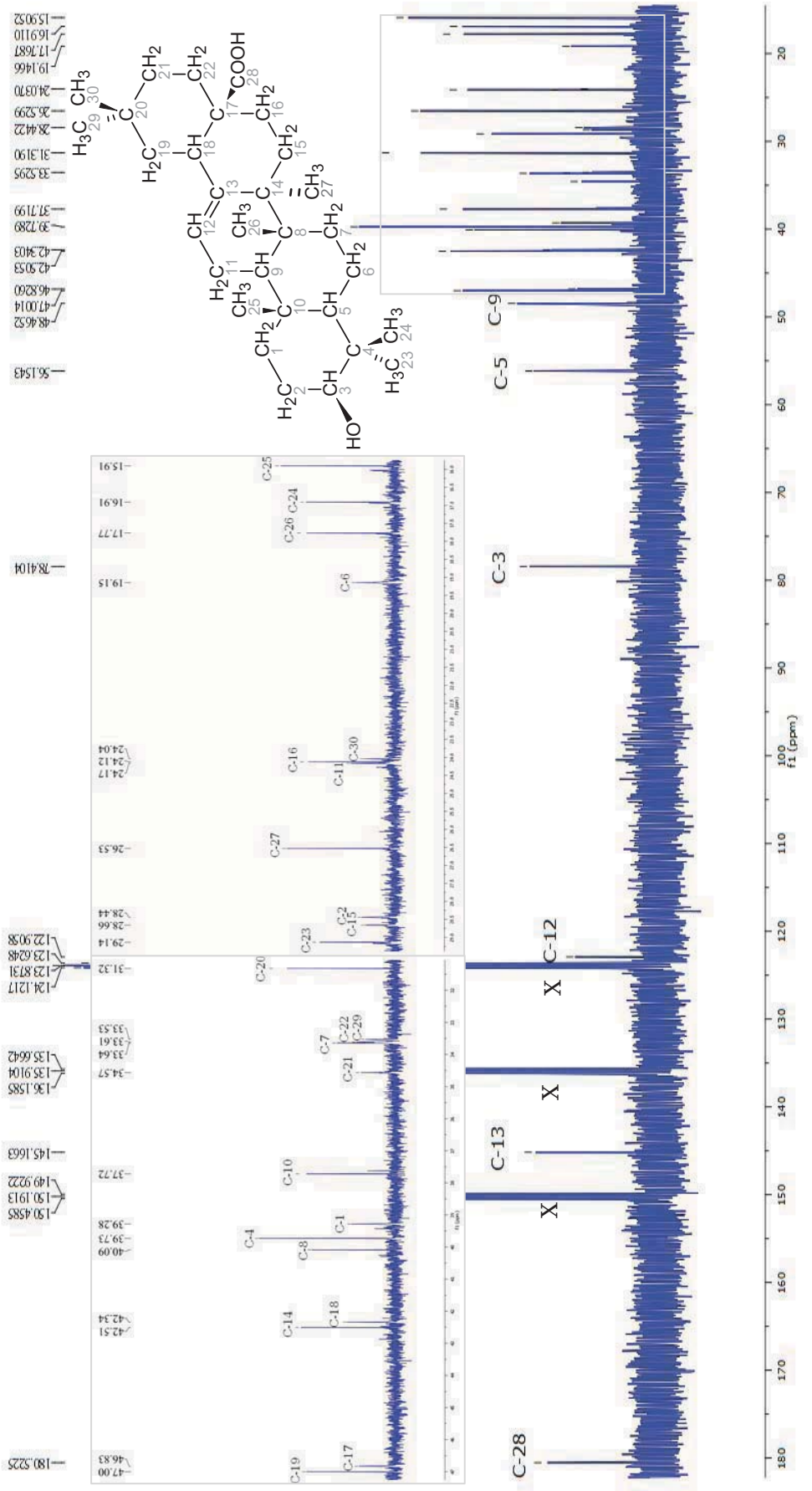


Figure 44. ^{13}C NMR spectrum of **(CV1)**, 100MHz, C_5D_5N (X)

3.3.4.4 Characterisation of **(CV2)** as epifriedelanol

(CV2) was isolated from the *n*-hexane extract of *Calluna vulgaris* aerial parts.

EIMS of **(CV2)** indicated a molecular ion $[M]^+$ at *m/z* 428.4 corresponding to the molecular formula $C_{30}H_{52}O$ (5 double bond equivalents).

Noticeable signals on the ^1H NMR spectrum (Figure45) included a broad singlet at δ3.72ppm, a secondary methyl group at δ0.92ppm and 7 tertiary methyl groups at δ0.84, 0.93, 0.95, 0.98, 0.98, 0.99 and 1.16ppm.

The ^{13}C NMR spectrum (Figure 46) confirmed the presence of 30 carbons among which one oxymethine at δ72.7 ppm.

On the HMBC spectrum (Figure 47), the signal at δ0.92ppm (Me-23) correlated with carbons at δ37.82 (C-5), 49.16 (C-4) and 72.75ppm (C-3). The methyl at δ0.95ppm (Me-24) correlated to carbons at δ37.8 (C-5), 41.7 (C-6), 49.2 (C-4) and 61.3ppm (C-10).

Two methyls at δ0.93 (Me-29) and 0.98ppm (Me-30) were identified as geminal methyls since they correlated with carbons at δ31.8 (C-30) and 35.0ppm (C-29), respectively.

The proton a δ3.72ppm (H-3) appears as a broad singlet which indicates only small coupling constant values and therefore an equatorial proton.

The above data led to the identification of **(CV2)** as 3β-friedelanol or epifriedelanol. Based on the analysis of the HMQC and HMBC data, the assignements previously reported for epifriedelanol (Kundu et al. 2000) were revised. This is the first report of the isolation of epifriedelanol from *Calluna vulgaris*.

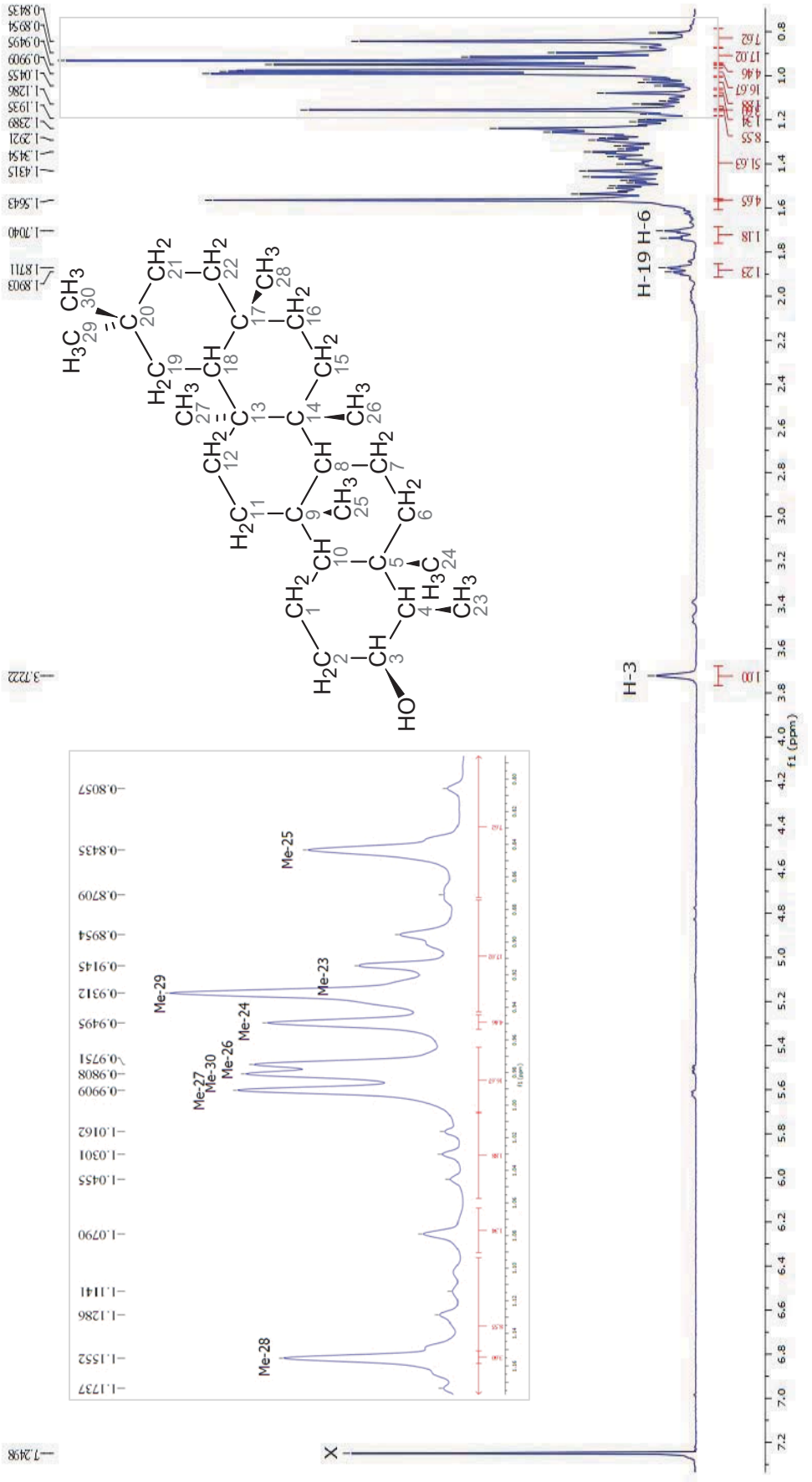


Figure 45. ^{1}H NMR spectrum of **(CV2)**, 400MHz, $CDCl_3$ (X)

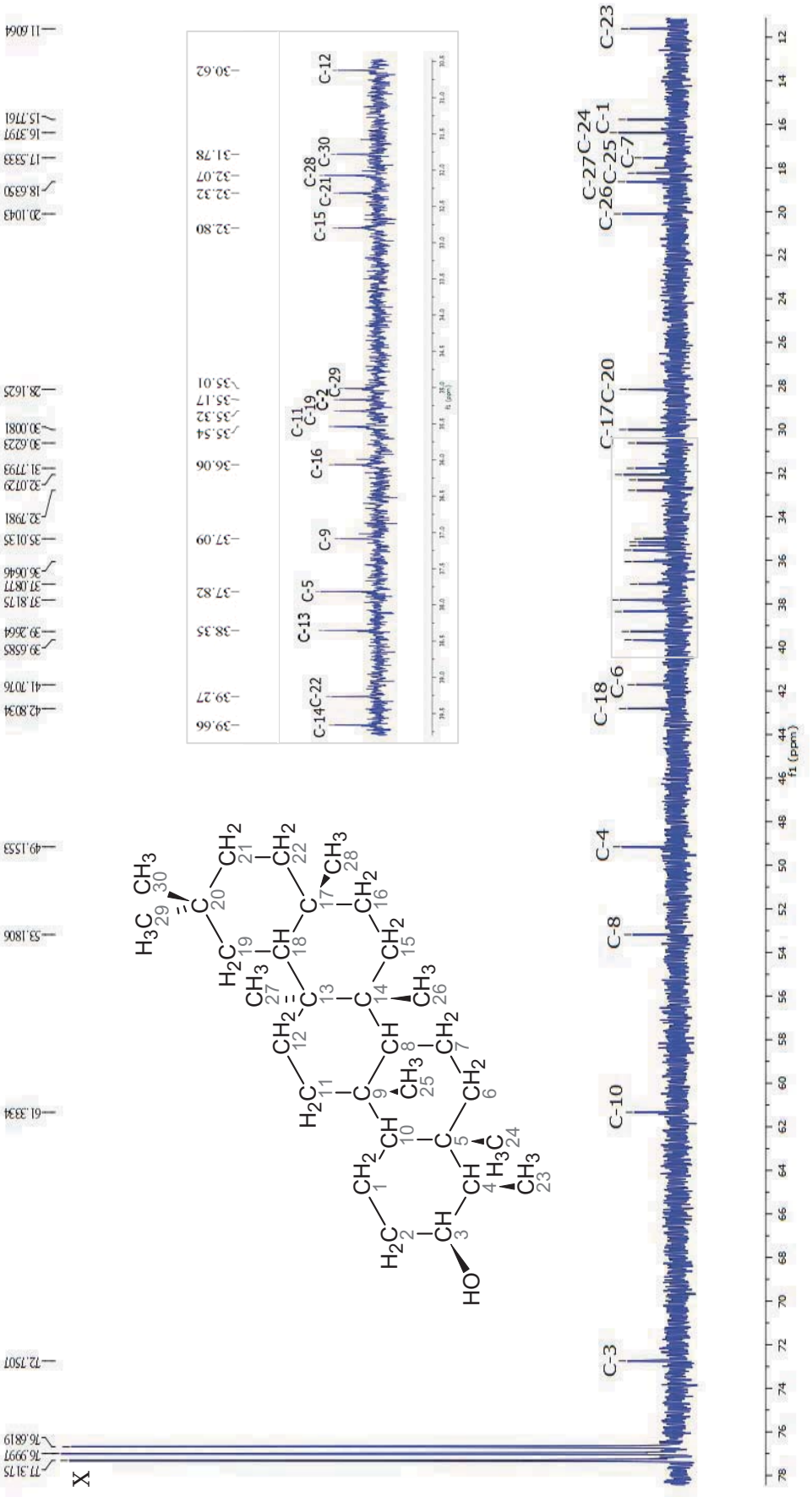


Figure 46. ^{13}C NMR spectrum of **(CV2)**, 100MHz, $CDCl_3$ (X)

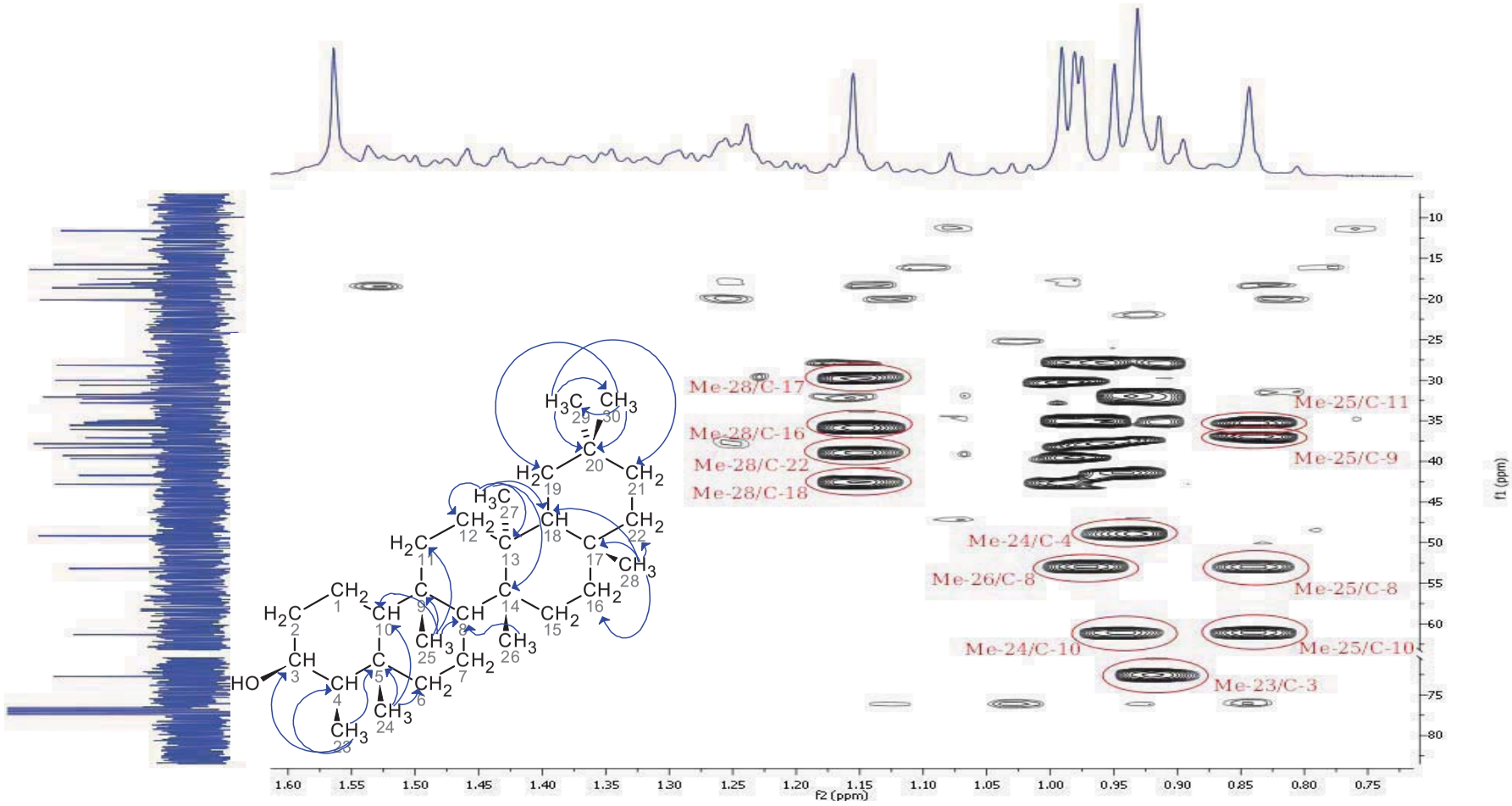


Figure 47. HMBC spectrum of **(CV2)** and selected HMBC correlations, 400MHz, $CDCl_3$ (X)

3.3.5 Characterisation of **(SJ2)** as a mixture of oxypeucedanin and meranzin

(SJ2) was obtained from the *n*-hexane extract of *Skimmia japonica* aerial parts as a yellow oil. On TLC, it showed a strong tailing spot under UV light (λ=254nm). After spraying with Dragendorff's, reagent the spot turned light orange.

The ^{1}H NMR spectrum (Figure 48) indicated 30 protons among which 4 tertiary methyl groups appearing as singlets at δ1.22, 1.29, 1.36 and 1.43ppm, a methoxy group at δ 3.87ppm and 10 aromatic protons between 6.0 and 8.2ppm. Two signals at δ6.18 (d, 9.5Hz) and 6.23ppm (d, 9.8Hz) were typical for coumarins. This was not incompatible with the TLC observations as coumarins due to the lactone ring are known to give false-positive alkaloid reaction with Dragendorff's reagent (Abdel-Azim 1980).

The ^{13}C NMR spectrum (Figure 50) indicated 31 carbons including 20 aromatic carbons and 5 aliphatic carbons between 15 and 25ppm. The spectrum also disclosed 6 oxygen-bearing carbons at δ72.1, 62.8, 61.0, 59.2, 58.2 and 56.0ppm. Chemical shifts values of 62.8, 61.0, 59.2, 58.2 suggested two epoxide groups. The HMQC spectrum (Figure 51) indicated 13 quaternary carbons that showed no correlations.

On the COSY spectrum (Figure 49), the doublets at δ6.23 (oxypeucedanin, H-3) and 8.14ppm (oxypeucedanin, H-4) correlated to one another with a coupling constant of 9.8Hz. Similarly, the broad singlet at δ6.90ppm (oxypeucedanin, H-3') and the doublet at δ7.55ppm (oxypeucedanin, H-2') correlated to one another.

The smaller coupling constant of 2.12Hz was compatible with *ortho* protons on a furan ring which suggested a furanocoumarin skeleton.

The COSY spectrum also revealed correlations between the protons at δ6.17 (meranzin, H-3) and 7.58 ppm (meranzin, H-4) and between the protons at δ6.81 (meranzin, H-6) and 7.30 ppm (meranzin, H-5) suggesting a substituted coumarin skeleton.

Although TLC analysis and 1D NMR spectra initially suggested a single pure compound, closer study of the 2D NMR data allowed distinction of two different sets of signals and identification of **(SJ2)** as a 1:1 mixture of a prenylated simple coumarin and a prenylated furanocoumarin.

The HMBC (Figures 52 and 53) revealed ^{3}J correlations between two methyl groups at δ1.29 (oxypeucedanin, Me-4") and δ1.22ppm (oxypeucedanin, Me-5") which correlated to the carbons at δ24.4 (oxypeucedanin, C-5") and 18.9ppm (oxypeucedanin, C-4"), respectively. These geminal methyls also showed ^{2}J correlations with the carbon at δ58.2ppm (oxypeucedanin, C-3") and ^{3}J correlations with the carbon at δ61.0ppm (oxypeucedanin, C-2"). The proton at δ4.37ppm (oxypeucedanin, H-1"a) showed ^{2}J correlation with the carbon at δ61.0ppm (oxypeucedanin, C-2") and ^{3}J correlation with the carbon at δ58.2ppm (oxypeucedanin, C-3").

Similarly, the methyls at δ1.36 (meranzin Me-5') and 1.43ppm (meranzin Me-4') showed ^{3}J correlations to the carbons at δ18.9 (meranzin C-4') and 24.6ppm (meranzin C-5'), respectively. These geminal methyls also showed ^{2}J correlations with the carbon at δ59.2ppm (meranzin, C-3') and ^{3}J correlations with the carbon at δ62.8ppm (meranzin, C-2').

The protons at δ3.13 (meranzin, H-1'b) and 2.95ppm (meranzin, H-1'a) showed ^{3}J correlation to the carbon at δ59.2ppm (meranzin, C-3') and ^{2}J correlation to the carbon at δ62.8ppm (meranzin, C-2').

The positions of the prenylated side chains were established following ^{3}J correlations between the protons at δ4.37 (oxypeucedanin, H-1"a) and 4.57ppm (oxypeucedanin, H-1"b) and the carbon at δ148.18ppm (oxypeucedanin, C-5). The protons at δ2.95 (meranzin, H-1'a) and 3.13ppm (meranzin, H-1'b) showed ^{2}J correlations to the carbon at δ113.8ppm (meranzin, C-8) and ^{3}J correlations with two carbons at δ153.1 (meranzin, C-9) and 160.5ppm (meranzin, C-7).

The position of the methoxy group was established following the correlation of the protons at δ3.87ppm (meranzin, OMe) to the carbon at δ160.5ppm (meranzin, C-7).

Oxypeucedanin has previously been isolated from *Skimmia japonica* (Atkinson et al. 1974, Reisch and Achenbach 1989, Reisch and Achenbach 1991, Reisch and Achenbach 1992a). The NMR data reported here are in good agreement with previous reports (Bergendorff et al. 1997). Meranzin has previously been reported from the bark of *Skimmia japonica* (Reisch and Achenbach 1991). However, this is the first unambiguous report of all ^{1}H and ^{13}C NMR assignements for this compound.

Table 27. ^{1}H (400MHz) and ^{13}C (100MHz) NMR spectral data, COSY and HMBC correlations for **(SJ2)** in $CDCl_3$.

Position	^{13}C	^{1}H	COSY	HMBC
oxypeucedanin				
2	160.9*			
3	112.8*	6.23 (d, 9.8)	4	2, 10
4	138.9	8.14 (d, 9.8)	3, 8	2, 5, 9
5	148.2			
6	113.9			
7	157.8			
8	94.5	7.08 (br s)	4, 3'	6, 7, 9, 10
9	152.3			
10	107.3			
2'	145.2	7.55 (d, 2.12)	3'	6, 7
3'	104.4	6.90 (br s)	8, 3'	6, 7, 2'
1"a	72.1	4.37 (m)	1"b, 2"	5, 2", 3"
b		4.57 (dd, 11.0, 4.0)	1"a, 2"	5
2"	61.0	3.19 (m)	1"a, 1”b	
3"	58.2			
4"	18.9	1.29 (s)		2", 3", 5"
5"	24.4	1.22(s)		2", 3", 4"

* overlapping signals

Table 27 (continued). ^{1}H (400MHz) and ^{13}C (100MHz) NMR spectral data, COSY and HMBC correlations for **(SJ2)** in $CDCl_3$.

Position	^{13}C	^{1}H	COSY	HMBC
meranzin				
2	160.9*			
3	112.8*	6.17 (d, 9.5)	4	2, 10
4	143.7	7.58 (d, 9.4)	3	2, 5, 9
5	127.1	7.30 (d, 8.6)	6	4, 7, 9
6	107.1	6.81 (d, 8.7)	5	8, 10
7	160.5			
8	113.8			
9	153.1			
10	112.7*			
1'a	22.3	2.95* (m)	1'b, 2'	7, 8, 9, 2'
b		3.13 (t, 8.0)	1'a, 2'	7, 8, 9, 3'
2'	62.8	2.95* (m)	1'a, 1’b	
3'	59.2			
4'	18.9	1.43 (s)		2', 3', 5'
5'	24.6	1.36 (s)		2', 3', 4'
OMe	56.0	3.87 (s)		7

* overlapping signals

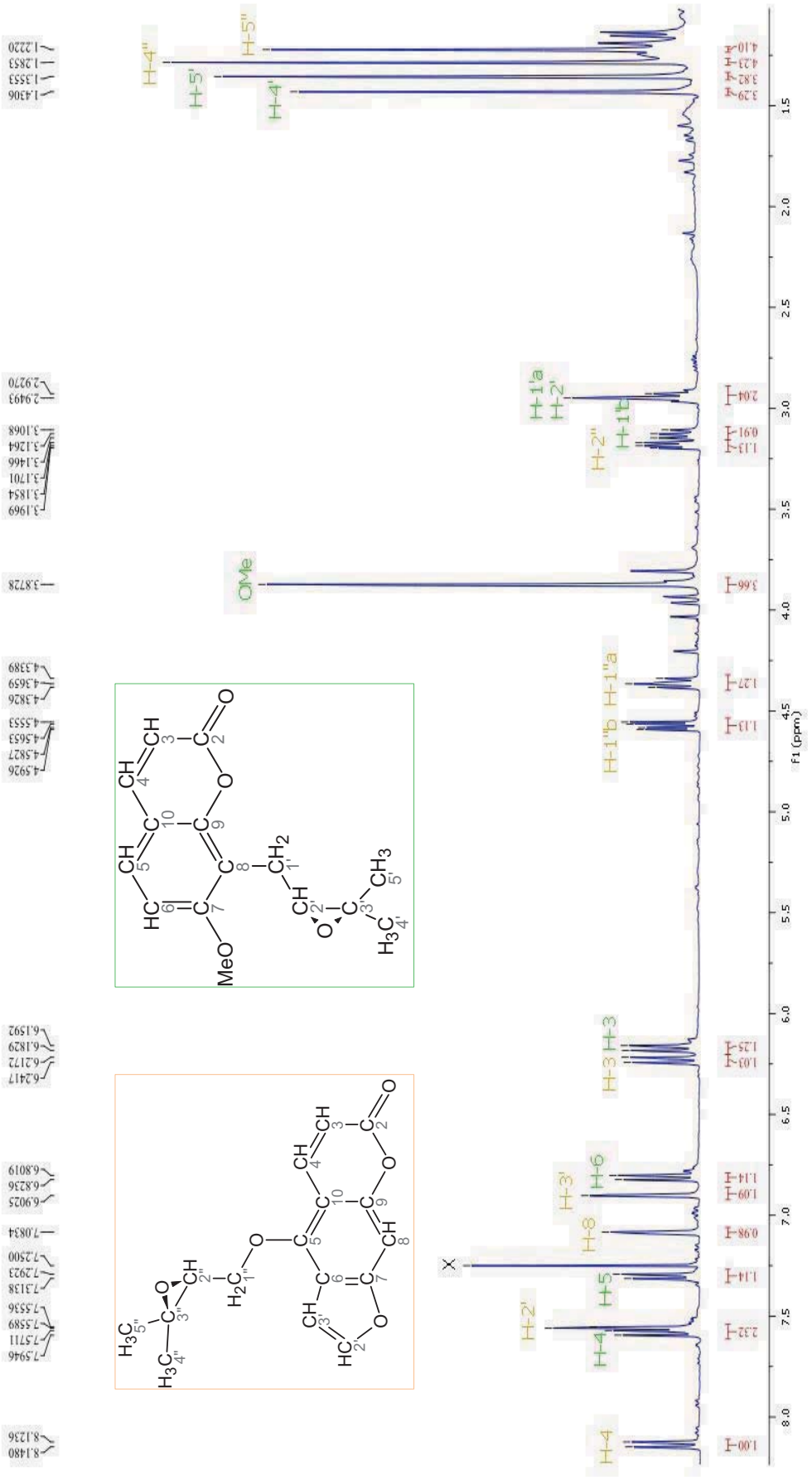


Figure 48. ^{1}H NMR spectrum of **(SJ2)**, 400MHz, $CDCl_3$ (X)

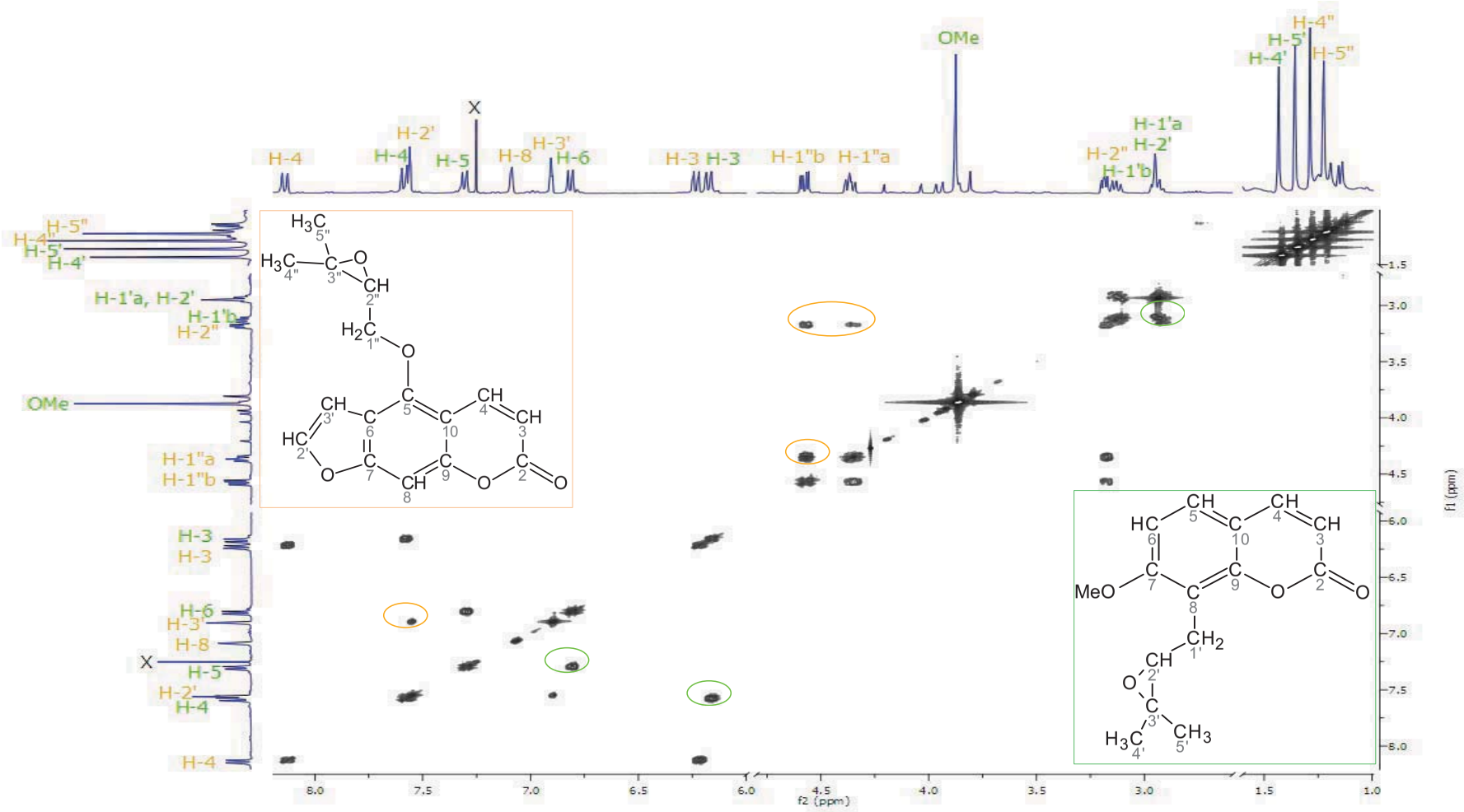


Figure 49. COSY NMR spectrum of **(SJ2)**, 400MHz, $CDCl_3$ (X)

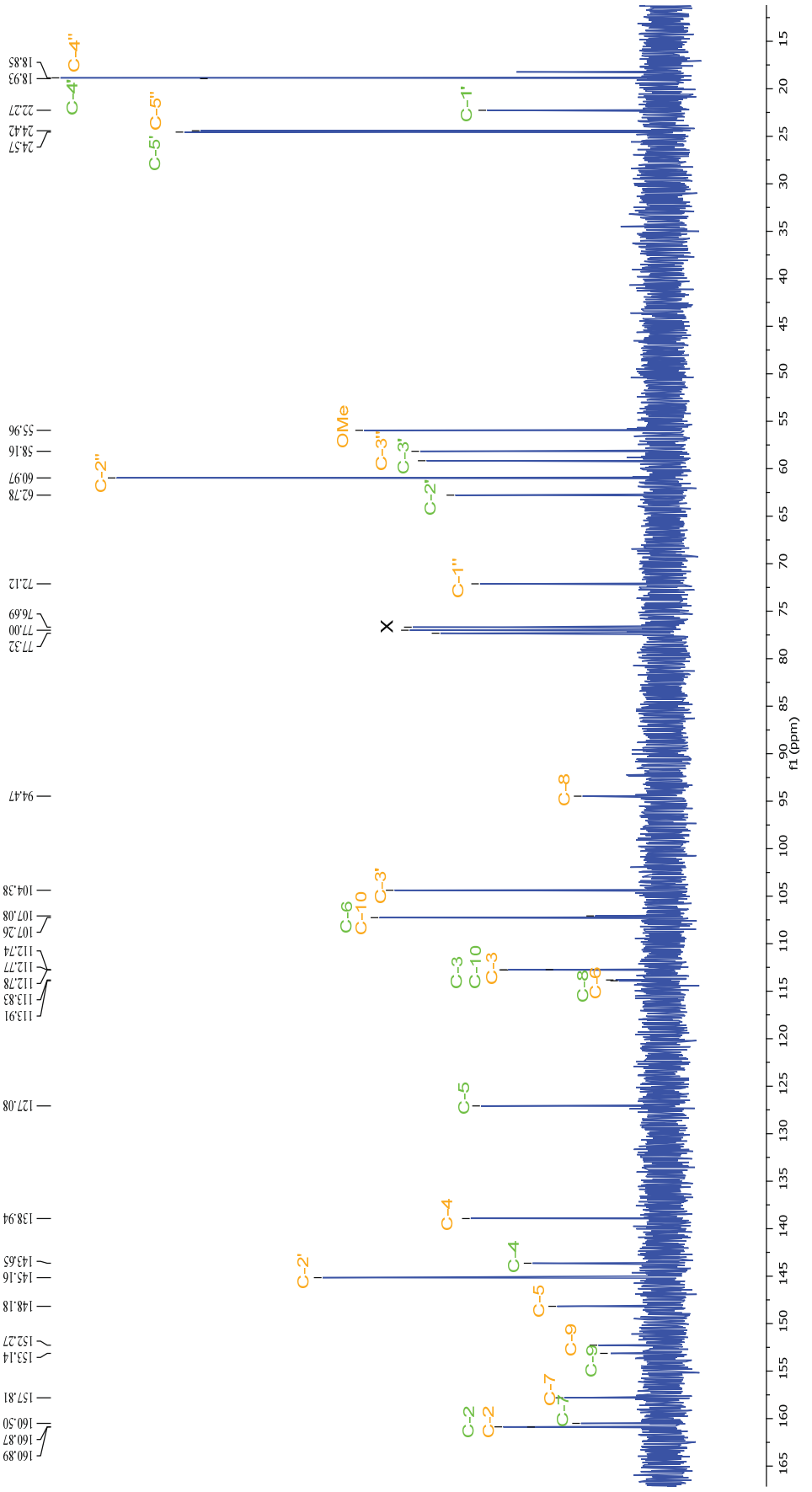


Figure 50. ^{13}C NMR spectrum of **(SJ2)**, 100MHz, $CDCl_3$ (X)

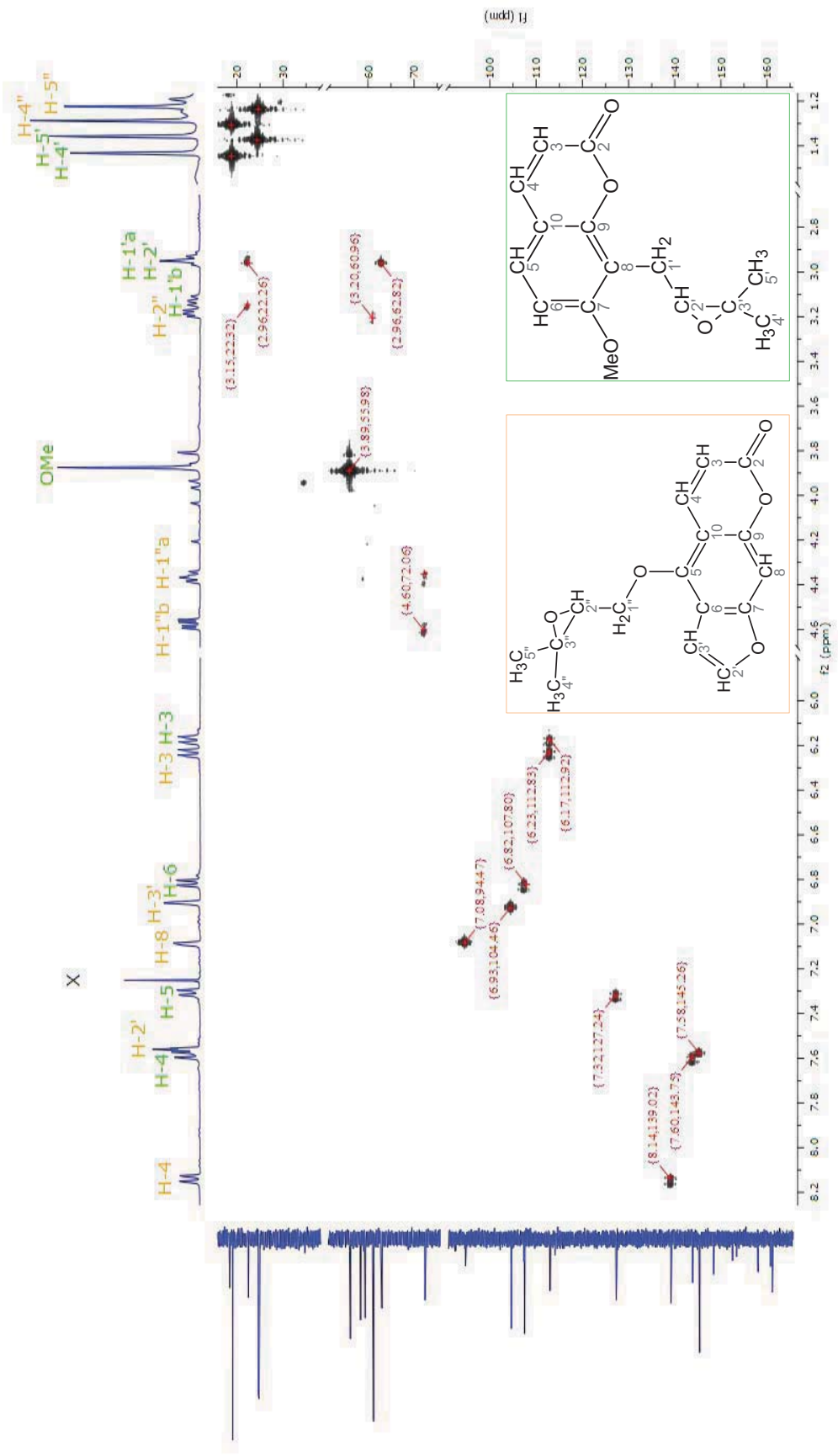


Figure 51. HMQC spectrum of **(SJ2)** (400MHz, $CDCl_3$)

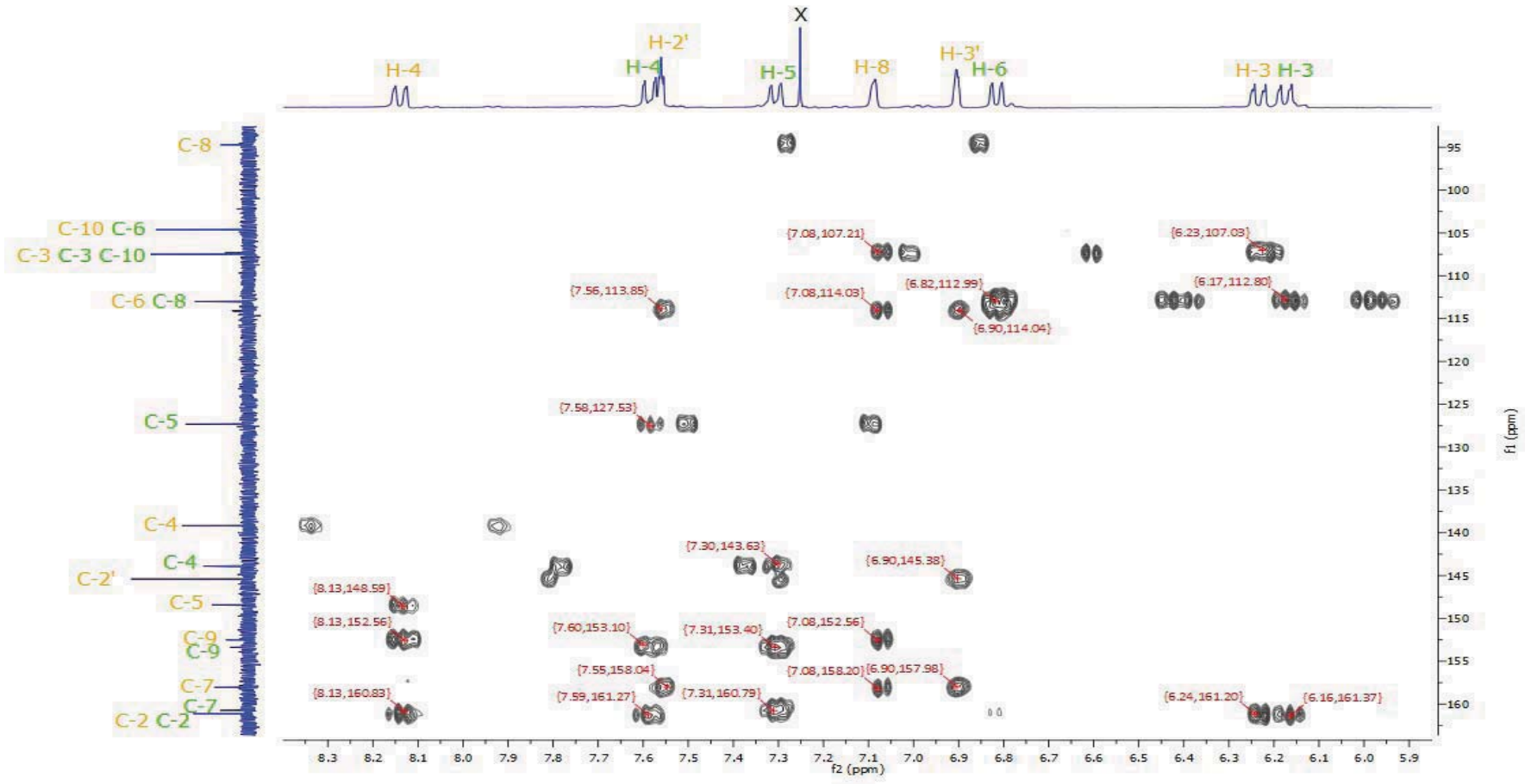
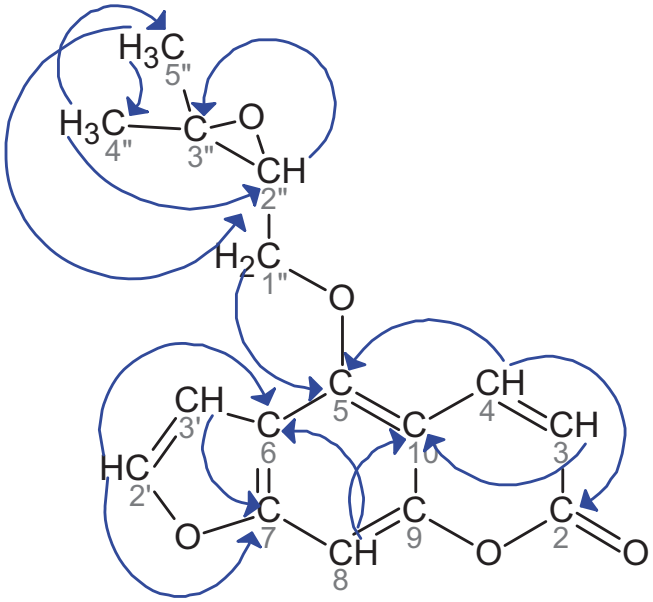
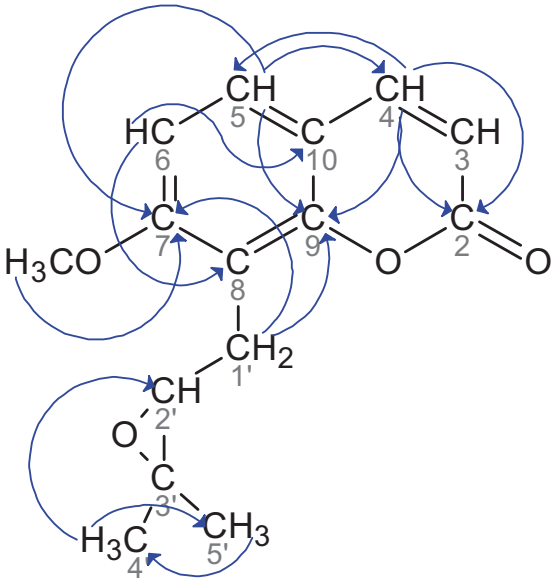


Figure 52. HMBC spectrum of **(SJ2)** (expansion of the the aromatic region), 400MHz, $CDCl_3$ (X)



Oxypeucedanin



Meranzin

Figure 53. Key HMBC correlations of **(SJ2)**

3.3.6 Characterisation of **(SJ3)** as bergapten

(SJ3) was obtained as white crystals from the *n*-hexane extract of *Skimmia japonica* aerial parts. On TLC, it showed a single spot under UV light (λ=254nm). After spraying with anisaldehyde-sulfuric acid reagent and heating, the spot turned brown.

The ^{1}H NMR spectrum (Figure 54) disclosed some features typical for a furanocoumarin as described previously. Only one methoxy group was present as a singlet at δ1.26ppm. Analysis of the proton spectrum suggested two possible structures; namely 5-methoxypsoralen or 8-methoxypsoralen. Comparison with previously reported data (Bergendorff et al. 1997) allowed to identify **(SJ3)** as 5-methoxypsoralen or bergapten. Bergapten has previsouly been reported from the bark and root of *Skimmia japonica* (Reisch and Achenbach 1989, Reisch and Achenbach 1991).

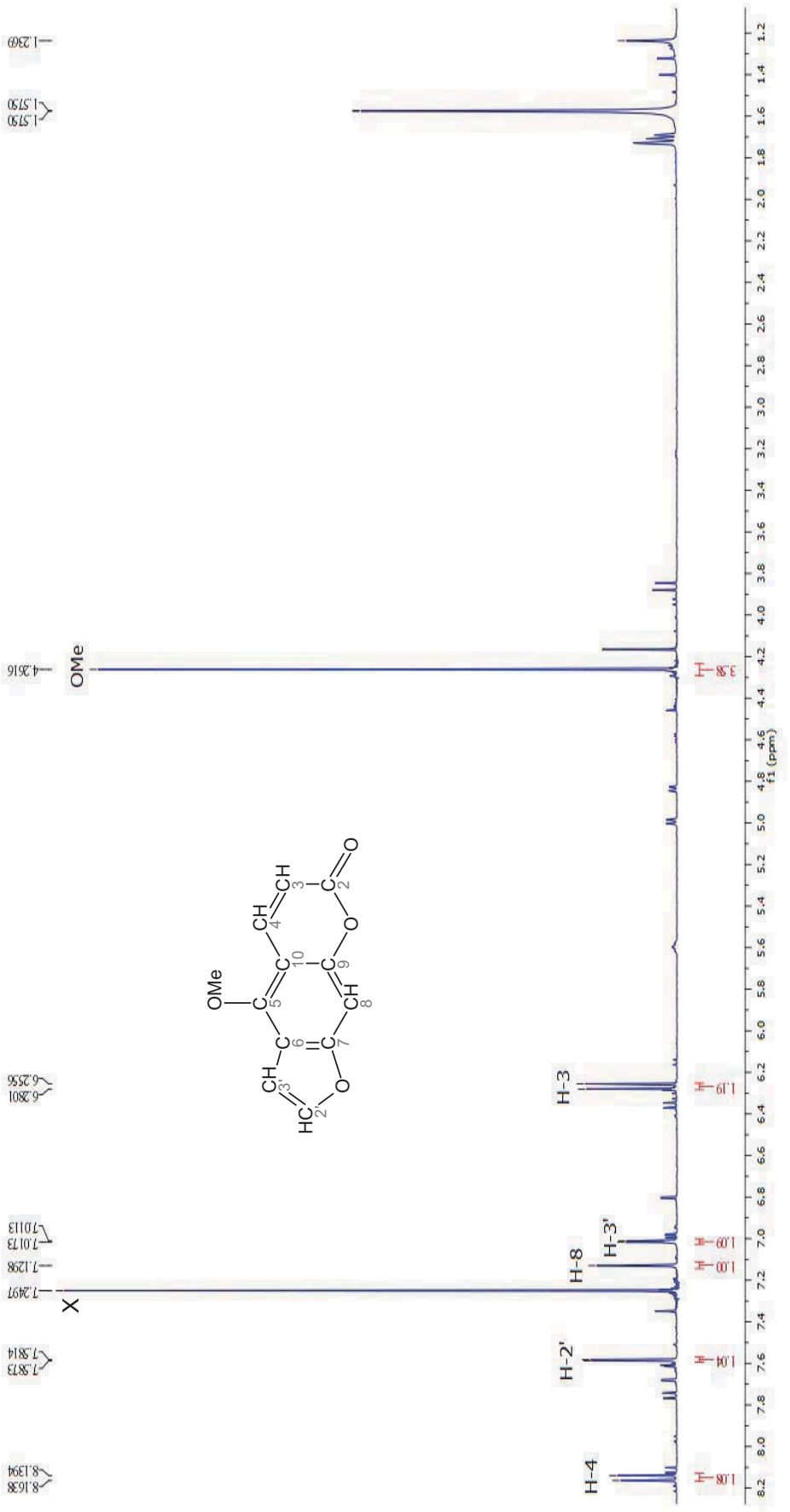


Figure 54. ^{1}H NMR spectrum of **(SJ3)**, 400MHz, $CDCl_3$ (X)

3.3.7 Characterisation of **(MG1)** as *n*-nonacosane

(MG1) was isolated from the *n*-hexane extract of *Myrica gale* stems as a white flaky solid. On, TLC, no spot was detected under short UV light (λ=254nm). Spraying with anisaldehyde-sulfuric acid reagent and heating revealed a brown spot.

The IR spectrum revealed peaks at 2955, 2917, 2848, 1473, 1463, 1376cm^{-1} typical for CH_2 and CH_3 bonding and vibrating and signals at 730 and 719cm-^{1} Typical for $(CH_2)_n$ rocking. EIMS disclosed a molecular ion $[M]^+$ at *m/z* 408.5 suggesting a molecular formula of $C_{29}H_{60}$. The fractionation pattern observed was typical for an *n*-alkane loosing successive CH_2 (*m/z* 14) fragments.

The ^{1}H NMR spectrum (Figure 55) also suggested an *n*-alkane. It only showed a sharp singlet at δ1.24ppm integrating for 54 protons and a triplet (J=6.9Hz) at δ0.87ppm integrating for 6 protons.

The ^{13}C and DEPT135 NMR spectra (Figure 56) disclosed five signals expected typical for an *n*-alkane with methyl groups at δ14.1ppm and methylenes at δ22.7, 29.4, 29.7 and 31.9ppm.

Based on these data, **(MG1)** was identified as *n*-nonacosane. All spectroscopic data showed good agreement with a previous report (Chen et al. 2008). This is the first report of the isolation of *n*-nonacosane from *Myrica gale*.

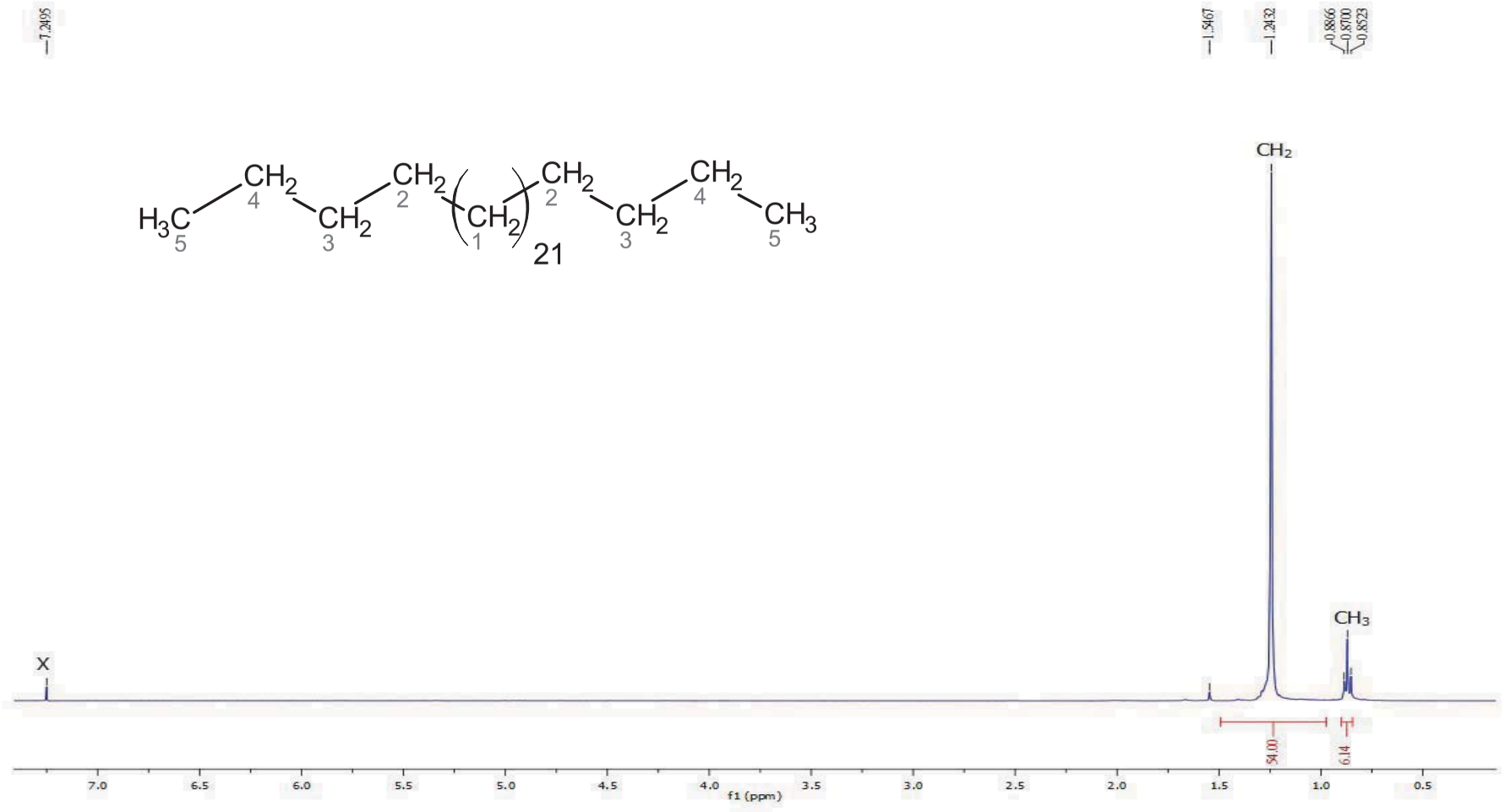


Figure 55. ^{1}H NMR spectrum of **(MG1)**, 400MHz, $CDCl_3$ (X).

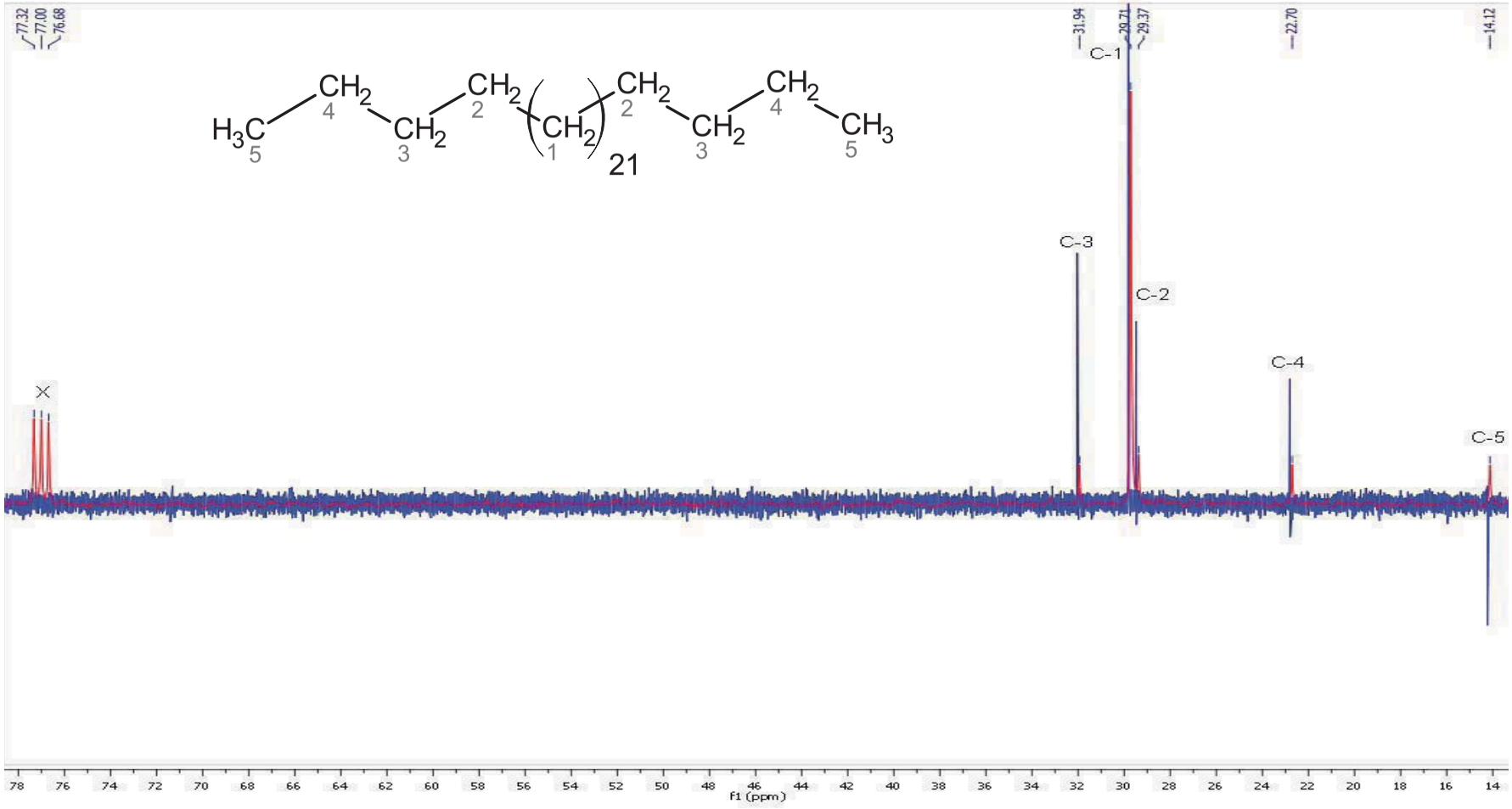


Figure 56. ^{13}C and DEPT 135 NMR spectra of **(MG1)**, 100MHz, $CDCl_3$ (X).

3.3.8 Characterisation of **(MG2)** as myrigalone B

(MG2) was isolated as a yellow amorphous solid from the *n*-hexane extract of *Myrica gale* stems. On TLC, it showed a single yellow spot, strongly absorbing under short UV light. After spraying with anisaldehyde-sulfuric acid reagent and heating, the spot took a bright orange colour.

CIMS disclosed a pseudo-molecular ion $[M+H]^+$ at *m/z* 301.4142 suggesting a molecular formula of $C_{18}H_{20}O_4$ (9 double bond equivalents).

On the ^1H NMR spectrum (Figure 57) a broad singlet at δ10.91ppm suggested an OH participating in intra-molecular hydrogen bonding. A multiplet at δ7.27ppm (4 protons) suggested a mono-substituted benzene ring. Other noticeable signals included a sharp singlet at 3.71ppm attributable to a methoxy group, two vicinal methylenes at δ3.48 and 2.99ppm, and a sharp singlet at 2.10ppm attributable to two equivalent methyl groups.

(MG2) was isolated in too little amount to obtain interpretable carbon and DEPT spectra. Carbon data were extracted from the HMQC and HMBC spectra (Figures 58 and 59).

On the HMBC spectrum, the methoxy protons at δ3.71ppm showed ^3J correlation to a carbon at δ162.6ppm. The methylenes protons at δ3.48ppm correlated with carbons at δ30.5 and 205.7ppm. The other methylenes protons at δ2.99ppm correlated to carbons at δ128.6 and 141.6pm. The methyls at δ2.10ppm correlated to three carbons at δ108.5, 158.2 and 162.6ppm.

These data indicated a dihydrochalcone with a fully substituted A-ring and an unsubstituted B-ring. The presence of two magnetically equivalent methyl groups appearing as a singlet at δ2.10ppm indicated symmetry in the subtituents position which could only be satisfied with the methoxy group at position 4', the hydroxyl groups at position 2', 6' and the methyl groups at position 3', 5'.

The above data and comparison with previous reports (Malterud et al. 1996, Mathiesen et al. 1997, Uyar et al. 1978) led to the characterisation of **(MG2)** as 2',6'-dihydroxy-4'-methoxy-3',5'-dimethyldihydrochalcone or myrigalone B.

Myrigalone B has previously been reported from the diethylether extract of *Myrica gale* fruits (Uyar et al. 1978). This is, however, the first report of its isolation from *Myrica gale* stems.

Table 28. ^{1}H (400MHz) and ^{13}C (100MHz) NMR spectral data and HMBC correlations for **(MG2)** in deuterated DMSO.

Position	^{13}C	^{1}H	HMBC
1	142.8	_	
2	129.3	7.27(m)	
3	129.3	7.27(m)	
4	126.5	7.18(m)	
5	129.3	7.27(m)	
6	129.3	7.27(m)	
1’	ND	_	
2’	159.7	_	
3’	109.8	_	
4’	164.0	_	
5’	109.8	_	
6’	159.7	_	
α	47.2	3.48(dd, 7.08, 8.49)	β, C=O
β	31.3	2.99(dd, 7.12, 8.44)	α, 1, 2, 6
β’	207.1	_	
4’-OMe	60.4	3.71(s)	4’
3’-Me	8.7	2.10(s)	2’, 4’, 6’
5’-Me	8.7	2.10(s)	2’, 4’, 6’
2’-OH	_	10.9(s)	

ND: not determined

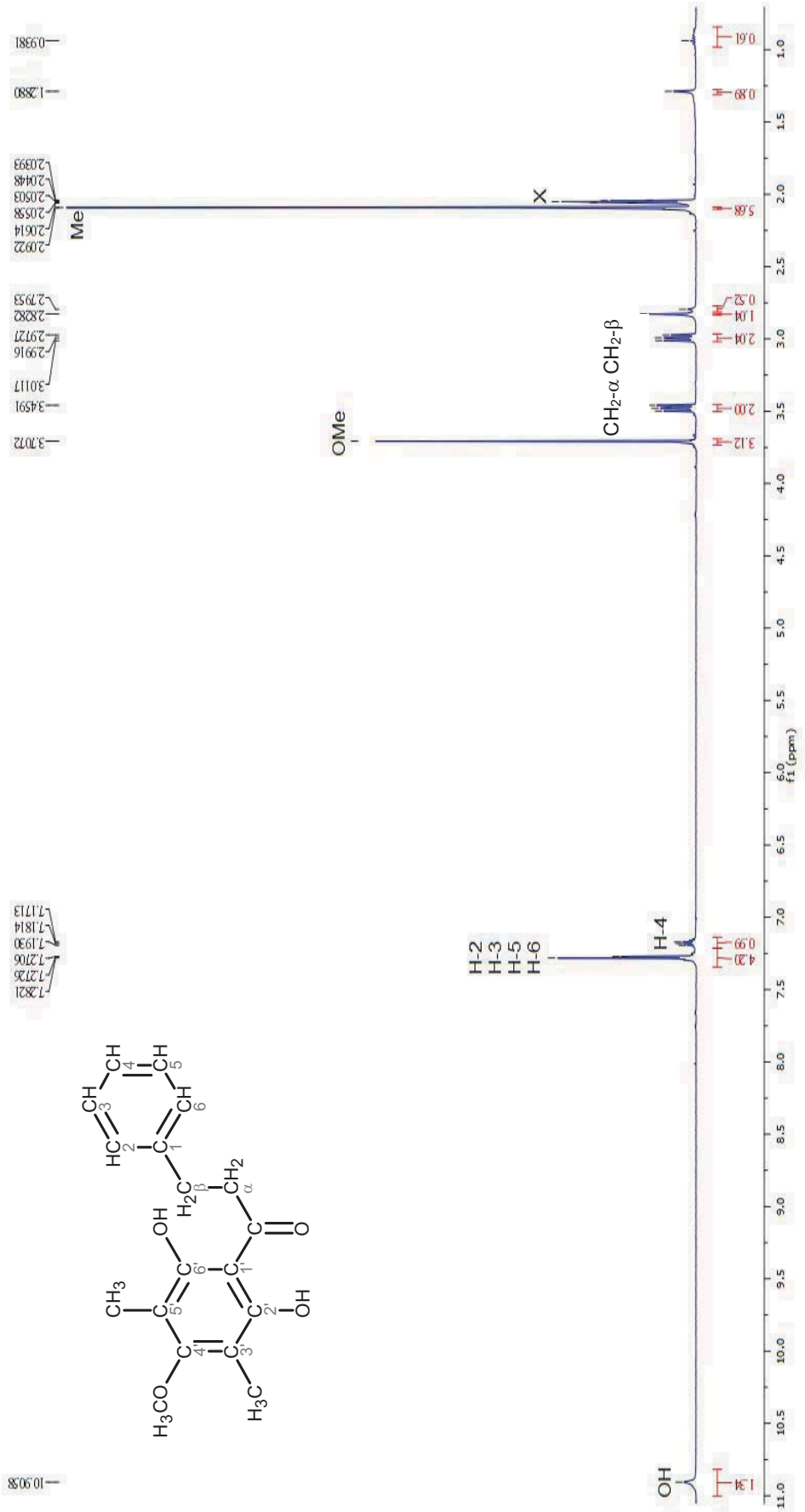


Figure 57. ^{1}H NMR spectrum of **(MG2)**, 400MHz, acetone-d_6.

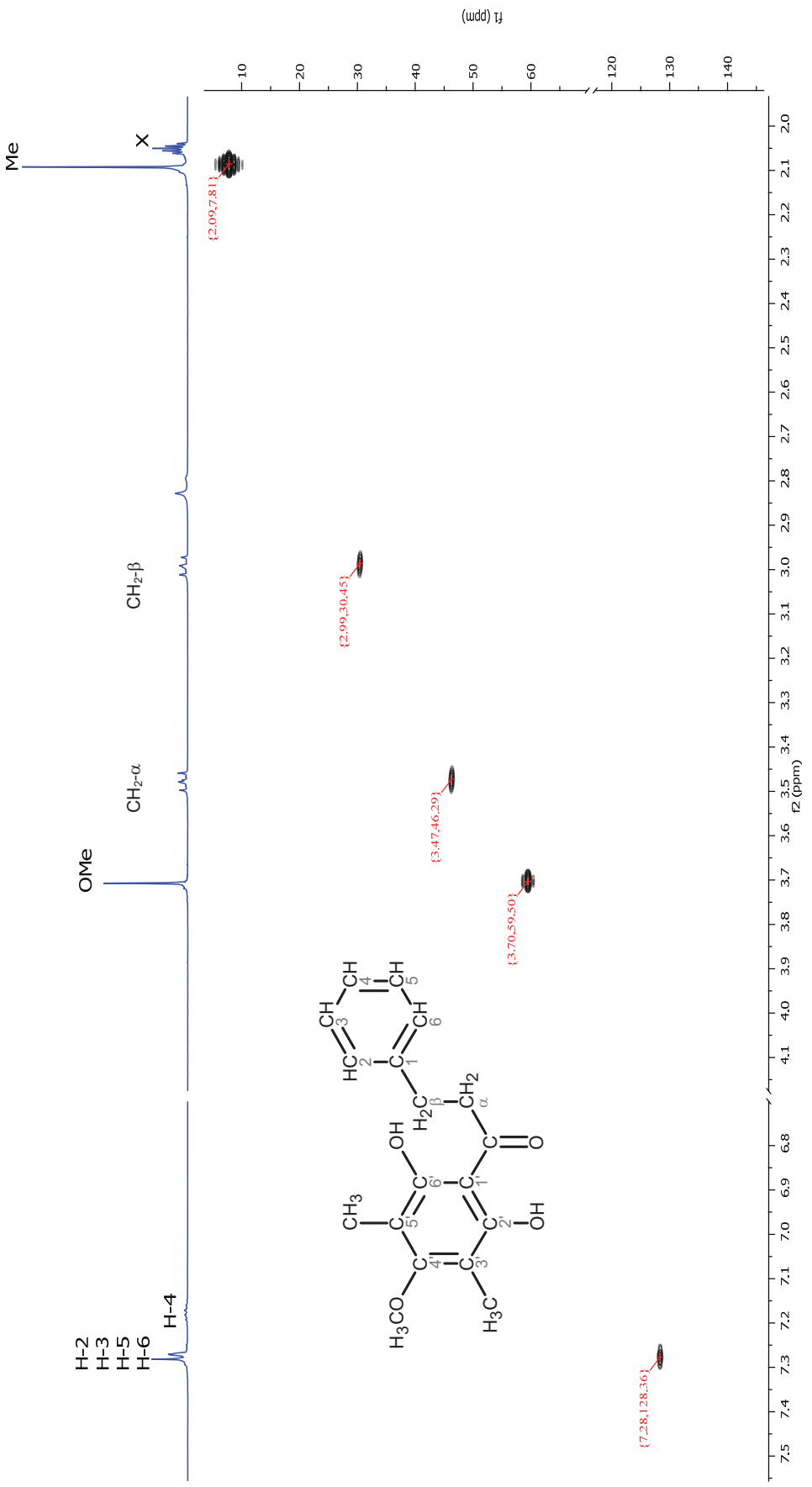


Figure 58. HMQC spectrum of **(MG2)**, 400MHz, acetone-d_6.

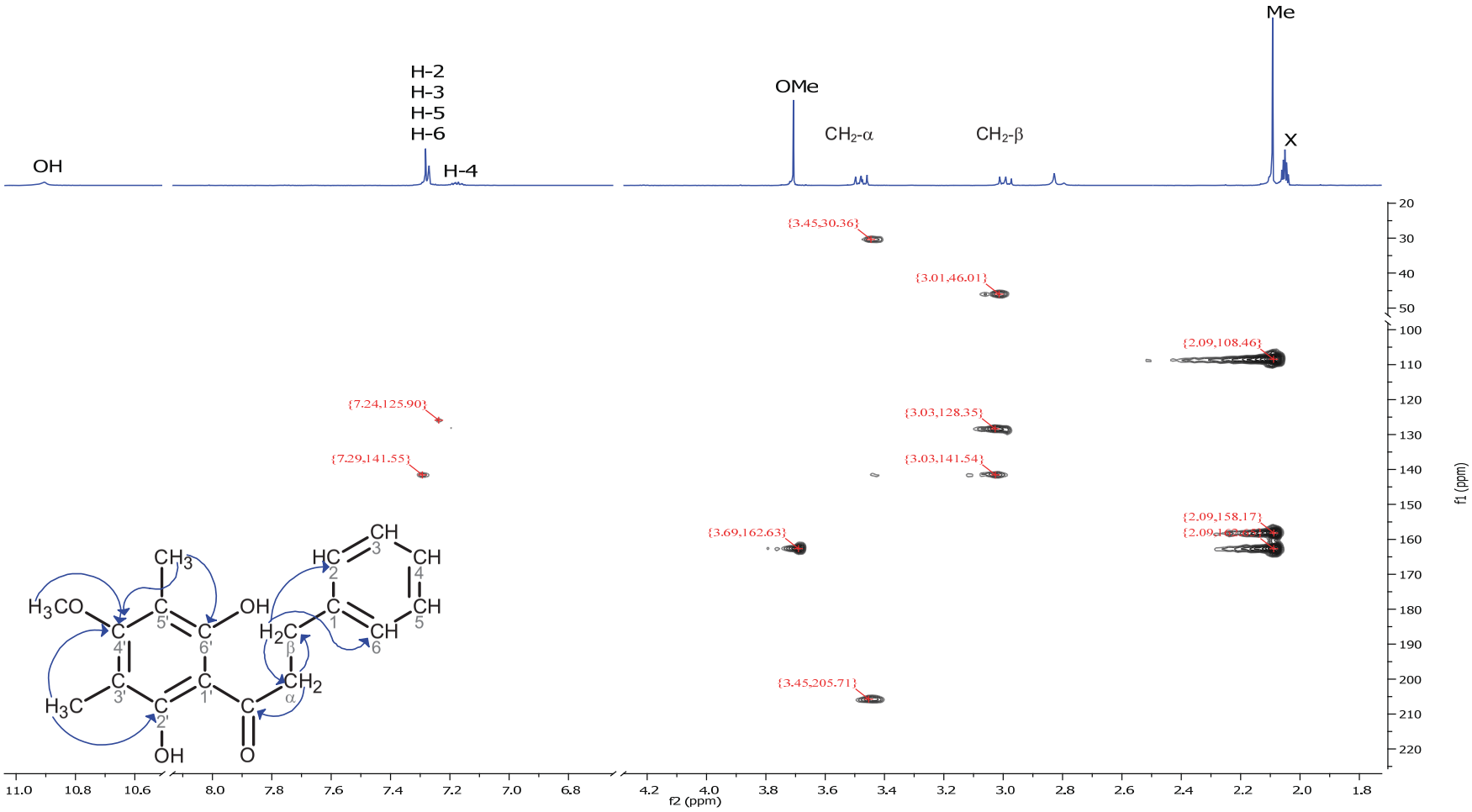


Figure 59. HMBC spectrum of **(MG2)** (400MHz, acetone-d_6) and selected HMBC correlations.

3.3.9 Physicochemical properties and spectroscopic data of isolated compounds

***(SJ1)**:* colourless crystals; **IR** ν_{max} (KBr) cm^{-1}: 3482 (O-H bonding), 3053 (=C-H bonding), 2998C-H_3 bonding) , 2934 (C-H_2 bonding), 2852 (C-H bonding), 1465 (C=C bonding), 1444 (C-H_3 vibrating), 1385 (C-H_3 vibrating), 1038 (C-O bonding), 1000 (C-H_2 vibrating); **HREIMS**: $[M]^+$ *m/z* 426.3859 (calcd. for $C_{30}H_{50}O$ 426.3862); **EIMS** (positive mode) *m/z* (%): 204.2 (11), 79.0 (100), 52.0 (27); **^{1}H NMR** ($CDCl_3$, 400MHz) (δ):3.18 (m, H-3), 0.76 (dd, H-5), 2.02 (dt, 12.7; 3.2, H-7β), 1.42 (H-9), 5.52 (dd, 8.2, 3.2, H-15), 1.90 (dd, 14.7, 3.0, H-16β), 0.92 (H-18), 0,97 (s, Me-23), 0,79 (s, Me-24), 0,91 (s, Me-25), 1,08 (s, Me-26), 0,89 (s, Me-27), 0,81 (s, Me-28), 0,94 (s, Me-29), 0,89 (s, Me-30); **^{13}C NMR** ($CDCl_3$, 100MHz): Table 26

***(SJ2)**: Y*ellow oil; **^{1}H NMR** ($CDCl_3$, 400MHz) (δ): Table 27; **^{13}C NMR** ($CDCl_3$, 100MHz): Table 27.

***(SJ3)**:* White crystals; **^{1}H NMR** ($CDCl_3$, 400MHz) (δ): 6.27 (d, 9.7, H-3), 8.15 (d, 10.1, H-4), 7.13 (br s, H-8), 7.58 (d, 2.2, H-2'), 7.01 (dd, 2.6, 0.9, H-3'), 4.26 (s, OMe)

***(JC1)**:* pale yellow oil; **IR** ν_{max} (KBr) cm^{-1}: 3064 (=C-H_2 bonding), 2953 (C-H_3 bonding), 2867 (C-H_2 bonding), 1656 (C=C bonding), 1456 (C-H_3 vibrating), 1375 (C-H_3 vibrating), 1361 (C-H_2 vibrating), 1295 (C-C bonding), 873 (C=C-H vibrating), 820 (C=C-H vibrating); **^{1}H NMR** ($CDCl_3$, 600MHz): Table 22. **^{13}C NMR** ($CDCl_3$, 150MHz): Table 22.

***(JC2)**:* White crystals; **$[\alpha]_D$** +70.4° (*c* 1.0; MeOH, 21°C); **IR** ν_{max} (KBr) cm^{-1}: 3462 (O-H bonding), 2940 (C-H_3 bonding), 2865 (C-H_2 bonding), 1715 (C=O bonding), 1586 (C=C bonding), 1269 (C-C bonding), 906 (C=C-H vibrating), 803 (C=C-H

vibrating); **UV** λ_{max} (EtOH) nm: 225.5 (sh), 280; **CIMS** (positive mode) *m/z* (%): 286. 4004 (100) $[M]^+$ (calcd. for $C_{20}H_{30}O$ 286.2296) 271.4 (50) 191.3 (19); **^{1}H NMR** ($CDCl_3$, 400MHz): Table 23. **^{13}C NMR** ($CDCl_3$, 100MHz): Table 23.

(JC3)*:* White amorphous solid; **$[\alpha]_D$** +92.5° (*c* 1.0; MeOH, 21°C); **IR** ν_{max} (KBr) cm^{-1}: 3413 (O-H bonding), 3082 (=C-H bonding), 2935 (C-H_3 bonding), 2872 (C-H_2 bonding), 2848 (C-H bonding), 1696 (C=O bonding), 1466, 1458.0 (C-H_3 vibrating), 1448 (C=C bonding), 1387 (C-H_3 vibrating), 1268 (C-C bonding), 1181 (C-O bonding), 905 (C=C-H vibrating), 887 (C=C-H vibrating); **UV** λ_{max} (EtOH) nm: 231.5; **HRCIMS**: $[M+H]^+$ *m/z* 303.2322 (calcd. for $C_{20}H_{31}O_2$ 303.2324) **CIMS** (positive mode) *m/z* (%): 303.4 (87), 85.2 (74), 69.1 (100); **^{1}H NMR** ($CDCl_3$, 400MHz): Table 24; **^{13}C NMR** ($CDCl_3$, 100MHz): Table 24.

(CV1)*:* White crystals; **$[\alpha]_D$** +76.0° (*c* 1.0; MeOH, 21°C); **IR** ν_{max} (KBr) cm^{-1}: 3422 (O-H bonding), 2941 (C-H_3 bonding), 2871 (C-H_2 bonding), 1690 (C=O bonding), 1459 (C-H_3 bonding), 1030 (C-C bonding); **HREIMS**: $[M]^+$ *m/z* 456.3602 (calcd. for $C_{30}H_{48}O_3$ 456.3603) **EIMS** (positive mode) *m/z* (%): 248.2 (100), 203.2 (47); **^{1}H NMR** (C_5D_5N, 400MHz): Table 25; **^{13}C NMR** (C_5D_5N, 100MHz): Table 26.

(CV2)*:* **EIMS** (positive mode) $[M]^+$ *m/z* 428.4 (40), 275.3 (48), 165.2 (74), 109.1 (82), 95.1 (100), 69.1 (82); **^{1}H NMR** ($CDCl_3$, 400MHz) (δ): 1.88 (dm, 9.6, H-2), 3.72 (br s, H-3), 1.25 (H-4), 1.72 (dt, 6.2, 2.9, H-6), 1.28 (H-8), 0.86 (H-10), 1.52 (H-18), 0.92 (d, 6.7, Me-23), 0.95 (s, Me-24), 0.84 (s, Me-25), 0.98 (s, Me-26), 0.99 (s, Me-27), 1.16 (s, Me-28), 0.93 (s, Me-29), 0.98 (s, Me-30); **^{13}C NMR** ($CDCl_3$, 100MHz): Table 26.

(MG1): White flaky solid; **IR** ν_{max} (KBr) cm^{-1}: 2955 (C-H_3 bonding), 2917 (C-H_2 bonding), 2848 (C-H_2 bonding), 1473 (C-H_2 vibrating), 1463 (C-H_3 vibrating), 1376 (C-H_3 vibrating), 730 ($(CH_2)_n$ rocking), 719 ($(CH_2)_n$ rocking); **EIMS** (positive mode) *m/z* (%): $[M]^+$ 408.5 (25), $[M\text{-}nCH_2]^+$ 365.4 to 155.2 (<10), 141.2 (12), 127.1 (15), 113.1 (19), 99.1 (26), 85.0 (64), 78.0 (100), 71.0 (77), 63.0 (96), 57.0 (74), 43.0 (22); **^{1}H NMR** ($CDCl_3$, 400MHz) (δ):1.24 (s, CH_3), 0.87 (t, 6.9, CH_2); **^{13}C NMR** ($CDCl_3$, 100MHz); 29.7 (C-1), 29.4 (C-2), 31.9 (C-3), 22.7 (C-4), 14.1 (C-5).

(MG2): yellow amorphous solid; **UV** λ_{max} (EtOH) nm: 206.0, 280.5, 350.0; **CIMS** (positive mode) *m/z* (%): $[M+H]^+$ 301.4142 (100), **^{1}H NMR** (acetone-d_6, 400MHz): Table 28; **^{13}C NMR** (acetone-d_6, 100MHz); Table 28.

(MG3): White flaky solid; **IR** ν_{max} (KBr) cm^{-1}: 3369 (OH), 2932 (C-H_3 bonding), 1472 (C-H_2 vibrating), 1386 (C-H_3 vibrating), 1080 (C-O bonding, secondary alcohol), 1028 (C-O bonding; primary alcohol), 1002 (C-C bonding); **Accurate FAB MS**: $[M+H]^+$ *m/z* 443.3885 (calcd. for $C_{30}H_{51}O_2$ 443.3889) **FAB MS** (positive mode) *m/z* (%): 443 (3), 232 (16), 154 (39), 136 (37), 79.0 (100); **^{1}H NMR** (C_5D_5N, 400MHz): Table 25; **^{13}C NMR** (C_5D_5N, 100MHz): Table 26.

3.4 Antimycobacterial activity of isolated compounds

Results of the evaluation of the activity of some of the isolated compounds against drug-susceptible, drug-resistant and non-replicating *Mycobacterium tuberculosis* are presented in Tables 29 to 31. Results of activity against non-tuberculous mycobacteria are presented in Table 32.

Epifriedelanol **(CV2)** was obtained in too little amount to be tested for antimycobacterial activity.

The isolated bergapten **(SJ3)** and the meranzin-oxypeucedanin mixture **(SJ2)** were not screened but results are reported for commercial samples of bergapten and oxypeucedanin.

Juniperus communis (roots and bark) is a plant reputed to be effective in the treatment of tuberculosis (Atkinson 2003, McCutcheon et al. 1997, UK CropNet Medicinal Plants of Native America Database 2009) and several studies have reported the activity of extracts, particularly against *Mycobacterium tuberculosis* (including some drug-resistant strains), *Mycobacterium avium*, *Mycobacterium aurum* and *Mycobacterium smegmatis* (Grange and Davey 1990, Jimenez-Arellanes et al. 2003, McCutcheon et al. 1997, Newton et al. 2002). Whilst several terpenoids have been identified as responsible for the antimycobacterial activity of Juniperus procera (another species traditionally used as an anti-TB remedy) (Mossa et al. 2004, Muhammad et al. 1995), the antimycobacterial activity of common juniper has never been attributed to any pure active substance(s). In this study, *trans*-communic acid **(JC3)** was isolated as the compound responsible for the antimycobacterial activity observed for the aerial part extract. It has previously been isolated from *Juniperus communis* berries (Arya et al. 1961) and is known to possess antibacterial activity (Muhammad et al. 1995, Smith et al. 2007).

This is here the first report of the isolation of *trans*-communic acid from the aerial parts (leaves and stems) of *Juniperus communis* and of its very good activity against *M. aurum* with an MIC of 4µg/mL (13.2µM). Communic acid was not active against *M. tuberculosis* H_{37}Rv and was neither tested against drug-resistant MTB nor other mycobacteria due to degradation. Although it was dried and stored at -20°C, the sample, initially white and amorphous, turned to a light yellow gum. A ^{1}H NMR experiment confirmed a modification of the structure.

The antimycobacterial activity of the *n*-hexane extract of *Juniperus communis* roots was attributed to longifolene **(JC1)** and totarol **(JC2)**.

Longifolene **(JC1)** showed weak activity against *M. tuberculosis* (MIC 452.1µM) and *M.aurum* (MIC 626.4µM) and no activity against other mycobacteria. Longifolene showed the second best activity against rifampicin-resistant MTB with (MIC of 24µM) and the best selectivity index (10.85). This is the first report of the antimycobacterial activity of longifolene.

Totarol **(JC2)** showed good activity against all tested strains of *Mycobacterium tuberculosis* and very good activity against all non-tuberculous mycobacteria. However it also showed poor selectivity compared to the antibiotic control with values ranging from 0.3 against streptomycin-resistant MTB to only 3.8 against *M. smegmatis*. The activity of totarol against *M. tuberculosi*s H_{37}Rv and *M. smegmatis* has previously been reported (Constantine et al. 2001, Mossa et al. 2004, Muhammad et al. 1995). This is the first report of the activity of totarol against *M. aurum*, *M. fortuitum* or *M. phlei*.

The activity of the ethyl acetate extract of *Calluna vulgaris* aerial parts and *Myrica gale* stems was attributed to oleanolic acid **(CV1)**. Oleanolic acid is a known antitubercular agent whose activity against drug-susceptible, drug-resistant and multidrug-resistant clinical isolates of *M. tuberculosis* has

previously been reported (Jimenez-Arellanes et al. 2006, Jimenez-Arellanes et al. 2003).

MyrigaloneB **(MG2)** isolated from the *n*-hexane extract of *Myrica gale* roots was only tested against *Mycobacterium tuberculosis*. It showed no activity against drug susceptible and non-replicating strains but showed good activity against the rifampicin and isoniazid drug resistant strains (MIC 35 and 34µM, respectively) but low selectivity (1.7). This is the first report of the antimycobacterial activity of myrigalone B.

Determination of MICs for a commercial sample of bergapten against drug-susceptible, drug resistant and non-replicating strains of *Mycobacterium tuberculosis* revealed bergapten is only a weak antitubercular agent. Oxypeucedanin tested against the same strains proved to be a better antitubercular agent especially against rimfanpicin-resistant *Mycobacterium tuberculosis* MIC (55.2µM) but showed low selectivity (2.17).

Taraxerol **(SJ1)**, *n*-nonacosane **(MG1)** and myricadiol **(MG3)** were inactive against all mycobacteria tested. Nevertheless the antimycobacterial screening of these compounds is reported here for the first time.

Table 29. Activity against *Mycobacterium tuberculosis*, cytotoxicity and selectivity indices for selected isolated compounds

Compound	Activity against *M. tuberculosis* $H_{37}Rv$ MIC in µg/mL(µM)	Cytotoxicity against Vero cells IC_{50} in µg/mL(µM)	Selectivity index IC_{50}/MIC
Myrigalone B **(MG2)**	>100 (>332.9)	17.33 (57.7)	<0.17
Longifolene **(JC1)**	92.4 (452.1)	53.21 (260.3)	0.58
Totarol **(JC2)**	21.1 (73.6)	7.51 (26.21)	0.36
Communic acid **(JC3)**	>100 (>330.6)	40.01 (132.3)	<0.40
Oxypeucedanin	47.0 (164.2)	34.25 (119.6)	0.72
Bergapten	>100 (462.5)	>100 (>462.5)	ND
Oleanolic acid **(CV1)**	47.1 (103.1)	>100 (>219.0)	>2.12
Rifampicin (RIF)	0.05 (0.06)	101.2 (123.0)	2049
Isoniazid (INH)	0.06 (0.41)	ND	ND
Moxifloxacin (MOX)	0.28 (0.63)	ND	ND
PA-824	0.14 (0.40)	ND	ND

ND: not determined

Table 30. MICs in µg/mL (µM) and selectivity indices (SI) of selected isolated compounds against single-drug resistant strains of *Mycobacterium tuberculosis*

Compounds	Rifampicin-resistant		Isoniazid-resistant		Streptomycin-resistant		Moxifloxacin-resistant	
	MIC	SI	MIC	SI	MIC	SI	MIC	SI
Myrigalone B **(MG2)**	10.5 (35.0)	1.65	10.2 (34.0)	1.70	>100 (>332.9)	<0.17	69.3 (230.7)	0.25
Longifolene **(JC1)**	4.9 (24.0)	10.85	22.7 (111.1)	2.34	93.8 (458.9)	0.57	76.6 (374.8)	0.69
Totarol **(JC2)**	5.8 (20.2)	1.30	11.0 (38.4)	0.68	23.9 (83.4)	0.31	17.2 (60.0)	0.44
Oxypeucedanin	15.8 (55.2)	2.17	42.3 (147.7)	0.81	23.6 (82.4)	1.45	40.8 (142.5)	0.84
Bergapten	>100 (462.5)	ND	86.9 (401.9)	>1.15	>100 (>462.5)	ND	>100 (>462.5)	ND
Oleanolic acid **(CV1)**	21.4 (46.9)	>4.67	23.3 (51.0)	>4.29	40.4 (88.5)	>2.47	23.9 (52.3)	>4.19
Rifampicin (RIF)	>3.29 (>4)	<31	0.06 (0.07)	1759	0.02 (0.03)	4098	0.12 (0.15)	820
Isoniazid (INH)	0.07 (0.48)	ND	>1.10 (>8)	ND	0.07 (0.48)	ND	0.11 (0.83)	ND
Moxifloxacin (MOX)	0.11 (0.24)	ND	0.14 (0.31)	ND	0.18 (0.42)	ND	>7.01 (>16)	ND
PA-824	0.04 (0.10)	ND	0.17 (0.48)	ND	0.04 (0.12)	ND	0.11 (0.30)	ND

ND: not determined

Table 31 Activity against non-replicating *Mycobacterium tuberculosis* and selectivity indices for selected isolated compounds

Compound	Activity against non-replicating *M. tuberculosis* MIC in µg/mL(µM)	Selectivity index IC_{50}/MIC
Myrigalone B **(MG2)**	>100 (>332.9)	<0.17
Longifolene **(JC1)**	93.4 (456.9)	0.57
Totarol **(JC2)**	23.3 (81.3)	0.32
Oxypeucedanin	>100 (>349.3)	<0.34
Bergapten	>100 (>462.5)	ND
Oleanolic acid	48.1 (105.3)	>2.08
Rifampicin (RIF)	1.07 (1.34)	92
Isoniazid (INH)	>18.67 (>128)	ND
Moxifloxacin (MOX)	>54.86 (>128)	ND
PA-824	0.09 (2.64)	ND

ND: not determined

Table 32. MICs in µg/mL (µM) and selectivity indices (SI) of selected isolated compounds against non-tuberculous mycobacteria

Compounds	*M. aurum*		*M. fortuitum*		*M. phlei*		*M. smegmatis*	
	MIC	SI	MIC	SI	MIC	SI	MIC	SI
Longifolene **(JC1)**	128 (626.4)	0.4	>128 (>626.4)	<0.4	>128 (>626.4)	<0.4	>128 (>626.4)	<0.4
Totarol **(JC2)**	2 (7.0)	3.8	4 (14.0)	1.9	4 (14.0)	1.9	2 (7.0)	3.8
Communic acid **(JC3)**	4 (13.2)	10.0	ND	ND	ND	ND	ND	ND
Taraxerol **(SJ1)**	>256 (>600.4)	ND	>256 (>600.4)	ND	>256 (>600.4)	ND	>256 (>600.4)	ND
Oleanolic acid **(CV1)**	64 (140.2)	>1.6	64 (140.2)	>1.6	256 (561.0)	>0.4	64 (140.2)	>1.6
n-nonacosane **(MG1)**	>256 (>626.7)	ND	>256 (>626.7)	ND	>256 (>626.7)	ND	>256 (>626.7)	ND
Myricadiol **(MG3)**	>256 (>577.4)	ND	>256 (>577.4)	ND	>256 (>577.4)	ND	>256 (>577.4)	ND
Rifampicin (RIF)	1 (1.2)	123.0	ND	ND	ND	ND	ND	ND
Isoniazid (INH)	0.125 (0.9)	ND	ND	ND	ND	ND	ND	ND
Ciprofloxacin (CIP)	ND	ND	1 (3.0)	ND	1 (3.0)	ND	ND	ND
Doxicyclin (DOX)	ND	ND	ND	ND	1 (2.3)	ND	ND	ND

ND: not determined

3.5 Antibacterial activity isolated compounds

The results of the evaluation of the activity of some of the isolated compounds against a panel of Gram-positive and Gram-negative bacteria are reported in Table 33.

Oleanolic acid **(CV1)** showed broad antibacterial activity. It has previsouly been reported to be active against *B. cereus* and *S. aureus* (Kamatou et al. 2007). This is the first report of its effect against *Enterococcus faecalis*, *Staphylococcus epidermidis* and *Streptococcus pyogenes*.

Taraxerol **(SJ1)**, *n*-nonacosane **(MG1)** and myricadiol **(MG3)** were inactive against all bacteria used. The bacterial screening of these compounds is nevertheless reported here for the first time.

Totarol **(JC2)** disclosed very good activity against *Bacillus cereus, Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis* and *Streptococcus pyogenes*. The activity of totarol against *S.aureus* has previously been reported (Kubo et al. 1992, Muhammad et al. 1995, Nicolson et al. 1999). Its effect against *Bacillus cereus, Enterococcus faecalis, Staphylococcus epidermidis* and *Streptococcus pyogenes* is reported here for the first time. Amongst the natural products screened during the course of this work, the diterpene totarol showed the best antimicrobial activity. Previous studies have indicated that totarol can inhibit cell proliferation by binding to FtsZ, a protein playing a major role in bacterial cell division and a novel target for antimicrobial drug development (Janin 2007, Jaiswal et al. 2007). This would explain the activity of totarol across the microbial species used in this study.

Table 33. Antibacterial activity for selected isolated compounds, MICs in µg/mL (µM)

	B. cereus	*E. faecalis*	*S. aureus*	*S. epidermidis*	*S.pyogenes*
Totarol **(JC3)**	2 (7.0)	4 (14.0)	8 (27.9)	2 (7.0)	1 (3.5)
Taraxerol **(SJ1)**	>256 (>600.4)	>256 (>600.4)	>256 (>600.4)	>256 (>600.4)	>256 (>600.4)
n-nonacosane **(MG1)**	>256 (>626.7)	>256 (>626.7)	>256 (>626.7)	>256 (>626.7)	>256 (>626.7)
Myricadiol **(MG3)**	>256 (>577.4)	>256 (>577.4)	>256 (>577.4)	>256 (>577.4)	>256 (>577.4)
Oleanolic acid **(CV1)**	256 (561.0)	32 (70.1)	>256 (>561.0)	64 (140.2)	16 (35.1)
Ciprofloxacin (CIP)	8 (24.2)	4 (12.1)	2 (6.0)	0.5 (1.5)	1 (3.0)

B. cereus: Bacillus cereus

E. faecalis: Enterococcus faecalis

S. aureus: Staphylococcus aureus

S. epidermidis: Staphylococcus epidermidis

S. pyogenes: Streptococcus pyogenes

3.6 Future work

It is interesting to note that although this study focuses on four plant species, our preliminary antimycobacterial screening identified several other extracts (plant, lichen, and endophytes) worthy of further investigation. In many cases, it would be worthwhile to find out whether the presence of antimycobacterial compounds could validate the use of plants with reported traditional anti-TB activity.

The insufficient amount of material recovered following the preparation of endophyte extracts did not allow for full bioassay-guided fractionation work to be undertaken. Nevertheless, further studies on the chemical composition of the endophyte extracts, for instance by analytical profiling and comparison of active/inactive samples, would be of particular interest.

Finally, it would be of interest to carry out some further optimisation studies on the compounds isolated during this study (i.e. modification of the natural template to generate compounds with better activity and lower cytotoxicity) as exemplified in recently published work on the development of novel anti-TB drugs (Al-Balas et al. 2009)

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APPENDICES

Appendices

Table 1. Origin of plant and lichen material and yields of extracts obtained

Code	Plant	Plant part	Origin	Amount extracted	Yields (% of dry material)		
					H	E	M
144	*Abies alba*	aerial parts	Abtrees, Oct 03	43.50g	1.90g (4.4%)	1.35g (3.1%)	8.34g (19.2%)
145	*Abies alba*	roots	Abtrees, Oct 03	38.33g	0.70g (1.8%)	0.32g (0.8%)	5.88g (15.3%)
142	*Abies fraseri*	aerial parts	Albatrees, Oct 03, Dec 05	743.47g	38.11g (5.1%)	15.67g (2.1%)	70.35g (9.5%)
143	*Abies fraseri*	roots	Albatrees, Oct 03, Dec 05	39.57g	0.55g (1.4%)	0.36g (0.9%)	3.35g (8.5%)
111	*Abies grandis*	aerial parts	Abtrees, Oct 03	43.66g	1.86g (4.3%)	0.95g (2.2%)	10.25g (23.5%)
112	*Abies grandis*	roots	Abtrees, Oct 03	46.23g	0.59g (1.3%)	0.22g (0.5%)	3.32g (7.2%)
131	*Abies nobilis*	aerial parts	Abtrees, Oct 03	37.84g	1.45g (3.8%)	3.17g (8.4%)	7.62g (20.1%)
132	*Abies nobilis*	roots	Abtrees, Oct 03	48.00g	1.18g (2.5%)	0.56g (1.2%)	6.54g (13.6%)
307	*Anemone nemorosa*	whole flowering plant	Roslin Glen, Apr 06	32.05g	1.10g (3.4%)	0.55g (1.7%)	7.11g (22.2%)
139	*Betula pendula*	aerial parts	Albatrees, Dec 05	38.43g	0.90g (2.3%)	0.68g (1.8%)	3.99g (10.4%)
138	*Betula pendula*	roots	Albatrees, Dec 05	20.49	0.26g (1.3%)	0.40g (2.0%)	2.93g (14.3%)

H: *n*-hexane extract E: ethyl acetate extract M: methanol extract

Table 1 (continued). Origin of plant and lichen material and yields of extracts obtained

Code	Plant	Plant part	Origin	Amount extracted	Yields (% of dry material)		
					H	E	M
309	*Calluna vulgaris*	aerial parts	Whim Moss, Apr 06	29.84g 688.98g	1.29g (4.3%) 21.82g (3.2%)	1.02g (3.4%) 55.94g (8.1%)	3.39g (11.4%)
302	*Cornus alba*	aerial parts	Albatrees, Dec 05	38.35g	1.11g (2.9%)	0.71g (1.9%)	8.31g (21.7%)
306	*Cornus alba*	roots	Albatrees, Dec 05	42.54g	0.85g (2.0%)	1.26g (3.0%)	12.29g (28.9%)
116	*Crataegus monogyna*	aerial parts	Abtrees, Oct 03	44.78g	0.47g (1.0%)	0.64g (1.4%)	5.43g (12.1%)
125	*Crataegus monogyna*	roots	Abtrees, Oct 04	25.50g	0.05g (0.2%)	0.10g (0.4%)	1.73g (6.8%)
310	*Empetrum nigrum*	whole	Whim Moss, Apr 06	31.35g	2.54g (8.1%)	0.94g (3.0%)	4.15g (13.2%)
123	*Fagus sylvatica*	aerial parts	Abtrees, Oct 03	39.42g	0.29g (0.7%)	0.50g (1.3%)	5.15g (13.1%)
122	*Fagus sylvatica*	roots	Abtrees, Oct 04	12.48g	0.04g (0.3%)	0.08g (0.6%)	1.25g (10.0%)
135	*Fraxinus excelsior*	stems	Abtrees, Oct 03	31.68g	0.21g (0.7%)	1.95g (6.2%)	5.96g (18.8%)
136	*Fraxinus excelsior*	roots	Abtrees, Oct 03	42.08g	0.51g (1.2%)	1.45g (3.4%)	8.58g (20.4%)
115	*Ilex aquifolium*	aerial parts	Albatrees, Oct 03, Dec 05	34.71g	0.87g (2.5%)	1.23g (3.5%)	7.06g (20.3%)

H: *n*-hexane extract E: ethyl acetate extract M: methanol extract

Table 1 (continued). Origin of plant and lichen material and yields of extracts obtained

Code	Plant	Plant part	Origin	Amount extracted	Yields (% of dry material)		
					H	E	M
128	*Ilex aquifolium*	roots	Abtrees, Oct 03	20.20g	0.46g (2.3%)	0.28g (1.4%)	3.20g (15.8%)
140	*Juniperus communis*	aerial parts	Albatrees, Oct 03, Dec 05	739.66g	50.41g (6.8%)	31.79g (4.3%)	ND
141	*Juniperus communis*	roots	Albatrees, Oct 03, Dec 05	24.62g 192.30g	1.57g (6.4%) 15.22g (7.9%)	1.44g (5.8%)	2.76g (11.2%)
121	*Knautia arvensis*	whole	Abtrees, Oct 03	48.96g	0.58g (1.2%)	4.40g (9.0%)	23.72g (48.4%)
150	*Larix leptoleptis*	aerial parts	Abtrees, Oct 03	41.27g	0.05g (0.1%)	0.85g (2.1%)	5.66g (13.7%)
159	*Larix leptoleptis*	roots	Abtrees, Oct 03	31.10g	0.76g (2.4%)	0.58g (1.9%)	0.58g (1.9%)
308	*Lonicera periclymenum*	aerial parts	Roslin Glen, Apr 06	28.49g	0.44g (1.5%)	1.01g (3.5%)	4.29g (15.1%)
114	*Malus sylvestris*	roots	Abtrees, Oct 03	61.89g	0.58g (0.9%)	3.16g (5.1%)	6.34g (10.2%)
126	*Malus sylvestris*	aerial parts	Abtrees, Oct 03	38.14g	0.44g (1.2%)	2.16g (5.7%)	4.90g (12.8%)
299	*Myrica gale*	aerial parts	Albatrees, Oct 03, Dec 05	46.39g	1.06g (2.3%)	0.83g (1.8%)	5.35g (11.5%)

H: *n*-hexane extract E: ethyl acetate extract M: methanol extract ND: not determined (sample not fully dried)

Table 1 (continued). Origin of plant and lichen material and yields of extracts obtained

Code	Plant	Plant part	Origin	Amount extracted	Yields (% of dry material)		
					H	E	M
305	*Myrica gale*	roots	Albatrees, Dec 05, Dec 06	497.52g	8.08g (1.6%)	16.97g (3.4%)	ND
157	*Phragmites australis*	whole	Abtrees, Oct 03	27.01g	0.23g (0.9%)	0.20g (0.7%)	4.75g (17.6%)
163	*Picea abies*	aerial parts	Abtrees, Oct 03	38.10g	2.13g (5.6%)	2.18g (5.7%)	3.49g (9.2%)
134	*Picea abies*	roots	Abtrees, Oct 03	32.27g	0.28g (0.9%)	0.66g (2.0%)	2.96g (9.2%)
169	*Pinus mugo*	aerial parts	Abtrees, Oct 03	38.44g	2.43g (6.3%)	0.89g (2.3%)	3.96g (10.3%)
147	*Pinus mugo*	roots	Abtrees, Oct 03	43.80g	1.56g (3.6%)	0.74g (1.7%)	7.32g (16.7%)
158	*Pinus nigra maritima*	aerial parts	Abtrees, Oct 03	43.03g	2.20g (5.1%)	2.88g (6.7%)	0.22g (0.5%)
168	*Pinus nigra maritima*	roots	Abtrees, Oct 03	40.61g	0.60g (1.5%)	0.95g (2.3%)	ND
170	*Pinus nigra nigra*	aerial parts	Abtrees, Oct 03	29.17g	2.20g (7.5%)	0.87g (3.0%)	2.77g (9.5%)
152	*Pinus nigra nigra*	roots	Abtrees, Oct 03	45.69g	1.46g (3.2%)	0.95g (2.1%)	4.50g (9.8%)
109	*Pinus sylvestris Scotica*	aerial parts	Abtrees, Oct 03	37.82g	1.49g (3.9%)	0.93g (2.5%)	4.30g (11.4%)
113	*Pinus sylvestris Scotica*	roots	Abtrees, Oct 03	37.25g	0.59g (1.6%)	0.17g (0.5%)	4.07g (10.9%)

H: *n*-hexane extract E: ethyl acetate extract M: methanol extract ND: not determined

Table 1 (continued). Origin of plant and lichen material and yields of extracts obtained

Code	Plant	Plant part	Origin	Amount extracted	Yields (% of dry material)		
					H	E	M
178	*Polygonum persicaria*	whole flowering plant	Glasgow, Jul 04	33.76g	0.19g (0.6%)	0.28g (0.8%)	3.14g (9.3%)
118	*Populus alba*	aerial parts	Abtrees, Oct 03	26.85g	0.38g (1.4%)	1.21g (4.5%)	6.50g (24.2%)
129	*Populus alba*	roots	Abtrees, Oct 03	27.61g	0.47g (1.7%)	1.08g (3.9%)	2.22g (8.0%)
117	*Populus nigra*	stems	Abtrees, Oct 03	37.90g	0.59g (1.6%)	2.18g (5.8%)	3.66g (9.7%)
130	*Populus nigra*	roots	Abtrees, Oct 03	45.17g	0.46g (1.0%)	1.12g (2.5%)	5.24g (11.6%)
153	*Populus tremula*	aerial parts	Abtrees, Oct 03	28.22g	0.37g (1.3%)	1.63g (5.8%)	2.85g (10.1%)
166	*Populus tremula*	roots	Abtrees, Oct 03	44.09g	0.67g (1.5%)	1.85g (4.2%)	5.63g (12.8%)
291	*Pulmonaria officinalis*	flowered aerial parts	Maryhill, Glasgow, Apr 04	31.13g	0.29g (0.9%)	0.33g (1.1%)	5.49g (17.6%)
119	*Quercus petraea*	aerial parts	Abtrees, Oct 03	43.96g	0.55g (1.3%)	0.45g (1.0%)	7.13g (16.2%)
127	*Quercus petraea*	roots	Abtrees, Oct 03	49.58g	0.16g (0.3%)	0.21g (0.4%)	4.38g (8.8%)
180	*Rumex obtusifolius*	aerial parts	Helensburg, Glasgow, Jul 05	31.39g	0.21g (0.7%)	0.14g (0.4%)	4.01g (12.8%)
177	*Rumex obtusifolius*	roots	Helensburg, Glasgow, Jul 05	151.84g	1.42g (0.9%)	2.78g (1.8%)	14.01g (9.2%)

H: *n*-hexane extract E: ethyl acetate extract M: methanol extract

Table 1 (continued). Origin of plant and lichen material and yields of extracts obtained

Code	Plant	Plant part	Origin	Amount extracted	Yields (% of dry material)		
					H	E	M
297	*Salix caprea*	aerial parts	Albatrees, Dec 05	27.74g	0.19g (0.7%)	0.56g (2.0%)	3.94g (14.2%)
304	*Salix caprea*	roots	Albatrees, Dec 05	41.98g	0.87g (2.1%)	0.47g (1.1%)	6.85g (16.3%)
162	*Salix cinerea*	aerial parts	Abtrees, Oct 03	22.91g	0.24g (1.0%)	0.65g (2.8%)	0.78g (3.4%)
155	*Salix cinerea*	roots	Abtrees, Oct 03	41.30g	0.60g (1.5%)	1.15g (2.8%)	4.90g (11.9%)
69	*Skimmia. japonica*	Aerial parts	Bishopbriggs, Jul05	34.78g	1.04g (3.0%)	1.14g (3.3%)	4.38g (12.6%)
124	*Sorbus aucuparia*	stems	Abtrees, Oct 03	37.09g	0.52g (1.4%)	0.78g (2.1%)	6.23g (16.8%)
120	*Sorbus aucuparia*	roots	Abtrees, Oct 03	41.63g	0.36g (0.9%)	1.22g (2.9%)	6.06g (14.6%)
104	*Taxus baccata*	aerial parts	Abtrees, Oct 03	40.98g	0.01g (<0.1%)	0.61g (1.5%)	9.12g (22.3%)
105	*Taxus baccata*	root	Abtrees, Oct 03	36.60g	0.14g (0.4%)	2.11g (5.8%)	4.05g (11.1%)
106	*Thuja plicata*	aerial parts	Abtrees, Oct 03	28.63g	0.02g (0.1%)	0.14g (0.5%)	6.20g (21.7%)
107	*Thuja plicata*	roots	Abtrees, Oct 03	33.01g	1.05g (3.2%)	0.73g (2.2%)	3.11g (9.4%)

H: *n*-hexane extract E: ethyl acetate extract M: methanol extract

Table 1 (continued). Origin of plant and lichen material and yields of extracts obtained

Code	Plant/Lichen	Plant part	Origin	Amount extracted	Yields (% of dry material)		
					H	E	M
149	*Tsuga heterophylla*	aerial parts	Albatrees, Dec 05	36.25g	1.31g (3.6%)	1.14g (3.1%)	7.43g (20.5%)
156	*Tsuga heterophylla*	roots	Albatrees, Dec 05	41.33g	0.56g (1.4%)	0.74g (1.8%)	4.73g (11.4%)
311	*Vaccinium myrtillus*	whole	Whim Moss, Apr 06	32.65g	0.73g (2.2%)	0.43g (1.3%)	3.91g (12.0%)
312	*Vaccinium myrtillus*	whole	Roslin Glen, Apr 06	33.63g	0.85g (2.5%)	0.63g (1.9%)	3.83g (11.4%)
108	*Vaccinium myrtillus*	whole	Abtrees, Oct 03	42.26g	1.08g (2.6%)	0.72g (1.7%)	4.50g (10.6%)
154	*Viburnum opulus*	aerial parts	Abtrees, Oct 03	51.38g	2.39g (4.7%)	7.25g (14.1%)	7.89g (15.4%)
151	*Viburnum opulus*	roots	Abtrees, Oct 03	48.56g	0.47g (1.0%)	2.08g (4.3%)	3.77g (7.8%)
315	*Sphagnum* species	whole	Whim Moss, Apr 06	17.42g	0.30g (1.7%)	0.10g (0.6%)	0.88g (5.1%)
314	*Cladonia arbuscula*	whole	Whim Moss, Apr 06	33.78g	0.52g (1.5%)	0.32g (0.9%)	1.43g (4.2%)

H: *n*-hexane extract E: ethyl acetate extract M: methanol extract

Table 2. Origin of endophyte material and yields of extracts obtained

Endophyte	Origin	Collection site	Weights (mg) and yields (% of dry material)				
			mycelium	H	C	B	E
Amanita muscaria #1	*P. sylvestris* (FB)	Glentress forest	400.2	27.2 (6.8%)	2.0 (0.5%)	23.1 (5.8%)	6.4 (1.6%)
Amanita muscaria #2	*P. sylvestris* (FB)	Tyninghame	123.2	<1 (<0.8%)	<1 (<0.8%)	14.4 (11.7%)	3.2 (2.6%)
Chalciporus piperatus	*P. sylvestris* (FB)	Glentress forest	292.6	<1 (<0.3%)	2.5 (0.9%)	26.0 (8.9%)	2.2 (0.8%)
Cortinarius semisanguineus	*P. sylvestris* (FB)	Rothiemurchus	96.5	<1 (<1.0%)	<1 (<1.0%)	<1 (<1.0%)	2.4 (2.5%)
Gyromitra esculenta	*P. sylvestris* (FB)	Blackwood of Rannoch	4953.0	<1 (<0.1%)	2.1 (0.1%)	30.9 (0.6%)	65.8 (1.3%)
Lactarius deliciosus	*P. sylvestris* (FB)	Yellowcraig	731.8	<1 (<0.1%)	1.1 (0.2%)	2.1 (0.3%)	37.3 (5.1%)
Leccinum holopus	*B. pendula* (FB)	Blackwood of Rannoch	579.8	23.5 (4.1%)	4.0 (0.7%)	67.0 (11.6%)	11.2 (1.9%)
Paxillus involutus	*P. sylvestris* (FB)	Whim Moss	179.4	12.5 (7.0%)	15.0 (8.4%)	80.9 (45.1%)	1.5 (0.8%)
Suillus bovinus	*P. sylvestris* (FB)	Rothiemurchus	351.0	5.0 (1.4%))	9.2 (2.6%)	19.3 (5.5%)	19.6 (5.6%)
Suillus luteus #1	*P. sylvestris* (FB)	Rothiemurchus	144.3	<1 (<0.7%)	3.2 (2.2%)	30.4 (21.1%)	1.2 (0.8%)
Suillus luteus #2	*P. sylvestris* (FB)	Yellowcraig	203.6	<1 (<0.5%)	1.5 (0.7%)	10.1 (5.0%)	2.2 (1.1%)
Suillus luteus #3	*P. sylvestris* (FB)	Glentress forest	588.9	1.6 (0.3%)	4.2 (0.7%)	16.2 (2.8%)	3.2 (0.5%)
Suillus variegatus	*P. sylvestris* (FB)	Whim Moss	277.7	0.6 (0.2%)	2.9 (1.0%)	31.9 (11.5%)	0.9 (0.3%)

FB: fruiting bodies H : *n*-hexane extract C : chloroform extract B : *n*-butanol extract E ethanol extract

Table 2 (continued). Origin of endophyte material and yields of extracts obtained

Endophyte	Origin	Collection site	Weights (mg) and yields (% of dry material)				
			mycelium	H	C	B	E
Tricholoma imbricatum	*P. sylvestris* (FB)	Bush Estate	1312.4	2.3 (0.2%)	4.2 (0.3%)	128.0 (9.8%)	62.2 (4.7%)
Xerocomus badius #1	*P. sylvestris* (FB)	Harlaw moor	194.2	2.1 (1.1%)	1.7 (0.9%)	6.6 (3.4%)	12.0 (6.2%)
Xerocomus badius #2	*P. sylvestris* (FB)	Blackwood of Rannoch	512.5	0.7 (0.1%)	3.1 (0.6%)	13.5 (2.6%)	1.8 (0.4%)
Xerocomus edulis	*P. sylvestris* (FB)	Harlaw moor	194.3	1.3 (0.7%)	1.2 (0.6%)	<1 (<0.5%)	3.8 (2.0%)
Cenococcum species	*B. pendula* roots	Blackwood of Rannoch	381.2	<1 (<0.3%)	5.0 (1.3%)	53.4 (14.0%)	20.2 (5.3%)
Rhizopogon species #1	*P. sylvestris* roots	Blackwood of Rannoch	333.3	9.9 (3.0%)	5.0 (1.5%)	37.8 (11.3%)	10.8 (3.2%)
Rhizopogon species #2	*P. sylvestris* roots	Glen Affric	514.2	<1 (<0.2%)	1.6 (0.3%)	8.1 (1.6%)	78.6 (15.3%)
Rhizopogon species #3	*P. sylvestris* roots	Whim Moss	210.2	<1 (<0.5%)	2.8 (1.3%)	12.1 (5.5%)	3.9 (1.9%)
Russula species	*P. sylvestris* roots	Whim Moss	1011.3	<1 (<0.1%)	2.7 (0.3%)	25.5 (2.5%)	<1 (<0.1%)
Tomentellopsis species	*P. sylvestris* roots	Blackwood of Rannoch	532.1	6.6 (1.2%)	1.4 (0.3%)	66.8 (12.6%)	16.1 (3.0%)
Ericoid root endophyte #1	*C. vulgaris* roots	Whim Moss	1149.8	<1 (<0.1%)	7.4 (0.6%)	10.4 (0.9%)	5.2 (0.5%)
Ericoid root endophyte #2	*C. vulgaris* roots	Rothiemurchus	2286.6	<1 (<0.1%)	2.2 (0.1%)	13.7 (0.6%)	62.5 (2.7%)

FB: fruiting bodies H : *n*-hexane extract C : chloroform extract B : *n*-butanol extract E ethanol extract

Table 2 (continued). Origin of endophyte material and yields of extracts obtained

Endophyte	Origin	Collection site	Weights (mg) and yields (% of dry material)				
			mycelium	H	C	B	E
Ericoid root endophyte #3	*C. vulgaris* roots	Glen Coe	2293.0	<1 (<0.1%)	2.2 (0.1%)	12.7 (0.6%)	77.6 (3.4%)
Ericoid root endophyte #4	*C. vulgaris* roots	Rannoch Moor	733.0	ND	ND	ND	29.6 (4.0%)
Ericoid root endophyte #5	*E. nigrum roots*	Glen Affric	955.9	ND	ND	ND	4.3 (0.4%)
Ericoid root endophyte #6	*V. myrtilis* roots	Rothiemurchus	2111.4	1.5 (0.1%)	1.3 (0.1%)	12.7 (0.6%)	93.6 (4.4%)
Ericoid root endophyte #7	*V. myrtilis* roots	Blackwood of Rannoch	503.0	1.2 (0.2%)	3.4 (0.7%)	79.7 (15.8%)	14.1 (2.8%)
Ericoid root endophyte #8	*V. vitis-idea* roots	Rothiemurchus	3857.2	<1 (<0.1%)	4.4 (0.1%)	10.1 (0.3)%	25.1 (0.7%)
Ericoid root endophyte #9	*C. vulgaris* roots	Whim Moss	1149.8	<1 (<0.%1)	7.4 (0.6%)	10.4 (0.9)%	6.2 (0.5%)
Ericoid root endophyte #10	*C. vulgaris* roots	Blackwood of Rannoch	552.4	1.0 (0.2%)	2.6 (0.5%)	25.2 (4.6)%	30.5 (5.5%)
Ericoid root endophyte #11	*C. vulgaris* roots	Glen Affric	1612.5	<1 (<0.1%)	1.2 (0.1%)	9.2 (0.6%)	29.1 (1.8%)
Ericoid root endophyte #12	*V. myrtilis* roots	Rannoch Moor	967.0	2.0 (0.2%)	7.7 (0.8%)	10.1 (1.0%)	26.1 (2.7%)
Ericoid root endophyte #13	*V. oxycoccus* roots	Whim Moss	907.8	2.4 (0.3%)	5.9 (0.6%)	7.7 (0.8%)	14.0 (1.5%)
Ericoid root endophyte #14	*E. tetralix* roots	Rannoch Moor	7105.2	3.4 (0.1%)	4.7 (0.1%)	60.4 (0.9%)	76.5 (1.1%)
Ericoid root endophyte #15	*E. tetralix* roots	Rannoch Moor	749.9	2.3 (0.3%)	4.5 (0.6%)	78.5 (10.5%)	20.5 (2.7%)
Ericoid root endophyte #16	*V. vitis-idea* roots	Glen Affric	384.8	<1 (<0.3%)	3.5 (0.9%)	55.5 (14.4%	33.5 (8.7%)

H : *n*-hexane extract C : chloroform extract B : *n*-butanol extract E ethanol extract

Figure 1. Fractionation scheme of the *n*-hexane extract of *Skimmia japonica* aerial parts

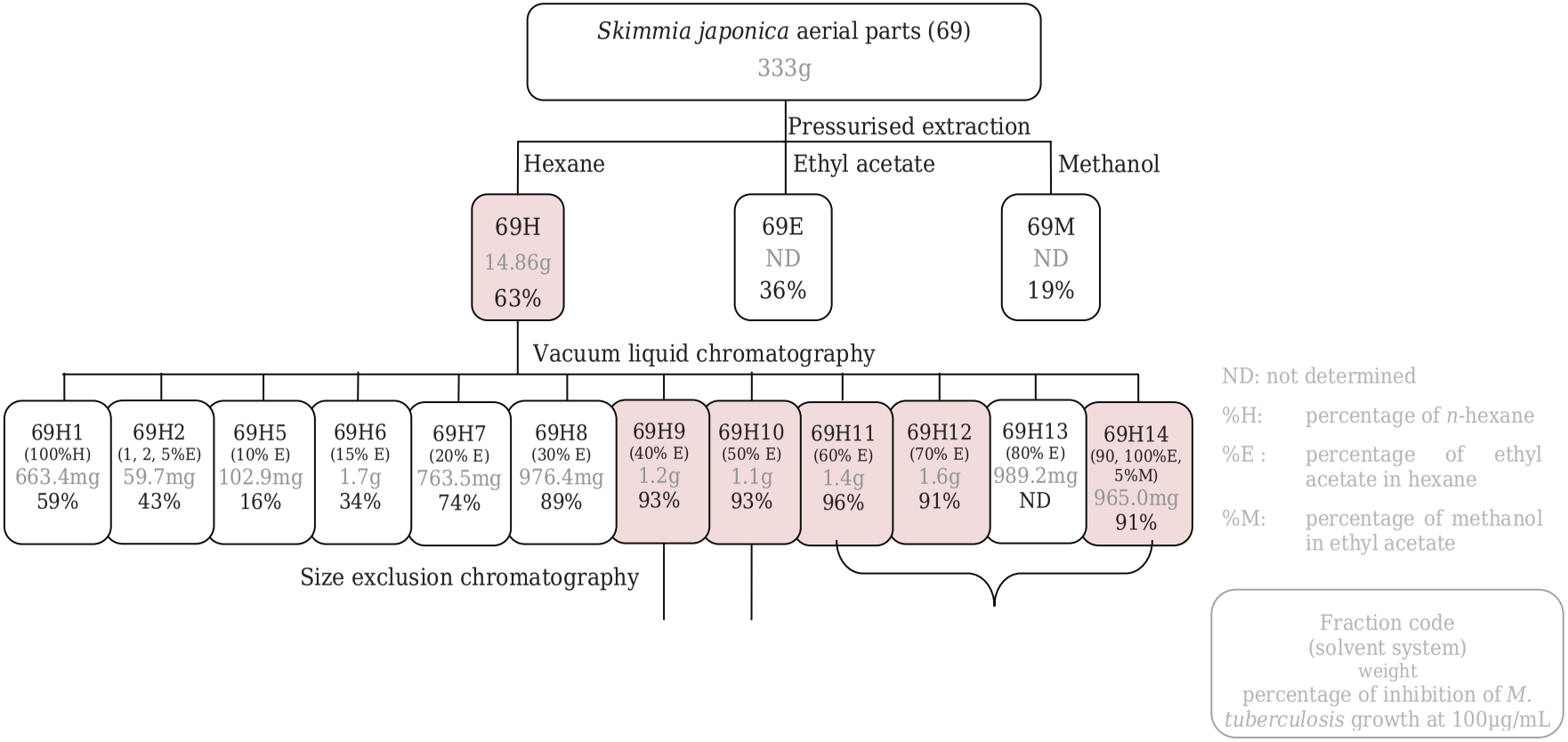


Figure 2. Fractionation scheme of fraction 69H9 from the *n*-hexane extract of *Skimmia japonica* aerial parts

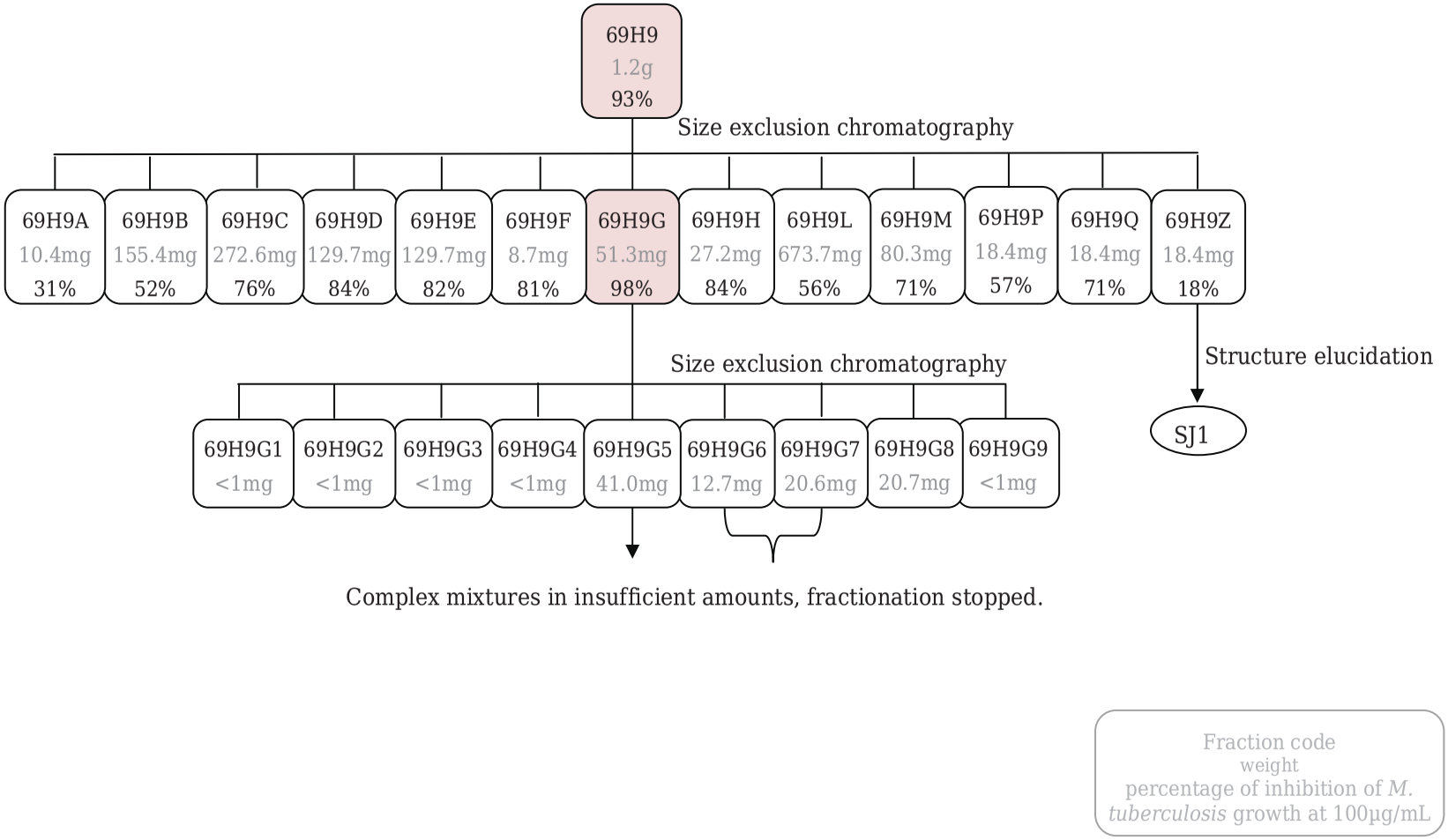
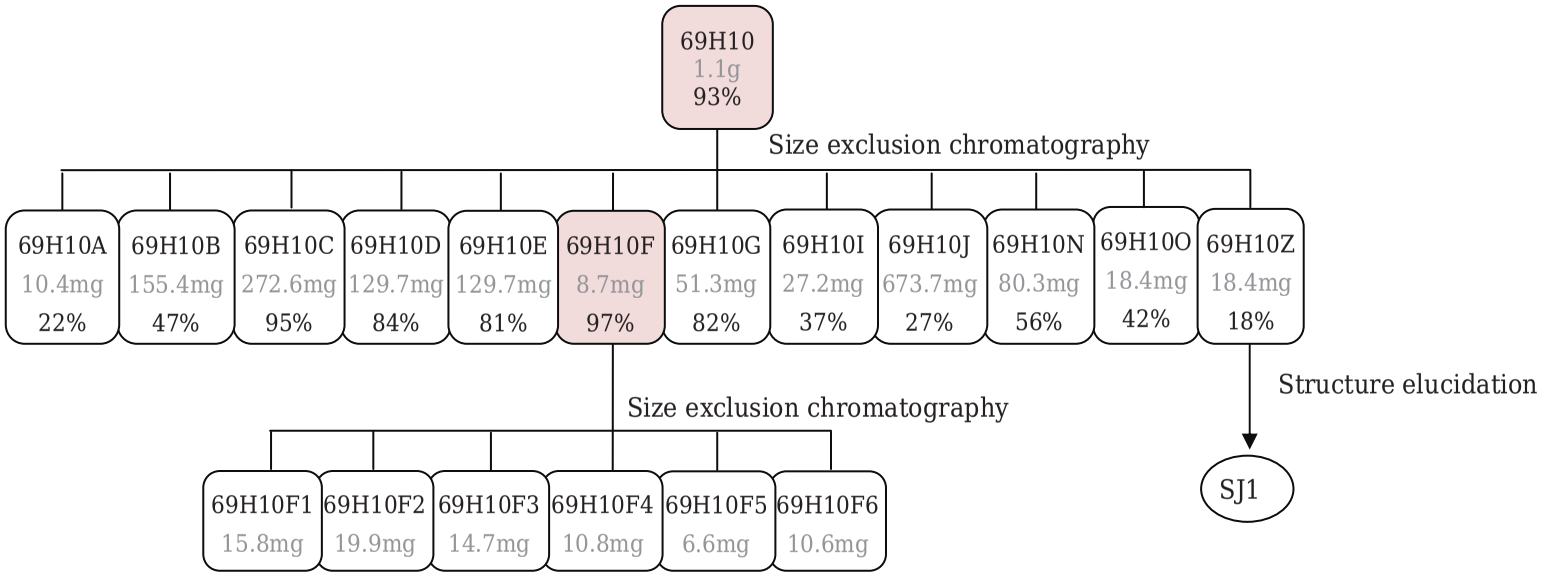


Figure 3. Fractionation scheme of fraction 69H10 from the *n*-hexane extract *Skimmia japonica* aerial parts



Complex mixtures in insufficient amounts, fractionation stopped.

Figure 4. Fractionation scheme of fractions 69H11 to 69H14 from the *n*-hexane extract of *Skimmia japonica* aerial parts

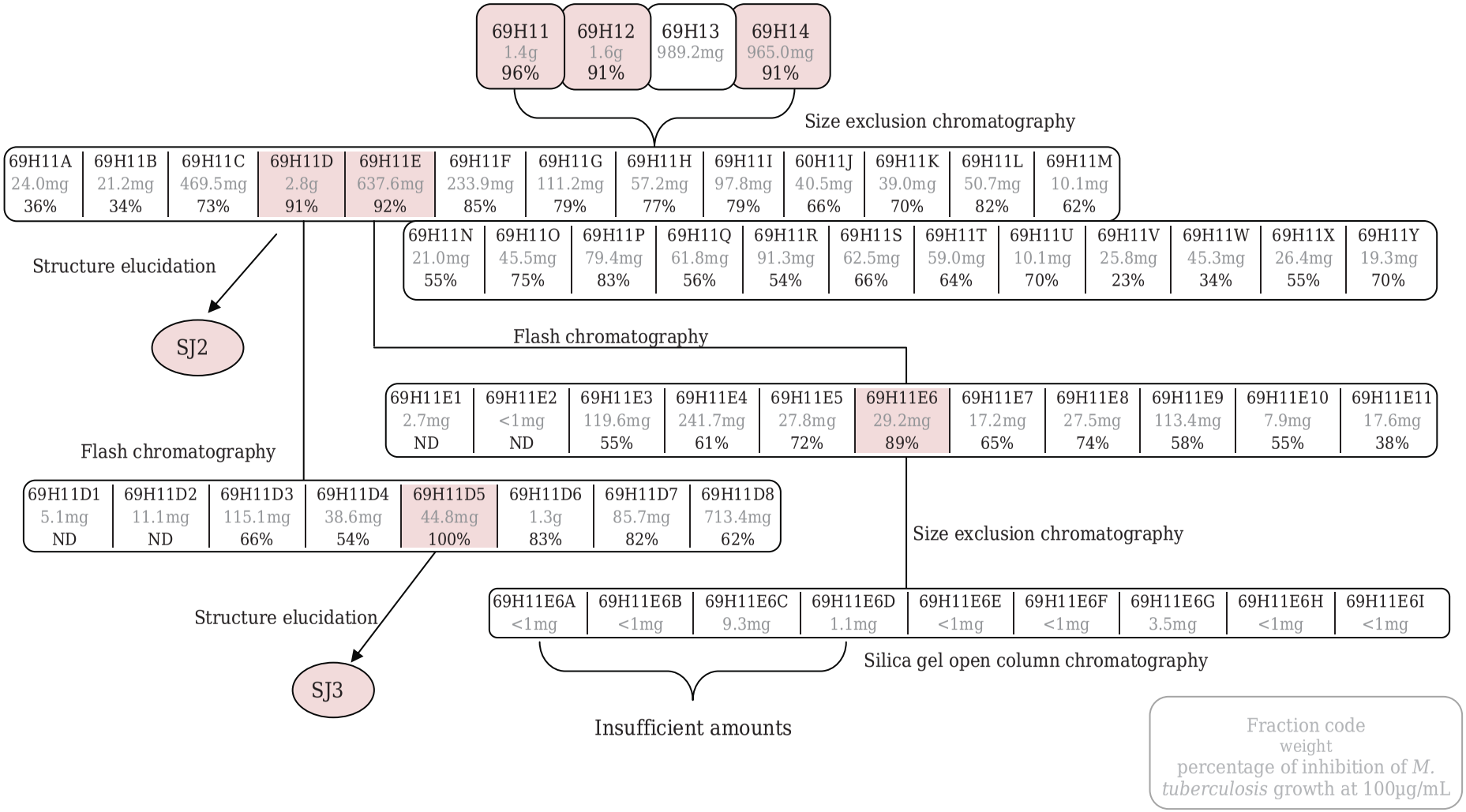


Figure 5. Fractionation scheme of the *n*-hexane extract of *Juniperus communis* roots

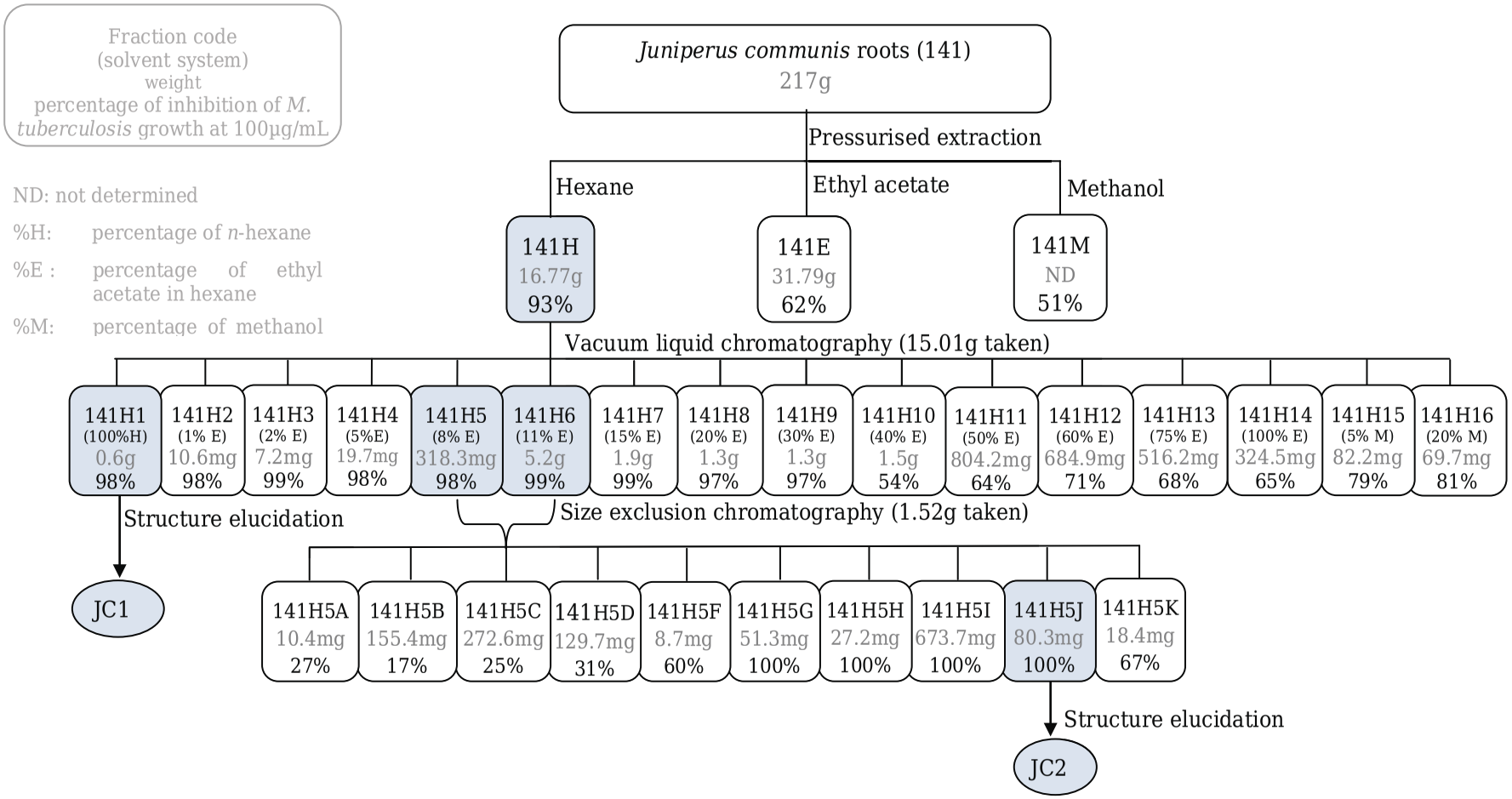


Figure 6. Fractionation scheme of the *n*-hexane extract of *Juniperus communis* aerial parts

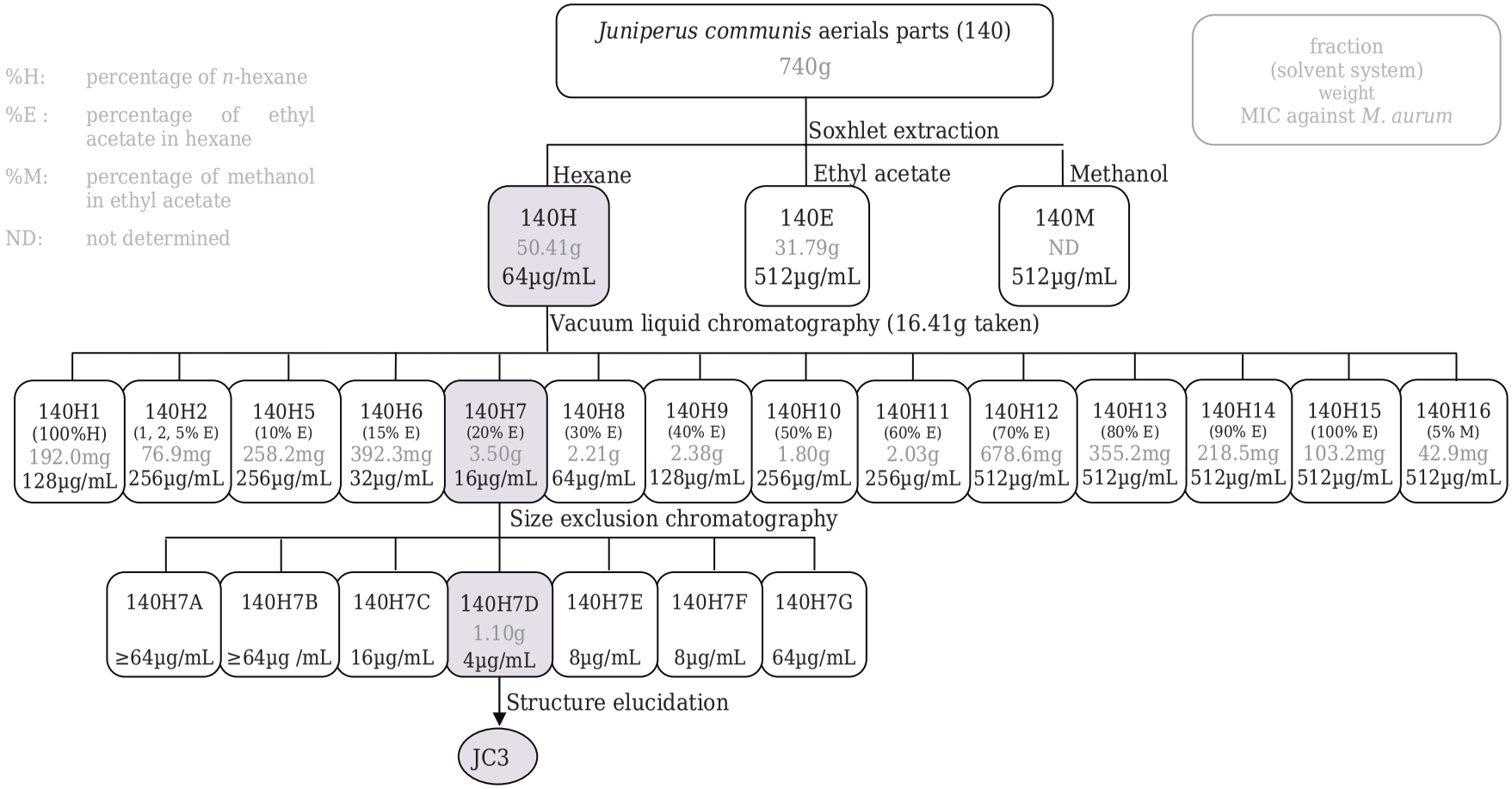


Figure 7. Fractionation scheme of the *n*-hexane and ethyl acetate extract of *Calluna vulgaris* aerial parts

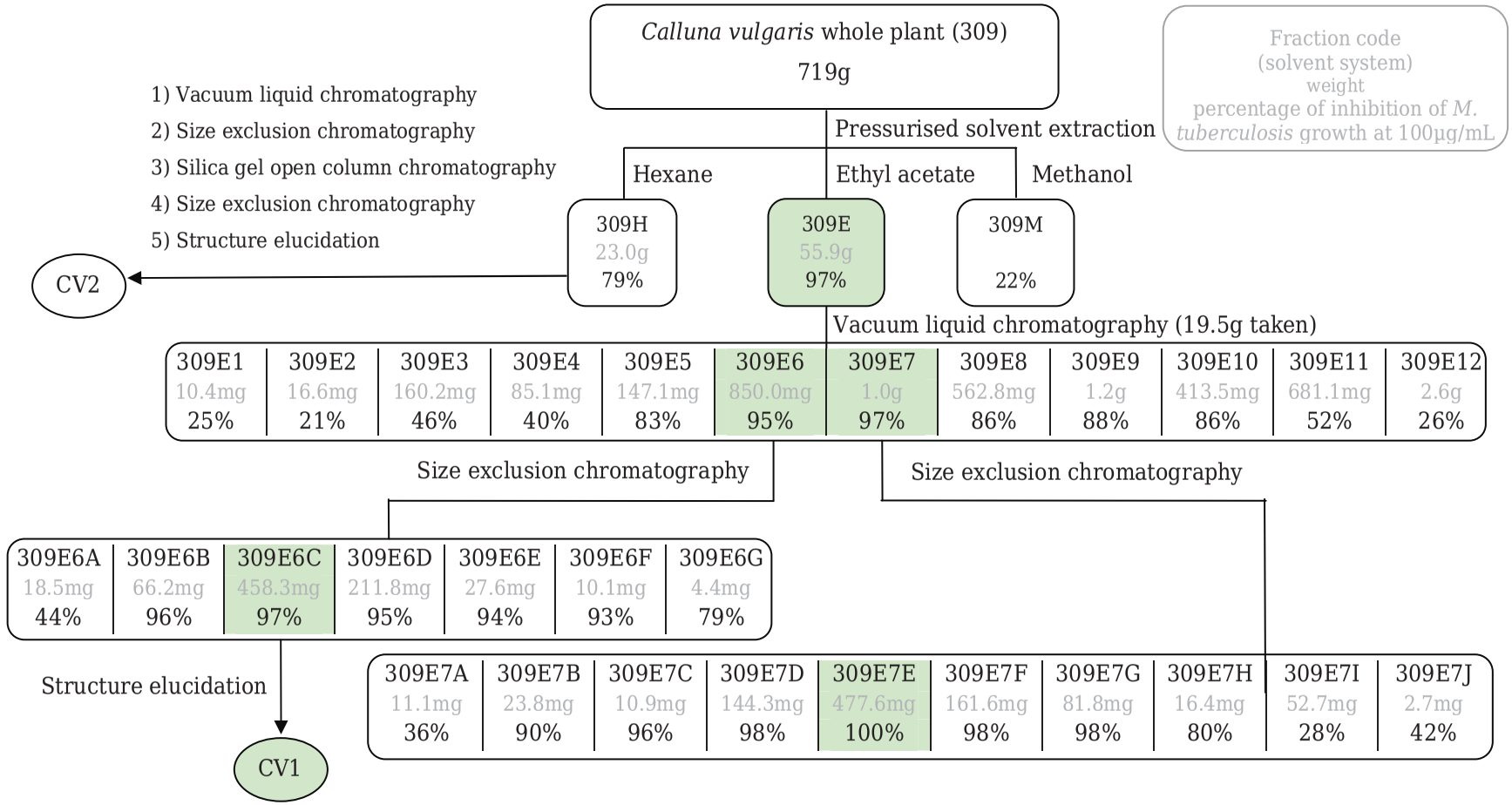


Figure 8. Fractionation scheme of the *n*-hexane extract of *Myrica gale* aerial parts.

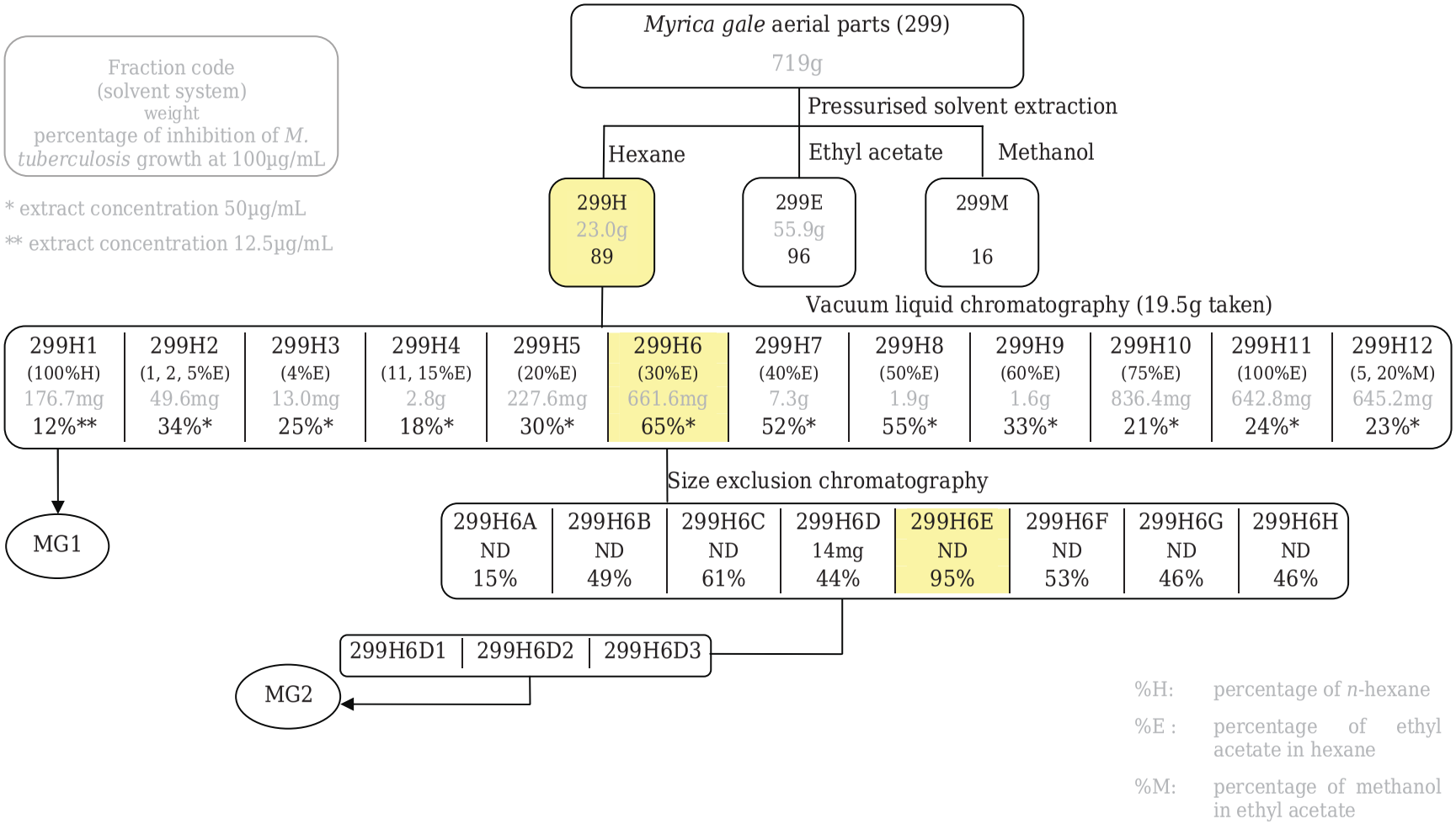


Figure 9. Fractionation scheme of the ethyl acetate extract *Myrica gale* aerial parts.

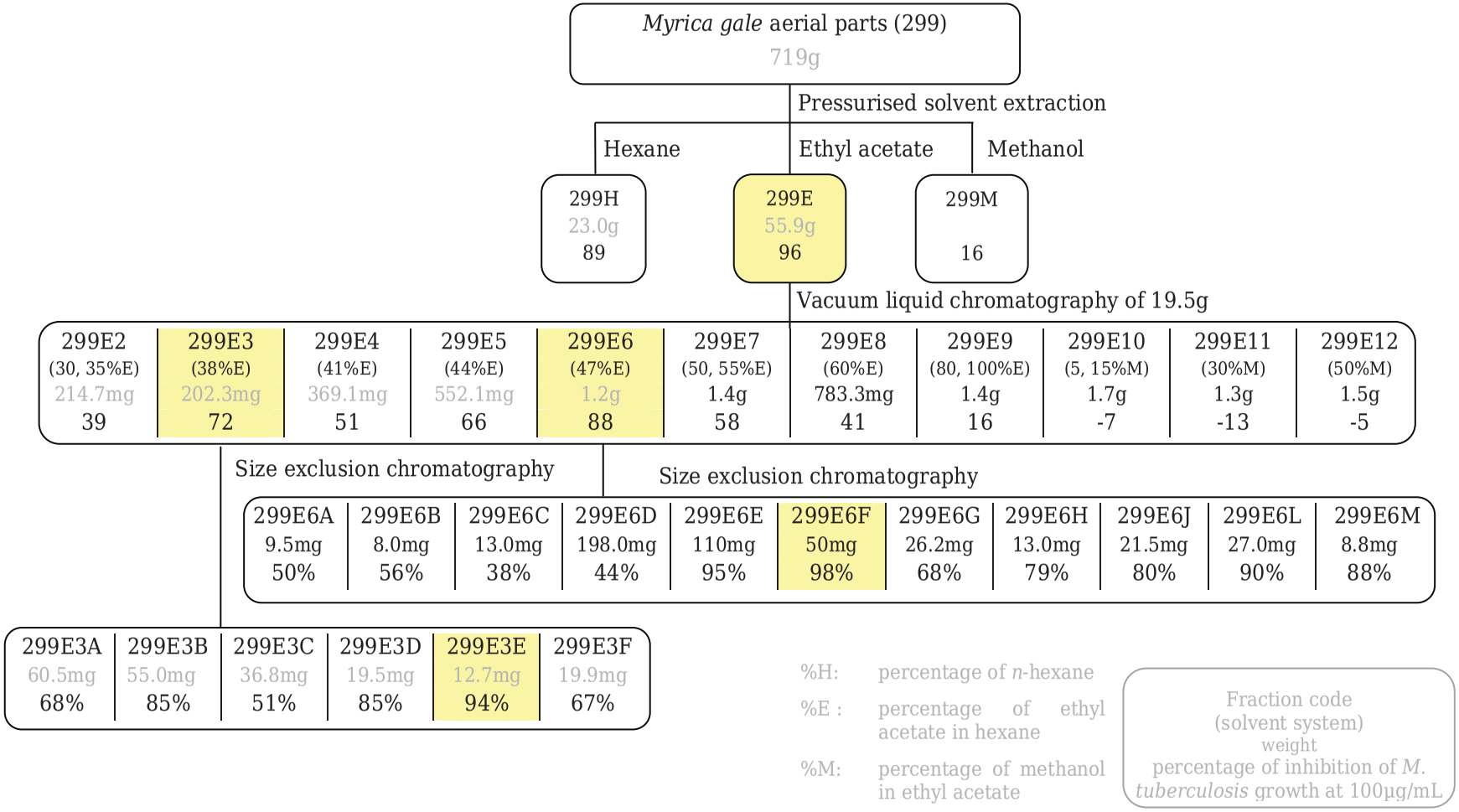
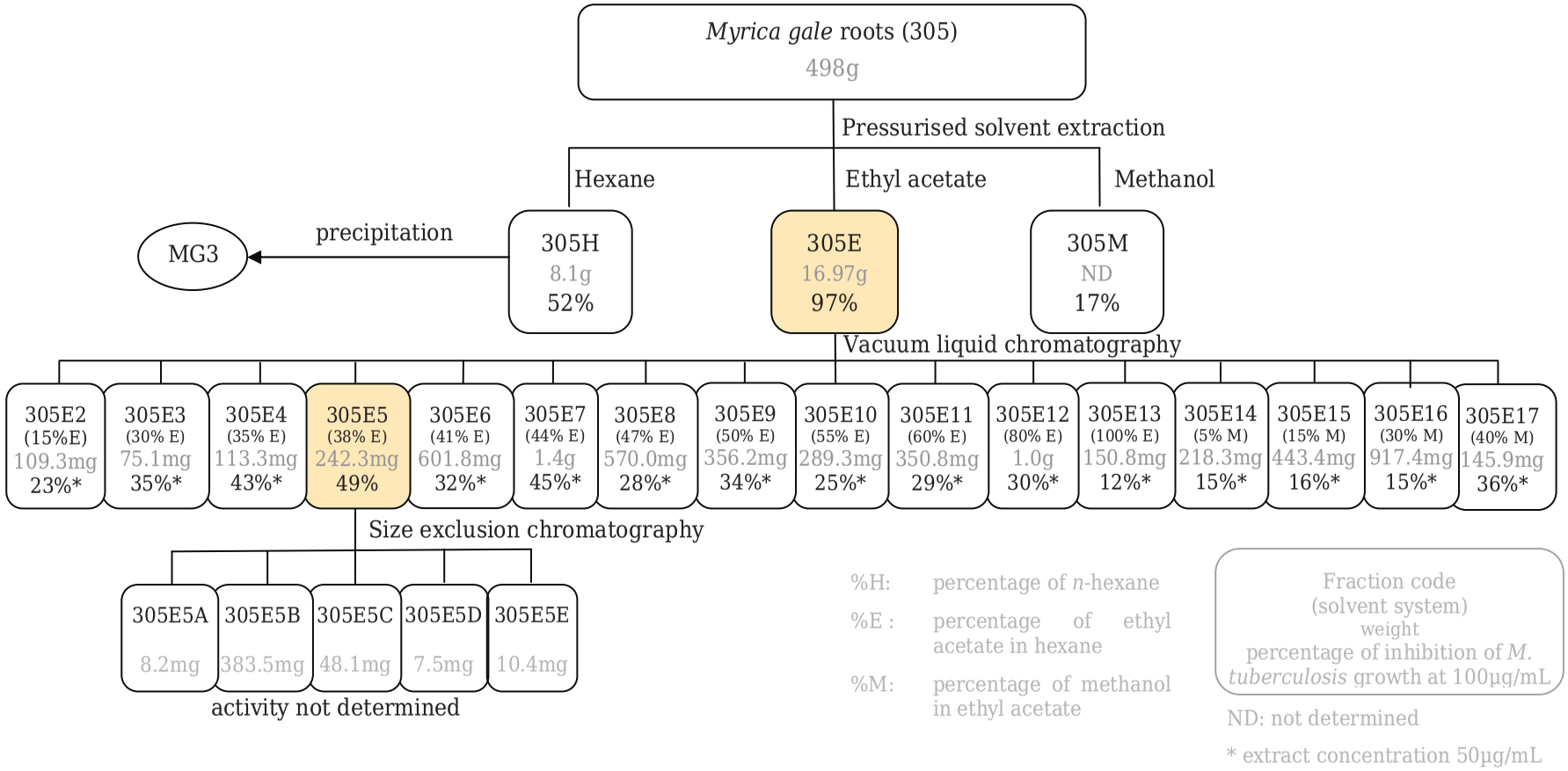


Figure 10. Fractionation scheme of the ethyl acetate extract of *Myrica gale* roots.



List of communications

Poster presentation:

Andréa Y. Gordien, Kevin Ingleby, Scott G. Franzblau, Alexander I. Gray, Véronique Seidel. Antimycobacterial Activity of Scottish Plants and Fungal Endophytes. PLANT-MICROBIAL INTERACTIONS 2008, 2-6 July 2008, Kraków Poland.

Journal articles:

Andréa Y. Gordien, Alexander I. Gray, Scott G. Franzblau, Véronique Seidel. Antimycobacterial terpenoids from *Juniperus communis* L. (Cuppressaceae). Journal of Ethnopharmacology Available online 13 September 2009. DOI:10.1016/j.jep.2009.09.007.

Andréa Y. Gordien, Alexander I. Gray, Kevin Ingleby, Scott G. Franzblau, Véronique Seidel. (2009). Activity of Scottish plant, lichen and fungal endophyte extracts against *Mycobacterium aurum* and *Mycobacterium tuberculosis*. Phytotherapy Research. Available online 13 October 2009. DOI: 10.1002/ ptr.2988.