

T.C.

YEDİTEPE UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

DEPARTMENT OF PHARMACOGNOSY

**ISOLATION AND STRUCTURE ELUCIDATION OF
CYTOTOXIC SECONDARY METABOLITES FROM
VALERIANA SPECIES AND INVESTIGATION OF
THEIR MECHANISMS OF ACTION**

DOCTOR OF PHILOSOPHY THESIS

MURAT ERDOĞAN, Pharm.

İstanbul-2022

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MURAT ERDOĞAN, Pharm.

SUPERVISOR

Prof. Dr. Hasan KIRMIZIBEKMEZ

İstanbul-2022

THESIS APPROVAL

Institute : Yeditepe University Institute of Health Sciences
Programme : Pharmacognosy
Title of the Thesis : Isolation and Structure Elucidation of the Cytotoxic Secondary Metabolites From *Valeriana* Species and Investigation of Their Mechanisms of Action
Owner of the Thesis : Murat Erdoğan
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This study have approved as a Doctorate Thesis in regard to content and quality by the Jury.

	Title, Name-Surname (Institution)
Chair of the Jury:	Prof. Dr. Erdem Yeşilada Yeditepe University
Supervisor:	Prof. Dr. Hasan Kırmızıbekmez Yeditepe University
Member/Examiner:	Prof. Dr. Hande Sipahi Yeditepe University
Member/Examiner:	Prof. Dr. Fatih Demirci Anadolu University
Member/Examiner:	Prof. Dr. Didem Şöhretoğlu Hacettepe University

APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated and numbered

Prof. Dr. Bayram YILMAZ

Director of Institute of Health Sciences

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

28/12/2022

Murat Erdoğan



Dedication

This thesis is dedicated to my family for their love, endless support and encouragement.

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LIST OF SYMBOLS and ABBREVIATIONS

†	Overlapping Signals
μ	Micro
1D	1-Dimensional
2D	2-Dimensional
Aav	α-Acetoxyisovaleroyl
Abv	β-Acetoxyisovaleroyl
Ac	Acetyl
ANOVA	Analysis of Variance
Aβ	Amyloid-Beta
Bak	Bcl-2 Homologous Antagonist Killer
Bax	Bcl-2 Associated X
Bcl-2	B-Cell Lymphoma-2
Bcl-2	B-cell Lymphoma-2
BMDM	Bone Marrow-derived Macrophage
Br	Broad
BSA	%1 Bovine Serum Albumin
BuOH	Butanol
Calcd	Calculated
Caspase	CysteinyI Aspartate Specific Proteinase
CC	Column Chromatography
CCK-8	Cell Counting Kit-8
CD ₃ OD	Deuterated Methanol
CDCl ₃	Deuterated Chloroform
CDK	Cyclin Dependent Kinase
CH ₂ Cl ₂	Dichloromethane
CHCl ₃	Chloroform
CNS	Central Nervous System
CoCl ₂	Phosgene, Cobalt(II) Chloride
COLO320	Cellosaurus Cell Line
COSY	Correlation Spectroscopy
COX	Cyclooxygenase
Cr	3-Methylcrotonyl
CRD	Caspase Recruitment Domain
d	Doublet
DED	Death Effector Domain
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
EEG	Electroencephalogram
ERS	Endoplasmic Reticulum Stress
Et	Ethyl
EtOAc	Ethyl Acetate
EtOH	Ethanol
F	Fluorescence indicator
FADD	Fas-associated Death Domain
FBS	Fetal Bovine Serum
FI	Fluorescein Isothiocyanate
Fr / Frs	Fraction / Fractions

FST	Forced-swimming Test
GABA	Gamma-aminobutyric Acid
Gent	Gentabiose
Glc	Glucose
GLOBACON	Global Cancer Observatory
Glu	Glutamic acid
GSC	Glioma Stem Cell
H1N1	Hemagglutinin Type 1 and Neuraminidase Type 1
H ₂ O	Water
HCl	Hydrochloric Acid
HCOOH	Formic Acid
HCV	Hepatitis C Virus
HEK 293T	Human Embryonic Kidney 293 Cells
HepG2	Human Liver Cancer Cells
HIV	Human Immunodeficiency Virus
Hiv	3-Hydroxyisovaleroyl
HMBC	Heteronuclear Multi-Bond Correlation
HPLC	High Performance Liquid Chromatography
HRESIMS	High Resolution-Electrospray Ionisation- Mass Spectrometry
HSQC	Heteronuclear Single Quantum Coherence
i.p.	Intraperitoneal
IC ₅₀	The Half Maximal Inhibitory Concentration
ICR	Institute of Cancer Research
IHD	Index of Hydrogen Deficiency
Iiv	α -Isovaleroxyisovaleroyl
iNOS	Inducible Nitric Oxide Synthase
IR	Infrared Spectroscopy
Iv	Isovaleroyl
LC ₅₀	Median Lethal Concentration
LD50	Median Lethal Dose
LN	Linarin
LPS	Liposaccharide
m	Multiplet
MA	6-Methylapigenin
MABA	Microplate Alamar Blue assay
MBC	Minimum Bactericidal Concentration
MDCK	Madin-Darby Canine Kidney cells
MeCN	Acetonitrile
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MLKL	Mixed Lineage Kinase Domain-like
MPLC	Medium Pressure Liquid Chromatography
MPP ⁺	1-Methyl-4-Phenylpyridinium
MS	Mass Spectrometry
mTOR	Mammalian Target of Rapamycin
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-Tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
Mv	3-Methylvaleroyl
MW	Molecular Weight

NF-κB	Nuclear Factor kappa B
NI	No Inhibition
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
NOESY	Nuclear Overhauser Enhancement Spectroscopy
p.o.	Per os, by mouth
PARP	Poly (ADP-ribose) Polymerase
PBS	Phosphate Buffered Saline
PC12	Pheochromocytoma Cells
PCD	Programmed Cell Death
PDK1	Phosphoinositide-dependent Kinase-1
PE	Petroleum Ether
PI	Propidium Iodide
PILO	Pilocarpine
PS	Phosphatidylserine
PTZ	Pentylentetrazolen
Rut	Rutinoside
q	Quartet
rH ₂ O	Remaining H ₂ O
Rha	Rhamnose
ROS	Reactive Oxygen Species
s	Singlet
SD	Standard Deviation
Sh	Shoulder
SH-SY5Y	Human Dopaminergic Neuroblastoma Cells
Si(CH ₃) ₄	Tetramethylsilane
SiO ₂	Silica Gel
TEAC	Trolox Equivalent Antioxidant Activity
TLC	Thin Layer Chromatography
TLE	Temporal Lobe Epilepsy
TNF	Tumor Necrosis Factor
TST	Tail Suspension Test
UV	Ultraviolet
Va	Valeroyl
Vis	Visible
WHO	World Health Organization
ZOI	Zone of Inhibition

ABSTRACT

Erdoğan, M. (2022). Isolation and Structure Elucidation of Cytotoxic Secondary Metabolites from *Valeriana* Species and Investigation of Their Mechanisms of Action. Yeditepe University, Institute of Health Science, Department of Pharmacognosy, Ph.D. Thesis, İstanbul.

The genus *Valeriana* L. is rich in diverse group of bioactive secondary metabolites. The aim of this thesis was to purify potential cytotoxic compounds from the underground parts of *V. alliariifolia* Adams, *V. sisymbriifolia* Vahl. and *V. speluncaria* Boiss. through cytotoxicity-guided fractionation, and unravel their cell death mechanisms. The *in vitro* cytotoxic activity of crude ethanolic extracts prepared from *Valeriana* species were evaluated against human cancer cell lines including lung (A549), breast (MCF7), gastric (HGC27), prostate (PC3) and glioblastoma (U87) as well as a healthy cell line (HUVEC) by MTS assay. The crude EtOH extracts of *V. alliariifolia* and *V. sisymbriifolia* showed pronounced cytotoxic activity against A549, MCF7, HGC27 and PC3 cancer cells, whereas EtOH extract of *V. speluncaria* inhibited cell proliferation in U87 and MCF7 cells. The crude extracts were further partitioned to yield CHCl₃, EtOAc, *n*-BuOH and rH₂O subextracts. Chromatographic separations on the cytotoxic subextracts (CHCl₃ and EtOAc) yielded 32 compounds, including six new ones, sisymbriifolivaltrate (**17**), sisymbriifolioside (**18**), sisymbriifolins A (**22**) and B (**23**), speluncarins A (**30**) and B (**31**). Their structures were elucidated by extensive NMR and HR-MS analyses. All the isolates were tested on the same cell lines and compounds **1-7** and **15-17** displayed remarkable cytotoxicity with IC₅₀ values close to or less than 10 µM. The mechanisms behind the cytotoxic activities of the most potent ones, IVHD-valtrate (**2**), valtrate (**3**), 1-β-acevaltrate (**4**), seneciovaltrate (**5**), valtrate hydrine B2 (**6**), 1-α-acevaltrate (**15**), 7-homovaltrate (**16**) and sisymbriifolivaltrate (**17**) were evaluated with Annexin V/PI staining, DNA content analysis, and protein levels analysis by using flow cytometry. Compounds **2-6** and **15-17** induced cell death through different mechanisms including apoptosis, necrosis, autophagy and cell cycle arrest, depending on the cancer cell lines.

Key Words: *Valeriana alliariifolia*, *V. sisymbriifolia*, *V. speluncaria*, cytotoxic activity, cell death mechanisms

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ÖZET

Erdoğan, M. (2022). *Valeriana* Türlerinden Sitotoksik Etkili Sekonder Metabolitlerin İzolasyonu, Yapılarının Tayini ve Etki Mekanizmalarının Araştırılması. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Farmakognozi ABD, Doktora Tezi, İstanbul.

Valeriana L. cinsi çeşitli biyoaktif sekonder metabolit grupları bakımından zengindir. Bu tezin amacı, *V. alliariifolia* Adams, *V. sisymbriifolia* Vahl. ve *V. speluncaria* Boiss. türlerinin toprak altı kısımlarından sitotoksikite yönlendirmeli fraksiyonlama ile potansiyel sitotoksik etkili bileşiklerin saflaştırılması ve hücre ölüm mekanizmalarının aydınlatılmasıdır. *Valeriana* türlerinden hazırlanan ham etanol ekstralarının *in vitro* sitotoksik etkileri, akciğer (A549), meme (MCF7), mide (HGC27), prostat (PC3) ve glioblastoma (U87) insan kanser hücre hatlarına ve sağlıklı hücre hattına (HUVEC) karşı MTS yöntemi ile araştırılmıştır. *V. alliariifolia* ve *V. sisymbriifolia*'nın ham EtOH ekstraları, A549, MCF7, HGC27 ve PC3 kanser hücrelerine karşı belirgin sitotoksik aktivite gösterirken, *V. speluncaria*'nın EtOH ekstresi, U87 ve MCF7 hücrelerinde hücre proliferasyonunu inhibe etmiştir. Ham ekstralar üzerinde yapılan partiyon işlemi sonucu CHCl_3 , EtOAc, *n*-BuOH ve H_2O alt ekstraları elde edilmiştir. Sitotoksik alt ekstralar (CHCl_3 ve EtOAc) üzerinde yapılan kromatografik ayrımlar sonucu sisimbrifolivaltrat (17), sisimbrifolozit (18), sisimbrifolin A (22) ve B (23), speluncarin A (30) ve B (31) olarak isimlendirilen altısı yeni toplam 32 bileşik elde edilmiştir. Bileşiklerin yapıları NMR ve HR-MS analizleri ile aydınlatılmıştır. Bütün bileşikler aynı hücre hatları üzerinde test edilmiş ve bunlardan bileşik 1-7 ve 15-17, 10 μM 'a yakın veya daha düşük IC_{50} değerleri ile önemli sitotoksik etki göstermiştir. Güçlü aktivite gösteren bileşikler olan IVHD-valtrat (2), valtrat (3), 1- β -asevaltrat (4), senecivaltrat (5), valtrat hidrin B2 (6), 1- α -asevaltrat (15), 7-homovaltrat (16) ve sisimbrifolivaltrat (17)'ın sitotoksik etkilerinin altında yatan mekanizmalar, Annexin V/PI boyaması, DNA içerik analizi ve protein seviyeleri analizi ile akış sitometrisi kullanılarak incelenmiştir. Bileşik 2-6 ve 15-17 kanser hücre hatlarına bağlı olarak apoptoz, nekroz, otofaji ve hücre döngüsünü durdurma gibi farklı mekanizmalar ile hücre ölümünü indüklemiştir.

Anahtar Kelimeler: *Valeriana alliariifolia*, *V. sisymbriifolia*, *V. speluncaria*, sitotoksik aktivite, hücre ölüm yolları

Bu çalışma Türkiye Bilimsel ve Teknolojik Araştırma Kurumu (TÜBİTAK) tarafından desteklenmiştir. Proje no: 119Z019

1. INTRODUCTION and AIM

Cancer is a serious public health issue, and one of the main causes of death and morbidity globally. According to Global Cancer Observatory (GLOBACON) report in 2020, 19.3 million new incidences and approximately 10 million deaths originated from different cancers types were recorded (1). World Health Organization (WHO) data also demonstrated that cancer is the second reason of death in the world after cardiovascular diseases (2). Although mortality rates of cancers declined by 32% globally from 1991 to 2019, this decline was found to be less than the reduction in rates of cardiovascular disease death (3). Generally, the burden of cancer mortality and incidence is rapidly increasing around the world, and it is estimated to occur 28.4 million cancer cases in 2040 (1).

Cancer is defined by unregulated and uncontrolled cell proliferation as a result of changes in signaling pathways caused by various epigenetic and genetic modifications. It has been revealed that there are multiple relationships between cancer and programmed cell death (PCD). PCD consists of three distinct mechanisms namely apoptosis, necrosis and autophagy, and it is responsible for the balance between cell proliferation and cell death to maintain cellular homeostasis (4,5). Recent studies have shown that PCD is closely related to the treatment of cancer, thus, it has become an important target for novel anticancer drug development (6). Current strategies to treat cancer include surgery, radiotherapy, chemotherapy as well as their combinations. Since chemotherapeutic agents cause substantial serious side effects and multi-drug resistance observed in patients, the cancer treatment process becomes very challenging. Therefore, more potent novel anticancer drugs with fewer side effects are required to fight cancer (7).

Natural resources, in particular medicinal plants and products obtained thereof, play a very important role in the discovery and development of new drug candidates, especially for cancer treatment. Between 1941 and 2014, approximately 49% of the approved small anticancer drugs were natural products or their semi-synthetic derivatives including paclitaxel, irinotecan, omacetaxine, camptothecin, vinorelbine, vinblastine, homoharringtonine, etoposide, vincristine, mepesuccinate, vindesine, topotecan and teniposide (8,9). The reason why natural-based compounds receive attention in cancer chemotherapy can be explained by the relatively less toxicity to healthy cells and induce multiple responses in tumor cells, including programmed cell death pathways (10).

Valeriana L. (Caprifoliaceae, formerly Valerianaceae family) consists of more than 200 species distributed in North America, Asia and Europe. Some members of this genus, particularly *V. jatamansi* and *V. officinalis* are used as folk medicines due to their anxiolytic and sedative properties (11,12). In traditional Turkish folk medicine, the roots of *V. alliariifolia* have been used as cardiostimulant, sedative and antispasmodic (13). A noteworthy number of studies on different extracts of *Valeriana* species as well as the isolated compounds revealed various pharmacological activities such as anticonvulsant, neuroprotective, anti-inflammatory, anxiolytic, antidepressant, antibacterial, cytotoxic and antitumor activities (14–20). Previous phytochemical investigation on the genus *Valeriana* resulted in the isolation of diverse groups of secondary metabolites belonging to iridoid, sesquiterpenoid, flavonoid, lignan and essential oil chemical classes (20,21). Among these secondary metabolites, non-glycosidic ester iridoids and sesquiterpenes have received much attention in recent years due to their remarkable *in vitro* cytotoxic activities (17,22–26). Amongst, valtrate, didrovaltrate, isovaltrate, IVHD-valtrate and jatamanin P were also found to be antitumor agents based on the *in vivo* studies (17,22,26,27). In addition, the decomposition products of valepotriates such as baldrinal, desacylbaldrinal, homobaldrinal, isovaltral, valtrals A-C and valejatanin A were reported to possess noteworthy cytotoxic activities on various cancerous cells (24,28–31). Similarly, the sesquiterpenes including kessyl glycol, kessyl glycol diacetate, 4 α ,5 α -epoxy-8 β -hydroxy-1 α -hydro- α -guaiene, 4 α ,5 α -epoxy-1-hydroxy- α -guaiene and *iso*-teucladiol displayed pronounced cytotoxic effects against different cancer cell lines (25,32).

The genus *Valeriana* is represented by 13 species in the flora of Türkiye, three of which are endemic (33,34). In the view of previous promising bioactivity results on different *Valeriana* constituents and the absence of a study aiming at the isolation of cytotoxic secondary metabolites from *V. alliariifolia*, *V. sisymbriifolia* and *V. speluncaria* through activity-guided fractionation, we aimed to isolate the cytotoxic metabolites from underground parts of these species. Moreover, the cell death mechanisms behind the cytotoxicity of the most potent compounds were also elucidated.

2. GENERAL DESCRIPTION

2.1. General Information about *Valeriana*

2.1.1. Botanical Information

2.1.1.1. Caprifoliaceae

The genus *Valeriana* formerly belonged to Valerianaceae family which was subfamily in Dipsacales order. However, it was placed to its new family Caprifoliaceae based on the phylogenetic studies as well as mitochondrial sequences analysis on members of Dipsacales order. In addition, it was determined that the Dipsacales order is divided into two main families, namely Caprifoliaceae and Adoxaceae (35).

Caprifoliaceae family consists of 41 genera and approximately 960 species which are widely distributed around the world, particularly in northern temperate areas (36). Members of this family are shrubs, small trees, herbs, or lianas. Leaves are simple, opposite, sometimes pinnately compound; stipules are absent; and margin can be entire to serrate and pinnate with pinnate venation. Flowers are bisexual and bilateral. Calyx usually consists of 5 sepals and connate. Corolla usually contains 5 petals (with 2 upper parts and 3 lower parts or a single inferior lobe and 4 superior lobes; lobes can be imbricate or valvate). Androecium have (1-) 4 or 5 stamens and filaments are adnate to corolla. Pollen grains are large and spiny, tricolporate or triplicate. Members contain 2-5 carpels which are connate and ovary is inferior with axile placentation. Fruits can be achene, drupe, berry, capsule with or without endosperm (37).

2.1.1.2. *Valeriana* L.

Valeriana species are perennial glabrous or pubescent herbaceous plants. Stem is simple and erect. Leaves are opposite, simple or pinnately compound, exstipulate. Calyx is composed of 5-16 linear segments which are enrolled in flower and calyx is transformed into pappus in fruit. Corollas are 5-lobed, tube-shaped sometimes gibbous. Inflorescence is umbel, 2- or 3-branched. Flowers are zygomorphic, white, pink, yellowish or slightly grey-blue colored. 3 stamen and 1 pistil are found in reproductive system. Fruits are achene (cypsela) bearing pappus (33,38).

The genus *Valeriana* L. consists of more than 200 species distributed, especially in Europe, Asia, North and South America (39). In flora of Türkiye, the genus is represented by 13 species, three of which are endemic namely *V. speluncaria*, *V. oligantha* and *V. bolkarica* (33,34).

Dichotomous key for Turkish *Valeriana* species is as follows (33,34).

1. Plant with tuber

2. Plant 45-100 cm; basal leaves mostly lobed or simple ovate; tuber fusiform ***dioscoridis***

2. Plant 25-55 cm; basal leaves simple lanceolate, rarely lobed; tuber

3. Flowers bluish-purple; bracts linear-lanceolate c. 5.5-10 mm ***leucophaea***

3. Flowers pink; bracts deltoid-lanceolate c. 3 mm ***tuberosa***

1. Plant with rhizome

4. Basal leaves simple

5. Leaves simple cordate ***allariifolia***

5. Leaves simple lanceolate, ovate or pinnatisect ***alpestris***

4. Basal leaves simple or lobed

6. Plant 15-40 cm; roots with stolon rhizome

7. Leaves glabrous

8. Droopy stem, diameter less than 1 mm; Inflorescence panicle ***oligantha***

8. Erect stem, diameter less than 1-1.5 mm; Inflorescence capitulum or corymbose

9. Inflorescence corymbose; fruit glabrous ***saxicola***

9. Inflorescence capitulum; fruit hairy ***bolkarica***

7. Leaves hairy

10. Plant hermaphrodite, fruit hairy ***speluncaria***

10. Plant dioecious, fruit glabrous ***dioica***

6. Plant (25-) 40-150 cm; roots with thick rhizome

11. Leaves glabrous, leaflets ovate, elliptical-orbicular and entire ***sisymbriifolia***

11. Leaves hairy or glabrous, leaflet lanceolate-ovate or -linear, and entire or dentate

12. Lower stem, leaves and nodes hairy ***phu***

12. Lower stem, leaves and nodes glabrous ***officinalis***

2.1.1.3. *Valeriana alliariifolia* Adams

Plants are perennial, glabrous and 80-140 cm tall. Rhizomes are cylindrical and branched. Leaves are simple; basal leaves cordate with long cauline; upper leaves triangular to ovate, sometimes lanceolate with short cauline. Inflorescence is very dense, two or three branched. Bracts are ovate to lanceolate; c. 1.5-2 mm. Flowers are white sometimes pale pink. Fruits are glabrous achene; c. 4 mm (33,38).

Flowering season: June-August

Habitat: Moist and shady forest grounds

Altitude: 600-3000 m

Distribution in Türkiye: All Anatolian regions particularly Northern Anatolian mountains; Bursa, Bolu, Kastamonu, Amasya, Niğde, Adana, Giresun, Ordu, Artvin, Hakkari Balıkesir, Kütahya, Erzincan, Denizli and Bingöl provinces.

Distribution in World: Caucasia, Greece, Northern Iran (33,38).



Figure 1. Picture of *Valeriana alliariifolia* Adams

2.1.1.4. *Valeriana sisymbriifolia* Vahl.

V. sisymbriifolia is a glabrous perennial plant with 30-60 cm tall. Rhizome is cylindrical which rise to horizontally. Leaves are pinnate (1-) 3-7 (-11)-lobed, leaflets are ovate, orbicular-elliptical. Inflorescence is dense, corymbose and 3-branched. Bracts are c. 2.5-3 mm. Flowers can be pink or white. Fruits are achene and sparsely pubescent (33,38).

Flowering season: June-July

Habitat: Shady steep cliffs and rock crevices

Altitude: 1500-3500 m

Distribution in Türkiye: Widespread in Eastern Anatolian provinces including Erzurum, Erzincan, Van, Sivas, Kahramanmaraş, Adana, Bitlis, Ağrı, Hakkari and Tunceli provinces.

Distribution in World: Southern and Eastern Caucasia, Central Asia, Iraq, Northern Iran and Greece (33,38).



Figure 2. Picture of *Valeriana sisymbriifolia* Vahl.

2.1.1.5. *Valeriana speluncaria* Boiss.

Perennial pubescent plant with 5-15 cm in size. Rhizomes are slender and cylindrical, which grown horizontally underground. Leaves are pinnate (1-5 ovate leaflets). Inflorescence is dense, bracts with c. 2-2.5 mm. Corolla tube is 4-6 mm in size and its color is pink or white. Fruit is a hairy achene. Pollens are in 60-micron size (33,38).

Flowering season: May-June

Habitat: Moist rock crevices and cave entrances

Altitude: 1300-3600 m

Distribution: Endemic. Distributed in the region between Konya-Mersin-Antalya and Hakkari provinces (33,38).



Figure 3. Picture of *Valeriana speluncaria* Boiss.

2.1.1.6. Etymology of *Valeriana*

Although there is no universally accepted etymological information about the word of "*Valeriana*", there are some explanations about the origin of the word. The name of "*Valeriana*" might come from the Latin word "Valere (Valerius)" meaning being healthy or good health. Also, the other meaning of "Valere" is being courages which might be required to drink the infusion prepared from *Valeriana* (40). On the other hand, the words "Phu" or "Fu" were also used instead of "*Valeriana*" (41). *Valeriana* species are called as "Kediotu" in Türkiye because of their attractive effects on cats (33). They are known as Baldrian, Katzenwurz,el,

Baldrianwurzel, Balderbrackenwurzel in German, as valériane in French, as valeriana and amantilla in Italian, as baldrion in Danish (40).

2.1.1.7. Traditional Use of *Valeriana* species

Valeriana species are used medicinally in different parts of the world. The herbal drug “*Valerianae radix*” is originated from the underground parts of *Valeriana officinalis* L., and is listed in many pharmacopoeias including European, German, French, British, Dutch and Turkish Pharmacopoeias. On the other hand, the underground parts of some other species of *Valeriana* are listed in the different pharmacopoeia. For instance, *V. edulis* Nutt. in the Mexican Pharmacopoeia, as *V. wallichii* DC in the Indian Pharmacopoeia, as *V. fauriei* Briq. in the Japanese Pharmacopoeia (40).

Members of the genus *Valeriana*, especially *V. officinalis* and *V. jatamansi* have been utilized for healing purposes since remote times (42,43). The physician Dioscorides Pedanius mentioned the several properties of *Valeriana* root such as diuretic, spasmolytic, relieving pain arising from sides to menses as well as treatment of liver and spleen diseases in his book *De Materia Medica* (44). In addition, another famous physician, Galen was the first to refer to “*Valeriana*” as sedative against sleep disturbances. The Italian herbalist Fabio Colonna, who suffered from epileptic seizures, stated in his book “*Phytobasanos*” that he controlled his own seizures using powdered valerian roots in 1592 (45). In 1597, English herbalist John Gerard suggested valerian for the treatment of minor wounds and hurts in the popular the herbal book “*Herball*”. Furthermore, the sedative effects of valerian were also determined by English doctor John Hill, and he also preferred to drink infusion with milk and sugar to relieve his stress-related headache. Moreover, Canadian Indian warriors put *V. sylvatica* (synonyms of *V. dioica*) in their bags to use as wound antiseptic. In both the First and Second World War, the roots of *V. officinalis* were given to soldiers suffering from “shell shock” as a tranquillizer (40).

Valeriana wallichii (= *V. jatamansi*) (Indian Valerian), known as “Tagara” in Ayurveda, is included in 39 Ayurvedic formulations to allative symptoms of various diseases (46). For instance, the dried-root of Tagara is used for the treatment of asthma, wounds, dry cough, chronic fever, cardiac fever, ulcers as well as the elimination of bad breath (47). Also, the crushed leaves have been topically applied to the forehead for severe headaches whereas rhizomes have been utilized for hair blackening (48). Furthermore, Tagara is one of the plants used to treat *Anidra* (insomnia) in the Ayurveda system (49). In addition, *V. wallichii* has been

preferred to cure snake poisoning, sciatica, anxiety, hysteria, skin diseases, epilepsy and urinary problems in India (47).

In Mexico, the aqueous extract prepared from underground parts of *Valeriana edulis* ssp. *procera* (Mexican Valerian) has been traditionally used as sedative and hypnotic agents (50).

In Peruvian folk medicine, *Valeriana pilosa*, native to the Peruvian and Chilean Andes mountains, were consumed to treat epilepsy and gastric distress as well as for its sedative, anti-inflammatory and sleep-enhancing properties (51).

The crude or baked form of aerial parts of *Valeriana alliariifolia*, have been used as sedative, anodyne and diuretic in Iran traditional medicine (52).

The infusion prepared from leaves and stems of *Valeriana officinalis* is used as demulcent as tea in the evening in Sivrice, Elazığ province at Türkiye (53). Moreover, the root of this species is utilized by local inhabitants in Siirt for its sedative effects (54). On the other hand, a decoction prepared from the roots and aerial parts of *V. alliariifolia* has been consumed internally as antispasmodic, sedative and cardiostimulant. In contrast, decocted aerial part of *V. officinalis* is utilized to treat kidney stones in Eastern Anatolia region (13). *V. dioscoridis*, is called “pisi otu” or “pisik otu” in Kahramanmaraş and the flowers (as infusion) were used for reducing hypertension (55).

2.1.2. Bioactivity Studies on *Valeriana* Species

Valeriana species are well known and widely used medicinal plants worldwide owing to their effects on the neurological diseases including depression, anxiety and epilepsy. It has been stated in many studies that extracts and compounds obtained from the *Valeriana* species show their activities by activating the gamma-aminobutyric acid (GABA)ergic system in the brain (56). Also, the genus *Valeriana* has been given great importance in recent years due to the potent cytotoxic and antitumor activities of non-glycosidic iridoid esters and sesquiterpenes found in their chemical contents against various cancers (26,42). In addition to these well-recognized pharmacological effects of *Valeriana* species, antimicrobial, antiprotozoal, antiviral, anti-inflammatory, enzyme inhibitory, and insecticidal activities were reported (57–62).

2.1.2.1. Studies on Sedative and Anxiolytic Activity

Murphy et al. investigated the *in vivo* anxiolytic activity of *V. officinalis* root extract (3 ml/kg), valerenic acid (3 mg/kg) and GABA-valerenic acid mixture (3.6 µg/kg and 75 µg/kg, respectively) in elevated plus maze test. Then the anxiolytic activities of the rats were evaluated according to the time spent in the open arm of the elevated plus-maze and the number of times they entered the open arm. While ethanol was used as vehicle at the concentration of 1 mg/kg, diazepam (1 mg/kg) was selected as positive control in this study. The root extract was prepared by using 50% EtOH. As a result of the experiment, it was shown that the animals treated with root extract or valerenic acid had a significant decrease in anxious behaviors on rats when compared to the ethanol control group. Moreover, GABA synergistically increased the anxiolytic activity of valerenic acid in this study (15).

The underground parts of *V. glechomifolia* were extracted with CHCl₃ and which was applied to vacuum column chromatography to obtain valepotriate fraction (VF). The anxiolytic and sedative activity of VF were evaluated with *in vivo* animal models including open-field behavior and the elevated plus-maze tests. Saline with a tween 80 (5%, v/v) mixture was used as a vehicle solution whereas diazepam was used as a positive control in these two animal models. In the pre-learning range before each behavioral tests, a single dose of 1, 3 or 10 mg/kg of VF or vehicle was administered intraperitoneally (i.p.) to adult animals. In the open field exploration, animals treated with 10 mg/kg of VF demonstrated a decrease in locomotion and exploratory behavior. On the other hand, mice treated with 10 mg/kg of VF displayed a significant increase in time spent in the open arms, without any significant change in the total number of arm entries. Although mice treated with 3 mg/kg of VF showed a broken novel-object the recognition memory, any VF concentration did not influence fear related memory assessed in an aversive memory test. The findings suggest that VF might show sedative activity and this fraction influences behavioral parameters related to recognition memory (63).

Zhang and co-workers investigated the anxiolytic activity of iridoid fraction (IEFV) obtained from the underground parts of *V. jatamansi*. For this purpose, IEFV were injected to animals at three different concentrations (3 mg/kg, 6 mg/kg and 12 mg/kg) to evaluate their anxiolytic activities with various *in vivo* tests including elevated plus maze, light-dark box, open field drink and Vogel's drinking conflict test. Furthermore, the mechanisms behind the anxiolytic activity were elucidated. As a result, IEFV extracted from *V. jatamansi* demonstrated anxiolytic activity in a dose-dependent manner via increasing GABA levels in CNS (64).

In a study conducted by Marder and colleagues, 6-methylapigenin (MA) and hesperidin (HN) isolated from underground parts of *V. wallichii* were evaluated for their effects on central nervous system by using different behavioral experiments including elevated plus maze test, horizontal wire test, locomotor activity assay, sodium thiopental-induced sleeping-time assay and hole-board test. HN was found as sleep-enhancer and sedative according to these aforementioned *in vivo* experiments. It was also reported that MA demonstrated anxiolytic activity by increasing the sleep-enhancing activity of HN by synergistic effect (65). In a similar study performed by same team in 2004, linarin (LN), isolated from underground parts of *V. officinalis*, was investigated for its anxiolytic activity by using sodium thiopental-induced sleeping-time assay and hole-board test. As a result of the experiment, it was shown that LN had sedative and sleep-increasing properties similar to those of hesperidin and these activities were potentiated when given to rats in combination with valerenic acid. It was also conducted that the hypnotic and sedative activity of Valerian extracts may be ascribed to the presence of linarin, hesperidin, 6-methylapigenin and valerenic acid acting synergistically in the extract (66).

Ugalde and colleagues evaluated the sedative activity of hydroalcoholic extract prepared from the roots of *V. edulis* in combination with six CNS depressants including diazepam, pentobarbital, ethanol, haloperidol, diphenhydramine and buspirone by isobolographic analysis on adult ICR mice. Results demonstrated that there were no synergistic or attenuating effects detected in any of the tested combinations. Thus, *V. edulis* extract did not enhance the sedative activity of CNS depressant drugs (67).

Taibe et al. tested the sleep enhancing effects of *V. officinalis* root extract on elderly women suffering from insomnia in a randomized, double-blind and cross-over controlled trial. Participants (average age= 69.4 ± 8.1) received 300 mg of valerian extract or placebo before 30 minutes of bedtime. At the end of two weeks, no statistically significant differences on sleep parameters including sleep efficiency, wake after sleep onset, self-rated sleep quality and sleep latency were not found in both valerian extract and placebo received groups, respectively. Authors concluded that valerian extract did not provide sleep enhancing properties on elderly women (68).

In a randomized double-blind and placebo-controlled clinical trial conducted on patients suffering from stress which was not severe enough for psychiatric diagnosis, totally 64 nonclinical participants were selected for this clinical trial. The subjects received valerian root extract (VRE) as capsule (100 mg) or a placebo three times each day for four weeks. The stress-

related psychological and anxiety construct of both groups were evaluated using EEG. The EtOH (70%) root extract of *V. officinalis* was standardized to include 0.8 % valerenic acid in 100 mg VRE in each capsule. According to the results, functional brain connectivity related to anxiety was changed in VRE used group (69).

2.1.2.2. Studies on Antidepressant Activity

In a study performed by Oshima et al., methanolic extract of *V. fauriei* roots was investigated in terms of its antidepressant activity on mice by *in vivo* forced-swimming test (FST). The extract showed antidepressant activity by reducing the immobility time of the mice in the FST. Six sesquiterpenoids were isolated from methanolic extract through activity-guided isolation. The antidepressant effects of the purified compounds were also investigated in the same method, and imipramine was used as positive control. Among the isolates, α -kessyl alcohol (30 mg/kg, i.p.) caused a reduction in immobility times in mice. Based on the findings, α -kessyl alcohol was determined as the compound responsible for the antidepressant activity of *V. fauriei* (70).

Sah and co-workers investigated the antidepressant activity of DCM extract prepared from underground parts of *V. wallichii* by FST and locomotor assays on animals. The extract (10, 20 and 40 mg/kg by p.o.) were applied to animals by acute or chronic (once a day for 2 weeks) administration. The results revealed that a single dose of 40 mg/kg acute administered extract significantly inhibited the immobility period in mice. Likewise, extracts administered mice as chronically at doses of 20 and 40 mg/kg, significantly decreased the immobility period, as well as increased the dopamine and norepinephrine levels in forebrain of mice, where it can be concluded that the DCM extract of *V. wallichii* exhibited significant antidepressant activity on mice (71).

In another study conducted by Sah et al., the antidepressant activity of the patchouli alcohol-dominant essential oil of *V. wallichii* (EOVW) was investigated by using FST and locomotor assay on mice. Mice received 10, 20 and 40 mg/kg dose of EOVW by p.o. as acute and chronic (once a day for two weeks) treatment. As a result, acute administration of EOVW to mice with the doses of 20 and 40 mg/kg, which significantly diminished the period of immobility (57.6 and 46.9 %, respectively), without alterations on locomotor activity. On the other hand, the immobility period of mice received 20 mg/kg of EOVW was reduced as 69.9 % in FST. Moreover, norepinephrine and serotonin levels significantly increased as 29 and 19%, respectively, in the chronic treatment of mice with 20 mg/kg (72).

Müller et al. studied the antidepressant activity of supercritical CO₂ extract (SCE) of *V. glechomifolia* by forced-swimming, and tail suspension tests. The effects of the extract on monoaminergic transmission including noradrenergic, dopaminergic and serotonergic systems were also investigated by using reference drugs. The results indicated that SCE (dose range between 0.5-20 mg/kg, p.o.) decreased immobility of the mice in the FST test in mice. In addition, the SCE demonstrated a biphasic dose-response relationship in the TST (1-20 mg/kg p.o.) with no change in locomotor activity as well as in motor coordination. The anti-immobility activity of SCE given to mice with the dose of 5 mg/kg was inhibited by yohimbine (α 2-adrenoreceptor antagonist), sulpiride (D₂ antagonist) and SCH23390 (D₁ antagonist). However, the inhibitory effect of SCE could not be blocked in mice pre-treated with prazosin (α 1-adrenoreceptor antagonist) and *p*-chlorophenylalanine methyl ester (serotonin synthesis inhibitor). Furthermore, the immobility time of the mice in the FST test was significantly reduced when desipramine (5 mg/kg), imipramine (10 mg/kg), and bupropion (3 mg/kg) were separately given to mice in dual combination with SCE (0.25 mg/kg). As a result, it has been stated that *V. glechomifolia* exhibited antidepressant activity through interaction with dopaminergic and noradrenergic systems (73).

Liu and colleagues isolated nine sesquiterpenes, including two new ones from *V. fauriei* roots through activity-guided isolation. In addition, four compounds namely Valerianin A and B, bicyclo [8, 1, 0] 5 β -hydroxyl-7 β -acetoxyl- 5 α ,11,11'-trimethyl-E-1(10)-ene-4 α -15-olide and 8 α -acetoxyl-3 α ,4 α ,10-trihydroxyl-guaia-1(2)-ene-12-6 α -olide were investigated for their *in vivo* antidepressant activities by FST on mice, where bicyclo [8, 1, 0] 5 β -hydroxyl-7 β -acetoxyl- 5 α ,11,11'-trimethyl-E-1(10)-ene-4 α -15-olide and 8 α -acetoxyl-3 α ,4 α ,10-trihydroxyl-guaia-1(2)-ene-12-6 α -olide showed significant antidepressant effect on tested mice of 20 mg/kg (74).

2.1.2.3. Studies on Anticonvulsant Activity

Oliva et al. extracted underground parts of *V. edulis* ssp. *procera* with 70% EtOH to obtain hydroalcoholic extract. Pentylenetetrazolen (PTZ)-induced seizure model was utilized in order to evaluate *in vivo* anticonvulsant activity of hydroalcoholic extract (100, 300 and 1000 mg/kg extracts, respectively). The results revealed that hydroalcoholic extract of all tested doses showed promising anticonvulsant activity in a dose-dependent manner. The duration to beginning of the clonic convulsions were extended from 0.8 to 1.85 min. The extract showed remarkable anticonvulsant activity for prolonging the onset of tonic convulsions from 2.5 to 11.3 min (75).

Hiller and Zetler investigated the anticonvulsant activity of *V. officinalis* root extracts by performing *in vivo* studies. The compounds like harman, pentetrazol and picrotoxin were applied to mice and wait for 30 minutes to observe convulsions on mice. Also, they prepared hydroalcoholic extract (70% EtOH) in order to evaluate its anticonvulsant activity. While the extract did not alter the spontaneous movement, body temperature or nociception on mice, did not generate palpebral ptosis. However, the extract only demonstrated anticonvulsant effect against picrotoxin with the ED₅₀ values ranging from 4.5 to 6 mg/kg. Afterwards, the hydroalcoholic extract were partitioned with *n*-hexane and DCM and their anticonvulsant activities were also tested with same methods. Among the subextracts, DCM subextract exhibited antipicrotoxin activity with ED₅₀ value of 0.25 mg/kg (76).

Zhang et al. evaluated the anticonvulsant activity of baldrinal in pilocarpine (PILO)-induced seizure model on mice. Before the PILO injection, mice were pre-treated with baldrinal or sodium valproate with the different doses. The evoked and spontaneous seizures were evaluated according to EEG recordings and their severity was investigated by using the Racine scale. In addition, the histological changes in the brain tissues were analyzed, and levels of glutamic acid (GLU) and gamma-aminobutyric acid (GABA) in the brain were also measured. A significant increase in GLU levels were observed in baldrinal-restored PILO-treated mice due to treatment with baldrinal. Also, baldrinal led to improved levels of inflammatory agents such as BDNF, TNF- α , NMDAR1 and IL-1 β , as well as reducing the rate of seizures in epileptic animals (18).

In another study by Rezvani et al., anticonvulsant effects of the aqueous extract (AE) and petroleum ether (PE) extracts prepared from *V. officinalis* roots were investigated using the temporal lobe epilepsy (TLE) experimental model on rats. Effects of AE (200, 500 and 800 mg/kg, given i.p.) and PE (50 and 100 mg/kg, given i.p.) and CPT (selective A₁ receptor antagonist, 10 and 20 μ M, given as i.c.) on stage 5 seizure duration (S5D), afterdischarge duration (ADD), and latency to onset of bilateral forelimb clones (S4L) were analyzed. The result revealed that AE (500 and 800 mg/kg) of *V. officinalis* demonstrated anticonvulsant activity by reducing in the ADD and S5D, as well as increasing the S4L. However, PE of *V. officinalis* led to increase seizures on rats. In addition, the anticonvulsant activity of AE to be mediated by the activation of adenosine systems, in line with reduction of the anticonvulsant activity of AE by CPT (77).

González-Trujano and co-workers investigated the anticonvulsant effect of crude EtOH extract of *V. edulis* roots and its valepotriate-rich fraction on the PTZ-induced seizures in Wistar

rats by *in situ* EEG analysis. Ethosuximide (100 mg/kg, i.p.), an anticonvulsant drug, was utilized as reference compound. To evaluate the anticonvulsant activity of *V. edulis* extract and valepotriate-rich fraction (100 mg/kg, i.p.). For this purpose, all rats were intraperitoneally pre-treated with a single dose of PTZ at 35 mg/kg and then waited for 15 and/or 30 minutes. EEG recordings were taken from the frontal and parietal cortices and were investigated to measure ictal behaviors over 60-75 minutes. According to results, a significant reduction on convulsive behavior as well as prohibited changes in the EEG profiles on animals were observed in both extracts, and valepotriate-rich fraction groups when compared to ethosuximide-treated group. Authors stated that anticonvulsant activity of *V. edulis* was associated to valepotriate-type compounds (78).

2.1.2.4. Studies on Neuroprotective Activity

Xu et al. isolated four bakkenolide-type sesquiterpenes from *V. jatamansi* roots. The neuroprotective effect of these compounds was evaluated against MPP⁺ induced cell death in SH-SY5Y (human dopaminergic neuroblastoma) cells by MTT assay. The cell viability rate increased to 88.1%, 97.7% and 105.8% when treated with valerilactone A, bakkenolide H and valerilactone B respectively, at the concentration of 15 μ M. Therefore, these compounds were regarded as potent neuroprotective agents (79).

In a similar study performed by Xu and co-workers, six iridoids were isolated from the *V. jatamansi* roots and their neuroprotective activities were determined with MPP⁺ induced cell death in SH-SY5Y cell line by MTT assay. Chlorovaltrate, valeriandoids A and C, and 1,5-dihydroxy-3,8-epoxyvalechlorine demonstrated moderate neuroprotective activity with IC₅₀ values in the range of 3-30 μ M (80).

In another study conducted by Tan et al., 11 secondary metabolites were purified from hydroalcoholic extract (75% EtOH) of *V. jatamansi* roots and their neuroprotective activities were tested against CoCl₂-induced neuronal cell death in rat pheochromocytoma (PC12) cells by CCK-8 assay. According to the observed results, Isopatrinoside and vibutinal displayed moderate neuroprotective effect against PC12 cells at 10 μ M concentration (81).

13 isolates were obtained from the *V. amurensis* underground parts in a study performed by Wang and colleagues in 2012 (82). In addition, neuroprotective effects of isolates were investigated on amyloid-beta (A β) induced- cell death in PC12 cells using MTT assay. Results showed that seven compounds namely heishuixiecaolines A–C, 8-hydroxypinoresinol-4,4'-di-O- β -D-glucopyranoside, pinoresinol-4,4'-di-O- β -D-glucopyranoside, pinoresinol-8-O- β -D-

glucopyranoside and volvalerenal C were found to be protective against A β ₂₅₋₃₅ induced-cell death in PC12 cells (82).

Wang et al. (83) investigated the neuroprotective activities of 17 compounds that were isolated from *V. amurensis* against A β ₁₄₂ induced-cell death in PC12 cells. According to the results phenolic substances like xiecaoside E, lariciresinol-4,4'-di-O- β -D-glucopyranoside, olivil-4'-O- β -D-glucopyranoside, olivil-4-O- β -D-glucopyranoside, lariciresinol-4-O- β -D-glucopyranoside, 8-hydroxylariciresinol-4'-O- β -D-glucopyranoside, massoniresinol 3a-O- β -D-glucopyranoside, lariciresinol-4'-O- β -D-glucopyranoside and neoarctin A were found as neuroprotective compounds among the tested compounds (83).

The neuroprotective effects of four iridoids from *V. jatamansi* were evaluated against MPP⁺-induced cell death in SH-SY5Y cell line. Among these iridoids, jatadoid A and jatamanvaltrate H demonstrated moderate neuroprotective activity in the concentration ranging from 3-30 μ M (84).

In a study investigating the neuroprotective activity of four sesquiterpenoids (Valeneomerins A-D) isolated from *V. officinalis* var. *latifolia*, Valeneomerins A, B and D displayed significant neuroprotective activity against oxidative stress produced by H₂O₂ in SH-SY5Y cells by increasing cell viabilities to 49.8, 40.9 and 44.6 % at the concentration of each compound as 50 μ M, respectively (85).

Yoo and co-workers demonstrated the neuroprotective activity of *V. officinalis* root extract (70% Ethanolic extract) on hippocampal CA-1 region in forebrain, ischemia-induced animals. The extract (25 and 100 mg/kg) was orally given to animals for 3 weeks (once a day). Reduced motor hyperactivity was observed in the group of which received 100 mg/kg extract after one day of ischemia. Also, extract led to not only decrease microglial activation, but also increase the amount of cresyl violet positive neurons on CA-1 region on animals after 4 days of ischemia (86).

2.1.2.5. Studies on Antimicrobial Activity

Liu et al. isolated eight secondary metabolites including four new ones from the roots of *V. jatamansi* and studied their antibacterial activities against Gram-positive (*Staphylococcus aureus* ATCC25923) and -Negative bacteria (*Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853). Among the isolates, 8-acetoxyl-pathchouli alcohol exhibited weak antibacterial effect on *S. aureus* and *P. aeruginosa* (MIC=128 and 64 μ g/mL, respectively) (42).

In a study conducted by Gu and co-workers, 13 compounds were acquired from CH₂Cl₂ and *n*-hexane extracts prepared from whole parts of *V. laxiflora* via activity-guided isolation. The antitubercular activity of the isolated compounds were evaluated against *Mycobacterium tuberculosis* by MABA (Microplate Alamar Blue assay). According to results, (+)-1-hydroxy-2,6-bis-*epi*-pinoresinol, ursolic acid, oleanolic acid, betulin, 5,7-dihydroxy-3,6,4'-trimethoxyflavone, betulinic acid, tricin and 23-hydroxyursolic acid demonstrated antitubercular activity at the concentration between 15.5 and 127 µg/mL (57).

The *in vitro* antibacterial effects of four extracts (hexane, chloroform, methanol and water extracts) prepared from the aerial parts of *V. wallichii* were tested against plant and animal pathogenic bacteria by utilizing disk diffusion test. Erythromycin (15 µg) and Ampicillin (15 µg) were chosen as positive controls in this study. Aqueous extract of *V. wallichii* demonstrated the highest effect on *S. aureus* [ZOI (zone of inhibition) =23±1.0 mm, MIC=250 µg/mL and MBC=500 µg/mL]. Methanolic extract also displayed important antibacterial activity against *Bacillus subtilis* (ZOI =20±1.0 mm, MIC=31.25 µg/mL and MBC= 500 µg/mL) and *S. aureus* (ZOI=19±0.8 mm, MIC=MBC=125 µg/mL). In addition, hexane extract exhibited remarkable antibacterial activity against *B. subtilis* (ZOI=18±1.2 mm, MIC=MBC=125 µg/mL). However, CHCl₃ extract indicated weak activity against tested bacteria (87).

Agnihotri et al. evaluated the antimicrobial activity of essential oil obtained from whole plant of *V. jatamansi* against 10 microorganisms namely *Candida albicans*, *Bacillus pumilus*, *Aspergillus niger*, *B. subtilis*, *S. aureus*, *Micrococcus epidermidis*, *Saccharomyces cerevisiae*, *S. epidermidis*, *E. coli* and *P. aeruginosa* by disc diffusion method. The essential oil demonstrated potent antimicrobial activity against *B. pumilus*, *C. albicans*, *P. aeruginosa*, *E. coli*, *S. aureus* and *S. epidermidis* in the range of ZOI 18-22 mm (88).

Fuzzati et al. extracted *V. capense* with CH₂Cl₂ and then evaluated its antifungal activity against pathogenic fungus *Cladosporium cucumerinum* by TLC bioautography. The CH₂Cl₂ displayed antifungal activity according to the TLC bioautography results. Therefore, sequential chromatographic separation on this active extract led to the purification of 10 non-glycosidic ester iridioids. Among these isolated compounds, valtrate, didrovaltrate, homovaltrate, homodidrovaltrate, diavaltrate, dihomovaltrate and IVHD-valtrate were tested for their antifungal activities against plant pathogenic fungi *Cercospora arachidicola*, *Venturia inequalis* and *Erysiphe graminis* using the same method. Results revealed that valtrate displayed antifungal activity against all tested fungi and also didrovaltrate exhibited antifungal

effects on *C. arachidicola* and *E. graminis*. In addition, all tested compounds except for homovaltrate, were antifungal against *C. arachidicola* (89).

15 non-glycosidic iridoids were isolated from underground parts of *V. jatamansi* in a study by Quan and colleagues. Their antibacterial effects were evaluated on three Gram-(+) bacteria (*Staphylococcus aureus*, *S. argenteus* and *Streptococcus agalactiae*) and three Gram-(-) bacteria (*Shigella flexneri*, *Salmonella enteritidis* and *Klebsiella pneumoniae*) by Oxford cup double plate assay. The results indicated that 9-*epi*-valtral C significantly inhibited *S. argenteus*, *S. aureus*, *Klebsiella pneumoniae* and *Shigella flexneri*, whereas valeridoid Q displayed promising inhibition against *S. enteritidis* (31).

Letchamo et al. obtained essential oils by hydrodistillation from underground parts of *V. officinalis* which were collected from two different regions (Select and Anthose region) at two different time periods (8th and 14th months). The antibacterial effects of these four essential oils were tested against *E. coli*, *S. aureus* and *P. aeruginosa* by agar overlay technique and *Thymus vulgaris* essential oil was used as a reference. As a result of the experiment, essential oils from the Select region showed strong antibacterial activity against *E. coli* and *S. aureus*, whereas essential oils from the Anthose region displayed weak or no effect. The reason for this situation was explained as the difference in the ratios of the components found in the essential oil due to the factors arising from the harvesting region and time of the plants (90).

In another study, the chemical composition of volatile oils separately obtained from aerial parts (Sample A) and rhizomes (Sample B) of *V. dioscoridis*, were analyzed with GC-MS and the main component of essential oils were identified as patchouli alcohol. The antifungal activities of rhizome essential oil and patchouli alcohol were tested against 15 different fungi strains. The oil from the rhizome and patchouli alcohol were found to exhibit different antifungal activity against the strains tested. Essential oil (MIC: 1.0-10.0 μ L/mL, MFC: 1.5-15.0 μ L/mL) showed stronger antifungal effect than patchouli alcohol (MIC: 5.0-10.0 μ L/mL, MFC: 5.0-15.0 μ L/mL). In addition, the essential oil was found to be more effective than bifonazole, which is known as an antifungal molecule. The main reason why the essential oil showed higher antifungal activity than its main component was explained as the synergistic effect of patchouli alcohol and the other components in essential oil (91).

2.1.2.6. Studies on Antiprotozoal Activity

Glaser et al. isolated 17 secondary metabolites from rhizomes of *V. wallichii* through antileishmanial activity guided isolation. Promastigotes of *Leishmania major* were used for evaluation of *in vitro* antileishmanial activity. Result indicated that four valtrate hydrine type iridoids were found as potent antileishmanial compounds with IC₅₀ values in the range of 0.8-2.3 µg/mL. In addition, two cinnamic acid derivatives including bornyl 3-hydroxycinnamate and bornyl isoferulate (IC₅₀: 12.2 and 16.7 µg/mL, respectively) exhibited moderate antileishmanial effect on *L. major* (59).

Ghosh et al. extracted *V. wallichii* with three different solvents (H₂O, CHCl₃ and MeOH) to obtain crude extracts and then antileishmanial activities of these extracts were evaluated against *L. major* and *L. donovani* by alamarBlue assay. The methanolic (IC₅₀: 44.1 µg/mL) and chloroform (IC₅₀: 9.9 µg/mL) extracts were found to be active on *L. donovani* promastigote, while only chloroform extract demonstrated antileishmanial activities on *L. major* promastigote and amastigote (IC₅₀: 9.0 and 0.8 µg/mL, respectively). Afterwards, chloroform extract was applied to prep-TLC to afford frs. F2 and F3. These two extracts were also investigated their antileishmanial activities on same *Leishmania* species. Results revealed that F2 (IC₅₀: 8.4 µg/mL) and F3 (IC₅₀: 4.1 µg/mL) displayed promising activities on *L. donovani* promastigote, whereas F2 (IC₅₀: 1.3 and 40.1 µg/mL) and F3 (IC₅₀: 0.3 and 7.1 µg/mL) exhibited important antileishmanial effect on *L. major* amastigote and promastigote (92).

Two cinnamic acid bornyl esters namely bornyl 3-phenylpropanoate and bornyl cinnamate were isolated from *V. wallichii* and their antiprotozoal activities were investigated *in vitro* and *in vivo* studies. Bornyl cinnamate demonstrated significant antileishmanial activity on *L. donovani* promastigotes and *L. major* amastigotes with the IC₅₀ values of 15.6 and 10.9 µM, respectively while bornyl 3-phenylpropanoate was moderately active against *L. major* promastigotes (IC₅₀: 50.2 µM). The cytotoxicity of these two compounds were also evaluated against BMDM, HEPG2 and HEK 293T cell lines. No cytotoxic effects of 3-phenylpropanoate was observed, thus this compound was selected for further *in vivo* study. Overall, 3-phenylpropanoate exhibited antileishmanial effect on *L. major*-infected mice at 50 mg/kg (93).

2.1.2.7. Studies on Antiviral Activity

Wang et al. isolated 15 compounds from *n*-BuOH subextract prepared from underground parts of *V. jatamansi*. Moreover, *in vitro* anti-influenza effects of isolated compounds were investigated against H1N1 strains (A/WSN/33/2009) on MDCK cells.

Oseltamivir, an antiviral drug, was used as a positive control. Among the isolates, β -sitosterol and 4-methylphenol displayed antiviral activities with IC_{50} values of 0.99 and 26.49 μ M. Also, β -sitosterol (IC_{50} : 0.99 μ M) was found to be a strong antiviral compound compared to oseltamivir (IC_{50} : 4.0 μ M) (60).

Murakami et al. purified valtrate from the roots of *V. fauriei*. The anti-HIV effect of valtrate was tested using fission-yeast expressing nuclear export signals of Rev (a HIV-1 protein). As a result, valtrate demonstrated anti-HIV activity by inhibiting the transport between the nucleus and cytoplasm. Also, it selectively inhibited 44% of p-24 antigen at 0.5 μ M concentration without exhibiting any cytotoxic activity against MT-4 cells (94).

In a similar study, valtrate was evaluated for its anti-influenza activity on MDCK cells. It significantly regulated the nuclear export of influenza vRNP and inhibited virus production with IC_{50} values of 0.19 μ M (95).

Antiviral activities of *V. wallichii* roots water, methanol and chloroform extracts were investigated in Huh-7.5 cells, infected with the hepatitis C virus (HCV) strain (J6/JFH strain). Among the tested extracts, only methanol extract inhibited the HCV replication with the ratio of 40, 50 and 59% at the concentration of 62.5, 125 and 250 μ g/mL, respectively. Therefore, methanol extract was subjected to column chromatography to afford four subfractions, F1-F4, which were also tested for their anti-HCV potentials. A significant inhibition on viral replication was found only in F4. Moreover, the action mechanism of F4 was revealed with fluorescence assay with NS5B (a viral protein in HCV, RNA dependent polymerase). The authors concluded that methanol extract and its active fraction, F4, exhibited the anti-HCV activities through binding with NS5B protein in HCV (96).

Quan and colleagues purified 14 secondary metabolites from underground parts of *V. jatamansi* and then tested *in vitro* anti-influenza effects of these compounds against the H1N1 influenza virus. However, all tested metabolites were inactive (IC_{50} : >20 μ M) (97).

In a study carried out by Quan et al., anti-influenza activities of 15 iridoids obtained from *V. jatamansi* were evaluated on H1N1 (A/WSN/33) virus infected MDCK cells and oseltamivir was chosen as positive control (IC_{50} : 4.0 μ M). Only Valeridoid Q demonstrated remarkable anti-viral activity with the IC_{50} value of 10.75 μ M (31).

Zhang and co-workers studied the antidiarrheal effect of hydroalcoholic extract (95% EtOH) of *V. jatamansi* in *in vivo* rotavirus (RV)- induced diarrhea models in mice. Animals

were orally treated with hydroalcoholic extract (10, 20 and 30 mg/kg) and combination of extract and ribavirin (30 mg/kg and 60 mg/kg, respectively), and also their antidiarrheal activities were measured with diarrhea scores. Among tested concentrations, only a combination of extract and ribavirin demonstrated significant antidiarrheal activity (98).

2.1.2.8. Studies on Anti-inflammatory Activity

Ming et al. investigated the anti-inflammatory activities of 12 secondary metabolites obtained from the *V. officinalis* var. *latifolia* aerial parts by screening *in vitro* inhibition of cyclooxygenase-2 (COX-2). Also, the possible mechanism between the most potent one and COX-2 was evaluated *in silico* molecular docking studies using some COX-2 proteins including TYR-341, PRO-500, ALA-502, ARG-499, VAL-509 and GLN-178 were performed. According to the results, two lignans namely syringaresinol and 1-hydroxypinoresinol demonstrated pronounced COX-2 inhibitory activity with IC₅₀ values of 0.67 and 6.77 μ M, respectively. In addition, syringaresinol was interacted with the activities of four proteins of COX-2 (GLN-178, TYR-341, ARG-499 and PRO-500) (61).

In another study, 33 compounds obtained from *V. jatamansi* underground parts were evaluated for their anti-inflammatory activities through lipopolysaccharide (LPS)-induced nitric oxide (NO) production in murine macrophage RAW264.7 cells. L-NMMA was used a positive control (IC₅₀: 21.80 μ M) Among the tested compounds, jatamanin U, jatamanin O, valejatadoids D- G, jatamanvaltrate E, jatamanvaltrate Q, jatamanvaltrate K, jatamanvaltrate W, isovaltrate IVHD-valtrate, valeriandoid F, valeriotetrate C and 10-isovaleroxy-valtrathydryn showed NO inhibition compared to L-NMMA. The most significant anti-inflammatory activity was observed in jatamanvaltrate K (IC₅₀: 0.62 μ M) and valeriandoid F (IC₅₀: 0.88 μ M) (20).

Quan et al. performed a similar experiment in order to evaluate the anti-inflammatory effects of 12 iridoids isolated from *V. jatamansi* on the NO inhibition in RAW264.7 cells. Authors declared that rupesin E (IC₅₀: 15.8 μ M) and jatamanin P (IC₅₀: 4.6 μ M) were found to be potent anti-inflammatory compounds due to the demonstration of higher inhibitory effects on NO release than L-NMMA (IC₅₀: 21.8 μ M) (99).

In a similar study, Wang and colleagues investigated the NO inhibitory activities of 12 compounds purified from *V. jatamansi* against LPS-induced BV-2 cells (mouse microglia cells). Among the tested compounds, jatamanvaltrate Q and jatadomins A-E showed anti-inflammatory effect with the IC₅₀ values less than 31 μ M. Amongst, jatamanvaltrate Q (IC₅₀: 0.4 μ M) and jatadomin B (IC₅₀: 9.2 μ M) were potent strong anti-inflammatory compounds.

Moreover, active iridoids demonstrated their activities through interaction with iNOS protein based on the molecular docking study (100).

The chromatographic separation on whole plants of *V. jatamansi* led to the isolation of 18 compounds. The anti-inflammatory effects of the isolates were evaluated via their inhibitory potential of tumor necrosis factor- α (TNF- α), a pro-inflammatory cytokine, released from LPS-stimulated RAW264.7 cells. Syringaresinol displayed promising inhibitory activity on TNF- α release in RAW264.7 cells with the IC₅₀ value of 8.56 μ M. Also, moderate anti-inflammatory activities were seen in jatamanside A, lariciresinol, prinsepiol 4-O- β -D-glucopyranoside, 8-hydroxypinoresinol 4-O- β -D-glucoside, pinoresinol 4-O- β -D-glucopyranoside, 8-hydroxypinoresinol 4'-O- β -D-glucoside and apigenin with IC₅₀ values between 13.85-45.70 μ M (101).

In the study of Jacobo-Herrera et al., three known sesquiterpenes namely acetylvalerenolic acid, valerenal and valerenic acid were purified from the underground parts of *V. officinalis* by bioactivity-directed fractionation technique. EtOAc extract (100 μ g/mL) of *V. officinalis* showed inhibitory activity against NF- κ B in the IL-6/Luciferase(Luc) test on HeLa cells and served for protection against excitotoxicity in primary brain cell cultures at μ M concentrations. In addition, among the isolated compounds, acetylvalerenolic acid and valarenic acid were reported to be as active as parthenolide, NF- κ B inhibitor, at a concentration of 100 μ g/mL. Acetylvalerenolic acid decreased NF- κ B activity to 4% while, valerenic acid decreased NF- κ B activity to 25% (102).

In a study which set out to determine the anti-inflammatory effects of 12 isolates obtained from *V. amurensis* whole parts, only (-)-matairesinol, a lignan derivative, demonstrated a remarkable inhibitory effect on 50% of NO release in LPS-induced RAW264.7 macrophage cells at 1.97 μ M concentration (103).

Wang et al. isolated 27 compounds from *n*-BuOH subextract prepared from *V. jatamansi* underground parts. The inhibitory effects of these isolates were investigated against NO production in LPS-induced RAW 264.7 macrophage cells. According to the result, only ferulaldehyde displayed moderate anti-inflammatory activity by inhibiting 50% of NO-production at the concentration of 27.22 μ M (104).

In a similar study conducted by Wang et al., 15 compounds were purified from the *n*-BuOH extract of *V. jatamansi* underground parts and assessed for their inhibitory potential on NO-production in LPS-stimulated RAW 264.7 cells. L-NMMA (IC₅₀: 20.75 μ M) was utilized

as a positive control. (+)-9'-isovaleroxylariciresinol, baldrinal and leachianone A showed potent anti-inflammatory molecules with their IC₅₀ values of 6.08, 4.21 and 20.36 μ M, respectively (60).

Khuda et al. evaluated the anti-inflammatory activity of methanolic extract prepared from *V. wallichii* leaves in the doses of 50, 100, 200 mg/kg using *in vivo* carrageenan-induced paw edema model. All doses of extract demonstrated significant inhibition on edema in a dose-dependent manner. Among these doses, a significant reduction of edema was observed in animals that received 200 mg/kg of crude extract (105).

Agnihotri et al. determined the topical anti-inflammatory activity of *V. jatamansi* essential oil using xylene-induced edema animal model. Authors described that essential oil demonstrated remarkable anti-inflammatory at the dose of 30 μ M when compared to positive diclofenac (positive control) in acute edema in mouse ear (88).

The anti-inflammatory effects of aqueous (AqE) and methanolic (MeE) extracts prepared from the rhizomes of *V. wallichii* were investigated by Subhan et al using the *in vivo* carrageenan-induced paw edema experimental model. In the experiment, four groups were designed with eight rats in each group. These groups included saline (0.2 mL) as negative control, aspirin (100, 150 and 200 mg/kg p.o.) as positive control, AqE (100, 150 and 200 mg/kg p.o.) and MeE extract (100, 150 and 200 mg/kg p.o.) are given to animals. The rates of edema were measured every hour within three hours after administration of each extract. The findings showed that both AqE and MeE significantly reduced edema, comparable to the reference drug aspirin (106).

2.1.2.9. Studies on Cytotoxic and Anticancer Activity

Tian and co-workers studied the *in vitro* cytotoxic effect of valtrate isolated from *V. jatamansi* against a panel of cancer cell lines including breast (MDA-MB-231, MDA-MB-468, MDA-MB-453, MCF7), ovary (A2780, SK-OV- 3), prostate (LNCaP) and pancreas (PANC1) cancer cells as well as a healthy breast epithelial (MCF-10A) cells by MTT assay. The results revealed that valtrate exhibited strong cytotoxic activity against breast cancer cell lines with the IC₅₀ values in the range of 7.81-9.27 μ M and also demonstrated selectivity without any significant cytotoxicity on MCF-10A (IC₅₀: 30.55 μ M). The mechanism of action behind the cytotoxic activity was elucidated to be induction of valtrate induced apoptosis in MCF7 and MDA-MB-231 cell lines via reducing the expression of caspase 8, p-Akt and cyclin B1 proteins

and also increasing the expression of p-cdc2, p21, cleaved-caspase 7, cleaved-caspase 3, and polypolymerase (PARP) proteins. Moreover, it accumulated the cells in the G₂/M phase (107).

In 2015, Lin et al. isolated five decomposition molecules of valepotriates namely valtrals A-C, homobaldrinal and baldrinal and evaluated their cytotoxic activities against colon (HCT-8), lung (A549), hepatoma (Bel7402) and prostate (PC-3M) cancer cell lines with MTT method. Valtrals A-C were found to exert remarkable cytotoxic activity against HCT-8 and PC-3M cell lines with the IC₅₀ values in the range of 2.8 and 6.5 μ M (29).

In another study of Lin and co-workers, 14 undescribed and 20 known compounds were obtained from the whole parts of *V. jatamansi*. The cytotoxic bioactivities of new compounds were tested against four tumor cell lines including PC-3M, A549, Bel7402 and HCT-8 cell lines. Among the tested compounds, only (+)-9'-isovaleroxylariciresinol, lignan derivative, possessed promising cytotoxicity on HCT-8 and PC-3M cell lines with the IC₅₀ values of 5.3 and 8.1 μ M, respectively (108). In the continuation of this study, the cytotoxic potentials of three new valepotriates (jatamanvaltrate Z1-Z3) were determined on the same cancer cell lines described above. All compounds were described to be strong cytotoxic molecules against tested cancer cell lines. (IC₅₀ for jatamanvaltrate Z1: 2.8- 7.6 μ M; IC₅₀ for jatamanvaltrate Z2: 3.0- 8.3 μ M; IC₅₀ for jatamanvaltrate Z3: 3.6- 6.8 μ M) (23).

Eleven compounds namely Jatamanvaltrate P-Y and nardostachin purified from *V. jatamansi* were assessed for their *in vitro* cytotoxic effects of compounds were determined against PC-3M cell line by using MTT assay. Nardostachin, jatamanvaltrate P and jatamanvaltrates X-Y and were found to have significant cytotoxic activity on PC-3M cell with the IC₅₀ values of 6.22, 1.66, 6.82 and 5.75 μ M, respectively. The structure activity relationships (SAR) of the tested compounds were also studied and, the presence of 10-chlorine, oxirane ring and double bond between C-3 and C-4 are required for the cytotoxicity (109).

Bos and colleagues prepared three valerian tinctures by extracting of underground parts of *V. officinalis*, *V. edulis* ssp. *procera* and *V. wallichii* with 70% EtOH as specified in the European Pharmacopoeia. A portion of freshly obtained tincture (FT) was kept at r.t. for 2 months to obtain stored tincture (ST). Together with these two tinctures, the cytotoxic activities of these two tinctures of each *Valeriana* species and 12 compounds (valtrate, IVHD-valtrate, acevaltrate, isovaltrate, didrovaltrate, baldrinal, isovaltral, homobaldrinal, methyl valerenate, valerenic acid, hydroxyvalerenic acid and acetoxvalerenic acid) predominantly found in *Valeriana* species were investigated against human colon cancer (COLO320) and human small-

cell lung carcinoma (GLC4) cell lines using MTT method. Monoene-type valepotriates namely isovaltrate, acevaltrate and valtrate not only demonstrated remarkable cytotoxic activity against GLC4 cancer cells (IC_{50} : 1.3-2.5 μ M) but also exhibited pronounced cytotoxicity on COLO320 cancer cell line (IC_{50} : 3.0-5.4 μ M). Although didrovaltrate and IVHD-valtrate, diene-type valepotriates, showed significant cytotoxic bioactivities on the tested cell lines (IC_{50} : 2.4-8.9 μ M for GLC4; IC_{50} : 7.2-15.2 μ M for COLO320), the activity was found to be lower than the monoene-type derivatives. Similarly, the cytotoxic activities of valepotriates were decreased to as 10- and 30-fold when they decomposed (baldrinal and homobaldrinal IC_{50} : 53.0 and 31.0 μ M for GLC4 and IC_{50} : 57.0 and 111.0 μ M for COLO320). However, isovaltral, an open-ring decomposition product, exhibited more cytotoxic effects (IC_{50} : 0.4 and 1.2 μ M) than its parent molecule, isovaltrate (IC_{50} : 2.5 and 5.4 μ M) in tested cancer cell lines. Valerenic acid derivatives showed low cytotoxicity against tested cell lines with IC_{50} values ranging from 100 to 200 μ M. When compared to the cytotoxic activities of tinctures, FTs were determined to be more cytotoxic than STs. According to quantitative analysis of tinctures, valepotriates transformed into baldrinals within the storage process. The decrease in the cytotoxic activities of tinctures during the storage period was explained by the decomposition of iridoids to baldrinals (28).

The inhibitory effects of 12 different 3,8-epoxy bearing iridoids isolated from underground parts of *V. jatamansi* on three glioma stem cells including GSC-3, GSC-12 and GSC-18 cancer cells were investigated in a study performed by Quan and workmates in 2019. While rupesin E and jatamanin P demonstrated remarkable cytotoxic effect against GSC-3 cell lines with the IC_{50} values of 6.35 and 10.88 μ g/mL, they displayed pronounced activities towards GSC-18 (IC_{50} : 4.44 and 1.35 μ g/mL, respectively). In GSC-12 cell line, only rupesin E was found to show relatively less toxicity with an IC_{50} value of 13.45 μ g/mL. Furthermore, authors emphasized that the presence of the isovaleroxy group at the first position in 3,8-epoxy iridoid core was found to be essential for the cytotoxic activity (99).

Tan et al. reported the cytotoxic activity of seven compounds from *V. jatamansi* roots against HCT116 cell line and determined the molecular mechanism of the most potent ones. Among the tested molecules, desoxidodidrovaltrate, valtral C and desacylvaltrate displayed promising cytotoxic bioactivities on HCT116 with the IC_{50} values of 2.2, 1.7 and 9.3 μ M, respectively. The mechanism behind the cytotoxicity of valtral C indicated that it inhibited the PDK1 phosphorylation as well as blocked the activation of Akt/mTOR. The Akt/mTOR signal is widely known to play an important role in autophagy inhibition. In addition, valtral C led to

an increase in the level of LC3B-II in HCT116 cancer cell line. Based on these findings, it was shown that valtral C induced cell death in HCT116 cancer cells through autophagy (24).

In another study carried out by Han et al., six previously unreported iridoids were obtained from *V. officinalis* var. *latifolia* and their cytotoxic bioactivities were investigated against A549, HCT116, HepG2 and human breast cancer (SK-BR-3) cell lines by MTT assay. Only (5S,6S,8S,9R)-3-isovaleroxy-6-isovaleroyloxy- Δ^4 ,11-1,3-diol was found to be active against tested cancer cell lines with the IC₅₀ values between 26.13 and 31.81 $\mu\text{g mL}^{-1}$ (11).

Wang et al. obtained 11 molecules after successful chromatographic separation on subterranean parts of *V. jatamansi*. Also, their toxic effects on lung (A549) and gastric (SGC7901) cancer cell lines were determined by MTT assay. However, 8-methoxy-4-acetoxy-3-chlormethyl-10-methylen-2,9-dioxa-tricyclo[4.3.1.0^{3,7}] and 3,8-epoxy-1-O-ethyl-5-hydroxyvalechlorine displayed weak cytotoxicity with the IC₅₀ values of 120.4 and 97.6 μM , respectively (110).

Xu et al. investigated cytotoxic effects of nine diene-type valepotriates from the aboveground parts of *V. sorbifolia* against PC-3M cancer cell line. Among the tested isolates, isovaltrate, valtrate and sorbifolivaltrate A exhibited notable activity with the IC₅₀ values of 3.1, 1.7 and 2.6 μM , respectively. In addition, remaining compounds were found to moderate cytotoxic with the IC₅₀ values ranging from 8.9 to 20.0 μM (111).

In another study conducted by Liu and co-workers, the cytotoxicity of seven compounds purified from *V. jatamansi* roots were evaluated against B16 (melanoma), K562 (leukemia), and HT29 (colon) cancer cells using *in vitro* MTT assay. Only valejatanin A, decomposition product of valepotriates, exhibited promising cytotoxic activities against the tested three cancer cell lines (IC₅₀: 3.53-22.17 μM) (42).

Kırmızıbekmez et al. isolated 12 secondary metabolites from *V. dioscoridis* roots and evaluated the antiproliferative effects of seven iridoids against three gynecological cancer cell lines namely A2780, T47D and HeLa at 10, 30 and 60 μM concentrations by using *in vitro* MTT method. Cisplatin was used as reference drug. As a result of the experiment, authors reported that all tested compounds moderately inhibited the cell proliferation and growth when compared with cisplatin. More than 50% cell growth inhibition was seen in A2780 and T47D cells treated with 10,2'-diacetylpatrinoside at the concentration of 30 μM . Also, 1-epi-bosnorol inhibited the cell growth in the range of 51.73 to 60.67% at 60 μM in all tested cell lines (12).

Chromatographic separations on the underground parts of *V. jatamansi* led to the purification of 15 secondary metabolites. The cytotoxic activities of these compounds were examined against three glioblastoma cancer cell lines (GSC-3, GSC-12 and GSC-18) as well as two healthy cell lines including human normal (HAC) and human glioma (U251) cell lines by MTS assay. The *in vitro* cytotoxicity result of compounds showed that baldrianal (IC₅₀: 21.16-61.81 μ M) and valtral C (IC₅₀: 12.50-23.59 μ M) inhibited the cell growth and proliferation of tested cancer cell lines. Although these two compounds demonstrated weak to moderate cytotoxicity, they were found as selective cytotoxic compounds by displaying without toxicity on HAC and U251 healthy cell lines (31).

Tang et al. reported the *in vitro* cytotoxic bioactivities of eight iridoids (jatavaleridoids A-H) against HeLa and PC3 cancer cell lines by utilizing MTT assay. Among the tested compounds, only jatavaleridioid G (IC₅₀: 19.64 μ M) displayed modest cytotoxic effect on PC3 cancer cell line (112).

Yang et al. studied the anticancer effects of jatamanvaltrate P against breast cancer cell lines including MCF7, MDA-MB-468 MDA-MB-453 and MDA-MB-231. The results revealed that jatamanvaltrate P inhibited the cell proliferation and growth in tested cell lines in concentration-dependent manner and also it exhibited relatively low toxicity on MCF-10A (healthy breast epithelial cell line). Also, it was found that jatamanvaltrate P arrested the cell cycle at G₂/M phase in MDA-MD-type cancer lines, and at G₀/G₁ phase in MCF7 cell line. Studies on mechanism behind the cytotoxicity revealed that jatamanvaltrate P not only induced the cleavage of caspases and PARP, but also reduced the expression of Cdc-2, cyclin D1 and cyclin B1 levels. Jatamanvaltrate P led to an increase in the levels of LC3-II and also promoted autophagosome formation. Further, an *in vivo* study showed that jatamanvaltrate P (15 mg/kg, i.p. three times per week for 30 day) inhibited the tumor growth and progression in mice bearing MDA-MB-231 cancer xenograft (17).

Zhu et al. prepared a crude EtOH extract from *V. jatamansi* and then obtained CH₂Cl₂ CH₂Cl₂ subextract (DEVJ) via partition. Further, DEVJ was subjected to ion-exchange chromatography to obtain F3 fraction. MTT assay was carried out to determine the cytotoxicity of F3 on cell growth in human breast cancer (MCF7, MDA-MB-231, MDA-MB-468 and MDA-MB-453) cell lines and a healthy cell line (MCF-10A). F3 significantly inhibited the cell growth in tested cancer cell lines (IC₅₀: 2.91-7.56 μ g/mL) without any cytotoxic effect on MCF-10A (IC₅₀: 24.34 μ g/mL). In addition, effects of F3 on ROS level, mitochondrial membrane potential and apoptosis were described via flow cytometry. In order to determine the DNA damage and

expression of phosphor-histone H2AX (γ -H2AX) of F3, immunofluorescence and comet assays were used. The expression of related proteins was evaluated with western blott test. Autophagy was followed by acridine orange (OA) staining as well as fluorescence microscope. Mechanistic studies revealed that F3 induced apoptosis through DNA damage occurred by γ -H2AX activation and DNA strand fracture. This situation might be related to ROS production. F3 also induced apoptosis by forming autophagosome and increasing LC3-II levels. Furthermore, *in vivo* xenograft model of MDA-MB-231 cell was used to evaluate the antitumor activity of F3. As a result, F3 significantly reduced the tumor growth compared to control group and this *in vivo* antitumor effect was mediated by DNA damage (113).

In a study by Li et al., the anticancer properties of IVHD-valtrate isolated from *V. jatamansi* was investigated against ovarian cancer cells both in *in vitro* and *in vivo* experiments. It has been found that IVHD-valtrate might be a selective cytotoxic compound against ovarian cancer cells due to its strong cytotoxic bioactivities against cancer cells as well as low cytotoxic effect on healthy ovarian cell line (IOSE144). According to mechanistic studies, IVHD-valtrate caused the cell cycle arrest in G₂/M phase and also induced apoptosis. Moreover, a significant reduction in OVCAR-3 and A2780 xenograft tumor was observed when animals treated with IVHD-valtrate. Further *in vitro* and *in vivo* mechanistic studies revealed that IVHD-valtrate increased the expression of p53, p21, p27 and Rb, while it decreased the level of Cyclin B1, Cdc25C, E2F1, Mdm2 and Cdc2. On the other hand, IVHD-valtrate induced the cleavage of caspases and PARP, and reduced the ratio of Bcl-2/Bad and Bcl-2/Bax (27).

Bounthan et al., purified baldrinal, valtrate and didrovaltrate from *V. wallichii* roots and evaluated their cytotoxic activities on liver cancer (HTC) cell line by trypan blue staining method. When all compounds tested at the same concentration (33 μ g/mL), valtrate displayed two-times more cytotoxicity than didrovaltrate and eight-times more cytotoxicity than baldrinal. Although valtrate was found the most potent compound, didrovaltrate was selected for further *in vivo* study because its crystalline form led to structure more stable. The treatment with didrovaltrate (1.25 mg/kg, i.p.) rapidly decreased the tumor growth in KREBS II induced ascites in mice. This rapid antitumor activity of didrovaltrate explained by the direct connection of didrovaltrate and tumor cells when administrated didrovaltrate as i.p. (22).

In a study investigating anticancer activity of valtrate, Chen et al. found that valtrate demonstrated notable cytotoxicity against pancreatic cancer cell lines (IC₅₀: 1.98-10.15 μ M for PC-1, PC-3, Capan-2, Hs 766t and PANC-1) without affecting healthy pancreatic cell line (HDPE). Valtrate activated significantly apoptotic pathway and also arrested cell cycle at G₂/M

phase. After mice were treated with 10 mg/kg valtrate, 61% inhibition of tumor growth was observed after 21 days in the PANC-1 xenograft mice. Mechanistic studies showed that valtrate led to a reduction in the expression of Stat3 and phosphorylated-Stat3 levels and to blocked the Stat3 transcriptional activity. As a result of *in silico* docking study, valtrate showed its cytotoxic effect by binding Cys712 in Stat3 protein (26).

2.1.2.10. Studies on Enzyme Inhibition

In another research conducted by Sarikurkcu et al., three extracts (MeOH, EtOAc and dH₂O) prepared from *V. dioscoridis* above grounds were investigated for their enzyme inhibitory effects including tyrosinase and α -amylase enzymes. Also, chemical profile of these three extracts were also described by LC-ESI-MS. The EtOAc extract (IC₅₀=2.17 mg/mL) displayed highest inhibitory effect on α -amylase while the MeOH extract (IC₅₀=2.53 mg/mL) exhibited moderate inhibitory activity on tyrosinase enzyme (43).

In another study conducted by Wang et al., valerianofal, volvalerenic acid A and actinidine were isolated from *V. officinalis* var. *latifolia* roots and then their AChE and BuChE inhibitory activities were determined with Ellman's method. Valerianofal and actinidine demonstrated significant BuChE inhibitory effects with the IC₅₀ values of 2.8 and 0.4 μ g/mL, respectively. Among the tested compounds, only actinidine exhibited AChE inhibitory activity (IC₅₀= 20.9 μ g/mL) (62).

Chen et al. evaluated the AChE inhibitory activities of five compounds isolated from *V. officinalis* var. *latifolia* by using *in vitro* Ellman assay. Among the isolated compounds, volvalerenal acid K exhibited pronounced AChE inhibitory effect with the IC₅₀ value of 0.161 μ M. Thus, the *in vivo* AChE inhibitory effect effects of volvalerenal acid K on animals were evaluated with Morris water maze test. The levels of acetylcholine (ACh), AChE, and acetylcholine transferase (AChT) were also determined by colorimetric assay. The authors concluded that volvalerenal acid K improved the memory and learning abilities of tested animals through regulating enzymes found in the cholinergic system (114)

2.1.2.11. Studies on Insecticidal Activities

Feng and co-workers investigated the insecticidal effects of *V. officinalis* volatile oil and its major constituents namely bornyl acetate and camphene against *Tribolium castaneum* (ref flour beetles) and *Liposcelis bostrychophila* (Booklice). Volatile oil exhibited significant contact and fumigant toxicity on *L. bostrychophila* (LD₅₀= 50.9 μ g/cm² and LC₅₀= 2.8 mg/L

air) and also the remarkable contact toxicity on *T. castaneum* with $LD_{50} = 10 \mu\text{g/adult}$. In addition, camphene and bornyl acetate showed promising contact and fumigant toxicity on both tested insects ($LD_{50} = 701.3, 32.9 \mu\text{g/cm}^2$ and $LC_{50} = 10.1, 1.1 \text{ mg/L}$ for *L. bostrychophila*; $LC_{50} = 21.6, 66.0 \mu\text{g/adult}$ and $LD_{50} = 4.1, >126.3 \mu\text{g/cm}^2$ for *T. castaneum*) (115).

In a similar study conducted by Liu et al., patchoulol, 3-methylvaleric acid and isovaleric acid isolated from essential oil through insecticidal activity-guided isolation study. The essential oil and isolates were investigated for their insecticidal activities against booklice by fumigant (FT) and contact toxicity (CT) tests. The essential oil demonstrated promising contact and fumigant toxicities against *L. bostrychophila* (FT, $LC_{50} = 6.0 \text{ mg/L}$; CT, $LC_{50} = 236.4 \mu\text{g/cm}^2$). Among the tested compounds, while patchoulol showed best acute toxicity on booklice with the LC_{50} value of $61.35 \mu\text{g/cm}^2$, isovaleric acid and 3-methylvaleric acid displayed significant fumigant toxicity ($LC_{50} = 5.67$ and 5.53 mg/L , respectively) (116).

Dua et al. evaluated the insecticidal activity of essential oil obtained from *V. jatamansi* root against five mosquitoes including *Culex quinquefasciatus*, *Anopheles culicifacies*, *Anopheles stephensi*, *Aedes albopictus* and *Aedes aegypti*. Essential oil demonstrated larvicidal activities against tested mosquitoes with the IC_{50} values ranging from 51.2 and 80.6 mg/L. The LC_{50} values of the essential oil were found in the range of 0.08-0.16 mg/cm², whereas essential oil killed 90% of population with the concentration range from 0.21 to 0.34 mg/cm². Authors concluded that *V. jatamansi* root essential oil demonstrated both adulticidal and larvicidal against the stated mosquitoes (58).

2.1.2.12. Studies on Other Activities

Antibronchospastic and anticoronaryspastic activities of aqueous and ethanolic extract prepared from roots of *V. officinalis* were evaluated on anaesthetized guinea pigs by Circosta and co-workers. For this purpose, pressor response and pitressin-induced coronary spasm experiments were used for the determination of protective effects of these two extracts with the concentration of 50, 100 and 200 mg/kg. Also, histamine- and antigen-induced broncospasm models were used for the evaluation of antibronchospastic effects of extracts. The results indicated that these two extracts had significant antihypertensive, antibronchospastic and anticoronaryspastic activities (117).

In a study by Piccinelli and colleagues, six compounds were obtained from *V. prinophylla* roots, and vasorelaxant activities of main extract (ethanolic) and isolates were also determined by organ bath experiment on Wistar albino male rats. Authors revealed that

ethanolic extract demonstrated more vasorelaxant activity than isolated compounds in animals. Among the isolates, 8-hydroxypinoresinol, a lignan aglycone, was found to be most potent vasorelaxant compound. In addition, antioxidant activity of isolates was also evaluated with chemiluminescence and Trolox equivalent antioxidant activity (TEAC) assays. As a result, 8-hydroxypinoresinol and prinsepiol demonstrated strong antioxidant effect (118).

2.1.3. Phytochemical Studies on *Valeriana* species

The previous phytochemical studies on *Valeriana* species revealed that the genus contains diverse group of secondary metabolites particularly iridoids, sesquiterpenoids, lignans, essential oils and flavonoids. The isolated compounds from *Valeriana* species were complied from literature and given in the figures as well as in tables (Tables 1-57). In addition, the chemical structures of the substituted groups in the compounds, which were given with their abbreviations, were presented below (Figure 4). Among the isolated compounds from the genus *Valeriana*, iridoids, especially non-glycosidic ester ones represent the main class of secondary metabolites.

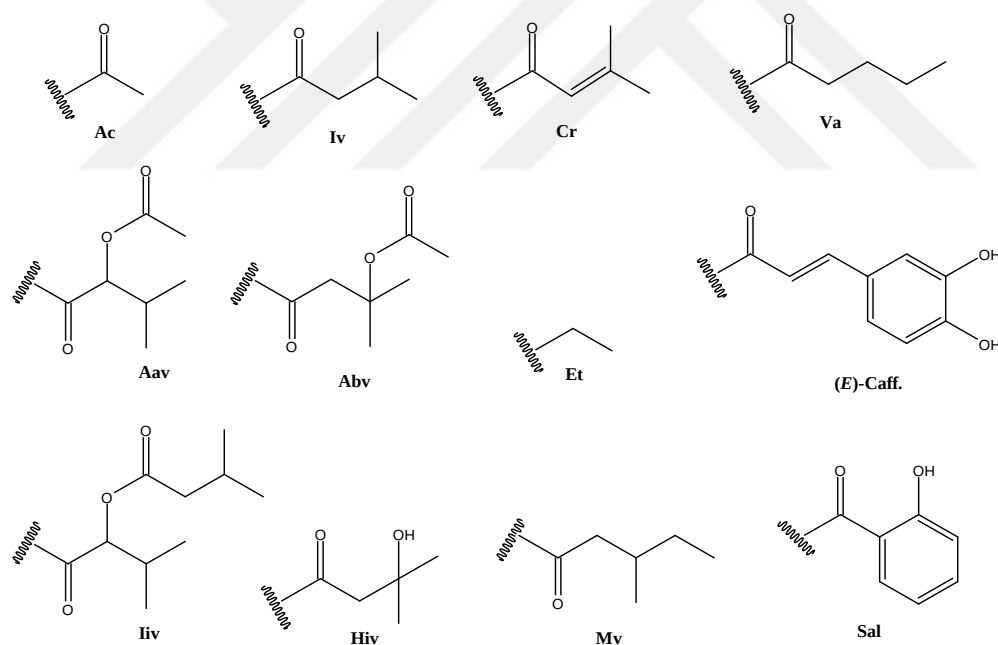


Figure 4. Substituted groups found in secondary metabolites of *Valeriana* species

2.1.3.1. Sesquiterpenes

2.1.3.1.A. Kessane-type Sesquiterpenes Isolated from *Valeriana* species

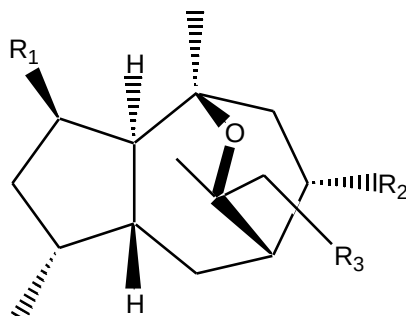


Table 1. Kessane-type sesquiterpenes isolated from *Valeriana* species

Compounds	R ₁	R ₂	R ₃	Species	Ref
Kessane	H	H	H	<i>V. wallichii</i> <i>V. himalayana</i> <i>V. hardvickii</i> <i>V. pyrolaefolia</i> <i>V. fauriei</i> <i>V. officinalis</i> <i>V. turkestanica</i> <i>V. javanica</i>	(119–122)
Kessyl-6-ol (kessanol)	H	OH	H	<i>V. fauriei</i>	(70)
Kessyl-6- acetate (kessanyl acetate)	H	OAc	H	<i>V. hardvickii</i> <i>V. fauriei</i>	(119,121)
Kessyl-2-ol (α -kessyl alcohol)	OH	H	H	<i>V. fauriei</i> <i>V. wallichii</i>	(59,70)
Kessyl glycol 8-acetate	OH	OAc	H	<i>V. fauriei</i> <i>V. officinalis</i>	(119,123)
Kessyl glycol	OH	OH	H	<i>V. fauriei</i> <i>V. officinalis</i> <i>V. turkestanica</i>	(70,119,122)
Kessyl glycol 8-O- glucopyranoside	OH	O- β -glc	H	<i>V. fauriei</i>	(119)

Table 1. Kessane-type sesquiterpenes isolated from *Valeriana* species (Continued)

Compounds	R ₁	R ₂	R ₃	Species	Ref
Kessyl-2-acetate (α -kessyl acetate)	OAc	H	H	<i>V. fauriei</i> <i>V. officinalis</i> <i>V. hardvickii</i> <i>V. himalayana</i> <i>V. turkestanica</i>	(119,122,123)
Kessyl glycol diacetate	OAc	OAc	H	<i>V. fauriei</i> <i>V. officinalis</i> <i>V. himalayana</i>	(70,119,121, 123)
Kessyl glycol 2-acetate	OAc	OH	H	<i>V. fauriei</i>	(119)
α -kessyl isovalerate	Olv	H	H	<i>V. amurensis</i>	(124)
Valerol A	H	H	OH	<i>V. officinalis</i>	(125)
Valeracetate	H	H	OAc	<i>V. hardvickii</i> <i>V. himalayana</i> <i>V. officinalis</i>	(121,123–125)

2.1.3.1.B. Valerane-type Sesquiterpenes Isolated from *Valeriana* species

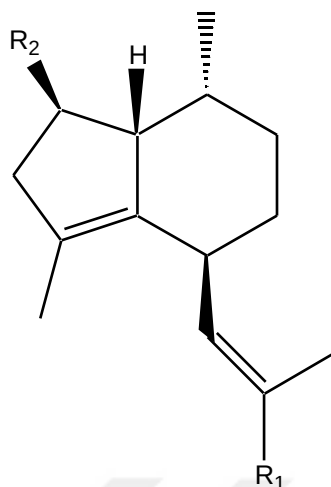
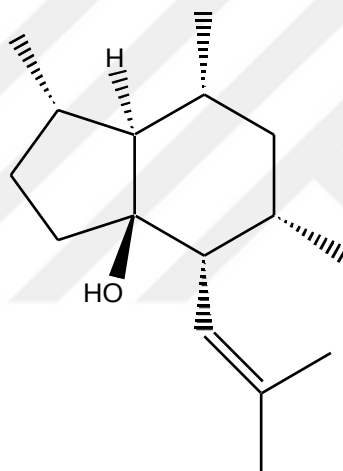


Table 2. Valerane-type sesquiterpenes isolated from *Valeriana* species

Compounds	R ₁	R ₂	Species	Ref
Valerenal	CHO	H	<i>V. officinalis</i> <i>V. turkestanica</i> <i>V. wallichii</i> <i>V. phu</i> <i>V. sambucifolia</i>	(122,126–130)
Valerenic acid	COOH	H	<i>V. officinalis</i> <i>V. fauriei</i> <i>V. turkestanica</i>	(126,131–133)
Hydroxyvalerenic acid	COOH	OH	<i>V. officinalis</i>	(133)
Acetoxyvalerenic acid	COOH	OAc	<i>V. officinalis</i> <i>V. turkestanica</i>	(122,132)
Valerenol	CH ₂ OH	H	<i>V. officinalis</i> <i>V. turkestanica</i> <i>V. italica</i> <i>V. pancicii</i> <i>V. celtica</i> <i>V. sambucifolia</i>	(122,127,128,132,134–136)

Table 2. Valerane-type sesquiterpenes isolated from *Valeriana* species (Continued)

Compounds	R ₁	R ₂	Species	Ref
(<i>E</i>)-Valerenyl acetate	CH ₂ OAc	H	<i>V. turkestanica</i> <i>V. officinalis</i> <i>V. collina</i> <i>V. celtica</i>	(122,137,138)
(<i>E</i>)- Valerenyl isovalerate	CH ₂ Oiv	H	<i>V. collina</i> <i>V. officinalis</i> <i>V. turkestanica</i>	(122,132,137)

**Table 3.** Valerane-type sesquiterpenes isolated from *Valeriana* species

Compound	Species	Ref
(-)- Pacifigorgiol	<i>V. phu</i> <i>V. collina</i> <i>V. celtica</i> <i>V. officinalis</i>	(129,137–139)

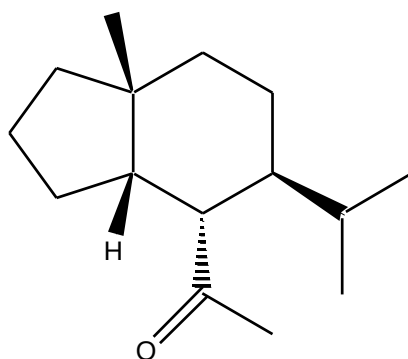


Table 4. Valerane-type sesquiterpenes isolated from *Valeriana* species

Compound	Species	Ref
Faurinone	<i>V. collina</i> <i>V. officinalis</i>	(137,140)

2.1.3.1.C. Elemene-type Sesquiterpenes Isolated from *Valeriana* species

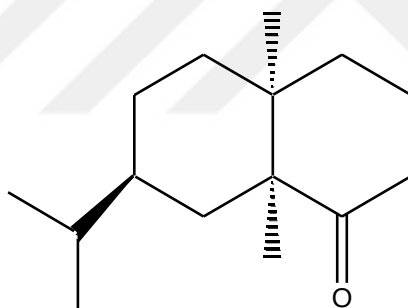


Table 5. Elemene-type sesquiterpenes isolated from *Valeriana* species

Compound	Species	Ref
Valeranone	<i>V. stenoptera</i> <i>V. wallichii</i> <i>V. himalayana</i> <i>V. pyrolaefolia</i> <i>V. officinalis</i>	(59,121,124,131)

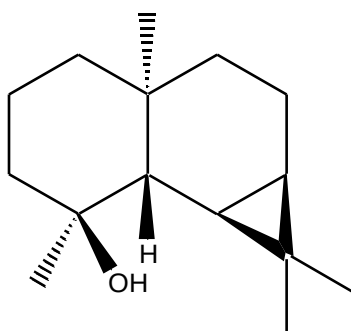


Table 6. Elemmane-type sesquiterpenes isolated from *Valeriana* species

Compound	Species	Ref
Maaliol	<i>V. wallichii</i>	(119,121,137,141–143)
	<i>V. himalayana</i>	
	<i>V. hardvickii</i>	
	<i>V. dioscoridis</i>	
	<i>V. sisymbriifolia</i>	
	<i>V. collina</i>	
	<i>V. fauriei</i>	

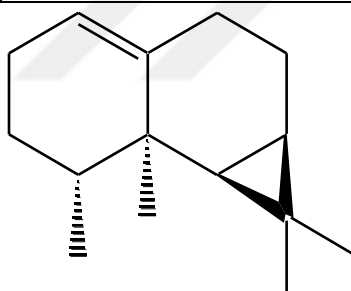


Table 7. Elemmane-type sesquiterpenes isolated from *Valeriana* species

Compound	Species	Ref
β -Gurjunene	<i>V. wallichii</i>	(90,121,141,144–146)
	<i>V. himalayana</i>	
	<i>V. hardvickii</i>	
	<i>V. pyrolaefolia</i>	
	<i>V. sisymbriifolia</i>	
	<i>V. montana</i>	
	<i>V. amurensis</i>	
	<i>V. officinalis</i>	

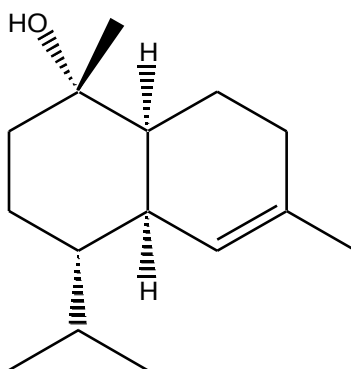


Table 8. Elemmane-type sesquiterpenes isolated from *Valeriana* species

Compound	Species	Ref
<i>Epi-α-muurolol</i>	<i>V. officinalis</i> <i>V. hardwickii</i>	(121,142,145)

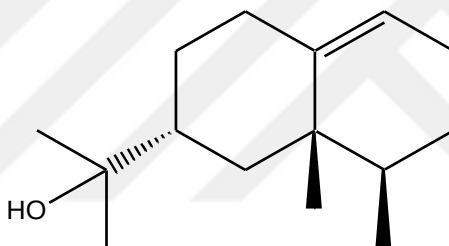


Table 9. Elemmane-type sesquiterpenes isolated from *Valeriana* species

Compound	Species	Ref
Valerianol	<i>V. phu</i> <i>V. hardwickii</i>	(121,129)

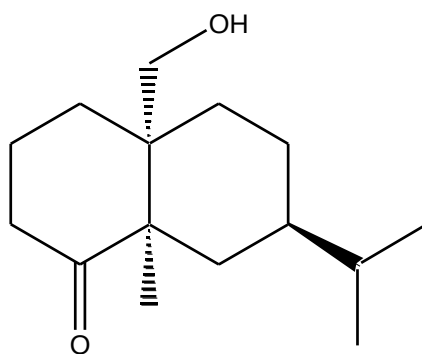


Table 10. Elemane-type sesquiterpenes isolated from *Valeriana* species

Compound	Species	Ref
Kanokonol	<i>V. fauriei</i>	(70)

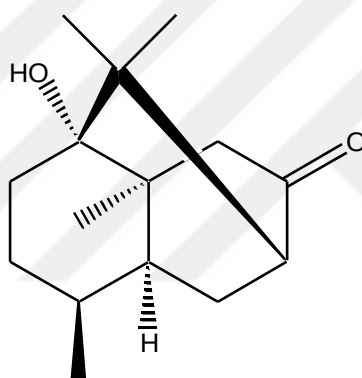


Table 11. Elemane-type sesquiterpenes isolated from *Valeriana* species

Compound	Species	Ref
Valeriananoid A	<i>V. wallichii</i>	(80,147)

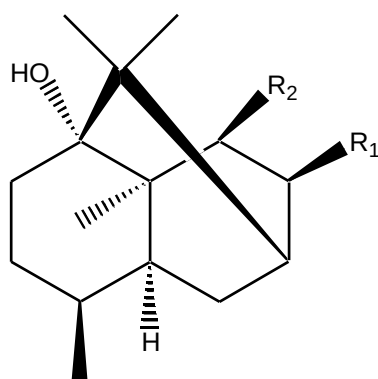


Table 12. Elemmane-type sesquiterpenes isolated from *Valeriana* species

Compounds	R ₁	R ₂	Species	Ref
Patchouli alcohol (Patchoulol)	H	H	<i>V. jatamansi</i> <i>V. stenoptera</i> <i>V. sisymbriifolia</i> <i>V. phu</i> <i>V. pyrolaefolia</i> <i>V. celtica</i> <i>V. fauriei</i> <i>V. officinalis</i>	(129,136,138,145,148–151)
8-acetoxy patchouli alcohol (Valeriananoid c)	OAc	H	<i>V. jatamansi</i> <i>V. stenoptera</i> <i>V. amurensis</i> <i>V. fauriei</i>	(80,124,148,150,152)
8-hydroxypatchoulol (Valeriananoid b)	OH	H	<i>V. fauriei</i> <i>V. stenoptera</i> <i>V. jatamansi</i> <i>V. amurensis</i>	(124,147,148,151)
9-hydroxypatchoulol	H	OH	<i>V. stenoptera</i>	(151)
9-acetoxypatchoulol	H	OAc	<i>V. stenoptera</i>	(151)

2.1.3.2. Iridoids

2.1.3.2.A. Valepotriates

2.1.3.2.A.1. Monoene-type Valepotriates Isolated from *Valeriana* species

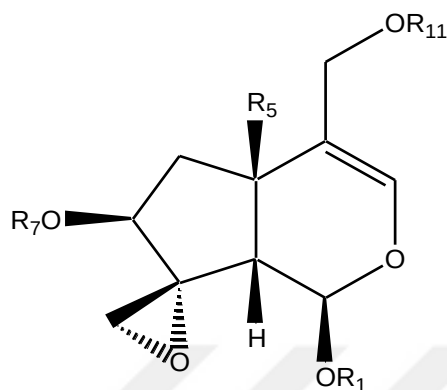


Table 13. Monoene-type valepotriates isolated from *Valeriana* species

Compounds	R ₁	R ₅	R ₇	R ₁₁	Species	Ref
Didrovaltrate	Iv	H	Ac	Iv	<i>V. jatamansi</i> <i>V. officinalis</i> <i>V. vaginata</i> <i>V. glechomifolia</i> <i>V. capense</i> <i>V. microphylla</i> <i>V. mexicana</i> <i>V. sambucifolia</i>	(22,89,153–160)
Isodidrovaltrate	Iv	H	Iv	Ac	<i>V. vaginata</i> <i>V. microphylla</i>	(157,158,161)
Homodidrovaltrate	Mv	H	Ac	Iv	<i>V. jatamansi</i> <i>V. capense</i> <i>V. microphylla</i>	(89,161,162)

Table 13. Monoene-type valepotriates isolated from *Valeriana* species (*Continued*)

Compounds	R ₁	R ₅	R ₇	R ₁₁	Species	Ref
Isovaleroxyhydroxy didrovaltrate (IVHD-valtrate)	Iv	OH	Ac	Iiv	<i>V. edulis</i> ssp. <i>procera</i> <i>V. microphylla</i> <i>V. capense</i> <i>V. sambucifolia</i> <i>V. wallichii</i> <i>V. officinalis</i> <i>V. edulis</i> <i>V. polystachya</i> <i>V. fauriei</i>	(28,117, 131,159, 161,163– 165)
IIHD-acevaltrate	Ac	OH	Ac	Iiv	<i>V. polystachya</i>	(164)
11- homohydroxydihydrovaltrate	Iv	OH	Ac	Mv	<i>V. wallichii</i>	(162)
5-hydroxydidrovaltrate	Iv	OH	Ac	Iv	<i>V. wallichii</i>	(166)
Jatamanvaltrate L	Iv	OH	H	Iiv	<i>V. wallichii</i>	(166)
Jatamanvaltrate M	Iv	OH	Ac	Et	<i>V. wallichii</i>	(166)

2.1.3.2.A.2. Diene-type Valepotriates Isolated from *Valeriana* species

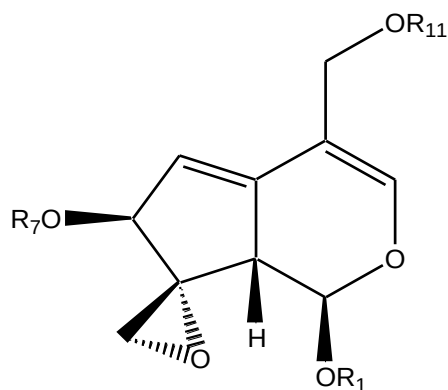


Table 14. Diene-type valepotriates isolated from *Valeriana* species

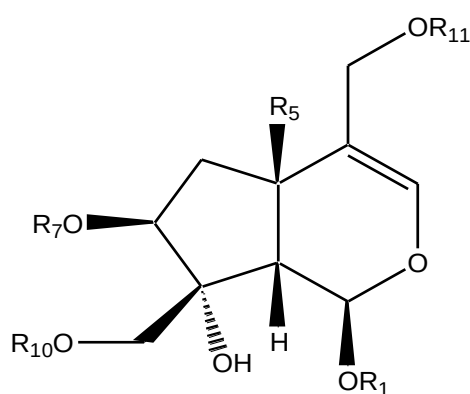
Compounds	R ₁	R ₇	R ₁₁	Species	Ref
Valtrate	Iv	Iv	Ac	<i>V. wallichii</i> <i>V. officinalis</i> <i>V. fauriei</i> <i>V. edulis</i> ssp. <i>procera</i> <i>V. alliariiifolia</i> <i>V. capanse</i> <i>V. javanica</i> <i>V. sambucifolia</i> <i>V. microphylla</i> <i>V. sisymbriifolia</i> <i>V. glechomifolia</i> <i>V. sorbifolia</i> <i>V. pseudofficinalis</i> <i>V. polystachya</i>	(22,78,89,111,120,153,156,158,161,164,165,167–173)

Table 14. Diene-type valepotriates isolated from *Valeriana* species (Continued)

Compounds	R ₁	R ₇	R ₁₁	Species	Ref
Isovaltrate	Iv	Ac	Iv	<i>V. mexicana</i> <i>V. wallichii</i> <i>V. thalictroides</i> <i>V. microphylla</i> <i>V. sambucifolia</i> <i>V. sorbifolia</i> <i>V. glechomifolia</i> <i>V. fauriei</i> <i>V. officinalis</i>	(111,161,162,165,170,174–178)
Diavaltrate	Iv	Ac	Ac	<i>V. wallichii</i> <i>V. sambucifolia</i> <i>V. microphylla</i> <i>V. glechomifolia</i>	(127,158,163,171)
11- α -acevaltrate	Iv	Ac	Aav	<i>V. microphylla</i>	(161)
1- α -acevaltrate	Aav	Iv	Ac	<i>V. wallichii</i>	(89,161)
1- β -acevaltrate	Abv	Iv	Ac	<i>V. alliariifolia</i> <i>V. glechomifolia</i>	(160,169,179)
Acevaltrate	Iv	Aav	Ac	<i>V. officinalis</i> <i>V. wallichii</i> <i>V. thalictroides</i> <i>V. microphylla</i> <i>V. glechomifolia</i> <i>V. pseudofficinalis</i> <i>V. fauriei</i>	(158,161–163,165,167,171,173,176)
1- β -aceacevaltrate	Abv	Abv	Ac	<i>V. glechomifolia</i>	(171)
1-homoacevaltrate	Mv	Abv	Ac	<i>V. wallichii</i>	(162)
1-homovaltrate	Mv	Iv	Ac	<i>V. wallichii</i>	(161,162,180)

Table 14. Diene-type valepotriates isolated from *Valeriana* species (Continued)

Compounds	R ₁	R ₇	R ₁₁	Species	Ref
7-homovaltrate	Iv	Mv	Ac	<i>V. wallichii</i> <i>V. thalictroides</i> <i>V. capense</i> <i>V. edulis</i>	(78,89,161,176,181)
Dihomovaltrate	Mv	Mv	Ac	<i>V. wallichii</i> <i>V. capense</i>	(89,162,163,180)
1-homoisoacevaltrate	Mv	Ac	Aav	<i>V. wallichii</i>	(162)
Deacetylisovaltrate	Iv	H	Iv	<i>V. wallichii</i>	(161)
Seneciovaltrate	Cr	Iv	Ac	<i>V. alliariifolia</i> <i>V. sorbifolia</i>	(111,179)
Sorbifolivaltrate A	Cr	Iv	Iv	<i>V. sorbifolia</i>	(111)
Sorbifolivaltrate B	Cr	Mv	Iv	<i>V. sorbifolia</i>	(111)

2.1.3.2.A.3. Monoene-type Valtrate Hydrines Isolated from *Valeriana* species**Table 15.** Monoene-type valtrate hydrines isolated from *Valeriana* species

Compounds	R ₁	R ₅	R ₇	R ₁₀	R ₁₁	Species	Ref
Jatamanvaltrate A	Iv	OH	Ac	Abv	Iiv	<i>V. wallichii</i>	(81,166)
Jatamanvaltrate B	Iv	OH	Ac	Iv	Iiv	<i>V. wallichii</i> <i>V. officinalis</i>	(166,182)

Table 15. Monoene-type valtrate hydrines isolated from *Valeriana* species (*Continued*)

Compounds	R ₁	R ₅	R ₇	R ₁₀	R ₁₁	Species	Ref
Jatamanvaltrate C	Iv	OH	Ac	Ac	Iiv	<i>V. wallichii</i> <i>V. officinalis</i>	(166,182)
Jatamanvaltrate D	Iv	OH	Ac	Sal	Iiv	<i>V. wallichii</i>	(166)
Jatamanvaltrate E	Iv	OH	Ac	CH ₃	Iiv	<i>V. wallichii</i>	(20,81,166)
Jatamanvaltrate F	Iv	H	Ac	Abv	Iv	<i>V. wallichii</i>	(81,166)
Jatamanvaltrate G	Iv	H	H	Ac	Iv	<i>V. wallichii</i>	(166)
Jatamanvaltrate H	Iv	H	Ac	H	Iv	<i>V. wallichii</i>	(166,183)
Valeriotetrate A	Iv	OH	Ac	Iiv	Iiv	<i>V. wallichii</i>	(166,184)
Valeriotetrate B	Iv	OH	H	Ac	Iv	<i>V. wallichii</i>	(166,185)
Valeriotetrate C	Iv	OH	Ac	Iiv	Iiv	<i>V. wallichii</i> <i>V. fauriei</i>	(20,81,165)
Didrovaltrate acetoxy hydrine	Iv	H	Ac	Ac	Iv	<i>V. wallichii</i>	(166)
Valeriotriate b	Iv	OH	H	Ac	Iiv	<i>V. wallichii</i> <i>V. fauriei</i>	(165,166, 186)

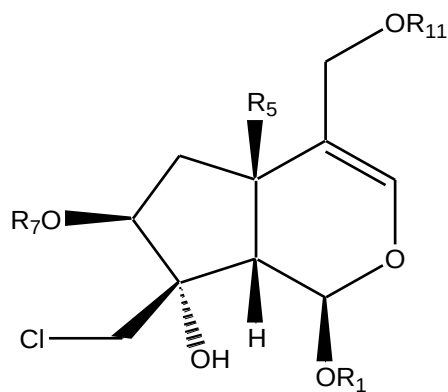


Table 16. Chlorinated monoene-type valtrate hydrines isolated from *Valeriana* species

Compounds	R ₁	R ₅	R ₇	R ₁₁	Species	Ref
Chlorovaltrate E	Iv	OH	H	Iiv	<i>V. wallichii</i>	(187)
Chlorovaltrate F	Iv	OH	Ac	Iv	<i>V. wallichii</i>	(187)
Chlorovaltrate G	Iv	OH	Ac	H	<i>V. wallichii</i>	(187)
Chlorovaltrate H	Iv	OH	Ac	CH ₃	<i>V. wallichii</i>	(187)
Chlorovaltrate I	Iv	OH	Ac	Et	<i>V. wallichii</i>	(187)
Chlorovaltrate J	Iv	H	H	Iv	<i>V. wallichii</i>	(187)
Rupesin B	Iv	H	Ac	Iv	<i>V. wallichii</i>	(187)
Volvaltrate B	Iv	OH	Ac	Iiv	<i>V. wallichii</i> <i>V. officinalis</i>	(151,182,187)

2.1.3.2.A.4. Diene-type Valtrate Hydrines Isolated from *Valeriana* species

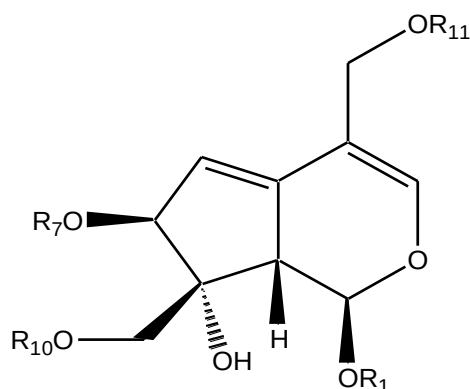


Table 17. Diene-type valtrate hydrines isolated from *Valeriana* species

Compounds	R ₁	R ₇	R ₁₀	R ₁₁	Species	Ref
Valtrate hydrine b1	Iv	Iv	Iv	Ac	<i>V. tiliaefolia</i> <i>V. wallichii</i> <i>V. pavonii</i>	(188–190)
10-acetoxyvaltrate hydrine	Iv	Iv	Ac	Ac	<i>V. tiliaefolia</i> <i>V. wallichii</i> <i>V. pavonii</i>	(166,188, 189)
Jatamanvaltrate K	Iv	Iv	H	Ac	<i>V. wallichii</i>	(166)
Jatamanvaltrate J	Iv	H	Iv	Ac	<i>V. wallichii</i>	(166)
10-Acetoxy-1-homovaltrate hydrine	Mv	Iv	Ac	Ac	<i>V. wallichii</i>	(162)
10-acetoxy-1-acevaltrate hydrine	Abv	Iv	Ac	Ac	<i>V. wallichii</i>	(162)
Isovaltrate isovaleroyloxyhydrine	Iv	Ac	Iv	Iv	<i>V. wallichii</i>	(80,161)
Valtrate hydrine b8	Iv	Ac	Abv	Iv	<i>V. wallichii</i> <i>V. alliariifolia</i>	(179,191)

Table 17. Diene-type valtrate hydrines isolated from *Valeriana* species (Continued)

Compounds	R ₁	R ₇	R ₁₀	R ₁₁	Species	Ref
Jatamanvaltrate P	Iv	Iv	Ac	Iv	<i>V. wallichii</i> <i>V. officinalis</i>	(21,192)
Jatamanvaltrate Q	Iv	Iv	Ac	Iiv	<i>V. wallichii</i>	(192)
Sorbifolivaltrate C	Hiv	Iv	Iv	Iv	<i>V. sorbifolia</i>	(111)
Sorbifolivaltrate D	Cr	Mv	Iv	Ac	<i>V. sorbifolia</i>	(111)
Valtrate hydrine b3	Hiv	Iv	Iv	Ac	<i>V. sorbifolia</i> <i>V. tiliaefolia</i>	(111, 188)
Valtrate hydrine b7	Cr	Iv	Iv	Ac	<i>V. sorbifolia</i> <i>V. alliariifolia</i>	(111,179)
Valerjatadoids A	Iv	Iv	Et	Ac	<i>V. wallichii</i>	(193)
Valeriotetrate B	Iv	Abv	Iiv	Ac	<i>V. wallichii</i>	(185)
1- α - acetoxysovaleroxy isovaltrate hydrine	Aav	Ac	Ac	Iv	<i>V. sisymbriifolia</i>	(172)

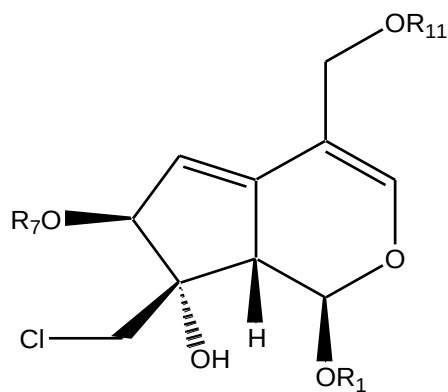


Table 18. Chlorinated diene-type valtrate hydrines isolated from *Valeriana* species

Compounds	R ₁	R ₇	R ₁₁	Species	Ref
Chlorovaltrate K	Iv	Iv	Hiv	<i>V. wallichii</i>	(187)
Chlorovaltrate M (Valeriandoid B)	Iv	Abv	Ac	<i>V. wallichii</i>	(80,187)
Chlorovaltrate N	Iv	Mv	Ac	<i>V. wallichii</i>	(187)
Chlorovaltrate L	Iv	Iv	H	<i>V. wallichii</i>	(187)
Valeriandoid A	Ac	H	Iv	<i>V. wallichii</i>	(80)
Valechlorin (Chlorovaltrate)	Iv	Iv	Ac	<i>V. officinalis</i> <i>V. wallichii</i> <i>V. capense</i> <i>V. fauriei</i>	(80,89,161,165,167)

2.1.3.2.B. Decomposition Products of Valepotriates (Baldrinals) Isolated from *Valeriana* species

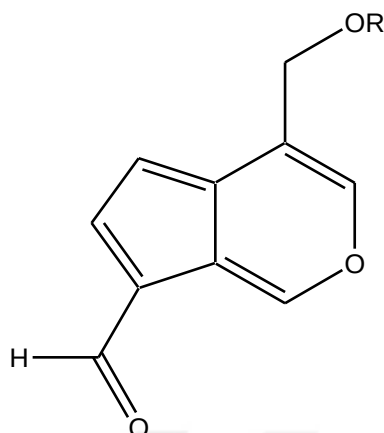


Table 19. Baldrinals isolated from *Valeriana* species

Compounds	R	Species	Ref
Desacylbaldrrinal	H	<i>V. wallichii</i>	(24,191)
Baldrinal	Ac	<i>V. wallichii</i> <i>V. mexicana</i> <i>V. polystachya</i>	(60,164,175,186,191)
Homobaldrinal	Iv	<i>V. wallichii</i> <i>V. polystachya</i>	(164,183)
11-Methoxyviburtinal	CH ₃	<i>V. wallichii</i>	(81,186)
11-Ethoxyviburtinal	Et	<i>V. officinalis</i> <i>V. wallichii</i>	(60,194)
Valejatanin A	liv	<i>V. wallichii</i>	(42)

2.1.3.2.C. 3,8-Epoxy Iridioids Isolated from *Valeriana* species

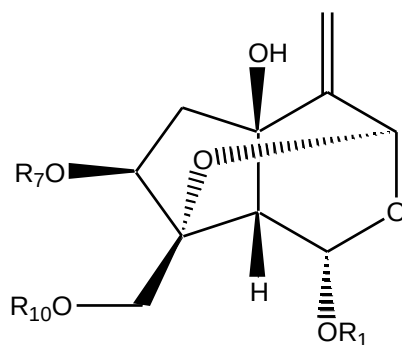


Table 20. 3,8-Epoxy iridoids isolated from *Valeriana* species

Compounds	R ₁	R ₇	R ₁₀	Species	Ref
Jatamanin R	CH ₃	H	H	<i>V. wallichii</i>	(195)
Jatamanin S	CH ₃	H	Ac	<i>V. wallichii</i>	(195)
Jatamanin T	CH ₃	Ac	CH ₃	<i>V. wallichii</i>	(195)
Jatamanin U	CH ₃	Ac	Iv	<i>V. wallichii</i>	(195)
Chlorovaltrate V	Et	Ac	Iv	<i>V. wallichii</i>	(24)

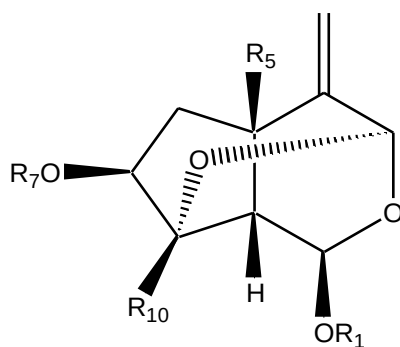


Table 21. 3,8-Epoxy iridoids isolated from *Valeriana* species

Compounds	R ₁	R ₅	R ₇	R ₁₀	Species	Ref
1,5-dihydroxy-3,8-epoxyvalechlorin	H	OH	Ac	CH ₂ Cl	<i>V. wallichii</i>	(182,195)
Chlorovaltrate Q	CH ₃	OH	H	CH ₂ Cl	<i>V. wallichii</i>	(195)

Table 21. 3,8-Epoxy iridoids isolated from *Valeriana* species (*Continued*)

Compounds	R ₁	R ₅	R ₇	R ₁₀	Species	Ref
3,8-epoxy-1- <i>O</i> -methyl-5-hydroxyvalechlorin	CH ₃	OH	Ac	CH ₂ Cl	<i>V. wallichii</i>	(195)
Jatamanin O (Chlorovaltrate A)	Iv	OH	Ac	CH ₂ Cl	<i>V. wallichii</i>	(24,187, 195,196)
Jatamanin P	Iv	H	Ac	CH ₂ Cl	<i>V. wallichii</i>	(187,195)
Jatamanin D	H	H	H	CH ₃	<i>V. wallichii</i>	(108,195)
(4β,8β)- 8-methoxy-3-methoxy-10-methylene-2,9-dioxatricyclo[4.3.1.0 ^{3,7}]decan-4-ol	CH ₃	H	H	CH ₃	<i>V. wallichii</i> <i>V. stenoptera</i>	(32,195, 197,198)
Rupesin E	Iv	H	H	CH ₃	<i>V. wallichii</i>	(108,195)
Jatairidoid C	H	OH	Ac	CH ₂ O-Iiv	<i>V. wallichii</i>	(84,195)
Valejatoid A	Iv	OH	Ac	CH ₂ O-Iiv	<i>V. wallichii</i>	(20)
Chlorovaltrate U	Et	H	Ac	CH ₂ Cl	<i>V. wallichii</i>	(24)
Chlorovaltrate W	CH ₃	OH	Ac	CH ₂ O-Iv	<i>V. wallichii</i>	(24)
Valeriandoid C	H	OH	Ac	CH ₂ O-Iv	<i>V. wallichii</i>	(80)
Volvaltrate A	Iv	OH	Ac	OH	<i>V. officinalis</i>	(182)
Jatamanvaltrate R	Et	OH	Ac	CH ₂ O-Iv	<i>V. wallichii</i>	(199)
Jatamanvaltrate S	Iv	OH	Ac	CH ₂ O-Va	<i>V. wallichii</i>	(199)

2.1.3.2.D. Pyrane Ring Opened Iridoids Isolated from *Valeriana* species

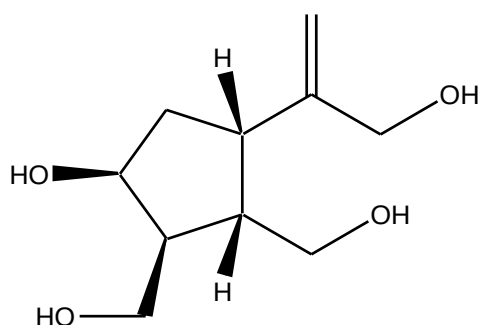


Table 22. Pyrane ring opened iridoids isolated from *Valeriana* species

Compound	Species	Ref
Dioscoridin A	<i>V. dioscoridis</i> <i>V. officinalis</i>	(12,200)

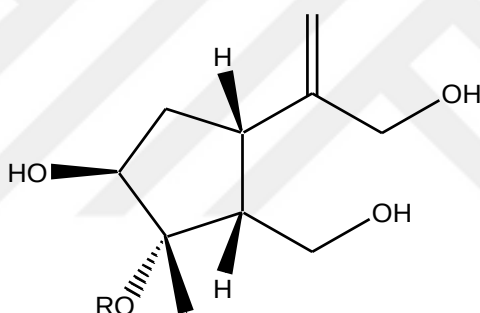


Table 23. Pyrane ring opened iridoids isolated from *Valeriana* species

Compounds	R	Species	Ref
Jatamanin J	H	<i>V. wallichii</i> <i>V. officinalis</i>	(108,200)
(5S,7S,8S,9R)-7-hydroxy- $\Delta^{4,11}$ -dihyronepeta-1,3-diol-8-O- β -D-glucopyranosyl	β -glc	<i>V. officinalis</i>	(200)

2.1.3.2.E. Non-glycosidic Iridioids Isolated from *Valeriana* species (Other)

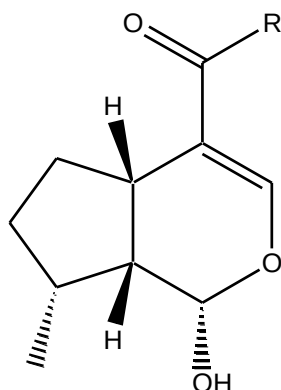


Table 24. Non-glycosidic iridioids isolated from *Valeriana* species

Compounds	R	Species	Ref
1- <i>epi</i> -bosnarol	H	<i>V. dioscoridis</i>	(12)
8- <i>epi</i> -deoxyloganin aglycone	OCH ₃	<i>V. dioscoridis</i>	(12)

2.1.3.2.F. Iridolactones Isolated from *Valeriana* species

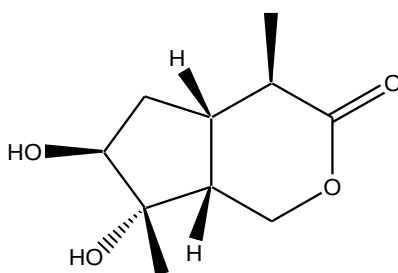


Table 25. Iridolactones isolated from *Valeriana* species

Compound	Species	Ref
Patriscabrol	<i>V. wallichii</i>	(108)

2.1.3.2.G. Iridoid Glycosides Isolated from *Valeriana* species

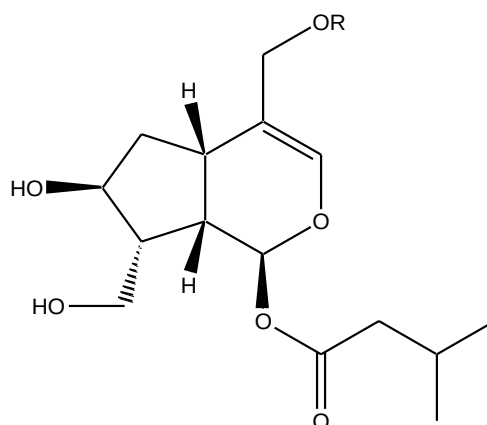


Table 26. Iridoid glycosides isolated from *Valeriana* species

Compounds	R	Species	Ref
Patrinocide	β -glc	<i>V. fauriei</i> <i>V. amurensis</i>	(82,119)
Kanokoside D	β -gent	<i>V. fauriei</i>	(119)

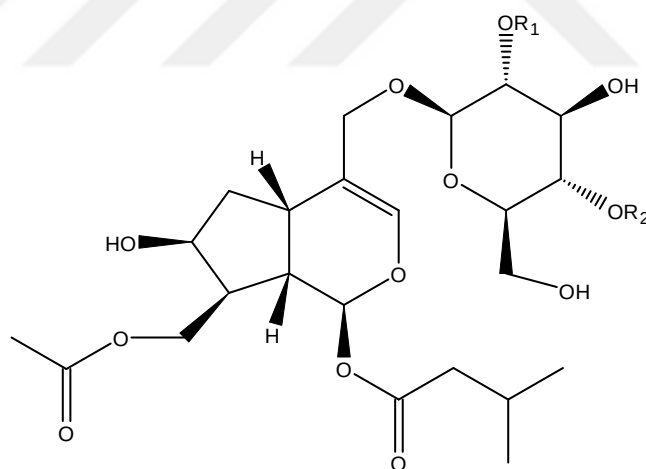


Table 27. Iridoid glycosides isolated from *Valeriana* species

Compounds	R ₁	R ₂	Species	Ref
Dioscorin B	Ac	β -glc	<i>V. dioscoridis</i>	(12)
10-acetylpatrinocide	H	H	<i>V. dioscoridis</i>	(12)
10,2'-diacetylpatrinocide	Ac	H	<i>V. dioscoridis</i>	(12)

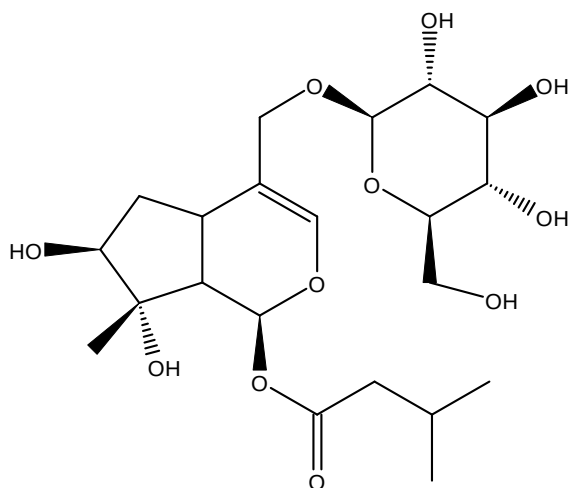


Table 28. Iridoid glycosides isolated from *Valeriana* species

Compound	Species	Ref
Valeroside (Valerosidatum)	<i>V. officinalis</i> <i>V. wallichii</i>	(184,201)

2.1.3.2.H. 6,7-Epoxy Iridoids Isolated from *Valeriana* species

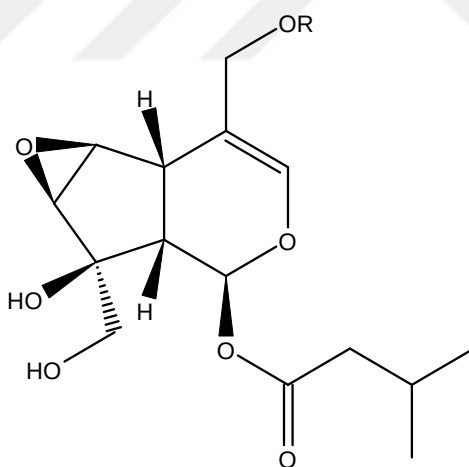


Table 29. 6,7-Epoxy iridoids isolated from *Valeriana* species

Compounds	R	Species	Ref
Monovalerianester A	H	<i>V. fauriei</i> <i>V. officinalis</i>	(119,202)
Kanokoside A	β -glc	<i>V. fauriei</i>	(119,202)
Kanokoside C	β -gent	<i>V. fauriei</i>	(119)

2.1.3.3. Monoterpene Esters

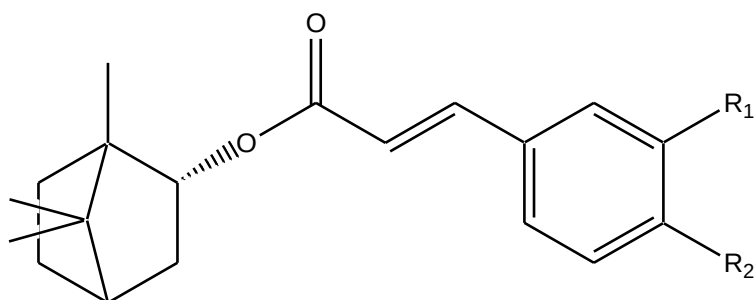


Table 30. Monoterpene esters isolated from *Valeriana* species

Compounds	R ₁	R ₂	Species	Ref
(<i>E</i>)-bornyl coumarate	H	OH	<i>V. wallichii</i>	(59)
(<i>E</i>)-bornyl caffeate	OH	OH	<i>V. wallichii</i>	(59)
(<i>E</i>)-bornyl ferulate	OCH ₃	OH	<i>V. wallichii</i>	(59)

2.1.3.4. Lignans

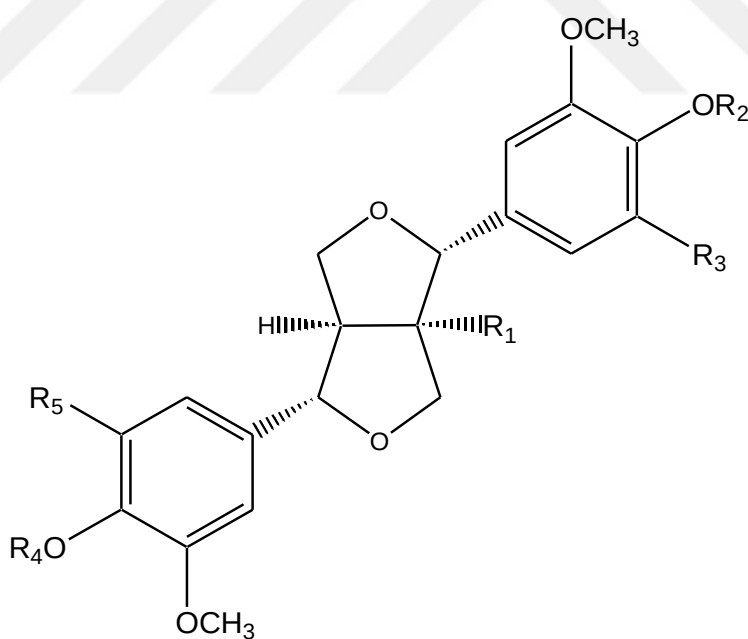


Table 31. Pinoresinol type lignans isolated from *Valeriana* species

Compounds	R ₁	R ₂	R ₃	R ₄	R ₅	Species	Ref
Pinoresinol	H	H	H	H	H	<i>V. officinalis</i> <i>V. wallichii</i> <i>V. microphylla</i>	(59,60,158,191, 194,202,203)

Table 31. Pinoresinol type lignans isolated from *Valeriana* species (Continued)

Compounds	R ₁	R ₂	R ₃	R ₄	R ₅	Species	Ref
8-hydroxypinoresinol	OH	H	H	H	H	<i>V. wallichii</i> <i>V. officinalis</i> <i>V. laxiflora</i> <i>V. prionophylla</i> <i>V. microphylla</i> <i>V. polystachya</i> <i>V. stenoptera</i>	(57,118, 125,158, 164,197, 203,204)
Pinoresinol 4-O-β-D-glucoside	H	β-glc	H	H	H	<i>V. wallichii</i> <i>V. officinalis</i>	(125, 204)
Pinoresinol 4,4'-di-O-β-D-glucoside	H	β-glc	H	β-glc	H	<i>V. wallichii</i> <i>V. officinalis</i>	(204, 205)
8-hydroxypinoresinol 4-O-β-D-glucoside	OH	β-glc	H	H	H	<i>V. wallichii</i> <i>V. officinalis</i>	(202)
8-hydroxypinoresinol 4'-O-β-D-glucoside	OH	H	H	β-glc	H	<i>V. wallichii</i> <i>V. officinalis</i> <i>V. prionophylla</i>	(118, 125,204)
Fraxiresinol 4'-O-β-D-glucoside	OH	H	OCH ₃	β-glc	H	<i>V. prionophylla</i>	(118)
8-acetoxypinoresinol	OAc	H	H	H	H	<i>V. officinalis</i> <i>V. wallichii</i>	(108, 194)
Medioresinol	H	H	OCH ₃	H	H	<i>V. wallichii</i>	(108)
Pinoresinol monomethyl ether	H	CH ₃	H	H	H	<i>V. wallichii</i>	(108)
Syringaresinol	H	H	OCH ₃	H	OCH ₃	<i>V. wallichii</i>	(108)

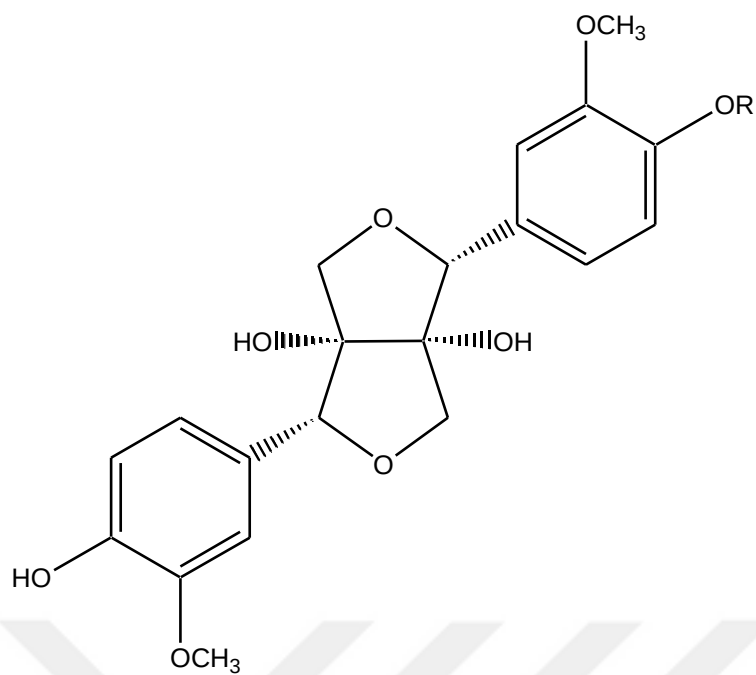


Table 32. Prinsepiol type lignans isolated from *Valeriana* species

Compounds	R	Species	Ref
Prinsepiol	H	<i>V. laxiflora</i> <i>V. prionophylla</i> <i>V. officinalis</i>	(21,57,118,125,194, 203)
Prinsepiol 4-O- β -D-glucoside	β -glc	<i>V. wallichii</i> <i>V. prionophylla</i> <i>V. officinalis</i>	(118,186,202)

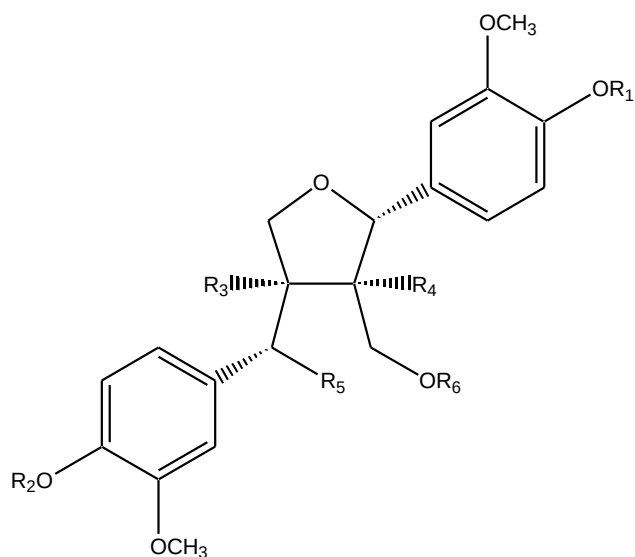
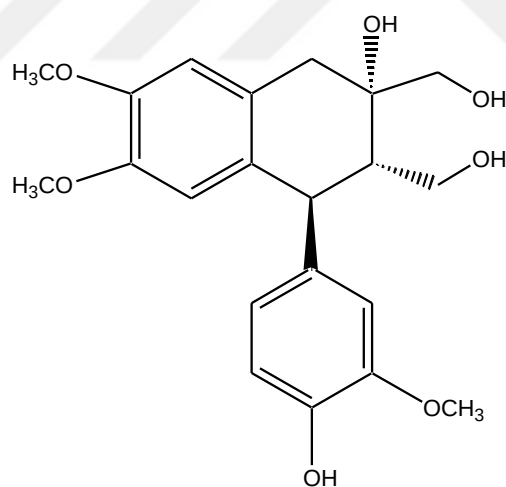


Table 33. Olivil type lignans isolated from *Valeriana* species

Compounds	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	Species	Ref
Olivil	H	H	OH	H	H	H	<i>V. stenoptera</i> <i>V. officinalis</i>	(197,203)
Olivil 4'-O- β-D- glucopyranoside	H	β-glc	OH	H	H	H	<i>V. amurensis</i>	(83)
Lariciresinol	H	H	H	H	H	H	<i>V. officinalis</i>	(203)
Lariciresinol 4,4'-di- O-β-D- glucopyranoside	β-glc	β-glc	H	H	H	H	<i>V. amurensis</i>	(83)
Olivil 4-O-β-D- glucopyranoside	β-glc	H	OH	H	H	H	<i>V. amurensis</i>	(83)
8- hydroxylariciresinol 4'-O-β-D- glucopyranoside	H	β-glc	H	OH	H	H	<i>V. amurensis</i>	(83)
Lariciresinol 4-O-β- D-glucopyranoside	β-glc	H	H	H	H	H	<i>V. amurensis</i>	(83)
Neoarctin A	β-glc	H	H	H	OH	H	<i>V. amurensis</i>	(83)

Table 33. Olivil type lignans isolated from *Valeriana* species (Continued)

Compounds	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	Species	Ref
Lariciresinol 4'-O- β-D- glucopyranoside	H	β-glc	H	H	H	H	<i>V. amurensis</i>	(83)
Massoniresinol	H	H	OH	OH	H	H	<i>V. wallichii</i>	(186)
(-)-Massoniresinol 3α-O-β-D- glucopyranoside	H	H	OH	OH	H	β-glc	<i>V. amurensis</i>	(83)
Massoniresinol 4'- O-β-D- glucopyranoside	H	β-glc	OH	OH	H	H	<i>V. wallichii</i>	(204)
Berchemol 4'-O-β- D-glucopyranoside	H	β-glc	H	OH	H	H	<i>V. wallichii</i>	(204)
Berchemol	H	H	H	OH	H	H	<i>V. wallichii</i>	(108)

**Table 34.** Lignans isolated from *Valeriana* species

Compound	Species	Ref
Cyclo-olivil	<i>V. wallichii</i> <i>V. officinalis</i>	(108,194)

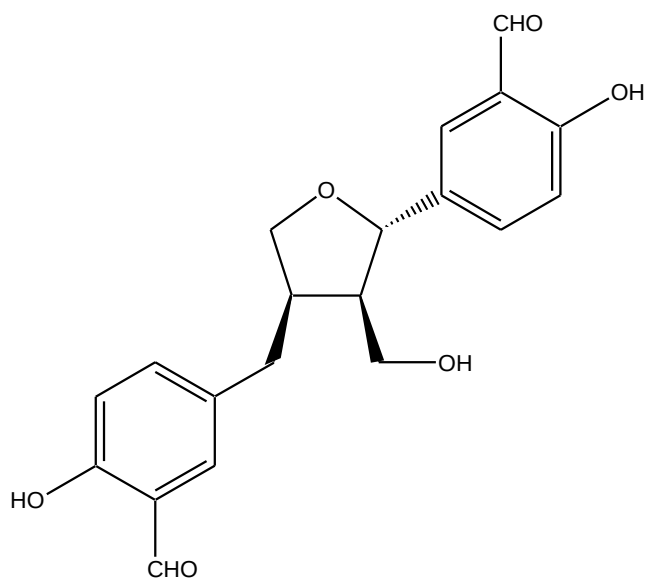


Table 35. Lignans isolated from *Valeriana* species

Compound	Species	Ref
Lariciresinol C	<i>V. officinalis</i>	(194)

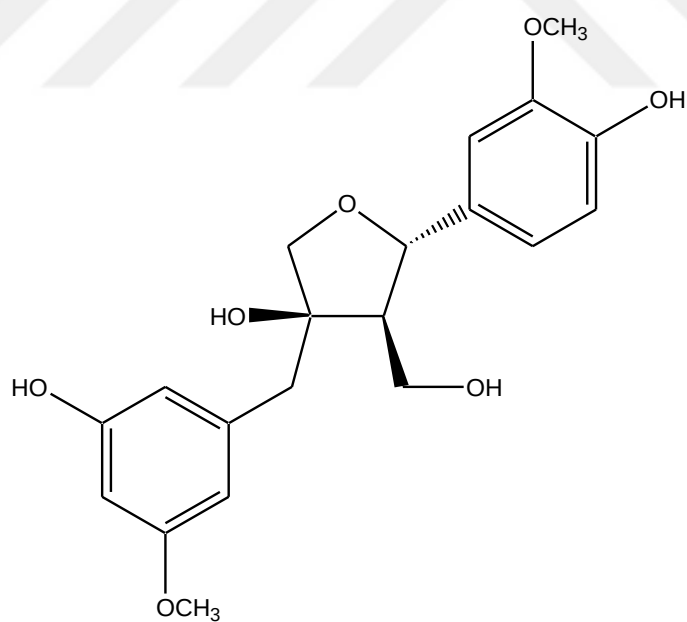


Table 36. Lignans isolated from *Valeriana* species

Compounds	Species	Ref
Ginkgool	<i>V. officinalis</i>	(194)

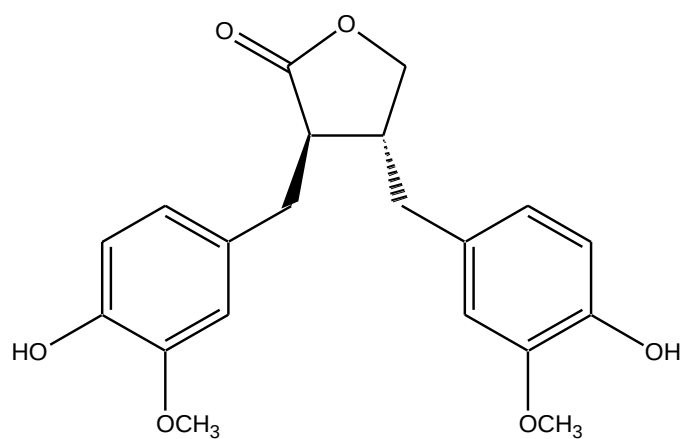


Table 37. Lignans isolated from *Valeriana* species

Compound	Species	Ref
Matairesinol	<i>V. amurensis</i>	(103)

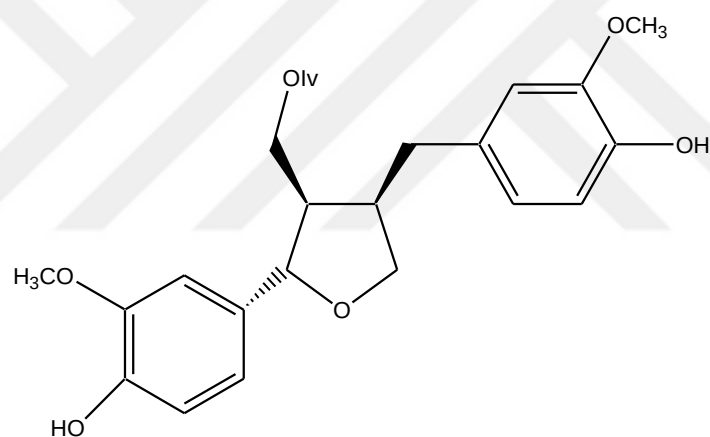


Table 38. Lignans isolated from *Valeriana* species

Compound	Species	Ref
(+)-9'-isovaleroloxylariciresinol	<i>V. wallichii</i>	(60)

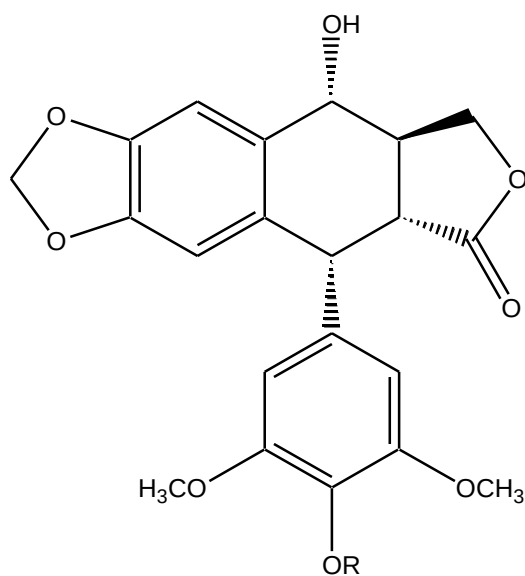


Table 39. Lignans isolated from *Valeriana* species

Compounds	R	Species	Ref
4'-demethylpodophyllotoxin	H	<i>V. wallichii</i>	(59)
Podophyllotoxin	CH ₃	<i>V. wallichii</i>	(59)

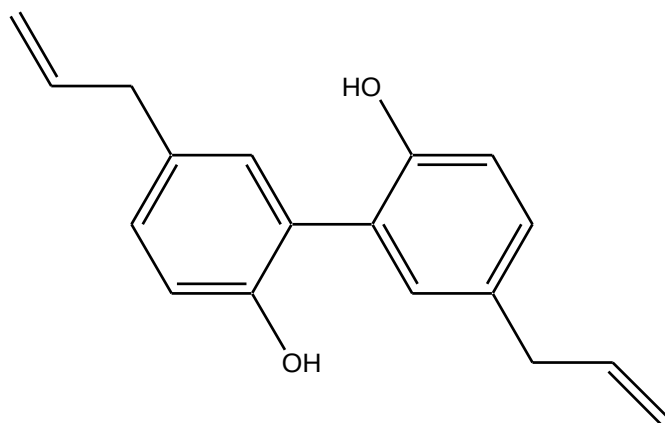


Table 40. Neolignans isolated from *Valeriana* species

Compound	Species	Ref
Magnolol	<i>V. wallichii</i>	(108)

2.1.3.5. Phenylpropane Derivatives

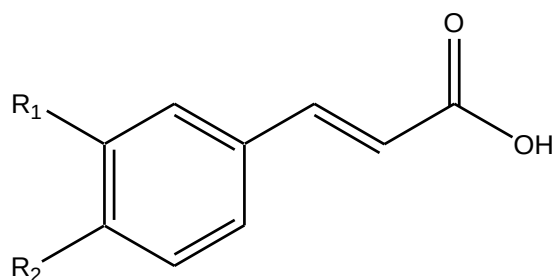


Table 41. Cinnamic acid derivatives isolated from *Valeriana* species

Compounds	R ₁	R ₂	Species	Ref
(<i>E</i>)-cinnamic acid	H	H	<i>V. officinalis</i>	(21)
(<i>E</i>)- <i>p</i> -coumaric acid	H	OH	<i>V. wallichii</i>	(206)
(<i>E</i>)-caffeic acid	OH	OH	<i>V. wallichii</i>	(206)
(<i>E</i>)-ferulic acid	OCH ₃	OH	<i>V. laxiflora</i> <i>V. officinalis</i>	(57,203)
(<i>E</i>)-isoferulic acid	OH	OCH ₃	<i>V. officinalis</i>	(203)

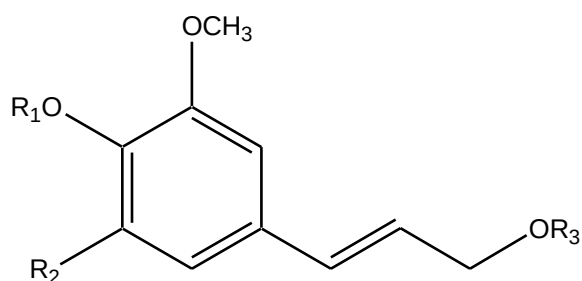


Table 42. Cinnamyl alcohol derivatives isolated from *Valeriana* species

Compounds	R ₁	R ₂	R ₃	Species	Ref
Coniferin	β-glc	H	H	<i>V. wallichii</i>	(186)
Coniferyl ethyl ether	H	H	Et	<i>V. wallichii</i>	(191)
Ethylconiferin	β-glc	H	Et	<i>V. wallichii</i>	(191)
Syringin	β-glc	OCH ₃	H	<i>V. wallichii</i>	(191)
Coniferyl alcohol methyl ether	H	H	CH ₃	<i>V. officinalis</i>	(21)

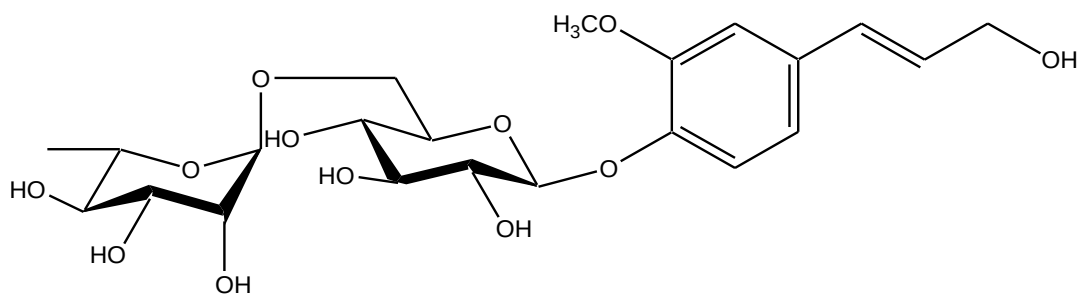


Table 43. Phenylpropane derivatives isolated from *Valeriana* species

Compound	Species	Ref
Xiecaoside E	<i>V. amurensis</i>	(83)

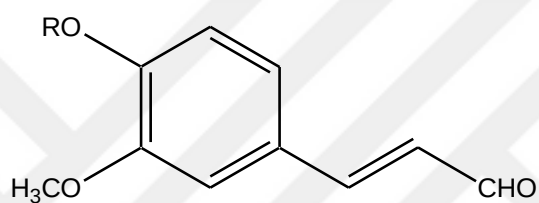


Table 44. Cinnamyl aldehyde derivatives isolated from *Valeriana* species

Compounds	R	Species	Ref
Coniferyl aldehyde	H	<i>V. wallichii</i>	(191)
Methylconiferyl aldehyde	CH ₃	<i>V. wallichii</i>	(191)

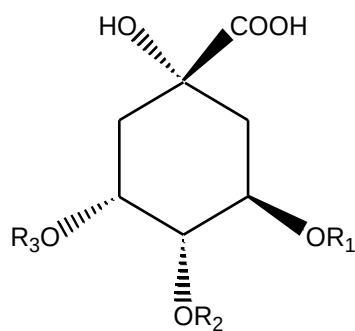


Table 45. Caffeoyl-quinic acid derivatives isolated from *Valeriana* species

Compounds	R ₁	R ₂	R ₃	Species	Ref
Chlorogenic acid	(<i>E</i>)-caff	H	H	<i>V. dioscoridis</i>	(12)
4,5-dicaffeoylquinic acid	H	(<i>E</i>)-caff	(<i>E</i>)-caff	<i>V. dioscoridis</i>	(12)

2.1.3.6. C₆C₁ Derivatives

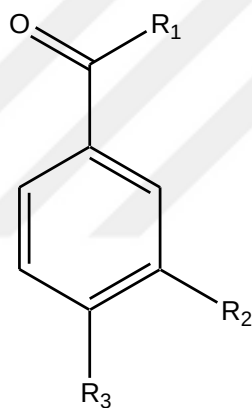


Table 46. Benzoic acid and derivatives isolated from *Valeriana* species

Compounds	R ₁	R ₂	R ₃	Species	Ref
Benzoic acid	OH	H	H	<i>V. officinalis</i>	(203)
Isovanillin	H	OH	OCH ₃	<i>V. officinalis</i>	(21)

2.1.3.7. Flavonoids

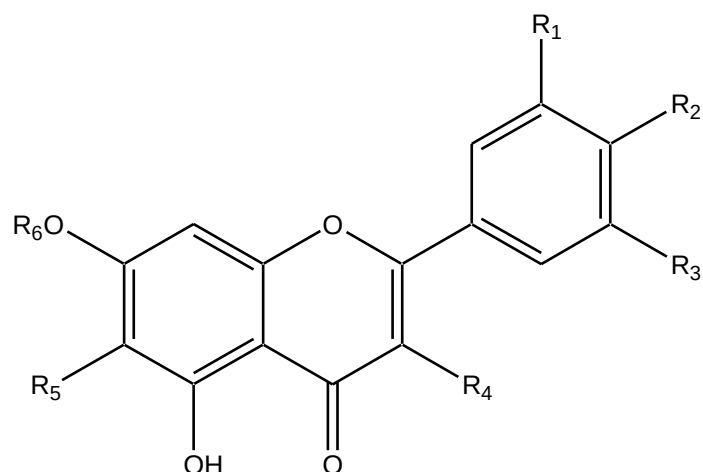
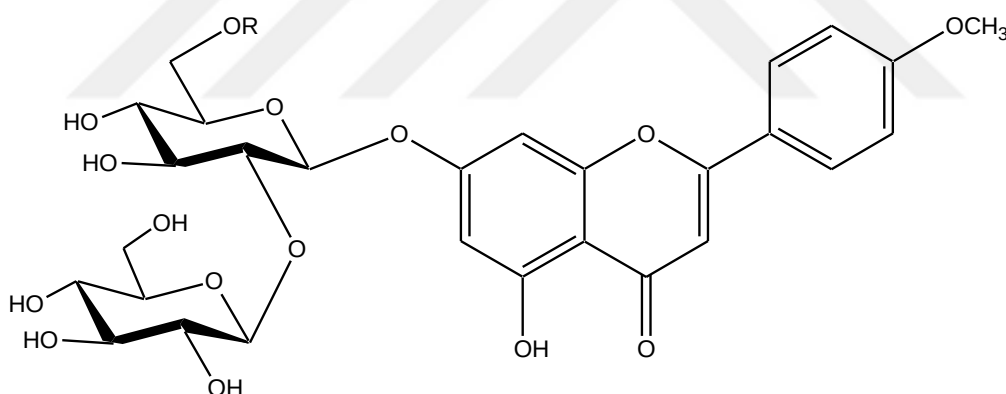


Table 47. Flavone and flavonol derivatives isolated from *Valeriana* species

Compounds	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	Species	Ref
Acacetin 7-O-β-D-glucopyranoside	H	OCH ₃	H	H	H	β-glc	<i>V. wallichii</i>	(206)
Apigenin 7-O-β-D-glucopyranoside	H	OH	H	H	H	β-glc	<i>V. wallichii</i>	(206)
Linarin	H	OCH ₃	H	H	H	Rut	<i>V. officinalis</i> <i>V. wallichii</i>	(66, 206)
Tricin	OCH ₃	OH	OCH ₃	H	H	H	<i>V. laxiflora</i>	(57)
5,7,3'-trihydroxy-4'-methoxyflavone	H	OCH ₃	OH	H	H	H	<i>V. laxiflora</i>	(57)
Quercetin	H	OH	OH	OH	H	H	<i>V. wallichii</i> <i>V. fauriei</i>	(206, 207)
Quercetin 7, 3', 4'-trimethyl ether	H	OCH ₃	OCH ₃	OH	H	CH ₃	<i>V. fauriei</i>	(207)

Table 47. Flavone and flavonol derivatives isolated from *Valeriana* species (Continued)

Compounds	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	Species	Ref
Quercetin 3-O-β-glucopyranoside	H	OH	OH	O-β-glc	H	H	<i>V. wallichii</i>	(206)
Rutin	H	OH	OH	O-rut	H	H	<i>V. wallichii</i>	(206)
Kaempferol	H	OH	H	OH	H	H	<i>V. wallichii</i> <i>V. fauriei</i>	(206, 207)
Kaempferol 3-O-β-glucopyranoside	H	OH	H	O-β-glc	H	H	<i>V. wallichii</i>	(206)
Kaempferol 3-O-rutinoside	H	OH	H	O-rut	H	H	<i>V. wallichii</i>	(206)
5,7-dihydroxy-3,6,4'-trimethoxyflavone	H	OCH ₃	H	OCH ₃	OCH ₃	H	<i>V. laxiflora</i>	(57)
6-methylapigenin	H	OH	H	H	CH ₃	H	<i>V. officinalis</i>	(66)

**Table 48.** Flavones isolated from *Valeriana* species

Compounds	R	Species	Ref
Acacetin 7-O- β-sophoroside	H	<i>V. wallichii</i>	(206)
Acacetin 7-O-(6'-O-α-L-rhamnopyranosyl)- β-sophoroside	α-rha	<i>V. wallichii</i>	(206)

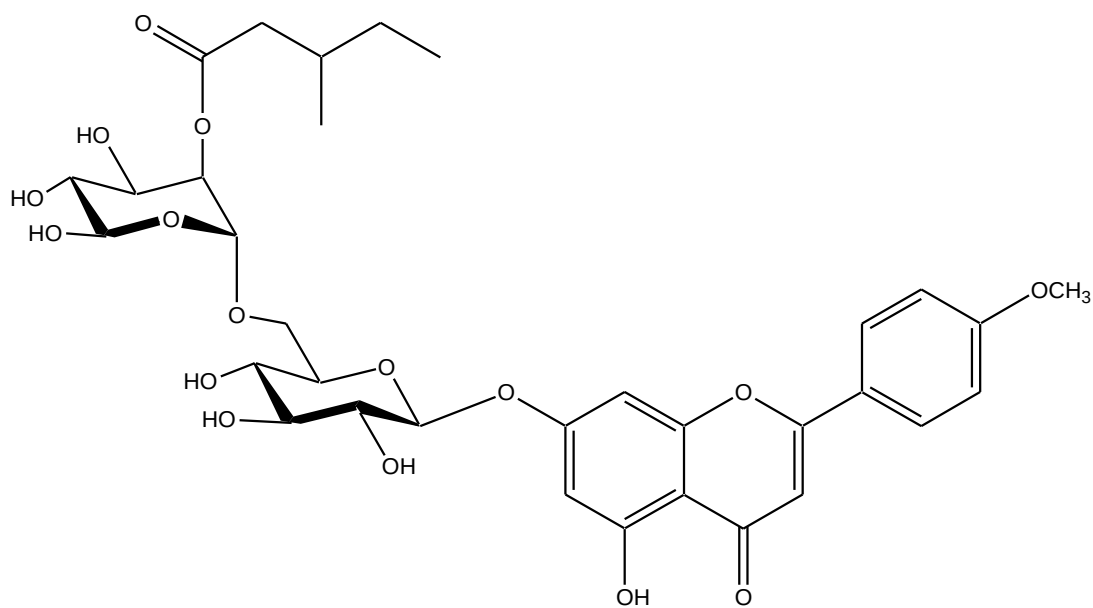


Table 49. Flavanones isolated from *Valeriana* species

Compound	Species	Ref
Linarin 2'''-methylbutyrate	<i>V. wallichii</i>	(59)

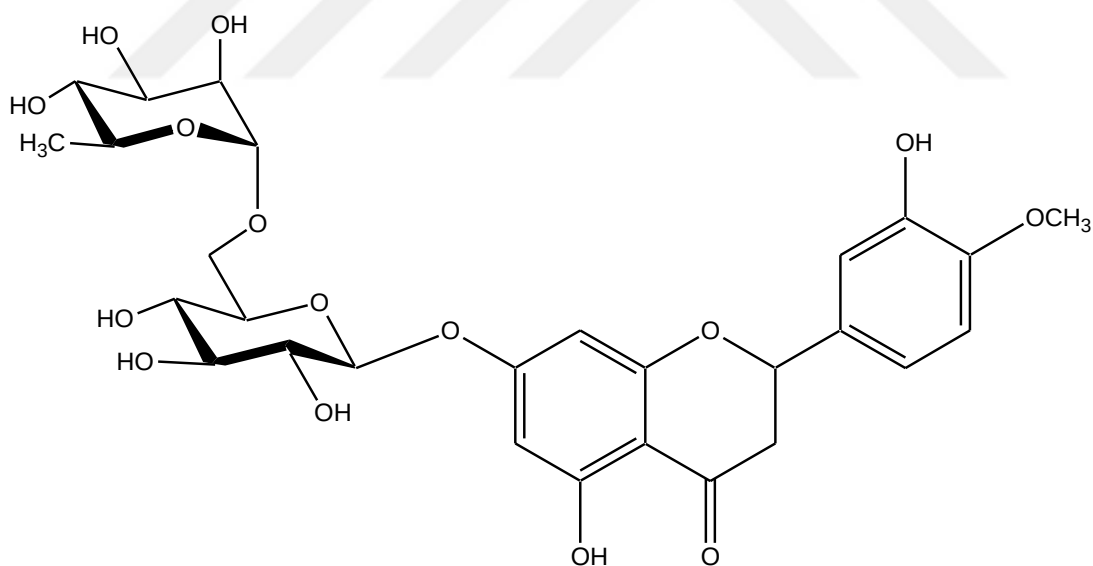


Table 50. Flavanones isolated from *Valeriana* species

Compound	Species	Ref
Hesperidin	<i>V. officinalis</i> <i>V. dioscoridis</i>	(12,66)

2.1.3.8. Phytosterol Derivatives

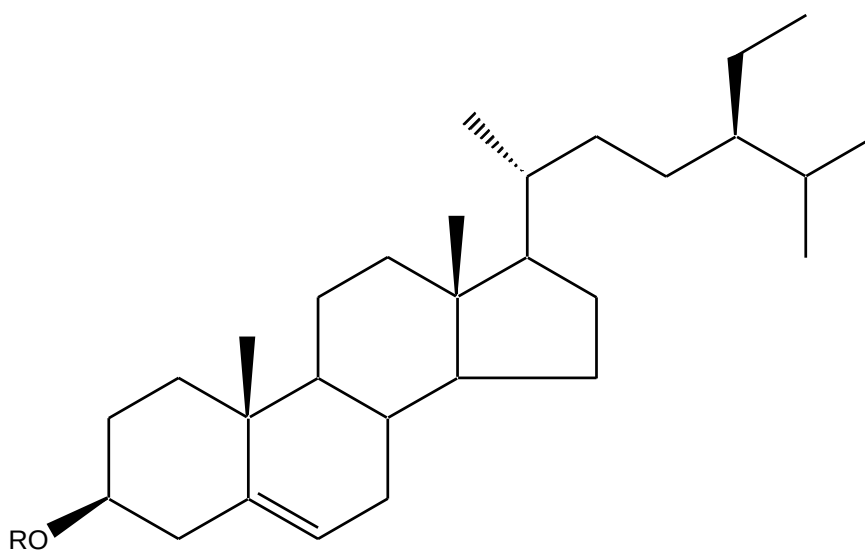


Table 51. Phytosterol derivatives isolated from *Valeriana* species

Compounds	R	Species	Ref
β -sitosterol	H	<i>V. wallichii</i> <i>V. officinalis</i> <i>V. dioscoridis</i> <i>V. officinalis</i> <i>V. polystachya</i>	(12,21,60,164,191,202)
β -sitosterol acetate	Ac	<i>V. wallichii</i>	(191)
Daucosterol	β -glc	<i>V. wallichii</i>	(191,206)

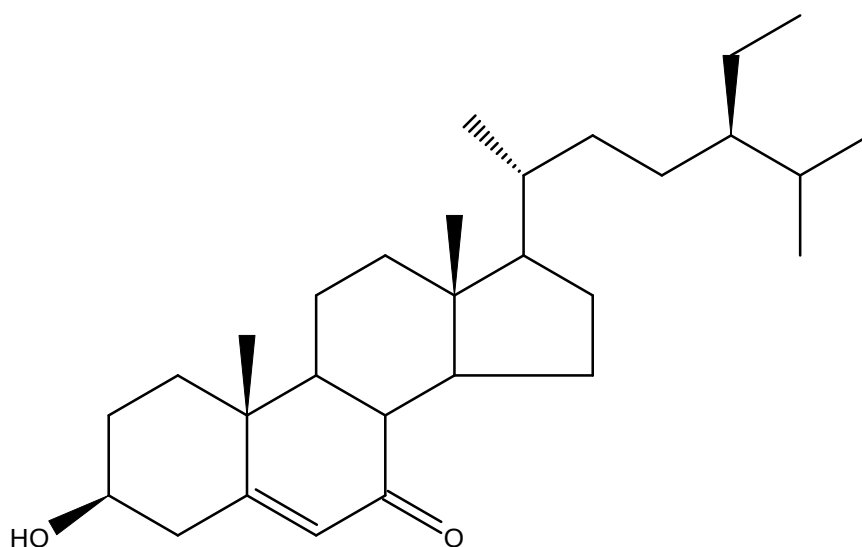


Table 52. Phytosterol derivative isolated from *Valeriana* species

Compound	Species	Ref
7-oxo- β -sitosterol	<i>V. officinalis</i>	(21)

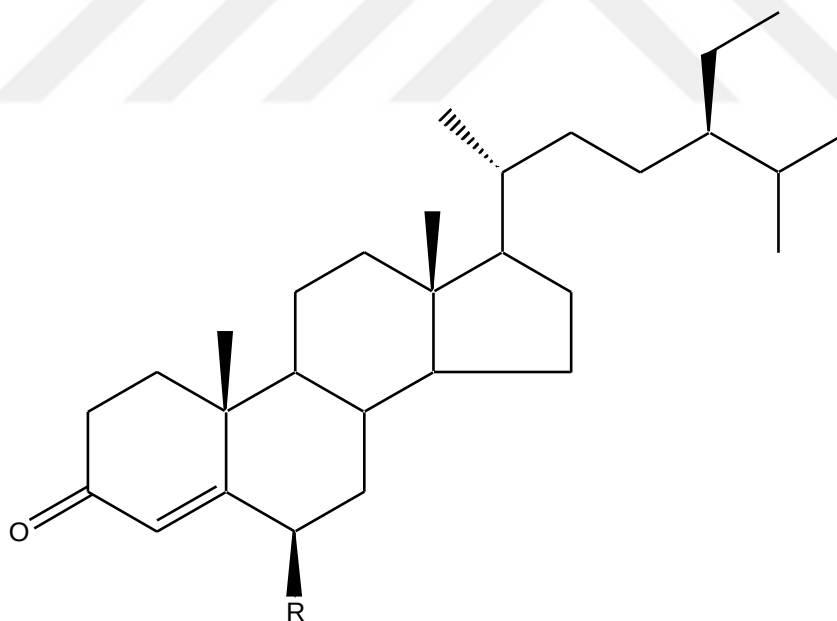


Table 53. Phytosterol derivatives isolated from *Valeriana* species

Compounds	R	Species	Ref
Stigmast-4-en-3-one	H	<i>V. officinalis</i>	(21)
6-hydroxystigmast-4-en-3-one	OH	<i>V. officinalis</i>	(21)

2.1.3.9. Triterpenes

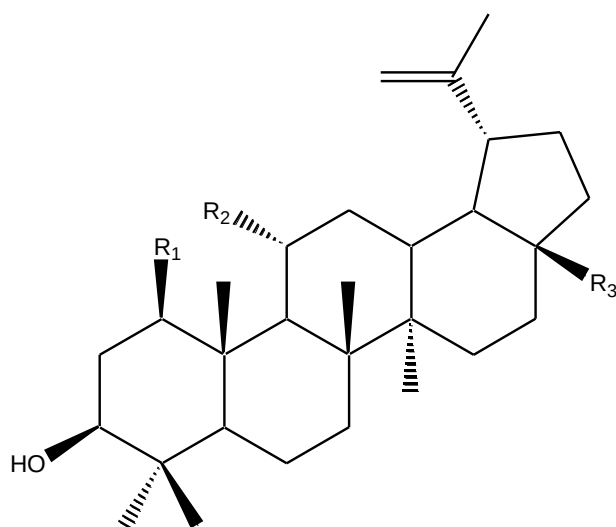


Table 54. Lupane-type triterpenes isolated from *Valeriana* species

Compounds	R ₁	R ₂	R ₃	Species	Ref
Betulin	H	H	CH ₂ OH	<i>V. laxiflora</i>	(57)
Betulinic acid	H	H	COOH	<i>V. laxiflora</i>	(57)
Nepetidin	OH	OH	CH ₃	<i>V. stenoptera</i>	(197)

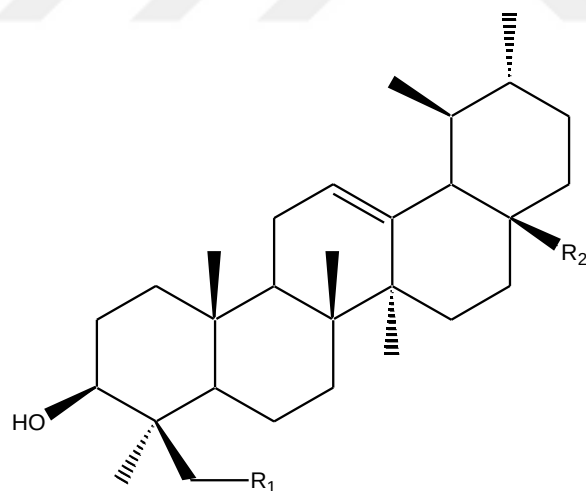


Table 55. Ursane-type triterpenes isolated from *Valeriana* species

Compounds	R ₁	R ₂	Species	Ref
Ursolic acid	H	COOH	<i>V. laxiflora</i> <i>V. polystachya</i>	(57,164)
23-hydroxyursolic acid	OH	COOH	<i>V. laxiflora</i>	(57)

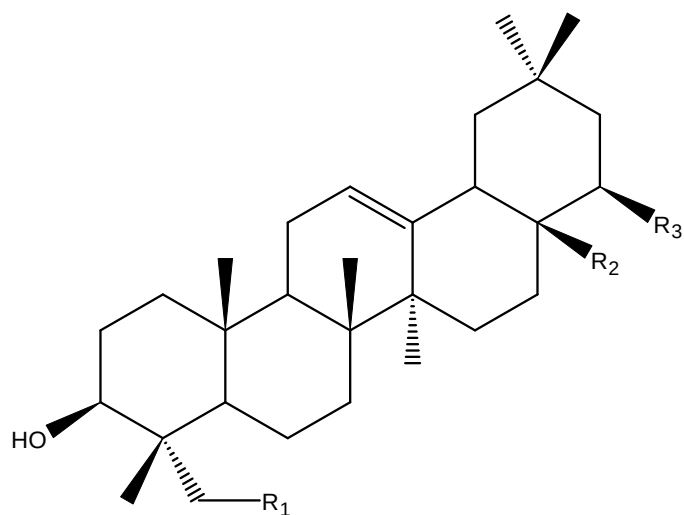


Table 56. Oleanane-type triterpenes isolated from *Valeriana* species

Compounds	R ₁	R ₂	R ₃	Species	Ref
Oleanolic acid	H	COOH	H	<i>V. laxiflora</i> <i>V. stenoptera</i>	(57,197)
Hedaregenin	OH	COOH	H	<i>V. stenoptera</i>	(197)
Sophoradiol	H	COOH	OH	<i>V. stenoptera</i>	(197)
β-amyrin	H	CH ₃	H	<i>V. stenoptera</i>	(197)

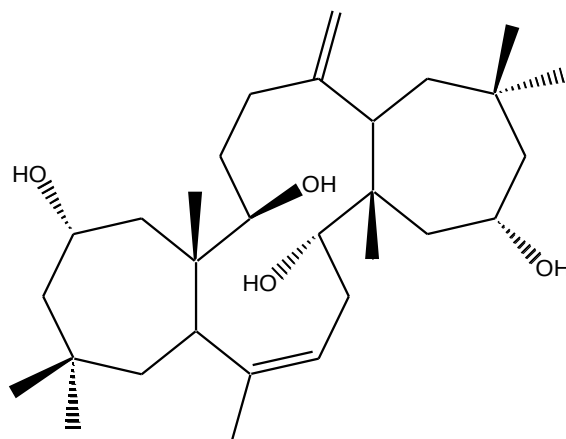


Table 57. Different type of triterpene isolated from *Valeriana* species

Compound	Species	Ref
Volvalerenol A	<i>V. hardwickii</i>	(208)

2.1.3.10. Essential Oils

Valeriana species are well-known for their unpleasant characteristic odors. Also, it is well-known that the chemistry of smell originates from isovaleric acid, which is formed as a result of enzymatic hydrolysis of some compounds, particularly non-glycosidic iridoid esters, during drying and storage process (40). In addition, isovaleric acid was found as the main component of essential oils obtained from some *Valeriana* species (209,210). The essential oils obtained from *Valeriana* species contain mixture of mono- and sesquiterpenes or almost completely sesquiterpenes. In general, *Valeriana* essential oils contain borneol, bornyl isovalerate, bornyl acetate, α -pinene, β -pinene, α -fenchene and myrcene as the main monoterpenes, while kessane, valerianol, valeranone, patchouli alcohol and guajene were found to be the major sesquiterpenes of the oil (132,134,136,146,209,211–214).

2.2. General Information about Cancer and Cell Death Pathways

Cancer is a serious public health issue and one of the main causes of death and morbidity globally. According to Global Cancer Observatory (GLOBACon) report in 2020, there have been predicted 19.3 million new incidences and approximately 10 million deaths originated from different cancers types. In the population, the incidence rate of breast cancer (11.7%) has surpassed over lung cancer (11.4%), with a predicted around 2.3 million new cancer cases. In addition, lung cancer is followed by colorectal (10%) and prostate (7.3%) cancers in population. Among the cancer types, lung cancer was reported to be major cause of cancer-related death, with a prediction of 1.8 million death that equals to 18% of cases, followed by colorectal cancer (9.4%) (1).

Cancer is defined by uncontrolled and unregulated cell proliferation. It can be occurred as a result of changes in various signaling pathways. In addition, it has been well-documented that cancer has multiple connections to programmed cell death (PCD). Programmed cell death maintains cellular homeostasis by balancing between cell survival and cell death (5). There are three different types of PCD according to their physiological and morphological features: apoptosis, necrosis and autophagy (215). Since every cell death program is a plain outcome of self-propagating signals and other signals that inhibit the other cell death processes, the sort of cell death that happens based on the cellular environment and the stimulus. Incomplete cell death is often related to early cancer progress and tumor resistance to radiotherapy or chemotherapy. As growing evidence showed that PCD is closely related to the treatment of cancer, it has become an important target for anticancer drug development studies (6).

2.2.1. Apoptosis

The term apoptosis was utilized for the first time to define a type of cell death linked to particular morphological properties in the year of 1972. It is well-documented that apoptosis has been deeply investigated, and also its underlying signaling processes are now well understood. From the morphological point of view, apoptosis is associated with membrane blebbing, cell shrinkage and chromatin condensation. Apoptosis can be determined as a programmed cell death pathway responsible for the elimination of cells that may damage the integrity of the organism. In addition, it is mediated by two major protein families: the B-cell lymphoma-2 (Bcl-2) family of proteins, that governs mitochondrial integrity, and the cysteine-aspartate proteases (caspases), that controls the execution phase of apoptosis (216). Caspases can be classified based on their prodomain length, that also related to their places in apoptotic-signaling pathway. Through this classification, caspase-1, -2, -4, -5, -8, -9, -10, -11 and -12 are defined as initiator caspases whereas, caspase-3, -6 and -7 are categorized as effector (executioner) caspases. Initiator caspases contains lengthy prodomains bearing one of two typical protein-protein interaction patterns, namely the caspase recruitment domain (CRD) and the death effector domain (DED). These caspases also participate in the interaction with upstream adapter proteins. On the contrary, the effector caspases possess short prodomains carrying out apoptosis downstream execution stages through cleavage of several cellular substrates and also, they are frequently activated and processed via upstream caspases. Many studies emphasized that the relationship between upstream and downstream is not certain and can only present temporarily in the very early stages of apoptosis (217). On the other hand, Bcl-2 protein family are crucial for controlling the mitochondrial pathway and also this protein family members can be classified into three groups according to their domain structure. The first group, known as anti-apoptotic proteins and suppressor proteins, is composed of Bcl-2, Bfl-1/A1, Bcl-W, Bcl-XL, MCL-1); the second group consists of pore-forming pro-apoptotic proteins such as Bok, Bak and Bax; The last group contains pro-apoptotic proteins bearing only BH(Bcl-2 homology)3 domains namely Bid, Bad, Bim, Bik, Hrk, Noxa, Puma (6,218). Moreover, Bcl-2 family members can possess four domains including BH1-4. All these domains are found in the pore-forming pro-apoptotic and anti-apoptotic proteins. The pore-forming pro-apoptotic and anti-apoptotic proteins have a quite conserved tertiary form that creates a hydrophobic BH3-binding groove and this groove serve like a receptor for BH3 belonging other protein family members (218). On the other hand, two apoptotic pathways, intrinsic (mitochondrial) and extrinsic (death receptor), are involved in apoptosis. (219,220). It has been well-documented that both pathways are associated with each other and also molecules

involved in one pathway have been shown to influence the molecules of other pathway (221). The intrinsic pathway of apoptosis is induced by stimuli from a variety of factors, such as chemotherapeutic agents, proinflammatory cytokines, DNA damage, and ROS-inducing compounds. (222). In this process, pro-apoptotic Bcl-2 protein family members such as Bax, Bok and Bak settle on the mitochondrial membrane and lead to the formation of pores in the organelle membrane that result in the release of cytochrome C (223). The intrinsic apoptosis pathway is also triggered dependently on processed caspase-12, the active form of caspase-12, as a result of endoplasmic reticulum stress (ER stress) mediated from an unfolded protein response (UPR) (224). Extrinsic pathway is initiated by death receptor of the tumor necrosis factor receptor (TNFR) like TNFR and TRAIL-r and Fas. These receptors-mediated signals cause a different cell response such as cell death, differentiation and proliferation. The development of a death-inducing signaling complex (DISC) causes apoptosis. Fas-associated death domain (FADD) regulates initiators of apoptosis namely caspase-8 and/or caspase-10 through homotypic death domain connections (216). In addition, the extrinsic apoptosis pathway is also triggered by the activation of the Bid protein by active caspase 8, producing the active form of the protein, truncated Bid (t-Bid) (225).

2.2.2. Autophagy

Autophagy is a programmed cell death process in which cytosolic components such as damaged organelles and intracellular aggregates are interacted with lysosomes in order to maintain homeostasis. Autophagic vacuoles are very important marker for autophagic cell death. Furthermore, there are various connections between autophagy and the other cell death mechanism including apoptotic or necrotic cell death. (216,226). Autophagy is classified into three subdivision; 1) macroautophagy leads to engulfment of cytoplasmic constituents within the double- membrane autophagosomes (227); 2) in microautophagy, lysosomal proteases cause the involvement of a substrate by degradation (228); 3) A pentapeptide domain directs the specific cargo to the lysosome in chaperone-originated autophagy (229). Among them, Beclin-1 and LC3 (microtubule-associated protein 1 light chain 3, or MAP1LC3), are the biomarker proteins that are included in macroautophagy (226). Recent researches showed that pharmacologically or genetically inhibition of autophagy increases the success of targeted therapy in cancer. Similarly, elevating the activity of autophagic pathway has also displayed that it is a different therapeutic approach to eradicate cancer cells with high autophagic threshold (230). That's why, autophagy is attributed as "double-edge sword" in cancer (231).

2.2.3. Necrosis

Necrosis was thought to be an uncontrolled and unintentional kind of cell death process that lacked underlying signaling actions for a long time. This situation might be correct due to cell death triggered by extreme physical damage such as cytolysis induced by detergents or hyperthermia. Nevertheless, several data suggest the presence of caspase-independent cellular death mechanisms which can work including in a properly organized developmental condition like interdigital cellular death (216). Necrotic cell death is defined by swelling of cellular organelles such as ER, Golgi apparatus, mitochondria, followed by the loss of cellular membrane rupture and then leakage of cellular components through to extracellular area (6).

Recent studies have demonstrated that necrosis is found to be a genetically programmed cell death pathway (232). Apoptotic and necrotic pathways can interact with each other, leading to cell death. Many regulatory molecules included in apoptosis and necrosis pathways are common, and also, they combine with receptor interaction protein kinase-1 (RIPK1) and -3 (RIPK3) in order to form a complex called as necrosome when caspase-8 mediated apoptosis cannot be occurred (233). This situation leads to phosphorylation (p-MLKL) of the mixed lineage kinase domain-like (MLKL) protein. After p-MLKL settles to the cell membrane, necrosis is initiated (234).

3. MATERIALS and METHODS

3.1. Materials

3.1.1. Chemicals and solvents

Table 58. Chemicals and solvents

Chemicals and Solvents	Trademark
%1 Bovine Serum Albumin (BSA)	Thermo Fisher
Acetic acid	Sigma-Aldrich
Acetone	Isolab
Annexin V binding buffer	BioLegend
Annexin V-FITC	BioLegend
Beclin-1	Novus Biology
Cyclin Dependent Kinase 4 (CDK4)	Novus Biology
Cyclin Dependent Kinase 6 (CDK6)	Novus Biology
Chloroform	Carlo Erba, Isolab, Sigma-Aldrich
Cleaved LC3B	Aviva System Biology
Cyclin D1	Novus Biology
Diaion HP20	Sigma Aldrich
Dichloromethane	Carlo Erba, Isolab, Sigma-Aldrich
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich
DMEM/F-12	Gibco
Dulbecco's Modified Eagle's Medium (DMEM)	Gibco
Dulbecco's Phosphate Buffered Saline (DPBS)	Thermo Fisher
Ethanol	Isolab
Ethyl acetate	Isolab, Sigma-Aldrich
Fetal bovine serum (FBS)	Thermo Fisher
Formic acid	Sigma Aldrich
Methanol	Carlo Erba, Isolab, Sigma-Aldrich
MLKL	Novus Biology
MTS reagent	Abcam
<i>n</i> -Butanol	Isolab
<i>n</i> -Hexane	Carlo Erba, Isolab, Sigma-Aldrich
p21	Novus Biology
p53	BioLegend
Paclitaxel	Sigma-Aldrich
Penicillin/streptomycin antibiotic mixture	Thermo Fisher
Petroleum ether	Sigma-Aldrich
p-MLKL	Abcam
Polyamide (for CC)	Fluka
Propodium Iodide (PI)	Sigma Aldrich
RediSep Rf MPLC columns (SiO ₂ and C ₁₈)	Teledyne Isco
Reverse phase (C ₁₈) F ₂₅₄ Aluminium TLC plate	Merck
Sephadex LH-20	Fluka
Silica gel 60 (for CC)	Merck
Silica gel 60 F ₂₅₄ Aluminium TLC plate	Merck
Sulphuric acid	Riedel de Haen

<i>tert</i> -Butanol	Isolab
Trypsin-EDTA	Thermo Fisher
Vanillin	Fluka

3.1.2. Equipments and Instruments

Table 59. Equipments and instruments

Equipments and Instruments	Trademark
96-well plate	Thermo Fisher
Balance	Ohaus Explorer
Basic laboratory glass equipments	Isolab
Centrifuge	Hitachi, Spectafuge, Hettic Rotina-35
Flow Cytometry System	Beckman Coulter
Freezer	Siemens
Infrared (IR) Spectrometer	Perkin-Elmer
Laminar Flow Cabinet	Flores Valles
Lyophilizator	Christ Alpha 2-4 LD
Mass Spectroscopy (ESI-MS)	Waters
Mass Spectroscopy (HR-MS)	Agilent
Medium Pressure Liquid Chromatography (MPLC)	Buchi
Nitrogen Blowing Instrument	Miulab
NMR device	Varian Mercury FT
Oven	Binder
Polarimeter	Perkin-Elmer
Rotary Evaporator	Buchi, Heidolph
Spectrophotometer	Epoch (Biotek, Agilent)
TLC Chamber	Camag
Ultrasonic Bath	Sonorex
Ultraviolet (UV) Spectrophotometer	HP Agilent
UV cabinet	CAMAG
Water bath	Memmert

3.1.3. Plant Materials

The underground parts (roots and rhizomes) of *V. alliariifolia* Adams were gathered from between Çankırı and Kastamonu road, Ilgaz Mountains in July 2016 at ca 1750 m height (41°04'23.9"N 33°44'21.3"E). The underground parts of *V. sisymbriifolia* Vahl. were collected from Gürün, Sivas in July 2018 at ca 1880 m height with the coordination of 38°49'53.4"N, 36°53'39.8"E. The underground parts of *V. speluncaria* Boiss. were gathered from Beşkuyu plateau, Kazancı Town, Ermenek, Karaman in July 2018 (ca 1830 m, 36°26'01.1"N 32°45'00.1"E). Plant materials were identified and authenticated by Prof. Dr. Hasan Kırmızıbekmez. Voucher specimens for *V. alliariifolia* Adams (YEF 16004), *V. sisymbriifolia*

Vahl. (YEF 18008) and *V. speluncaria* Boiss. (YEF 18009) have been stored at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Yeditepe University, İstanbul, Türkiye.



Figure 5. *Valeriana alliariifolia* roots and rhizomes



Figure 6. *Valeriana sisymbriifolia* roots and rhizomes



Figure 7. *Valeriana speluncaria* roots and rhizomes

3.2. Methods

3.2.1. Chromatographic Methods

3.2.1.1. Thin Layer Chromatography (TLC)

TLC method was carried out to obtain information about the general chemical profiles of the extracts, subextracts, and main fractions as well as the purity of the isolated compounds. Also, it was used for mobile phase selection in preparative separations and monitoring the collected fractions. SiO₂-coated (Normal phase) and C₁₈-coated (Reverse phase) aluminum plates were utilized as stationary phases in TLC analyses. Development of TLC plates was performed in TLC chamber and used solvent mixtures were given in Table 60.

Mobile Phases Used for TLC Analysis:

Mobile phases with different ratios of solvents (Table 60) were used in development process. In some cases, one or two drops of acetic acid were added to the mobile phase mixture in order to prevent tailing which negatively affected to resolution of spots.

Table 60. Mobile phases utilized in TLC

Stationary Phase	Mobile phases	Ratio
SiO ₂	CH ₂ Cl ₂ :MeOH:H ₂ O	90:10:1, 80:20:2, 70:30:3, 61:32:7
SiO ₂	CH ₂ Cl ₂ :EtOAc	7:1, 4:1, 3:1, 2:1, 1:1
SiO ₂	EtOAc:MeOH:H ₂ O	100:16:13, 100:10:5
SiO ₂	<i>n</i> -hexane:EtOAc	4:1, 3:1, 2:1, 1:1, 1:2
SiO ₂	Petroleum ether:EtOAc	1:1, 2:1, 3:1, 1:2
SiO ₂	Petroleum ether:Acetone	4:1, 3:1, 2:1, 1:1, 1:2
C ₁₈	MeOH:H ₂ O	1:3, 1:2, 1:1, 2:3

Developing Distance: 7-10 cm

Derivatization: 1% Vanillin/Sulphuric acid and heating in an oven at 105 °C for 2-3 min.

Visualization: UV light at 254 and 366 nm under UV cabinet (CAMAG)

3.2.1.2. Column Chromatography (CC)

3.2.1.2.A. Polyamide Column Chromatography

Polyamide column chromatography is generally preferred for the separation of phenolic compounds from non-phenolic ones in the extract. Also, this technique is mostly used for the main fractionation of the extracts that are rich in phenolic compounds which are capable of forming hydrogen bonds with polyamide filler material. For this reason, polyamide CC was chosen for the main fractionation of *n*-BuOH subextract of *V. alliariifolia* to separate phenolics and non-phenolics from each other. The appropriate amount of polyamide was weighed, mixed with dH₂O and kept over the night at room temperature. Then it was transferred into a suitable sized glass column with cotton placed at the bottom, and washed with dH₂O for a while to eliminate monomeric layers. The sample was dispersed in dH₂O and then applied onto the top of the column. When the sample was fully absorbed into the column, cotton was placed on top of it. The elution was initiated with dH₂O, and followed by increasing ratio of MeOH in dH₂O in a stepwise gradient until reaching 100 % MeOH.

3.2.1.2.B. Sephadex (Gel Filtration) Column Chromatography

In this technique, also known as molecular sieve or size-exclusion chromatography, the separation is based on the principle that the compounds in the mixture have different molecular

weights. Compounds with high-molecular weight are expected to be the first eluted when compared to low-molecular compounds. Because the low-molecular compounds travel longer distances in the column by passing through the dextran pores that constitute to the Sephadex LH-20 material. Sephadex LH-20 CC was utilized for preliminary fractionation of EtOAc subextract of all plants as well as the separation of diverse groups and particularly in purification of compounds at final stage. The adequate amount of filler material (Sephadex LH-20) was kept in MeOH or MeOH:CH₂Cl₂ (1:1) for one day in order to swell the dextran pores. The Sephadex LH-20 was then transferred and settled on an appropriately sized glass column. The sample to be purified was dissolved in proper volume of MeOH or MeOH:CH₂Cl₂ (1:1), then subjected to the Sephadex LH-20 column. Generally, isocratic elution was performed in Sephadex LH-20 column chromatography with MeOH or MeOH:CH₂Cl₂ (1:1) as mobile phases.

3.2.1.2.C. Silica Gel (SiO₂) Column Chromatography

SiO₂ column chromatography was carried out for the separation of compounds in the fractions based on their polarities. The proper amount of SiO₂ was suspended in mobile phase to start elution and then transferred into suitable-sized glass column with cotton placed bottom. After, the silica gel settled in the column, the sample, which was dissolved in proper volume of starting solvent system, was applied onto a SiO₂. During the isolation process, the polarity of mobile phase was incrementally increased by changing ratio of polar solvents in the solvent system.

Table 61. Mobile phases utilized in SiO₂ column chromatography

Mobile phases	Ratio
<i>n</i> -Hexane:EtOAc	100:0, 97.5:2.5, 92.5:7.5, 90:10, 87.5:12.5, 85:15, 82.5:17.5, 80:20, 77.5:22.5, 70:30, 65:35, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100
CH ₂ Cl ₂ :MeOH	100:0, 98:2, 97.5:2.5, 95:5, 92.5:7.5, 90:10, 87.5:12.5, 85:15, 80:20, 70:30, 60:40, 50:50
CH ₂ Cl ₂ :MeOH:H ₂ O	95:5:0, 90:10:1, 85:15:1, 85:15:2, 80:20:2, 70:30:3, 65:35:4, 61:32:7
CHCl ₃ :MeOH	95:5, 92.5:7.5, 89:11
CH ₂ Cl ₂ :EtOAc	100:0, 97.5:2.5, 92.5:7.5, 90:10, 87.5:12.5, 85:15, 82.5:17.5, 80:20, 75:25, 70:30, 65:35, 60:40, 55:45, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100
Petroleum ether:Acetone	95:5, 92.5:7.5, 90:10, 87.5:12.5, 85:15, 80:20, 75:25, 70:30
EtOAc:MeOH	1:1

3.2.1.2.D. Diaion HP-20 (Ion Exchange) Column Chromatography

Diaion HP-20 is an adsorbent resin which is composed of nonpolar styrene-divinylbenzene matrix and it used as a filler material in column chromatography owing extremely large pores in its structure. In this study, Diaion HP-20 CC was preferred for the prefractionation of *n*-BuOH subextract of *V. speluncaria* in order to get rid of sugar molecules in the extract. Adequate amount of Diaion HP-20 material was allowed to swell pores in the MeOH and left for one day. Afterwards, the material was transferred to a proper sized glass column and then material was placed in the column. Organic impurities in the material were eliminated by passing MeOH from the prepared column and then column was washed with sufficient amount of dH₂O. Sample was subjected to column before dissolved in dH₂O and H₂O-MeOH (100:0 to 0:100, increasing stepwise MeOH ratio as 20%) mixtures were used as elution solvent.

3.2.1.2.E. Medium Pressure Liquid Chromatography (MPLC)

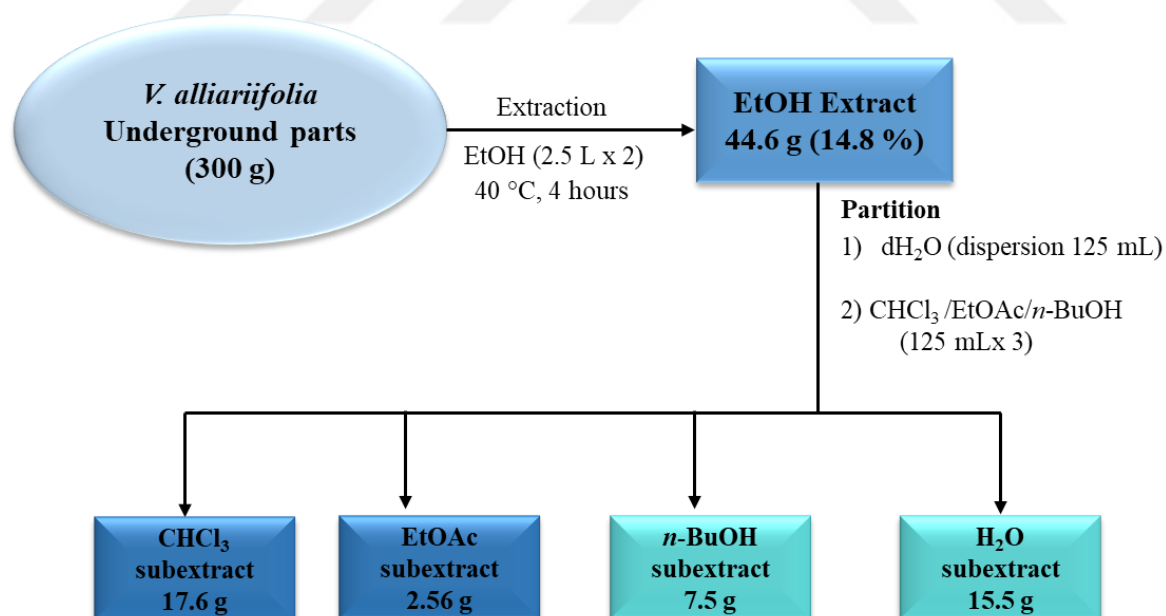
MPLC was performed for fractionation and purification of the fractions. SiO₂ and LiChroprep C₁₈ packed plastic columns were used for separation process. The column selection

was chosen by considering criteria such as the chemical profiles and amount of the extract or fraction, as well as the distances between R_f values of compounds from each other in TLC. Before sample was injected to the system, the column was initially conditioned with solvent mixture which was used as starting mobile phase. While MeOH-H₂O mixture in different ratios was used as mobile phase in LiChoroprep C₁₈-MPLC separations, CHCl₃-MeOH or CH₂Cl₂-MeOH solvent systems were utilized as elution solvents in the normal phase MPLC studies.

3.2.1.3. Extraction and Partition

3.2.1.3.A Extraction and Partition Studies on *V. alliariifolia*

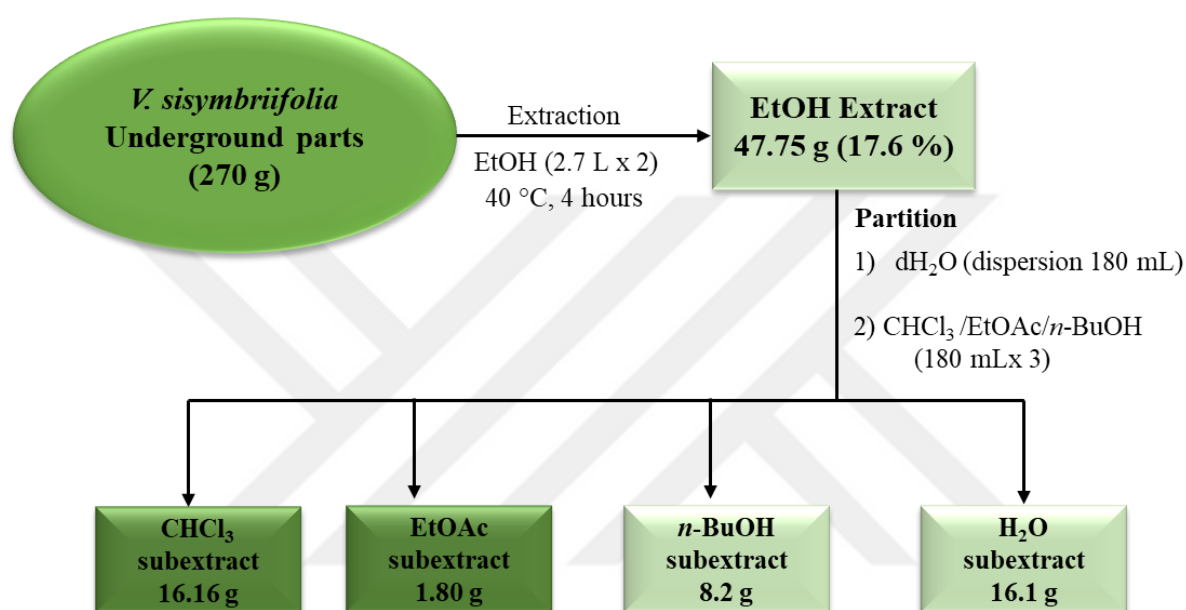
The air-dried underground parts of *V. alliariifolia* (300 g) were powdered and macerated with 2.5 L of EtOH for one day at room temperature (r. t) and then extracted at 40 °C for 4 hours. After filtration, the remaining marc was extracted again with the same amount of solvent under the same conditions. The combined extracts were concentrated under reduced pressure in rotary evaporator at 40 °C to yield crude EtOH extract (44.6 g, yield 14.8 %). Afterwards, the crude EtOH extract was suspended in 125 ml of dH₂O and then partitioned against CHCl₃ (125 ml x 3), EtOAc (125 ml x 3) and *n*-BuOH (125 ml x 3), respectively to obtain CHCl₃ (17.6 g), EtOAc (2.56 g), *n*-BuOH (7.5 g) and rH₂O (15.5 g) subextracts (Schema 1).



Schema 1. Extraction and partition studies on *V. alliariifolia*

3.2.1.3.B. Extraction and Partition Studies on *V. sisymbriifolia*

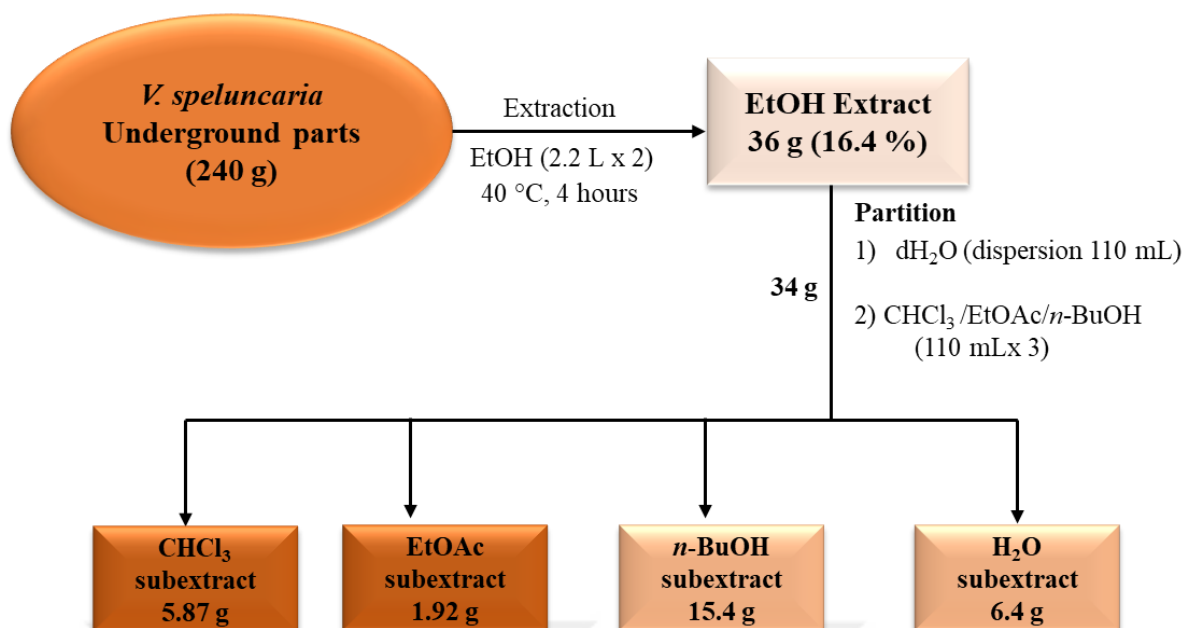
The shade-dried and powdered underground parts of *V. sisymbriifolia* (270 g) were macerated with 2.7 L of EtOH at room conditions for one day followed by extraction twice at 40 °C for 4 hours. The pooled EtOH extracts (47.75 g, yield: 17.6 %) were dried in *vacuo*. The crude EtOH extract was dispersed in 180 mL of dH₂O and then partitioned successively with equal volumes (each, 180 mL x 3) of CHCl₃, EtOAc, and *n*-BuOH, respectively to yield of CHCl₃ (16.16 g), EtOAc (1.8 g), *n*-BuOH (8.2 g) and rH₂O (16.1 g) subextracts (Schema 2).



Schema 2. Extraction and partition studies on *V. sisymbriifolia*

3.2.1.3.C. Extraction and Partition Studies on *V. speluncaria*

The air-dried and ground parts of *V. speluncaria* (240 g) were macerated with EtOH (2.2 L) at r.t for one day and then extracted exhaustively two times at 40 °C for 4 h. The organic solvent was concentrated under vacuum to give crude extract of *V. speluncaria* (36 g, yield 16.4 %). An aliquot of EtOH extract (34 g) was dispersed in rH₂O (110 mL) and then partitioned consecutively with equal volumes (each, 110 ml x 3) of CHCl₃, EtOAc and *n*-BuOH, respectively to afford CHCl₃ (5.87 g), EtOAc (1.92 g), *n*-BuOH (15.4 g) and rH₂O (6.4 g) subextracts (Schema 3).



Schema 3. Extraction and partition studies on *V. speluncaria*

3.2.1.4. Activity-guided Fractionation Studies on the Extracts

3.2.1.4.A. Main Fractionation and Isolation Studies on Cytotoxic Subextracts of *V. alliariifolia* (Schemas 4-7)

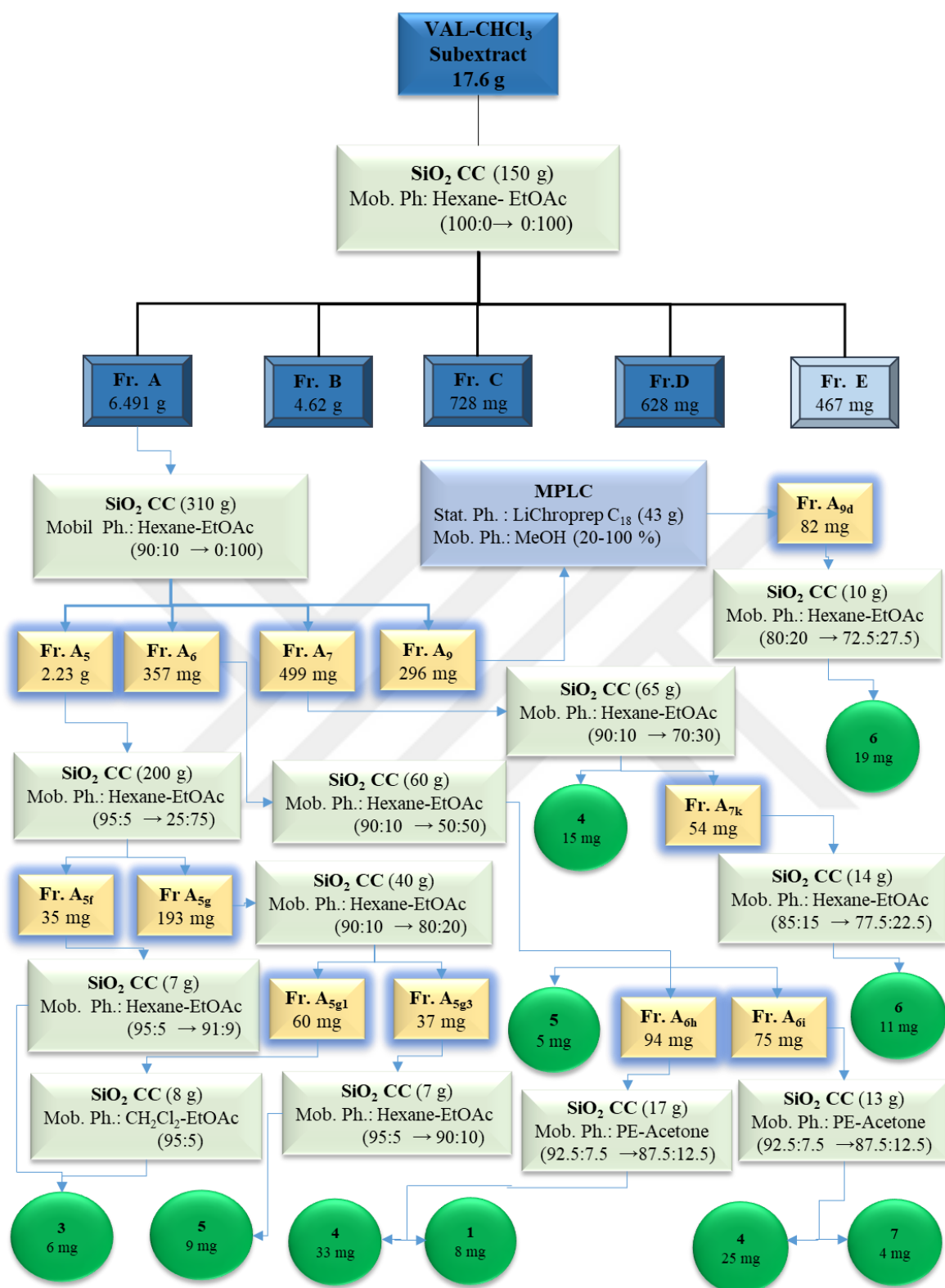
The CHCl_3 and EtOAc subextracts, which were found to be cytotoxic against the tested cancer cell lines, were fractionated to obtain main fractions. The CHCl_3 subextract (17.6 g) of *V. alliariifolia* was subjected to SiO_2 (150 g) column eluting with *n*-hexane-EtOAc mixture (0–100 %, increasing in steps of 25 % of EtOAc) to give five main fractions (frs. A-E). The EtOAc subextract (2.56 g) was loaded onto Sephadex LH-20 (100 g) CC eluting with 700 mL of MeOH to yield four main fractions (frs. 1-4).

Among the tested fractions, frs. A-D from CHCl_3 subextract and fr. 1 from EtOAc subextract displayed the most cytotoxic ones and thus they were purified by chromatographic methods. Fr. A (6.491 g) of CHCl_3 subextract was subjected to SiO_2 (310 g) column using *n*-hexane-EtOAc mixtures (10–100 %) to obtain 11 subfractions, fr. A₁₋₁₁. Similarly, fr. A₅ (2.23 g) was chromatographed over SiO_2 (200 g) CC, eluting with *n*-hexane:EtOAc (95:5 to 25:75) to give seven fractions, frs. A_{5a-g}. Compound **3** (6 mg) was purified from fr. A_{5f} (35 mg) by SiO_2 (7 g) CC eluting with *n*-hexane-EtOAc (95:5 to 91:9). Fr. A_{5g} (193 mg) was applied to SiO_2 (40 g) column using *n*-hexane-EtOAc (90:10→80:20) mixtures to yield three fractions, frs. A_{5g1-3}. Amongst, fr. A_{5g1} (60 mg) was loaded onto column that contains 8 g SiO_2 eluted with CH_2Cl_2 -EtOAc (95:5) to yield **3** (6 mg). Compound **5** (9 mg) was purified from fr. A_{5g3} (37 mg)

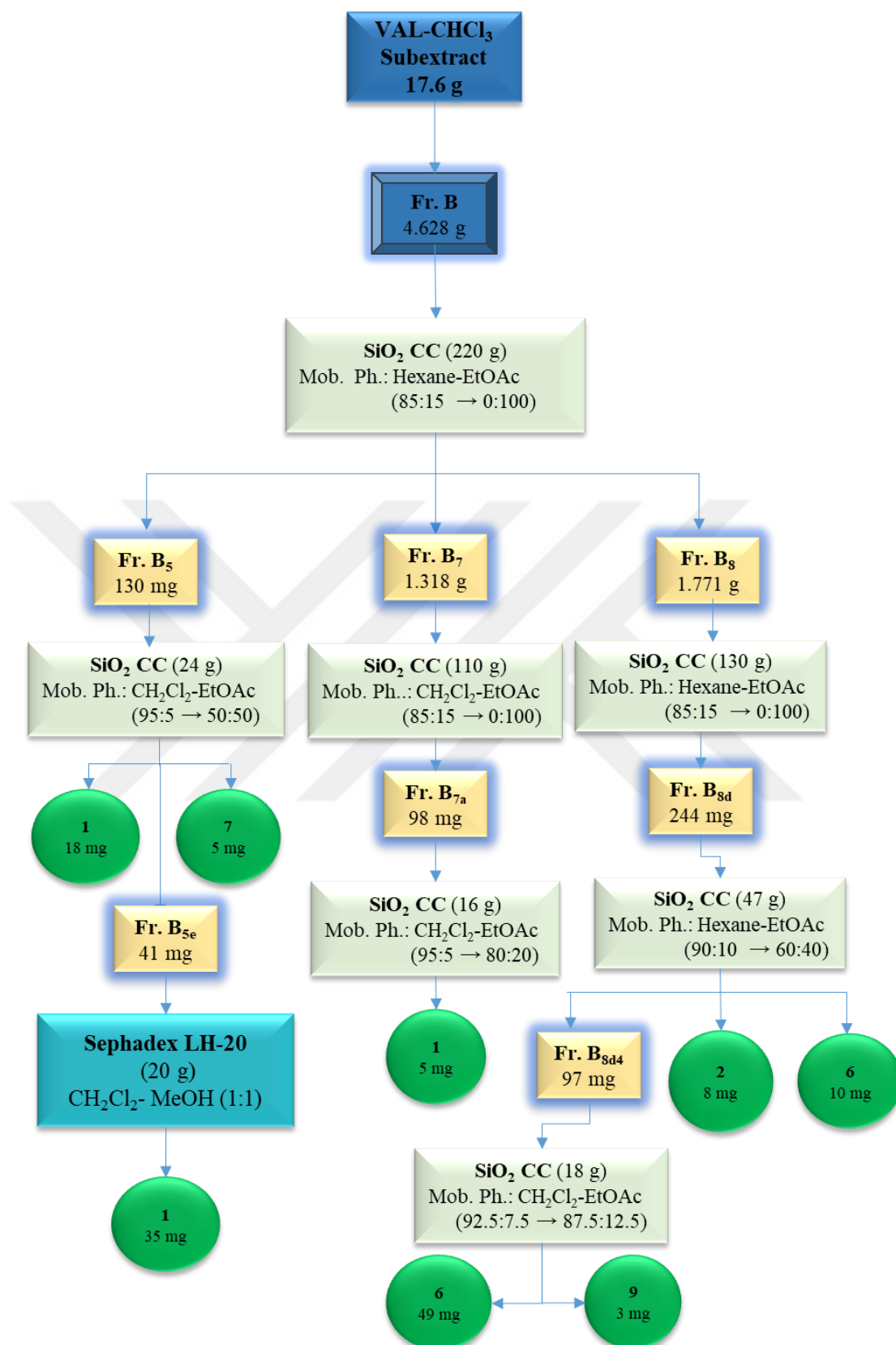
by SiO₂ (7 g) CC eluting with *n*-hexane:EtOAc (95:5→90:10). Fr. A₆ (357 mg) was chromatographed over SiO₂ (60 g) eluting with *n*-hexane:EtOAc (90:10 to 50:50) to afford **5** (5 mg) along with 12 fractions, frs. A_{6a-l}. Among these fractions, fr A_{6h} (94 mg) was submitted to SiO₂ (17 mg) CC eluted with petroleum ether (PE)-acetone (92.5:7.5→87.5:12.5) mixture to give **4** (33 mg) and **1** (8 mg). Likewise, purification of **4** (25 mg) and **7** (4 mg) was carried out from fr. A_{6i} (75 mg) by SiO₂ (13 g) CC eluting with stepwise PE-acetone gradient (7.5 to 12.5 % acetone). Fr. A₇ (499 mg) was further purified by SiO₂ (65 g) CC eluting with *n*-hexane-EtOAc (90:10 to 70:30) to afford **4** (15 mg) together 12 fractions, frs. A_{7a-m}. Fr. A_{7k} (54 mg) was separated by silica gel (14 g) column with *n*-hexane-EtOAc (85:15 to 77.5:22.5) to obtain **6** (11 mg). Fr. A₉ (296 mg) was chromatographed on a C₁₈-MPLC column (43 g) using MeOH-H₂O (20–100% MeOH) as the eluting solvent to yield seven fractions, frs. A_{9a-g}. Compound **6** (19 mg) was purified from fr. A_{9d} (82 mg) by SiO₂ (10 g) CC eluting with *n*-hexane:EtOAc (80:20→72.5:27.5). Fr. B (4.628 g) was similarly applied to SiO₂ (220 g) CC eluting with *n*-hexane-EtOAc mixture (85:15→0:100) to give 12 fractions, frs. B₁₋₁₂. Among these fractions, fr. B₅ (130 mg) was further chromatographed over silica gel (24 g) eluting with CH₂Cl₂-EtOAc (95:5→50:50) to yield **1** (18 mg), **7** (5 mg) together with five fractions, frs. B_{5a-e}. Fr. B_{5e} (41 mg) was subjected to Sephadex LH-20 column (20 g) eluting with CH₂Cl₂: MeOH (1:1) to afford **1** (35 mg). Fr. B₇ (1.318 g) was fractionated over SiO₂ (110 g) CC using CH₂Cl₂-EtOAc (85:15→0:100) as the mobile phase to give eight fractions, frs. B_{7a-h}. Compound **1** (5 mg) was purified from fr. B_{7a} (98 mg) via silica gel column (16 g) by elution of CH₂Cl₂-EtOAc (95:5→80:20). Fr. B₈ (1.771 g) was separated over silica gel (130 g) CC eluted with *n*-hexane-EtOAc (15-100%) mixture to afford ten fractions (frs. B_{8a-j}). Fr. B_{8d} (244 mg) was loaded to SiO₂ (47 g) CC eluting with *n*-hexane-EtOAc (15-100%) to yield **2** (8 mg), **6** (10 mg) together with seven fractions, frs. B_{8d1-7}. From these fractions, fr. B_{8d4} (97 mg) was rechromatographed over SiO₂ (18 g) CC eluted with CH₂Cl₂-EtOAc (92.5:7.5→87.5:12.5) to afford **6** (49 mg) and **9** (3 mg). Fr. C (728 mg) was separated by SiO₂ (110 g) CC eluted with *n*-hexane-EtOAc (80:20 to 0:100) to obtain ten fractions, frs. C₁₋₁₀. Fr. C₁ (35 mg) was subjected to SiO₂ (7 g) CC eluting with CH₂Cl₂-EtOAc (92.5:7.5 to 90:10) to obtain three fractions (frs. C_{1a-c}). Compound **2** (16 mg) and **9** (3 mg) were purified from fr. C_{1a} (20 mg) by Sephadex LH-20 (10 g) CC eluting with CH₂Cl₂: MeOH (1:1). Fr. C₂ (87 mg) was applied to SiO₂ (16 g) CC by elution of CH₂Cl₂-EtOAc (100:0 to 60:40) to yield **9** (1.5 mg) and **10** (3 mg). Fr. C₈ (216 mg) was chromatographed by SiO₂ column (32 g) eluting with CH₂Cl₂-EtOAc (90:10→30:70) to afford **12** (3 mg). Fr. D (628 mg) was subjected to C₁₈-MPLC (100 g) using H₂O-MeOH gradient (15–100% MeOH) as the eluting solvents to give eight subfractions, frs. D₁₋₈. Purification of

14 (2.5 mg) was carried out from fr. D₄ (24 mg) by SiO₂ (13 g) CC eluting with CH₂Cl₂-MeOH mixture (97.5:2.5 to 95:5). Similarly, fr. D₈ (105 mg) was applied to SiO₂ (18 mg) CC and using CH₂Cl₂-MeOH (98:2→50:50) to yield three fractions, frs. D_{8a-c}. Among these fractions, fr. D_{8b} (42 mg) was loaded onto column containing 8 g SiO₂ and eluted with CH₂Cl₂-EtOAc (85:15 to 65:35) to give three fractions, frs. D_{8b1-3}. Fr. D_{8b1} (18 mg) and fr. D_{8c} (33 mg) were combined and then applied to SiO₂ (9 g) CC eluting with CH₂Cl₂-EtOAc mixture (85:15→75:25) to yield **13** (10 mg).

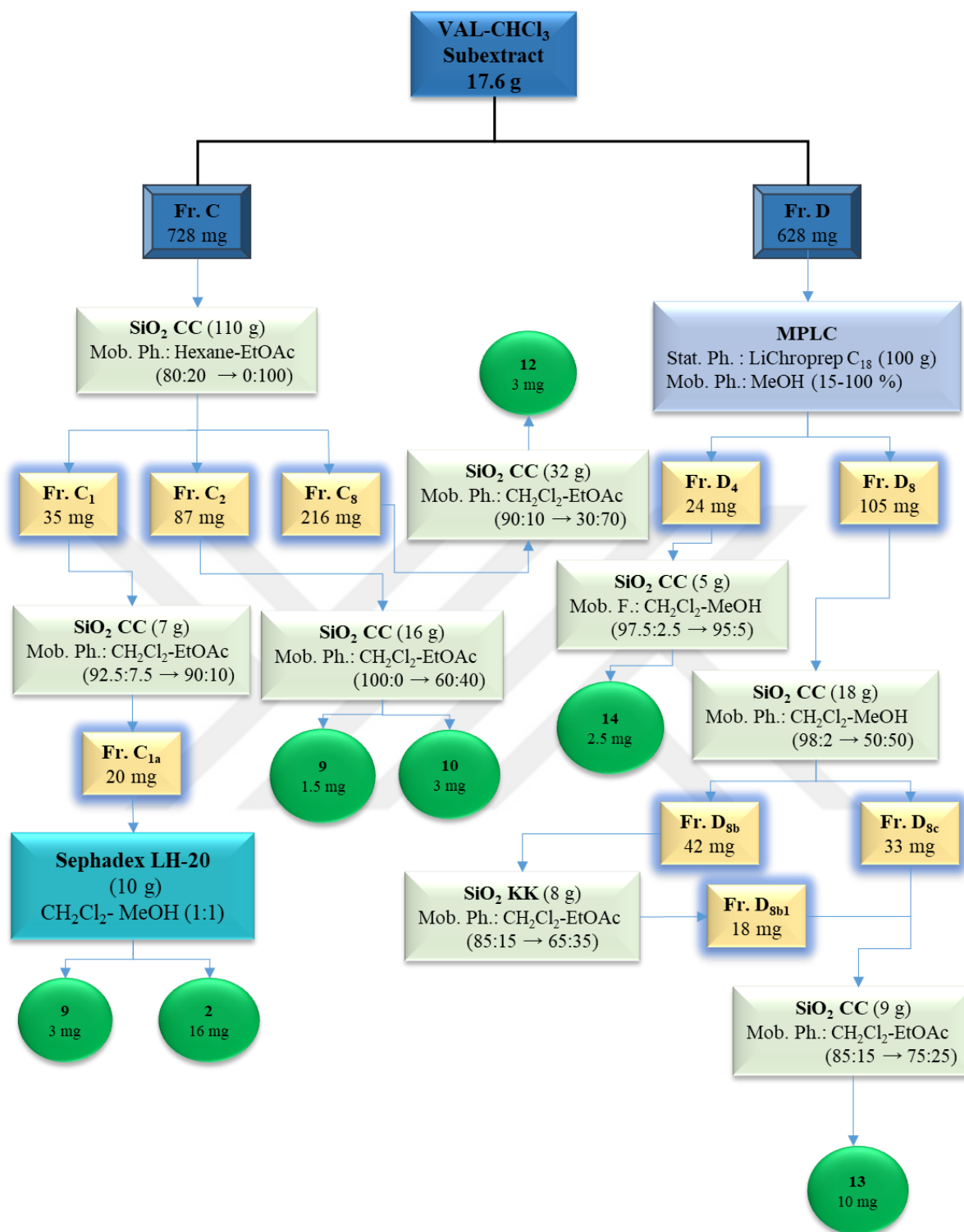
Fraction 1 (600 mg) was subjected to C₁₈-MPLC (43 g) using H₂O-MeOH (10 to 70% MeOH) to give five subfractions, frs. 1_{a-e}. Fr. 1_a (140 mg) was chromatographed over SiO₂-MPLC (24 g) eluting with stepwise CH₂Cl₂-MeOH gradient (100:0→70:30) to obtain **13** (6 mg). Compound **10** (6 mg) was purified from fr. 1_d (90 mg) by SiO₂-MPLC (12 g) eluted with increasing MeOH proportion in CH₂Cl₂ (0→75%).



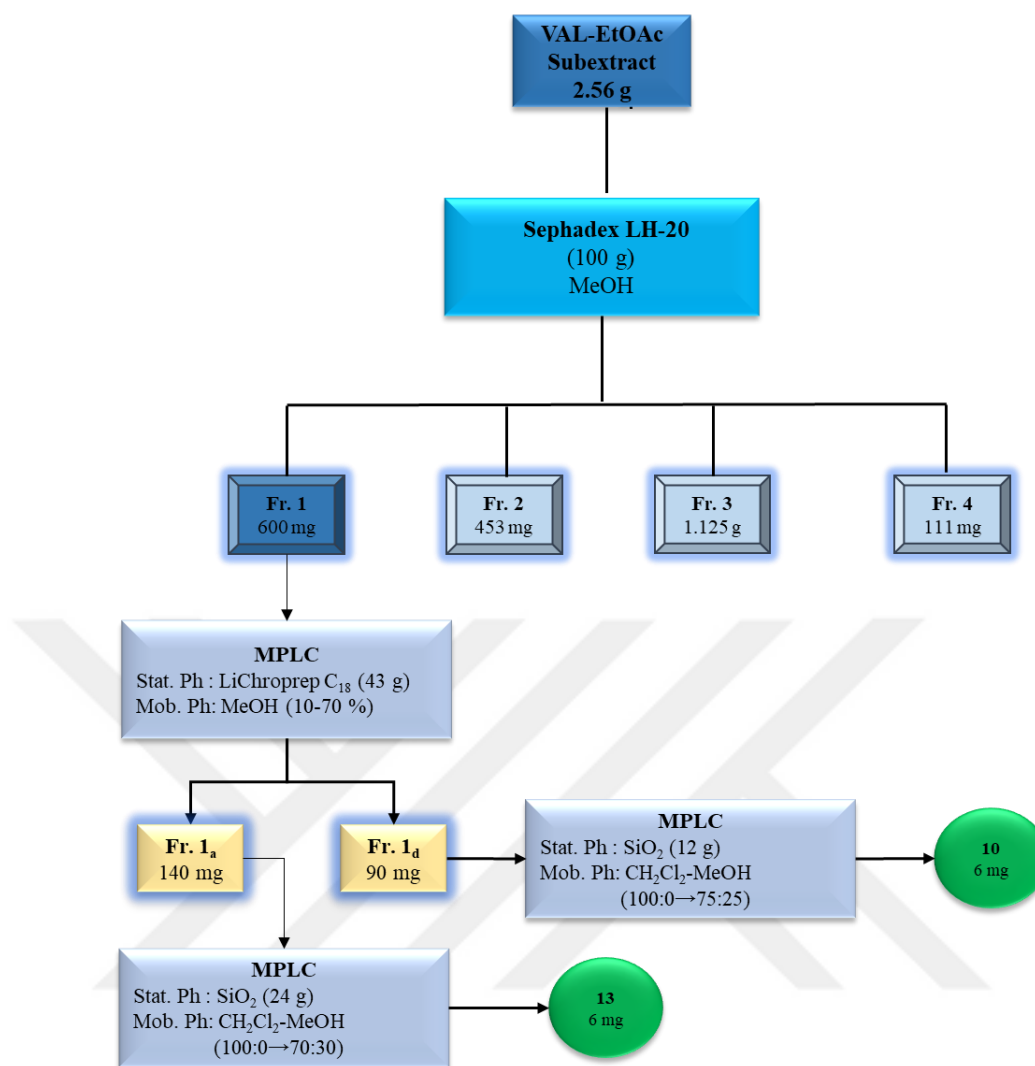
Schema 4. The isolation of compounds from *V. alliariifolia*



Schema 5. The isolation of compounds from *V. alliariiifolia*



Schema 6. The isolation of compounds from *V. alliariifolia*

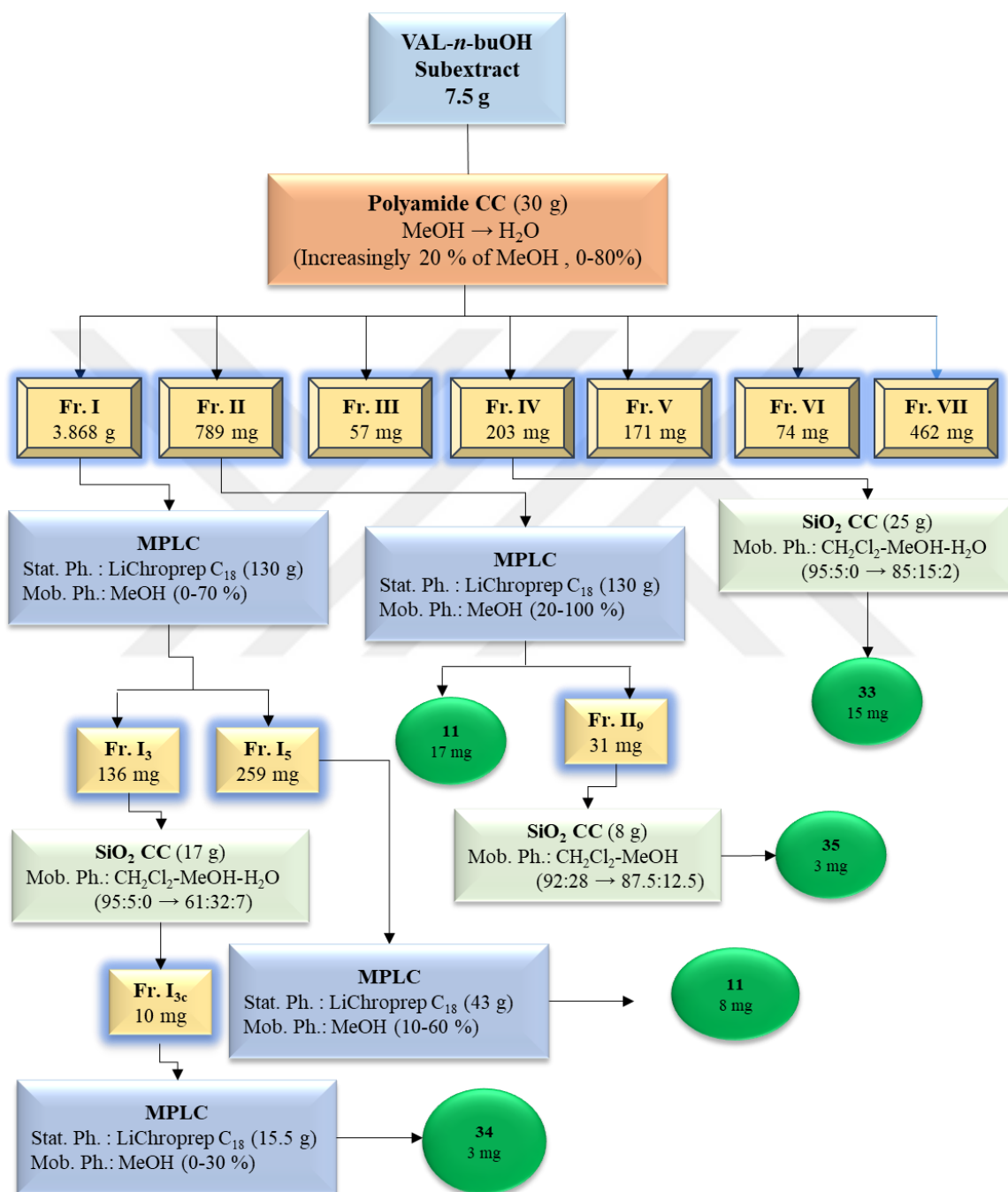


Schema 7. The isolation of compounds from *V. alliariifolia*

3.2.1.4.A.1 Main Fractionation and Isolation Studies on *n*-BuOH Subextracts of *V. alliariifolia* (Schema 8)

The *n*-BuOH subextract (7.5 g) was subjected to polyamide column (30 g) chromatography (CC) eluting H₂O-MeOH mixtures (0-100% MeOH) to give seven fractions (Fr. I-VII). Fraction I (3.868 g) was separated by C₁₈-MPLC (130 g) using MeOH-H₂O mixtures (0-70% MeOH) to obtain nine fractions (frs. I₁-9). Fr. I₃ (136 mg) was applied to SiO₂ (17 g) CC eluting with CH₂Cl₂:MeOH:H₂O (95:5:0→61:32:7) mixtures to obtain five fractions (Frs. I_{3a}-e). Among these compounds, fr. I_{3c} (10 mg) was separated by C₁₈-MPLC (15.5 g) with increasing amount of MeOH in H₂O (0-30%) to give **34** (3 mg). Fr. I₅ (259 mg) was applied to C₁₈-MPLC (43 g) by using MeOH-H₂O gradient (10-60%) to yield **11** (8 mg). Fraction II (789 mg) was separated by C₁₈-MPLC (130 g) eluting with MeOH-H₂O gradient (20-100% MeOH)

to yield 16 fractions (Frs. II₁₋₁₆) along with **11** (17 mg). Fr. II₉ (31 mg) was applied to SiO₂ (8 g) CC eluting with CH₂Cl₂-MeOH (92:8→87.5:12.5) to yield **35** (3 mg). Fr. IV (203 mg) was implemented to SiO₂ (25 g) CC eluting with CH₂Cl₂-MeOH-H₂O (95:5:0→85:15:2) to give **33** (15 mg).



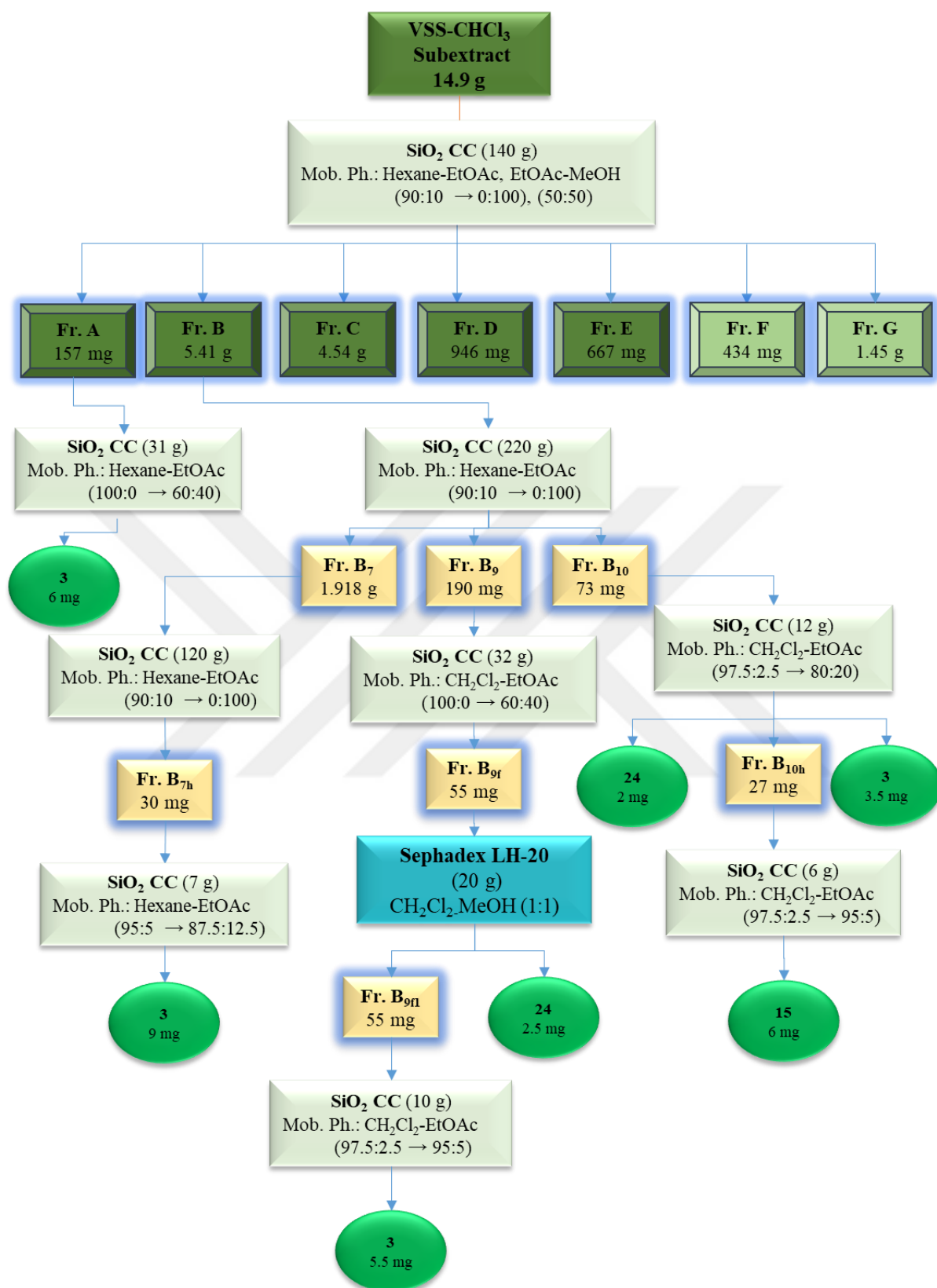
Schema 8. The isolation of compounds from *n*-BuOH subextract of *V. alliariifolia*

3.2.1.4.B. Main Fractionation and Isolation Studies on Cytotoxic Subextracts of *V. sisymbriifolia* (Schemas 9-12)

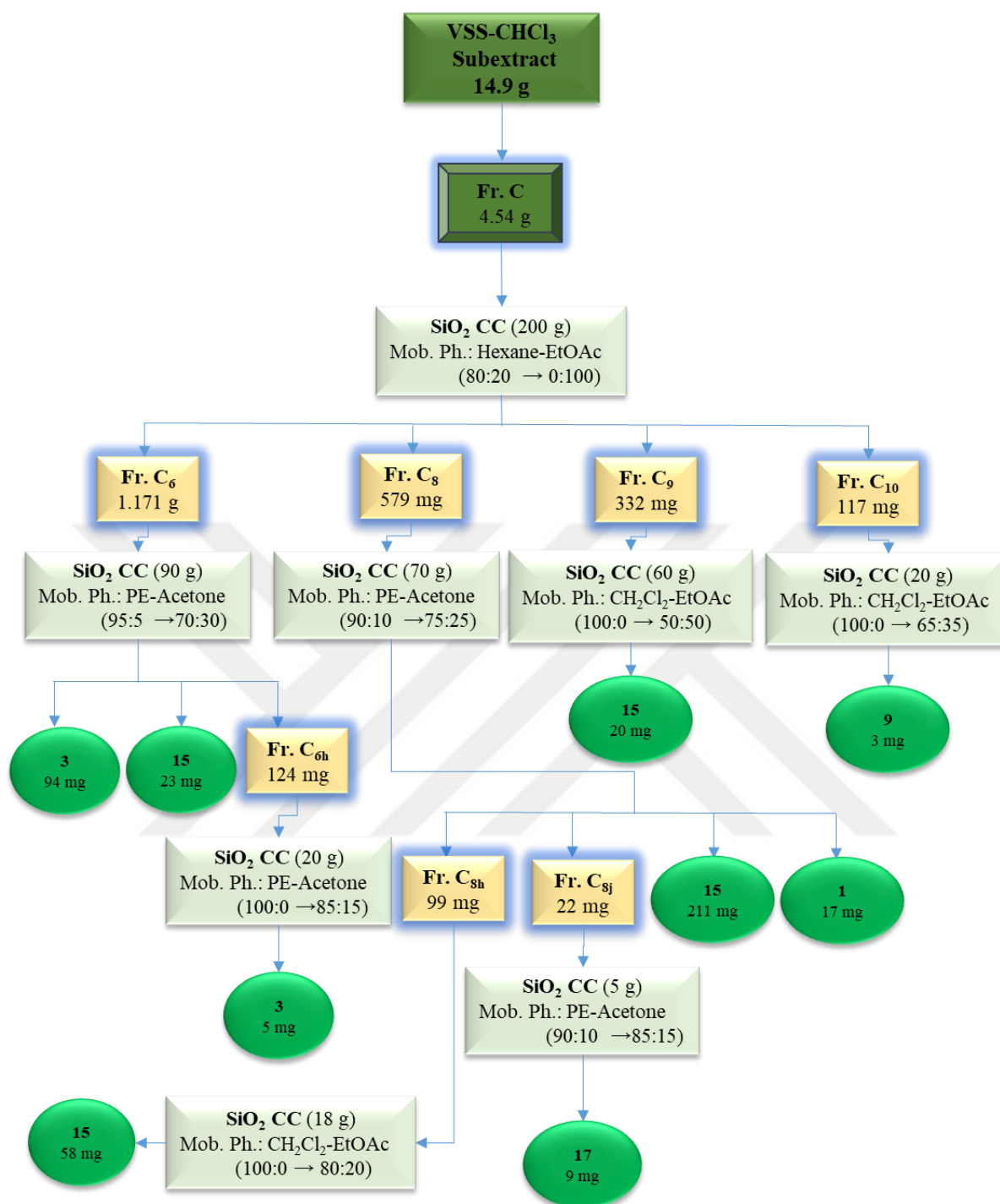
The CHCl₃ and EtOAc subextracts were selected for the further chromatographic separation to obtain main fractions since they exhibited cytotoxic activity against tested cancer cell lines. *V. sisymbriifolia* CHCl₃ subextract (14.9 g) was chromatographed over SiO₂ (140 g) CC eluting with *n*-hexane-EtOAc (0–100%, in steps of 20% EtOAc) and EtOAc-MeOH (1:1) solvent mixtures to obtain seven main fractions (frs. A-G). The EtOAc subextract (1.75 g) was subjected Sephadex LH-20 CC eluting with MeOH (1000 mL) to yield four main fractions (frs. 1-4).

Among the tested fractions, frs. A-E from CHCl₃ subextract and, frs. 1 and 2 from EtOAc subextract exhibited cytotoxicity. Therefore, they were submitted to consecutive chromatographic separations to purify compounds. Fr. A (157 mg) was applied to SiO₂ (31 g) CC using *n*-hexane-EtOAc mixture (100:0→60:40) as mobile phase to obtain **3** (6 mg). Fr. B (5.41 g) was fractionated by using SiO₂ (220 g) CC eluting with (90:10→0:100) to yield 12 fractions, frs. B₁₋₁₂. Fr. B₇ (1.918 g) was applied to SiO₂ (110 g) CC eluting with *n*-hexane-EtOAc mixture (90:10→0:100) to give 13 further fractions (frs. B_{7a-m}). Purification of **6** (9 mg) was carried out from fr. B_{7h} (30 mg) by SiO₂ (7 g) CC eluting with stepwise *n*-hexane-EtOAc gradient (95:5→87.5:12.5). Fr. B₉ (190 mg) was subjected to SiO₂ (32 g) CC eluted with CH₂Cl₂:EtOAc (100:0→60:40) to afford 12 fractions, frs. B_{9a-l}. Fr. B_{9f} (59 mg) was loaded to Sephadex LH-20 (20 g) CC eluting with CH₂Cl₂-MeOH (1:1) to obtain **24** (2.5 mg) and fr. B_{9f1}. Compound **3** (5.5 mg) was purified from fr. B_{9f1} (55 mg) by SiO₂ (10 g) CC with a gradient of CH₂Cl₂-EtOAc mixtures (97.5:2.5→95:5). Fr. B₁₀ (73 mg) was subjected to column containing 12 g SiO₂ and eluted with CH₂Cl₂-EtOAc (97.5:2.5→80:20) solvent system to afford 12 fractions (frs. B_{10a-l}) as well as **24** (2 mg) and **3** (3.5 mg). Fr. B_{10h} (27 mg) was applied to SiO₂ (6 g) CC using CH₂Cl₂-EtOAc (97.5:2.5→95:5) to yield **15** (6 mg). Fr. C (4.54 g) was further separated on SiO₂ (200 g) column eluting with a *n*-hexane-EtOAc gradient system (20–100%) to yield 12 fractions, frs. C₁₋₁₂. Fr. C₆ (1.171 g) was chromatographed over SiO₂ (90 g) CC, using Petroleum ether (PE):Acetone (95:5→70:30) mixture to give **3** (94 mg), **15** (23 mg) together with 10 fractions, frs. C_{6a-j}. Compound **6** (5 mg) was purified from fr. C_{6h} (124 mg) by SiO₂ (20 g) CC eluted with PE:Acetone (100:0 to 85:15). Fr. C₈ (579 mg) was separated by SiO₂ (70 g) CC by using PE:Acetone mixture (90:10→75:25) to afford 11 fractions (frs. C_{8a-k}) including **1** (17 mg) and **15** (211 mg). Fr. C_{8h} (99 mg) was separated by SiO₂ (18 g) CC eluted

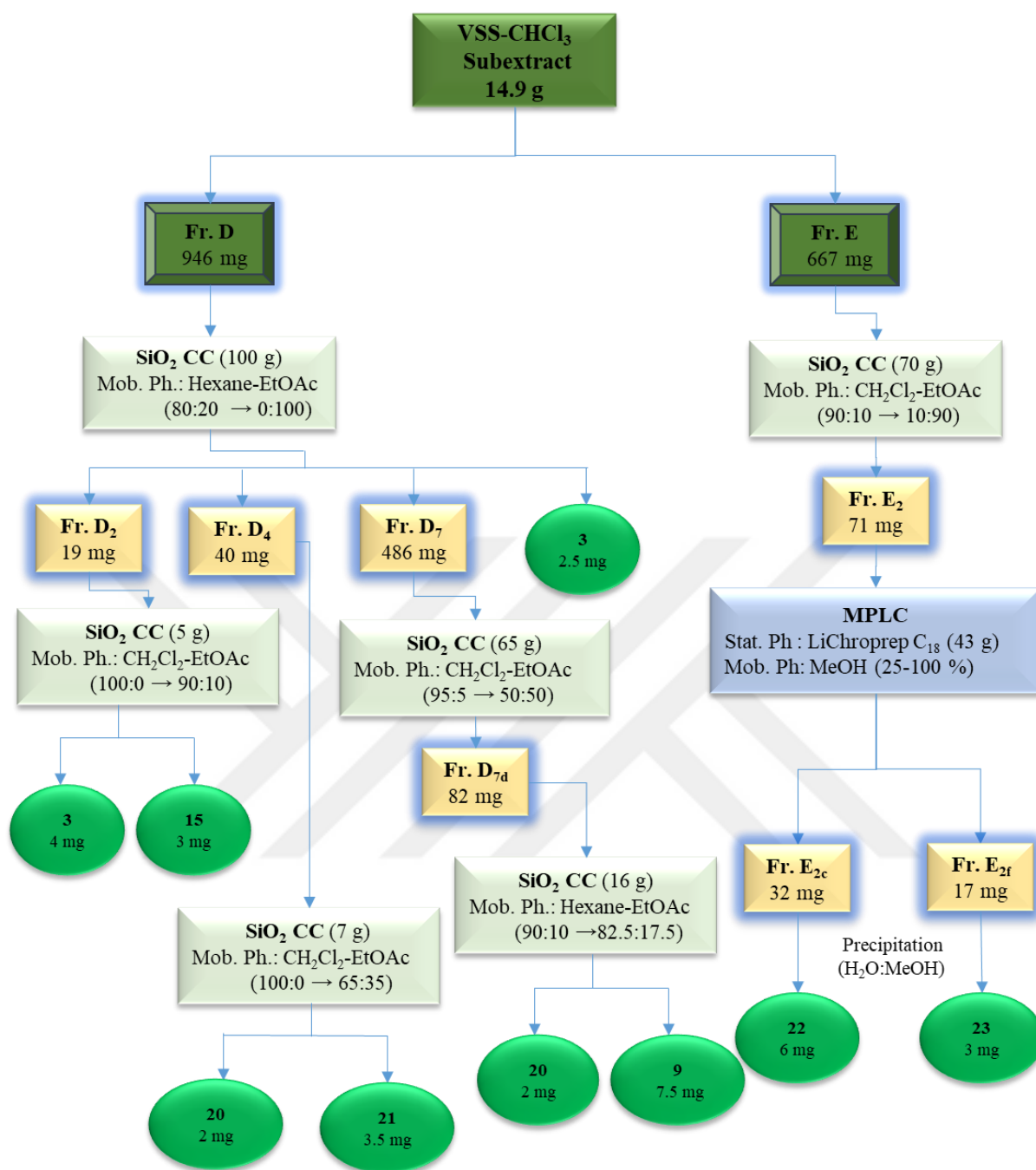
with CH₂Cl₂-EtOAc (100:0 to 80:20) to yield **15** (58 mg). Fr. C_{8j} (22 mg) was chromatographed over SiO₂ (5 g) CC eluting with PE:Acetone (90:10 to 85:15) to afford **17** (9 mg). Fr. C₉ (332 mg) was subjected to SiO₂ (60 g) CC using CH₂Cl₂-EtOAc (100:0 to 50:50) as mobile phase to obtain **15** (20 mg). The purification of **9** (3 mg) was accomplished by separation of fr. C₁₀ (117 mg) on SiO₂ (20 g) column with CH₂Cl₂-EtOAc (100:0 to 65:35) mobile system. Fr. D (946 mg) was separated over SiO₂ (100 g) CC using *n*-hexane-EtOAc (80:20→0:100) as the eluting solvent to give **3** (2.5 mg) together with 14 fractions (frs. D₁₋₁₄). Compounds **3** (4 mg) and **15** (3 mg) were purified from fr. D₂ (19 mg) eluted with CH₂Cl₂-EtOAc (100:0→90:10) mixture. Fr. D₄ (40 mg) was applied to SiO₂ (7 g) CC using CH₂Cl₂-EtOAc (100:0→65:35) to afford **20** (2 mg) and **21** (3.5 mg). Similarly, fr. D₇ (486 mg) was fractionated by SiO₂ (65 g) CC eluting with CH₂Cl₂-EtOAc (95:5→50:50) to give 13 fractions, frs. D_{7a-m}. Among these fractions, fr. D_{7d} (82 mg) was subjected to SiO₂ (16 g) CC using *n*-hexane-EtOAc (90:10→82.5:17.5) as solvent system to afford **9** (7.5 mg) and **20** (2 mg). Fr. E (667 mg) was chromatographed over SiO₂ (70 g) column eluting with CH₂Cl₂-EtOAc (90:10→10:90) to give seven fractions (frs. E₁₋₇). Fr. E₂ (71 mg) was applied to C₁₈-MPLC (43 g) with a gradient of H₂O-MeOH (25 to 100% MeOH) to give seven subfractions, frs. E_{2a-g}. Compounds **22** (6 mg) and **23** (3 mg) were successively precipitated during the concentration process of frs. E_{2c} (32 mg) and E_{2f} (17 mg) respectively. Fr. 1 from EtOAc subextract (600 mg) was applied to C₁₈-MPLC (130 g) with a gradient of H₂O-MeOH (10 to 100% MeOH) to give 10 subfractions, frs. 1_{a-j}. Fr. 1_a (108 mg) was chromatographed over Sephadex LH-20 (20 g) CC eluting with MeOH to obtain **19** (14 mg). Fr. 1_d (85 mg) was rechromatographed on Sephadex LH-20 (35 g) column using MeOH to afford two fractions, frs. 1_{d1-2}. Compound **18** (4 mg) was purified from fr. 1_{d1} (44 mg) by SiO₂ (7 g) CC eluted with increasing MeOH proportion in CH₂Cl₂ (100:0 to 80:20). Purification of fr. 1_i (239 mg) over SiO₂ (45 g) CC using *n*-hexane-EtOAc (90:10→0:100) mixtures yielded **3** (6 mg) and **15** (13 mg), together with nine fractions, frs. 1_{i1-9}. Fr. 1_{i4} (24 mg) was subjected to SiO₂ (5 mg) CC using *n*-hexane-EtOAc (90:10) mixture to afford **15** (2.5 mg). Fr. 1_j (113 mg) was loaded into SiO₂ (20 g) CC eluting with *n*-hexane-EtOAc (85:15→0:100) to yield seven fractions (frs. 1_{j1-7}) including **3** (6 mg). Fr. 1_{j1} (34 mg) was subjected to SiO₂ (8 g) CC eluted with *n*-hexane-EtOAc (82.5:17.5) to obtain **3** (2 mg), **16** (2 mg) and fr. 1_{j1} (28 mg). Purification of **16** (2 mg) was carried out from fr. 1_{j1a} (28 mg) by SiO₂ (6 g) CC eluting with *n*-hexane-EtOAc gradient (92.5:7.5→90:10). Fr. 2 (116 mg) was applied to SiO₂ (20 g) CC eluting with *n*-hexane-EtOAc gradient (90:10→0:100) to give 11 fractions, fr. 2_{a-k}. Compound **9** (2 mg) was purified from fr. 2_d (34 mg) by SiO₂ (6 g) CC using *n*-hexane-EtOAc mixtures (90:10→70:30).



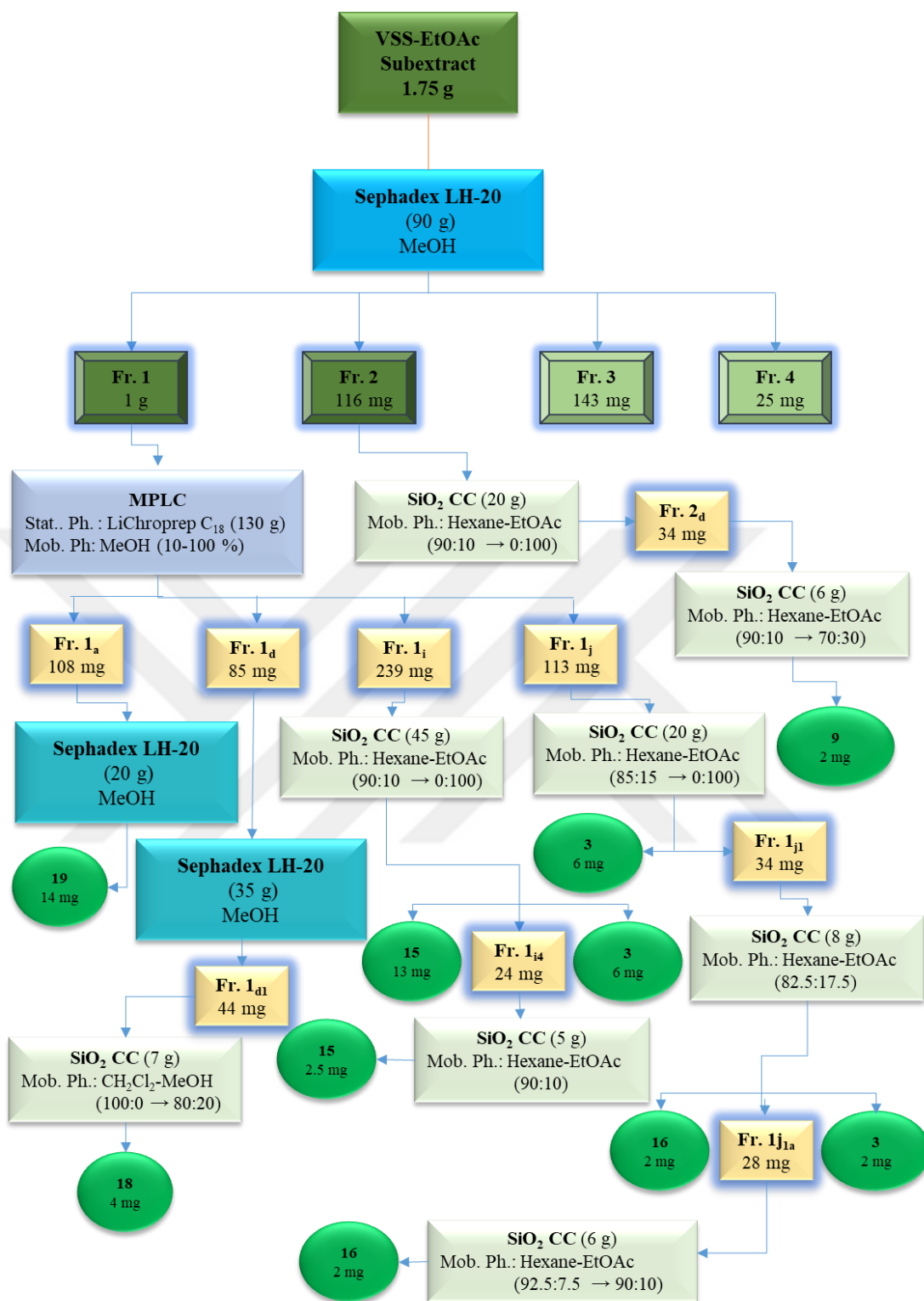
Schema 9. The isolation of compounds from *V. sisymbriifolia*



Schema 10. The isolation of compounds from *V. sisymbriifolia*



Schema 11. The isolation of compounds from *V. sisymbriifolia*



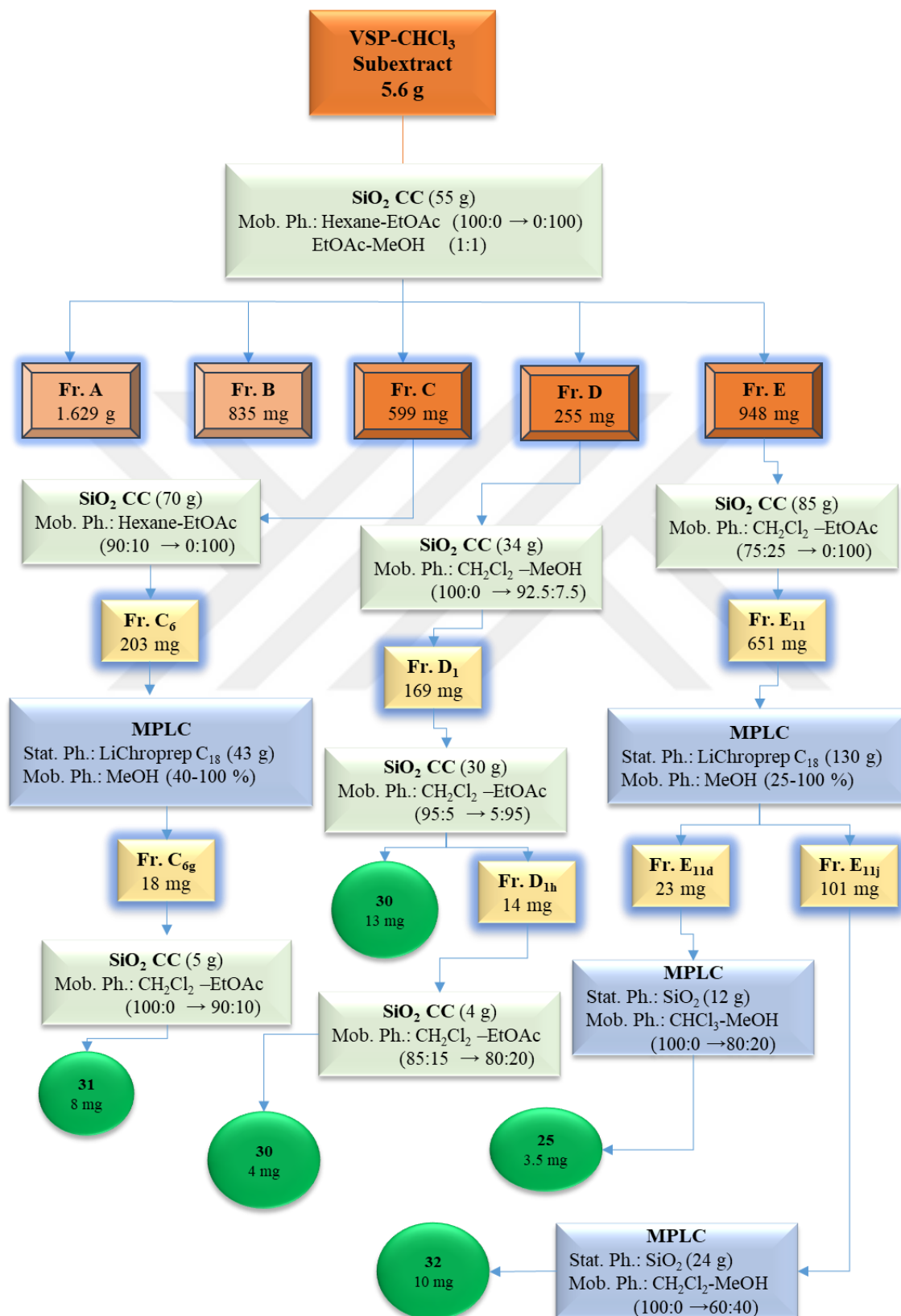
Schema 12. The isolation of compounds from *V. sisymbriifolia*

3.2.1.4.C. Main Fractionation and Isolation Studies on Cytotoxic Subextracts of *V. speluncaria* (Schemas 13 and 14)

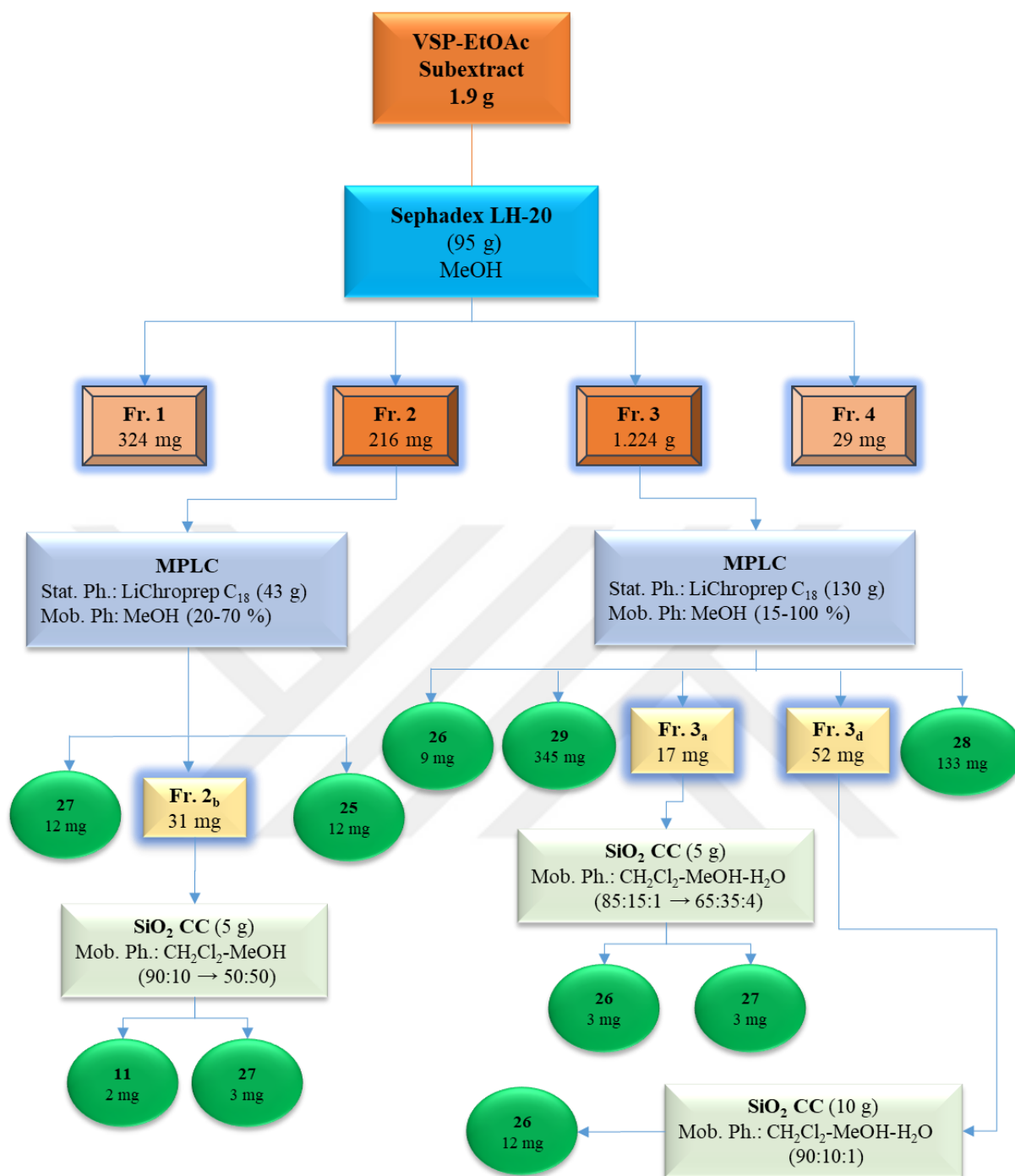
As CHCl_3 and EtOAc subextracts showed cytotoxicity against some of the cancer cell lines, they were submitted to chromatographic separations. The CHCl_3 layer (5.6 g) of *V. speluncaria* was subjected to SiO_2 (55 g) column then eluting with gradient of *n*-hexane-EtOAc (0–100% in steps of 20%) and EtOAc-MeOH (1:1) mixtures to afford five fractions (frs. A-E). To fractionate EtOAc subextract, 1.9 g of extract was loaded onto Sephadex LH-20 (95 g) CC eluting with MeOH (1.5 L) to give four main fractions (frs. 1-4).

Among the tested fractions, frs. C-E from CHCl_3 subextract and frs. 2 and 3 from EtOAc subextract were selected for further purification. Fr. C (599 mg) was fractionated with SiO_2 (70 g) CC and eluted with *n*-hexane-EtOAc mixture (90:10→0:100) to give nine fractions, frs. C_{1-9} . Fraction C_6 (203 mg) was separated by C_{18} -MPLC (43 g) with the increasing amount of MeOH in H_2O (40-100%) to afford nine fractions, frs. C_{6a-i} . Purification of **31** (8 mg) was carried out from fr. C_{6g} (18 mg) by SiO_2 (5 g) CC eluting with stepwise *n*-hexane-EtOAc gradient (100:0→90:10). Fr. D (255 mg) was applied to SiO_2 (34 g) CC eluting with CH_2Cl_2 -MeOH (100:0→92.5:7.5) to yield two fractions (frs. D_{1-2}). Fr. D_1 (169 mg) was loaded onto SiO_2 (30 g) CC using with CH_2Cl_2 -EtOAc (95:5→5:95) as mobile phase to obtain **30** (13 mg) along with 13 fractions, frs. D_{1a-m} . Compound **30** (4 mg) was also purified from fr. D_{1h} (14 mg) by SiO_2 (4 g) CC using CH_2Cl_2 -EtOAc mixtures (85:15→80:20). Fr. E (948 mg) was applied to SiO_2 (85 g) CC eluting with CH_2Cl_2 -EtOAc (75:25→0:100) mixtures to give 12 fractions, frs. E_{1-12} . Fr. E_{11} (651 mg) was separated by C_{18} -MPLC (130 g) with increasing amount of MeOH in H_2O (25-100%) to yield 10 fractions (frs. E_{11a-j}). Purification of **25** (3.5 mg) was carried out from fr. E_{11d} (23 mg) by SiO_2 -MPLC (12 g) eluting with CHCl_3 :MeOH (100:0→80:20) mixtures. Fr. E_{11j} (101 mg) was subjected to SiO_2 -MPLC (24 g) using CH_2Cl_2 -MeOH (100:0→60:40) as solvent system to afford **32** (10 mg). Fraction 2 of EtOAc (216 mg) was separated by C_{18} -MPLC (43 g) using MeOH- H_2O mixtures (20-70% MeOH) to obtain 11 fractions (frs. 2_{a-k}) in addition to **25** (12 mg) and **27** (12 mg). Purification of **11** (2 mg) and **27** (3 mg) were carried out by SiO_2 (5 g) CC from fr. 2_b (31 mg) eluting with CH_2Cl_2 -MeOH gradient (90:10→50:50). Fraction 3 (1.124 g) was separated by C_{18} -MPLC (130 g) using MeOH- H_2O gradient (15-100%, MeOH) to give **26** (9 mg), **28** (133 mg) and **29** (345 mg) together with nine fractions, frs. 3_{a-i} . Compounds **26** (3 mg) and **27** (3 mg) were purified from fr. 3_a (17 mg) by SiO_2 (5 g) CC eluted with CH_2Cl_2 -MeOH- H_2O gradient (85:15:1→61:35:4).

Fr. 3_d (52 mg) was applied to SiO₂ (10 g) CC eluting with CH₂Cl₂-MeOH-H₂O solvent system in the ratio of 90:10:1 to yield **26** (12 mg).



Schema 13. The isolation of compounds from *V. speluncaria*

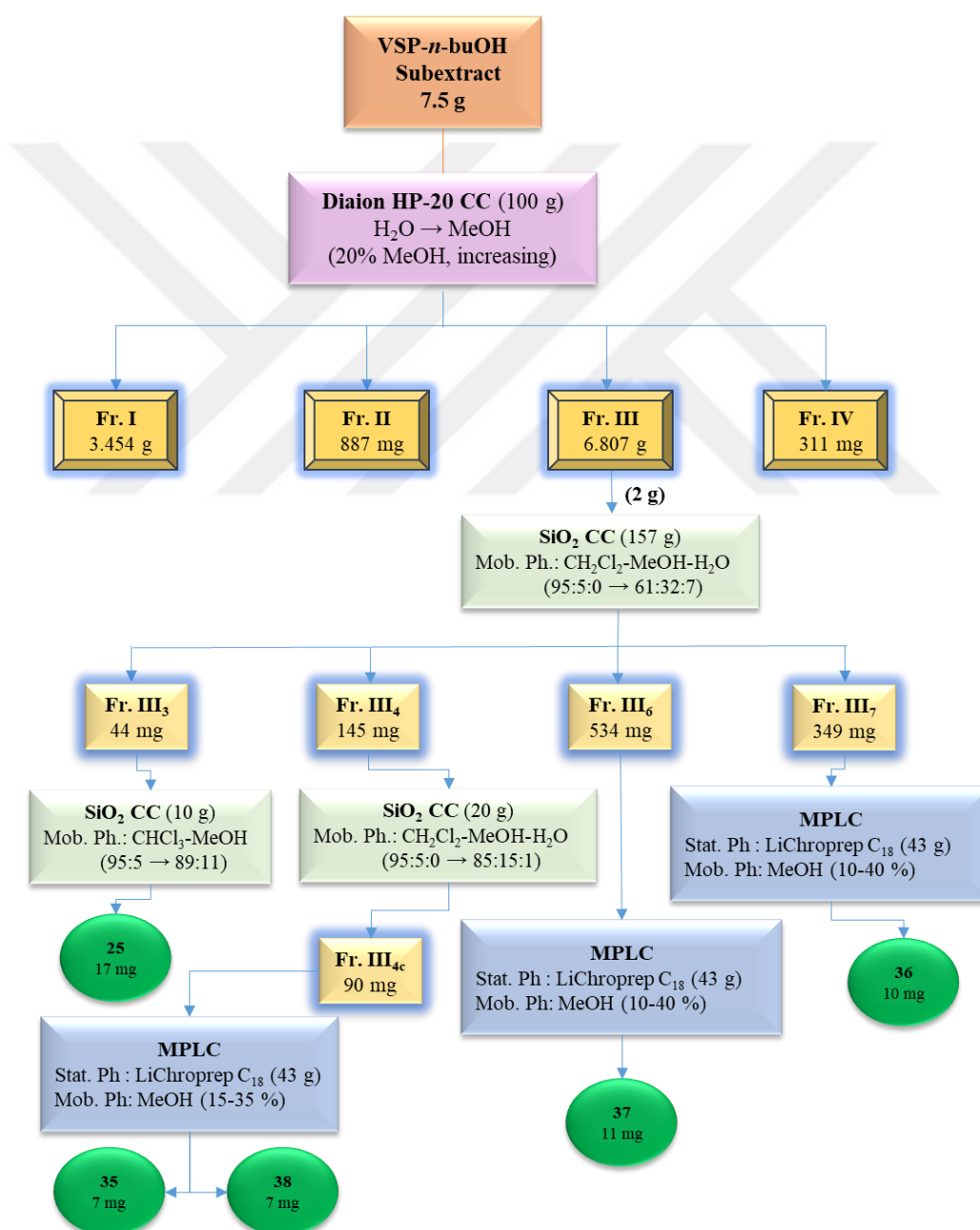


Schema 14. The isolation of compounds from *V. speluncaria*

3.2.1.4.C.1 Main Fractionation and Isolation Studies on *n*-BuOH Subextracts of *V. speluncaria* (Schema 15)

n-BuOH subextract (15.4 g) was fractionated over 100 g of Diaion HP-20 column with eluting H₂O-MeOH mixtures (0-100% MeOH, increasing 20% MeOH) to give four fractions (Fr. I-IV). An aliquot of fr. III (2 g) was subjected to SiO₂ (150 g) CC eluting with CH₂Cl₂-MeOH-H₂O (95:5:0→61:32:7) to obtain eight fractions (Frs. III₁₋₈). Fraction III₄ (145 mg) was

loaded onto SiO₂ (20 g) CC using CH₂Cl₂–MeOH–H₂O (95:5:0→85:15:1) as mobile phase to obtain three fractions (frs. III_{4a-c}). Fraction III_{4c} (90 mg) was separated by C₁₈-MPLC (43 g) using MeOH–H₂O mixtures (15–35% MeOH) to yield **35** (7 mg) and **38** (7 mg). Purification of **25** (17 mg) was accomplished by application of fr. III₃ (44 mg) on SiO₂ (10 g) CC using CHCl₃–MeOH gradient (95:15→89:11). Fraction III₆ (534 mg) was applied to C₁₈-MPLC (43 g) by using MeOH–H₂O gradient (10–40% MeOH) to obtain **37** (11 mg). Fr. III₇ (349 mg) was subjected to C₁₈-MPLC (43 g) eluting with MeOH–H₂O (10→ 40% MeOH) to afford **36** (10 mg).



Schema 15. The isolation of compounds from *n*-BuOH subextract of *V. speluncaria*

3.2.1.5. Structure Elucidation Studies on the Isolates

The exact structures of the isolated compounds were elucidated based on the comprehensive spectroscopic techniques including IR, UV, 1D (^1H and ^{13}C) and 2D (HSQC, COSY, HMBC and NOESY) NMR as well as MS or HR-ESI-MS analysis. Firstly, ^1H and ^{13}C NMR experiments were performed. The known compounds were identified by comparison of their ^1H and ^{13}C NMR and MS data with those of published in the literature. For the complex and new compounds, further 2D experiments including COSY, HSQC, HMBC and NOESY as well as HR-MS analysis were carried out to determine their exact structures.

3.2.2 Bioactivity Studies

3.2.2.1. Cell lines and Cell Cultures

Human lung (A549), gastric (HGC27), breast (MCF7), prostate (PC3), glioblastoma (U87) cancer cell lines and healthy cell line, HUVEC (human endothelial vein cell line), were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) and DMEM/F12 media, respectively. Both of these basal mediums were impregnated with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotic mixture. All cell lines were seeded into 96-well tissue culture plates as 5×10^3 cells per well and maintained in under cell culture incubator which provides humidified environment at 37 °C and %5 CO_2 to provide cellular attachment.

3.2.2.1. MTS Assay

To evaluate the *in vitro* cytotoxic activities of the crude extracts, subextracts, main fractions and isolated compounds, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay were performed. The MTS assay relies on the mitochondrial activity of the cell converting a tetrazolium salt (MTS reagent) into a coloured watersoluble formazan salt. The quantities of formazan generated by mitochondrial dehydrogenases is directly related to the amount of living cells in culture and the absorbance of formazan salts are evaluated at 490 nm (235). Phenol red-free DMEM basal media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution (antibiotics) was used for determination of concentration. Cells were placed in triplicated into 96-well plates at 5×10^3 cells per well into as triplicates and incubated overnight in cell culture incubator which provides humid environment (37 °C and 5% CO_2) for attachment of cells. The crude extracts, main fractions and isolated compounds were dissolved in dimethyl sulfoxide (DMSO) with the concentrations of 100 mg/mL for extracts and fractions and 100 mM for

compounds in order to prepare stock solutions. Afterwards, cells were then impregnated with extracts and fractions at the concentrations of 1, 5, 25, 50 and 100 µg/mL or with isolated compounds at the concentrations of 1, 5, 25, 50 and 100 µM for 48 hours. The concentration of DMSO in the medium was kept at less than 0.1% in order not to interfere with cellular activity. Untreated cells were used as control while paclitaxel was utilized as positive control. After the incubation period of the extracts or compounds was completed, 10% of the well medium volume which equals to 10 µL of MTS reagent was added into wells and then plates were reincubated for 2 hours in the incubator. The absorbance at 490 nm was measured by using a spectrophotometer at the end of the incubation period. The cells containing only medium were used as controls. Cell-free culture medium treated with equal concentration of extract/compound was used as the blank for the tested cells, and the cell-free culture medium was utilized as the blank for the control cells. The viability ratios of cells were calculated with formula given below, and IC₅₀ values of extracts or compounds were calculated in GraphPad Prism 6.

$$Viability = \frac{treatment\ OD\ at\ 490\ nm - blank\ OD\ at\ 490\ nm}{control\ OD\ at\ 490\ nm - blank\ OD\ at\ 490\ nm} \times 100$$

3.2.2.2. Evaluation of Apoptosis, Necrosis and Viability by Annexin V/Propidium Iodide Staining

Annexin V/Propidium Iodide (PI) analysis is a widely used method in the evaluation of apoptosis, based on simultaneous analysis of membrane asymmetry and permeability (236). The loss of membrane asymmetry is observed in all cells from the early stages and it is characterized by the localization of phosphatidylserine (PS) residues on the outer membrane leaflets. Annexin V, conjugated with a fluorochrome, selectively and strongly binds to PS residues located on the outer membrane leaflets in the presence of calcium and is used to detect of apoptosis from the early events by flow cytometry (237). The PI is a red fluorescent intercalating agent and is often used to detect dead cells in a population due to its impermeability to living cells. The capability of the PI to penetrate a cell is based on the permeability of the cell membrane. While the PI cannot penetrate the cell due to the protection of the plasma membrane in healthy cell conditions, it can pass through the membranes when the integrity of the plasma and nuclear membranes degrades in late apoptotic and necrotic cells and then it exhibits red fluorescence by intercalating into nucleic acids in the cell. (236).

Apoptosis, necrosis and viability of isolated compounds were evaluated with Annexin V/ PI staining by flow cytometry. In this method, cells were observed in four different areas: Viable cells in Annexin V-PI-, early apoptotic cells in Annexin V+PI-, late apoptotic cells in Annexin V+PI+ and necrotic cells in Annexin V-PI+ (Figure 8).

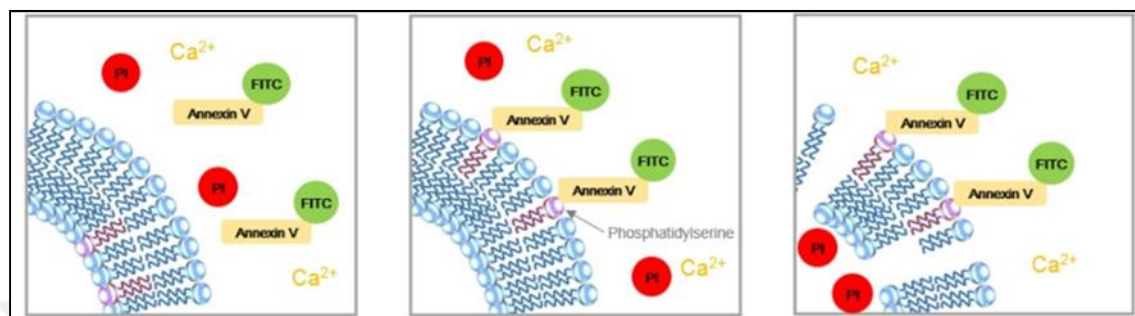


Figure 8. Schematic representation of Annexin V/Propidium Iodide analysis.

For Annexin V/PI staining, cells were dispersed in 500 μ L Annexin V binding buffer, labelled with Annexin V – FITC (2.5 μ L per test) and PI (5 μ g/mL per test, diluted with DPBS), by incubating at 4°C for 15 minutes under dark, and then instantly analyzed. 2.5×10^4 cells were evaluated per tube. Cells were gated from the Forward Scatter/Side Scatter plot, and doublets were discriminated according to Forward Scatter – Height/Forward Scatter – Area plot. Evaluations were carried out with CytExpert for DxFLEx software (Version 4.0).

3.2.2.3. DNA Content Analysis

DNA content analysis is based on measuring the differences in the amount of DNA content that cells have during the cell division phases such as $G_{0/1}$ (pre-replicative cells) phase, S (dividing cells) phase and $G_2 + M$ (post replicative plus mitotic cells) phase. DNA fragmentation arising from during apoptosis, the low-molecular DNA fragments like mono- and oligo-nucleosomes are extracted at the cell preparation and staining parts and these cells can be characterized as the apoptotic cells with fractional DNA content. Also, they are generally known as “Sub- G_1 ” population. The most common DNA fluorochromes used in DNA content analysis are 4',6-diamidino-2-phenylindole (DAPI), PI, and 7-aminoactinomycin D (7-AAD). Staining with fluorochromes that link to both RNA and DNA, like PI, demands enzymatic degradation of RNA by either pre-incubation with ribonuclease (RNase) or involvement of RNase A in a salt solution used for staining (for instance, PBS) (238).

Effects of the tested compounds on cell cycle progression and cellular division were performed with DNA content analysis. For this purpose, cells were fixed with 1 mL ice cold ethanol (70%) by incubating at 4°C for an hour. Fixative solution was discarded by centrifugation at 350g for 5 minutes, cells were washed once with DPBS, and stained with 500 µL Beckman Coulter Cell Cycle Kit (#C03551) reagent by incubating at r.t for 40 min under dark conditions. 5×10^4 cells were analyzed per tube. Evaluations were done with ModFit LT (Version 4.0) software.

3.2.2.4. Evaluation of Protein Levels by Flow Cytometry

The flow cytometry allows the analysis of extensive data sets in single cell suspensions where different parameters per cell can be evaluated simultaneously. It is possible to analyze various parameters on thousands of cells in seconds by using this method (239,240). Western blot and flow cytometry analysis are prevalently used for the analysis of protein expression. Studies comparing Western Blot and flow cytometry-based protein expression analysis revealed that the results of the two methods were consistent with each other and the reproducibility of protein level analysis using the flow cytometry found as higher than Western Blot analysis (241,242). Moreover, analysis of intracellular protein expression, particularly for cytokine levels is a widely preferred method in a flow cytometry (243).

Due to the presence of the phosphorylated parameter (p-MLKL) to be measured within the scope of the evaluation of the necrotic pathway, Tris-buffered saline (TBS) solution was used as a buffer solution instead of PBS in all washing, fixation, permeabilization and marking process after the cells were removed from petri dishes. For protein expression analysis, cells were fixed with 1 mL cold 1% paraformaldehyde solution (pH:7.4-7.6) by incubating at 4°C for an hour. Fixative solution was removed by centrifuging cells at 350g for 5 minutes, and cells were permeabilized with 1 mL tris-buffered saline solution (pH:7.5-7.6) containing 1% *Blocker™ BSA in TBS* and 0.1% Triton X-100. Fixed and permeabilized cells were labelled with primary antibodies by incubating at r.t. under dark conditions for 30 min. It has been shown that incubation over 30 minutes can lead to nonspecific binding of cells (244). Primary antibody solutions were discarded by centrifugation at 350 g for 5 minutes, and cells were stained with secondary antibodies by incubating at r.t. under dark conditions for 15 minutes. 25000 cells were evaluated per tube. Evaluations were carried out with CytExpert for DxFLEx software (Version 2.0). Fixation, permeabilization, marking and reading processes were performed on the same day, since long-term storage of the cells caused a decrease in signal levels (244).

Prior to experiments, ideal antibody concentrations were determined by staining either autophagy-induced (by treating cell with 100 μ M rapamycin) or paclitaxel-treated (10 nM) HUVECs with respective antibodies at five different concentrations; ideal concentrations of each antibody per test was selected according to their mean fluorescence intensities and presented in Table 62.

Table 62. Antibodies (with their Catalog numbers, hosts, clones and fluorochromes) and their test concentrations used in protein expression analyses

Antibody	Catalog Number	Host	Clone	Fluorochrome	Test concentration (μ L)
p53	645804	Mouse	DO-7	FITC	4
p21	NBP2-33084	Mouse	WA-1	Alexa Fluor® 750	1
Cyclin D1	NBP2-34816	Mouse	SPM587	Alexa Fluor® 647	0.25
CDK4	NBP1-31308	Rabbit	Polyclonal	-	1
CDK6	H00001021-M01	Mouse	8H4	-	1
Beclin-1	NB500-249	Rabbit	Polyclonal	Alexa Fluor® 750	1
cleaved LC3B	OAAB13518	Rabbit	Polyclonal	-	2.5
MLKL	H00197259-M02	Mouse	3B2	-	0.5
p-MLKL	ab187091	Rabbit	EPR9514	-	0.5

3.2.2.5. Statistical Analyses

Statistical analyses were done with GraphPad Prism 6 software (USA). The Shapiro-Wilk test was used to determine whether the data suitable to the normal distribution. Normally distributed data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test, and data that did not corresponding to normal distribution were analyzed by Dunn's multiple comparison test followed by Kruskal-Wallis' test. Results with a P value less than 0.05 were considered statistically significant.

4. RESULTS

The aim of this study was the isolation of cytotoxic metabolites from underground parts of *Valeriana alliariifolia*, *V. sisymbriifolia* and *V. speluncaria* naturally grown in Türkiye by activity-guided fractionation. For this reason, firstly crude EtOH extracts of these three *Valeriana* species were prepared separately, and then their subextracts (CHCl₃, EtOAc, *n*-BuOH and H₂O) were obtained, as a result of the liquid-liquid extraction. The crude EtOH extracts and subextracts were investigated for their cytotoxic effects by *in vitro* MTS assay against A549, MCF7, HGC27, PC3 and U87 cancer cell lines as well as a healthy cell line (HUVEC). According to MTS assay findings, the active subextracts were fractionated by chromatographic methods to yield main fractions which were also assayed for their cytotoxic potentials on the same cell lines in order to determine the active fractions. Chromatographic separations on the active main fractions led to the isolation of compounds. The structures were elucidated by spectroscopic methods. The cytotoxic bioactivities of the isolates were evaluated against same cell lines and the mechanisms behind the cytotoxic activities of the most active ones were elucidated. The detailed results of the study are given below.

4.1. Extraction

4.1.1. Extraction of *V. alliariifolia*

The dried and powdered underground parts of *V. alliariifolia* (300 g) were extracted with EtOH to afford crude EtOH extract (44.6 g, 14.8 %). The crude EtOH extract was firstly dissolved in dH₂O and then successively partitioned with CHCl₃, EtOAc, *n*-BuOH to obtain four subextracts; CHCl₃ (17.6 g), EtOAc (2.56 g), *n*-BuOH (7.5 g) and rH₂O (15.5 g).

4.1.2. Extraction of *V. sisymbriifolia*

The dried *V. sisymbriifolia* underground parts (270 g) were grounded and then extracted with EtOH to obtain crude EtOH extract (47.75 g, 17.6 %). The CHCl₃ (16.16 g), EtOAc (1.8 g), *n*-BuOH (8.2 g) and rH₂O (16.1 g) subextract were yielded from EtOH extract as a result of partition process as described previously.

4.1.3. Extraction of *V. speluncaria*

The air-dried and powdered underground parts of *V. speluncaria* were extracted with EtOH to yield EtOH extract (36 g, 16.4 %). The crude EtOH extract was firstly dispersed in dH₂O and then continued with partition process to yield CHCl₃ (5.87 g), EtOAc (1.92 g), *n*-BuOH (15.4 g) and rH₂O (6.4 g) as described in detail previously.

4.2. *In vitro* Cytotoxic Activity Results of Crude Extracts and Subextracts

The *in vitro* cytotoxic effects of the crude EtOH extracts and their subextracts acquired from *V. alliariifolia*, *V. sisymbriifolia* and *V. speluncaria* were tested on A549, MCF7, HGC27, PC3 and U87 cancer cell lines as well as HUVEC healthy cell line by MTS method. Paclitaxel, a clinically proven anticancer drug, was used as a positive control.

4.2.1. *In vitro* Cytotoxic Activity Results of *V. alliariifolia* Extracts

The *in vitro* MTS assay results (Table 63) revealed that crude EtOH extract of *V. alliariifolia* (VAL) displayed significant cytotoxic bioactivities with the IC₅₀ values ranging from 12.04-30.75 µg/mL against A549, MCF7, HGC27 and PC3 cancer cell lines. The subextracts were not tested against U87 cancer cell line, as the crude extract did not exhibit cytotoxicity on U87 cancer cell line (IC₅₀: >100 µg/mL). Among the subextracts, CHCl₃ and EtOAc subextracts exhibited remarkable cytotoxicity against all tested cell lines with IC₅₀ values between 2.5-21.5 µg/mL while EtOAc subextract displayed cytotoxic activity markedly only against MCF7 cell line with IC₅₀ value of 12.2 µg/mL. However, such activities were not observed in *n*-BuOH and rH₂O subextracts (Table 63).

Table 63. Cytotoxic activities of crude EtOH extract and subextracts from *V. alliariifolia* on A549, MCF7, HGC27, PC3, U87 and HUVEC cell lines.

Extracts	Cell lines, IC ₅₀ (µg/mL)					
	A549	MCF7	HGC27	PC3	U87	HUVEC
EtOH	15.1±0.21	12.04±0.7	30.75±0.11	14.13±1.7	>100	39.09±1.7
CHCl₃	21.5±0.21	2.5±0.09	12±0.29	3.5±0.09	NT	6.2±0.16
EtOAc	85.8±0.16	12.2±0.13	66.6±0.25	65.04±0.10	NT	31.9±0.15
<i>n</i>-BuOH	>100	>100	>100	>100	NT	>100
rH₂O	>100	>100	>100	>100	NT	>100
Paclitaxel*	40.5±2.7	2.3±0.6	35.1±2.6	20.5±2.7	>100	75.2±3.2

Data are mean ± SD of three independent experiments carried out in triplicate. NT: Not Tested

* Positive Control (nM)

4.2.2. *In vitro* Cytotoxic Activity Results of *V. sisymbriifolia* Extracts

As given in Table 64, the crude EtOH of *V. sisymbriifolia* (VSS) showed cytotoxicity on tested cancer cell lines, except for U87 cancer line, with the IC₅₀ values in the range of 7.46 to 12.56 µg/mL. Thus, A549, MCF7, HGC27 and PC3 cancer cell lines were used for the determination of cytotoxic effects of the subextracts. Among tested subextracts, the CHCl₃ and EtOAc subextracts displayed pronounced cytotoxic activity against MCF7, HGC27 and PC3 cancer cell lines with the IC₅₀ values in range of 2.4 to 44.1 µg/mL.

Table 64. Cytotoxic activities of crude EtOH extract and subextracts from *V. sisymbriifolia* on A549, MCF7, HGC27, PC3, U87 and HUVEC cell lines.

Extracts	Cell lines, IC ₅₀ (µg/mL)					
	A549	MCF7	HGC27	PC3	U87	HUVEC
EtOH	7.46±0.18	12.2±0.7	10.16±0.8	12.56±0.1	>100	17.6±1.02
CHCl₃	11.6±0.11	2.4±0.10	5.3±0.43	4.3±0.10	NT	3.6±0.13
EtOAc	44.1±0.13	7.5±0.09	16.3±0.48	11.8±0.11	NT	9.7±0.08
<i>n</i>-BuOH	>100	>100	>100	>100	NT	>100
rH₂O	>100	>100	>100	>100	NT	>100
Paclitaxel	40.5±2.7	2.3±0.6	35.1±2.6	20.5±2.7	>100	75.2±3.2

Data are mean ± SD of three independent experiments carried out in triplicate. NT: Not Tested

* Positive Control (nM)

4.2.3. *In vitro* Cytotoxic Activity Results of *V. speluncaria* Extracts

Unlike other crude extracts, *V. speluncaria* (VSP) EtOH extract was not active against HGC27 and PC3 cell lines. However, it displayed a strong cytotoxic effect against MCF7 and U87 cancer cell lines with IC₅₀ values of 5.09 and 2.01 µg/mL, respectively, whereas it showed weak cytotoxicity on A549 cell line (IC₅₀: 59.9 µg/mL). Therefore, MCF7 and U87 cell lines were selected for further cytotoxic activity studies of subextract/main fractions/isolates of *V. speluncaria* based for MTS assays (Table 65). Although the crude EtOH extract elicited remarkable activity on U87 and MCF7 cell lines, the subextracts showed relatively less cytotoxicity. Among the tested subextracts, CHCl₃ and EtOAc subextracts (IC₅₀: 48.8-81.0 µg/mL) were selected for further purification studies.

Table 65. Cytotoxic activities of crude EtOH extract and subextracts of *V. speluncaria* on A549, MCF7, HGC27, PC3, U87 and HUVEC cell lines.

Extracts	Cell lines, IC ₅₀ (µg/mL)					
	A549	MCF7	HGC27	PC3	U87	HUVEC
EtOH	59.92±0.11	5.09±2.1	>100	>100	2.01±0.1	83.17±0.5
CHCl₃	NT	73.7±0.10	NT	NT	70.7±0.15	66.9±0.16
EtOAc	NT	48.8±0.04	NT	NT	81.0±0.07	21.5±0.10
<i>n</i>-BuOH	NT	65.5±0.12	NT	NT	>100	>100
rH₂O	NT	>100	NT	NT	>100	>100
Paclitaxel	40.5±2.7	2.3±0.6	35.1±2.6	20.5±2.7	>100	75.2±3.2

Data are mean ± SD of three independent experiments carried out in triplicate. NT: Not Tested

* Positive Control (nM)

4.3. Main Fractionation of Cytotoxic Subextracts

The CHCl₃ and EtOAc subextracts of three *Valeriana* species were determined as cytotoxically active subextracts based on the MTS assay results. While the CHCl₃ subextracts were subjected to SiO₂ column chromatography (CC) for the main fractionation process, Sephadex LH-20 CC was used for the fractionation of the EtOAc subextracts.

4.3.1. Main Fractionation of VAL-subextracts

The CHCl₃ and EtOAc subextracts of VAL were separated into their main fractions by SiO₂ and Sephadex LH-20 column chromatography, respectively as described detailed before. The amounts of acquired main fractions from both two subextracts are presented in Table 66.

Table 66. The amount of the main fractions obtained from cytotoxic fractions of *V. alliariifolia*

Subextract	Main Fraction	Amount
CHCl ₃ subextract	Fr. A	6.49 g
	Fr. B	4.62 g
	Fr. C	0.728g
	Fr. D	0.628 g
	Fr. E	0.467 g
EtOAc subextract	Fr. 1	0.6 g
	Fr. 2	0.453 g
	Fr. 3	1.125 g
	Fr. 4	0.111 g

4.3.2. Main Fractionation of VSS-subextracts

The SiO₂ and Sephadex LH-20 column chromatographies were used for the fractionation of the CHCl₃ and EtOAc subextracts of *V. sisymbriifolia* as mentioned detailed previously. The amounts of main fractions are given in Table 67.

Table 67. The amount of the main fractions obtained from cytotoxic fractions of *V. sisymbriifolia*

Subextract	Main Fraction	Amount
CHCl ₃ subextract	Fr. A	0.157 g
	Fr. B	5.41 g
	Fr. C	4.54 g
	Fr. D	0.946 g
	Fr. E	0.667 g
	Fr. F	0.434 g
	Fr. G	1.45 g
EtOAc subextract	Fr. 1	1.04 g
	Fr. 2	0.116 g
	Fr. 3	0.143 g
	Fr. 4	0.025 g

4.3.3. Main Fractionation of VSP-subextracts

The CHCl₃ and EtOAc subextracts of VSP were fractionated with the SiO₂ and Sephadex LH-20 column chromatography, respectively as described exhaustively previously. Main fraction codes and their amounts are provided in Table 68.

Table 68. The amount of the main fractions obtained from cytotoxic fractions of *V. speluncaria*

Subextract	Main Fraction	Amount
CHCl ₃ subextract	Fr. A	1.629 g
	Fr. B	0.835 g
	Fr. C	0.599 g
	Fr. D	0.255 g
	Fr. E	0.948 g
EtOAc subextract	Fr. 1	0.324 g
	Fr. 2	0.216 g
	Fr. 3	1.224 g
	Fr. 4	0.029 g

4.4. In vitro Cytotoxic Activity Results of Main Fractions

The cytotoxic activities of the main fractions obtained from active subextracts of *V. alliariiifolia* and *V. sisymbriiifolia* were evaluated against A549, MCF7, HGC27 and PC3 cell lines, while MCF7 and U87 cell lines were used for the cytotoxic determination of main fractions of *V. speluncaria*.

4.4.1. Cytotoxic Activity Results of Main Fractions Obtained from VAL

The MTS assay findings of main fractions (Table 69) suggested that frs. A and B demonstrated noteworthy cytotoxic bioactivities against all tested cancer cell lines with the IC₅₀ values in the range of 3.62 to 12.04 µg/mL, whereas frs. C and D were found to be cytotoxic fraction against MCF7, HGC27 and PC3 cancer cells with IC₅₀ values ranging from 10.62 to 38.36 µg/mL. In the main fractions of VAL-EtOAc, only fr. 1 exhibited significant cytotoxic effect. Overall, frs. A-D of CHCl₃ and fr. 1 of EtOAc were chosen for isolation studies.

Table 69. Cytotoxic activities of the main fractions from *V. alliariifolia* on A549, MCF7, HGC27, PC3 and HUVEC cell lines

Fractions	Cell lines, IC ₅₀ (µg/mL)				
	A549	MCF7	HGC27	PC3	HUVEC
CHCl₃					
Fr. A	8.98±1.44	3.94±2	5.49±1.36	7.85±3.78	2.66±0.9
Fr. B	12.04±1.02	4.31±1.73	3.62±0.9	7.05±3.90	5.60±0.89
Fr. C	42.26±1.77	11.54±2.25	13.36±1.39	10.62±1.49	9.46±2
Fr. D	>100	38.36±2.39	26.17±0.7	27.3±1.60	39.91±1.62
Fr. E	>100	>100	>100	>100	>100
EtOAc					
Fr. 1	43±1.03	21.37±2.23	18.67±1.45	19.35±7.04	30.06±1.44
Fr. 2	>100	>100	96.05±1.36	>100	>100
Fr. 3	93.98±1.03	>100	59.86±1.18	>100	>100
Fr. 4	>100	>100	84.46±1.66	>100	>100
Paclitaxel*	40.5±2.7	2.3±0.6	35.1±2.6	20.5±2.7	75.2±3.2

Data are mean ± SD of three independent experiments carried out in triplicate.

* Positive Control (nM)

4.4.2. Cytotoxic Activity Results of Main Fractions Obtained from VSS

Among the tested main fractions of CHCl_3 subextract, frs. A-E were determined as active fractions due to cytotoxic activities of fr. A against MCF7 and PC3 cancer cells (IC_{50} : 20.96 and 17.12 $\mu\text{g/mL}$, respectively), of frs. B-D against MCF7, HGC27 and PC cancer cells (IC_{50} : 3.9 and 31.55 $\mu\text{g/mL}$), of fr. E against A549, MCF7 and PC3 (IC_{50} : 31.69, 15.89 and 28.72 $\mu\text{g/mL}$). In addition, fr.1 of EtOAc subextract displayed significant cytotoxic effects against all tested cancer cell lines with IC_{50} values ranging from 7.86 to 26.98 $\mu\text{g/mL}$, while fr.2 of EtOAc subextract demonstrated pronounced cytotoxicity on MCF7, HGC27 and PC3 cancer cells (IC_{50} : 15.08-38.35 $\mu\text{g/mL}$) (Table 70).

Table 70. Cytotoxic activities of the main fractions from *V. sisymbriifolia* on A549, MCF7, HGC27, PC3 and HUVEC cell lines

Fractions	Cell lines, IC_{50} ($\mu\text{g/mL}$)				
	A549	MCF7	HGC27	PC3	HUVEC
CHCl_3					
Fr. A	>100	20.96 $\pm$ 1.83	42.84 $\pm$ 3.37	17.12 $\pm$ 1.05	34.56 $\pm$ 4.12
Fr. B	44.9 $\pm$ 1.16	12.63 $\pm$ 1.68	13.79 $\pm$ 2.77	3.9 $\pm$ 0.8	9.87 $\pm$ 4.85
Fr. C	50.84 $\pm$ 1.84	13.08 $\pm$ 2.01	4.79 $\pm$ 1.57	13.33 $\pm$ 1.65	15.81 $\pm$ 4.66
Fr. D	53.19 $\pm$ 1.81	19.87 $\pm$ 2.05	22.76 $\pm$ 2.7	31.55 $\pm$ 2.09	12.77 $\pm$ 4.7
Fr. E	31.69 $\pm$ 1.52	15.89 $\pm$ 2.33	52.87 $\pm$ 3.92	28.72 $\pm$ 1.8	8.08 $\pm$ 6.31
Fr. F	73.79 $\pm$ 2.26	26.72 $\pm$ 2.10	88.35 $\pm$ 3.2	89.01 $\pm$ 2.61	29.93 $\pm$ 4.55
Fr. G	90.18 $\pm$ 3.3	90.15 $\pm$ 2.98	>100	>100	>100
EtOAc					
Fr. 1	26.98 $\pm$ 1.36	12.32 $\pm$ 3.01	11.13 $\pm$ 1.43	7.86 $\pm$ 1.28	9.173 $\pm$ 1.03
Fr. 2	>100	15.08 $\pm$ 1.11	38.35 $\pm$ 1.8	32.62 $\pm$ 0.6	37.14 $\pm$ 1.63
Fr. 3	>100	>100	>100	>100	>100
Fr. 4	>100	>100	>100	>100	>100
Paclitaxel*	40.5 $\pm$ 2.7	2.3 $\pm$ 0.6	35.1 $\pm$ 2.6	20.5 $\pm$ 2.7	75.2 $\pm$ 3.2

Data are mean $\pm$ SD of three independent experiments carried out in triplicate.

* Positive Control (nM)

4.4.3. Cytotoxic Activity Results of Main Fractions Obtained from VSP

According to the cytotoxic activities findings obtained from MTS assay, frs. C-E of CHCl₃ extract showed pronounced cytotoxic effects against MCF7 and U87 cancer cell lines (IC₅₀: 8.44-35.05 µg/mL), whereas frs. 2 and 3 displayed moderate cytotoxicity against only MCF7 cancer cell line with the IC₅₀ values of 63.63 and 32.31 µg/mL (Table 71).

Table 71. Cytotoxic activities of the main fractions from *V. speluncaria* on MCF7, U87 and HUVEC cell lines

Fractions	Cell lines, IC ₅₀ (µg/mL)		
	U87	MCF7	HUVEC
CHCl₃			
Fr. A	>100	>100	>100
Fr. B	45.65±1.94	44.51±1.26	>100
Fr. C	23.82±1.74	8.44±2.45	>100
Fr. D	8.86±0.91	10.52±1.01	6.01±2.27
Fr. E	35.05±1.24	29.61±1.28	2.248±2.51
EtOAc			
Fr. 1	>100	>100	86.2±1.04
Fr. 2	>100	63.63±1.37	>100
Fr. 3	>100	32.31±1.16	12.12±2.62
Fr. 4	>100	>100	15.02±2.58
Paclitaxel*	>100	2.29±0.6	75.19±3.20

Data are mean ± SD of three independent experiments carried out in triplicate.

* Positive control (nM)*

4.5. Phytochemical Findings

Consecutive chromatographic separations on cytotoxically active main fractions of *V. alliariifolia* (frs. A-D and fr. 1), *V. sisymbriifolia* (frs. A-E and frs. 1 and 2) and *V. speluncaria* (frs. C-E and frs. 2 and 3) led to the purification of potent cytotoxic molecules (Figure 9). In addition to activity-guided isolation studies, *n*-BuOH subextracts of *V. alliariifolia* (frs. I, II and IV) and *V. speluncaria* (fr. III) were also investigated for their main secondary metabolites to make a contribution to chemotaxonomy of the genus *Valeriana*. Consequently, 18

compounds (**1-14** and **32-35**) from *V. alliariifolia*, 13 compounds (**1, 3, 9** and **15-24**) from *V. sisymbriifolia* and 13 compounds (**11, 25-31** and **35-38**) from *V. speluncaria* were isolated and categorized as iridoid and derivatives (**1-9, 12-13, 15-20**), lignans (**14, 25, 30, 31** and **35-38**), phenylpropanes (**10, 11, 26** and **34**), quinic acid ester (**27-29**), monoterpene ester (**21**), sesquiterpene lactones (**22** and **23**), fatty acid (**24**), phytosterol (**32**) and flavonoid (**33**). The list of isolated compounds are given in Table 72 and Figure 9. Among the isolates, compounds **17, 18, 22, 23, 30** and **31** are previously unreported compounds, while the spectroscopic data of **12** are being reported for the first time by this study.

Table 72. Isolated compounds from *V. alliariifolia*, *V. sisymbriifolia* and *V. speluncaria*

Compound No	Compound Name	Species
1	Didrovaltrate	<i>V. alliariifolia</i> <i>V. sisymbriifolia</i>
2	IVHD-valtrate	<i>V. alliariifolia</i>
3	Valtrate	<i>V. alliariifolia</i> <i>V. sisymbriifolia</i>
4	1- β -acevaltrate	<i>V. alliariifolia</i>
5	Seneciovaltrate	<i>V. alliariifolia</i>
6	Valtrate hydrine B2	<i>V. alliariifolia</i>
7	Isovaleroxyvaltrate hydrine	<i>V. alliariifolia</i>
8	Valerosidate	<i>V. alliariifolia</i>
9	Baldrinal	<i>V. alliariifolia</i> <i>V. sisymbriifolia</i>
10	Coniferyl aldehyde	<i>V. alliariifolia</i>
11	Coniferin	<i>V. alliariifolia</i> <i>V. speluncaria</i>
12	7-deisovaleroylvaltrate	<i>V. alliariifolia</i>
13	Valeriotriate B	<i>V. alliariifolia</i>
14	8-hydroxypinoresinol	<i>V. alliariifolia</i>
15	1- α -acevaltrate	<i>V. sisymbriifolia</i>

Table 72. Isolated compounds from *V. alliariifolia*, *V. sisymbriifolia* and *V. speluncaria*
(Continued)

16	7-homovaltrate	<i>V. sisymbriifolia</i>
17	Sisymbriifolivaltrate	<i>V. sisymbriifolia</i>
18	Sisymbriifolioside	<i>V. sisymbriifolia</i>
19	Patriscabrol	<i>V. sisymbriifolia</i>
20	11-ethoxyviburtinal	<i>V. sisymbriifolia</i>
21	Bornyl caffeate	<i>V. sisymbriifolia</i>
22	Sisymbriifolin A	<i>V. sisymbriifolia</i>
23	Sisymbriifolin B	<i>V. sisymbriifolia</i>
24	9-decenoic acid (caproic acid)	<i>V. sisymbriifolia</i>
25	Pinoresinol 4-O- β -D-glucopyranoside	<i>V. speluncaria</i>
26	Caffeic acid	<i>V. speluncaria</i>
27	Chlorogenic acid	<i>V. speluncaria</i>
28	3,4-dicaffeoylquinic acid	<i>V. speluncaria</i>
29	3,5-dicaffeoylquinic acid	<i>V. speluncaria</i>
30	Speluncarin A	<i>V. speluncaria</i>
31	Speluncarin B	<i>V. speluncaria</i>
32	β -sitosterol 3-O- β -D-glucopyranoside (Daucosterol)	<i>V. speluncaria</i>
33	Eriocitrin	<i>V.alliariifolia</i>
34	Xiecaoside E	<i>V.alliariifolia</i>
35	8-hydroxypinoresinol 4'-O- β -glucopyranoside	<i>V.alliariifolia</i> <i>V. speluncaria</i>
36	Pinoresinol 4,4'-di-O- β -D-glucopyranoside	<i>V. speluncaria</i>
37	Medioresinol di-O- β -D-glucopyranoside	<i>V. speluncaria</i>
38	Fraxiresinol 1-O- β -D-glucopyranoside	<i>V. speluncaria</i>

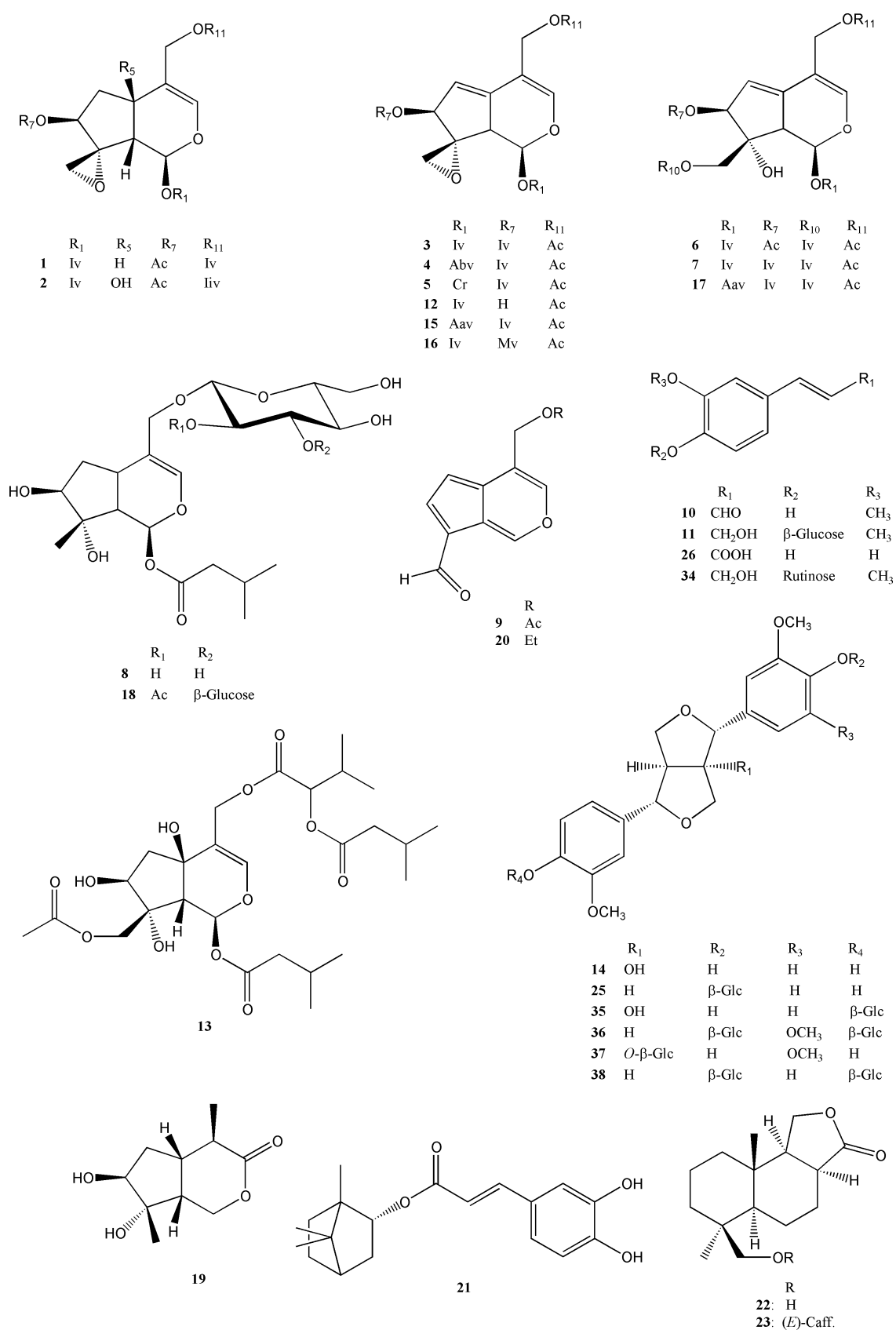


Figure 9. Chemical structures of isolated compounds (1-38)

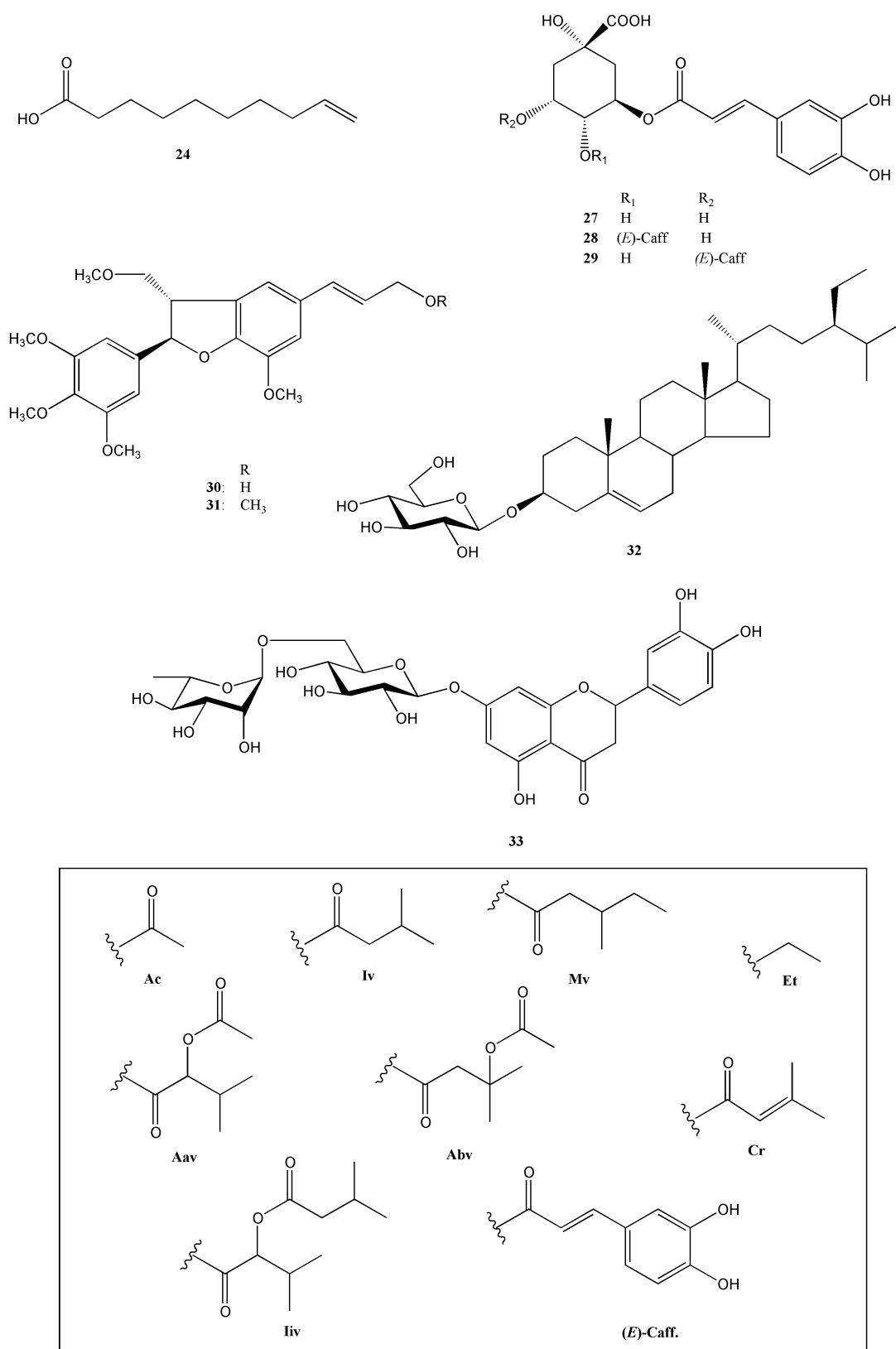


Figure 9. Chemical structures of isolated compounds (1-38) (Continued)

4.5.1. Structure Elucidation of Isolated Compounds

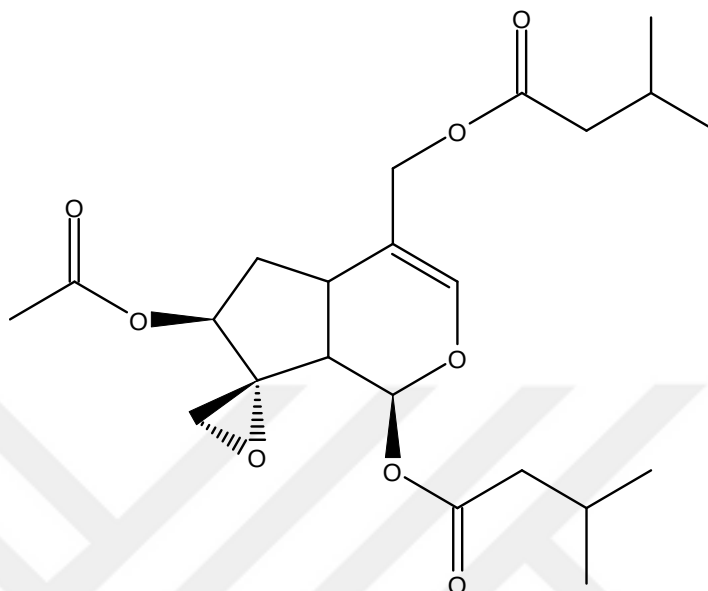
The exact structures of the isolated compounds were elucidated based on the comprehensive spectroscopic techniques including 1D (^1H and ^{13}C) and 2D (HSQC, COSY, HMBC and NOESY) NMR as well as MS or HRESIMS analysis. In addition, UV analysis and IR spectroscopy were used to provide supportive information about the structure of compounds. The structural elucidations of the new compounds (**17**, **18**, **22**, **23**, **30** and **31**) are extensively discussed considering their spectroscopic findings, while the structures of the known compounds discussed briefly and referred to related articles. The spectroscopic data as well as the NMR spectra of all compounds were provided in this section.



4.5.1.1. Compounds Isolated from Cytotoxic Main Fractions of *V. alliariifolia*

4.5.1.1.1. Iridoids

4.5.1.1.1.A Non-glycosidic Iridoids



DIDROVALTRATE (1): C₂₂H₃₂O₈ (MW: 424.48)

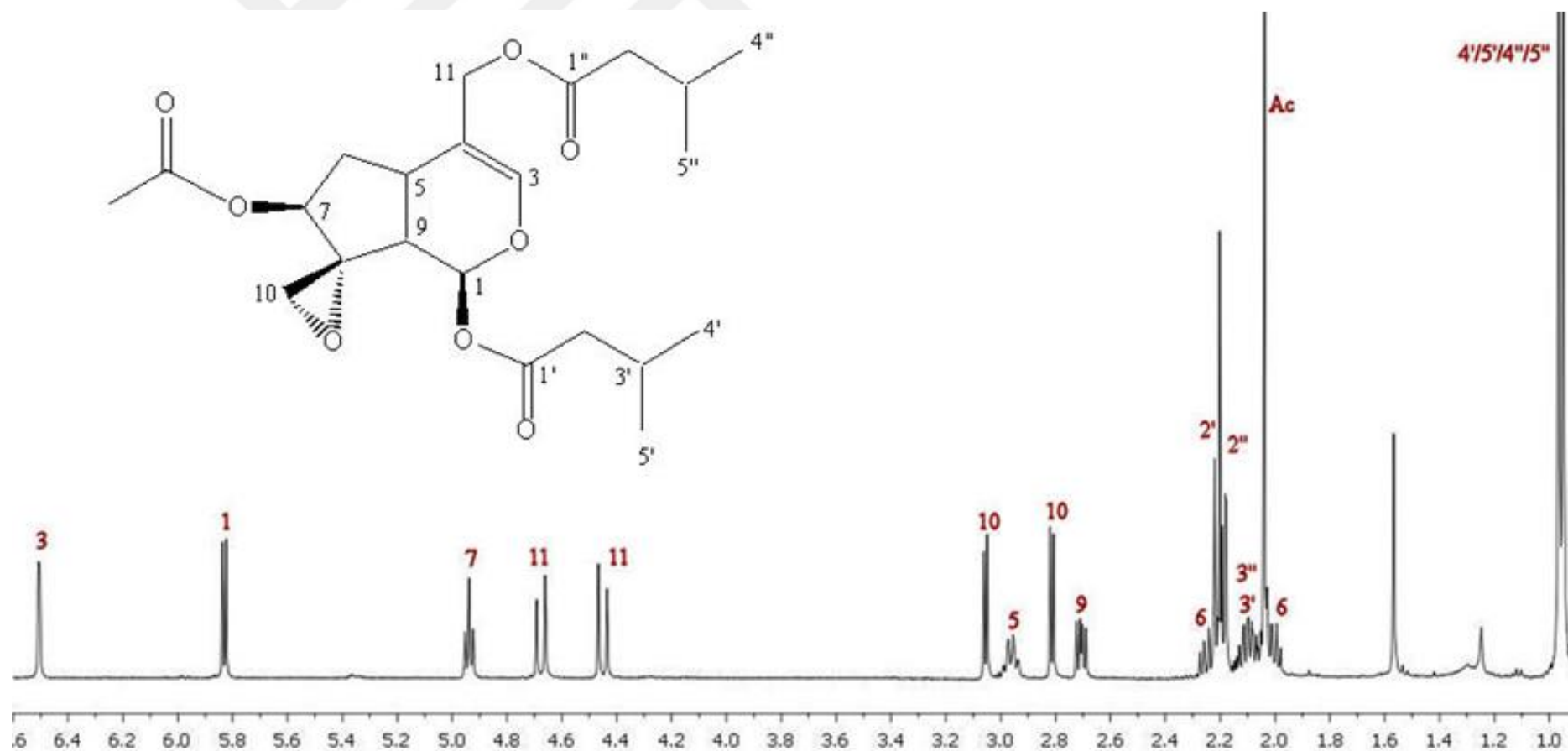
UV λ_{max} (MeOH) nm:	207
IR ν_{max} (KBr) cm ⁻¹ :	1776, 1737 (C=O), 1639, 1607 (C=C),
ESI-MS m/z :	448.11 [M+Na] ⁺
¹ H NMR:	Table 73, Spectrum 1
¹³ C NMR:	Table 73, Spectrum 2
COSY:	Spectrum 3
HSQC:	Spectrum 4
HMBC:	Spectrum 5

Compound **1** was obtained as colorless oil from CHCl₃ subextract of *V. alliariifolia*. The UV spectrum of **1** demonstrated absorption maximum at 207. Its IR spectrum showed absorption bands due to ester carbonyl (1776 and 1737 cm⁻¹) and olefinic (1639 and 1607 cm⁻¹) groups. Analysis of 1D (¹H and ¹³C) and 2D (COSY, HSQC and HMBC) NMR spectra (Table 73, Spectra 1-5) of **1** indicated that **1** was a monoene-type valepotriate. The spectroscopic findings were superimposed with those reported for didrovaltrate (161).

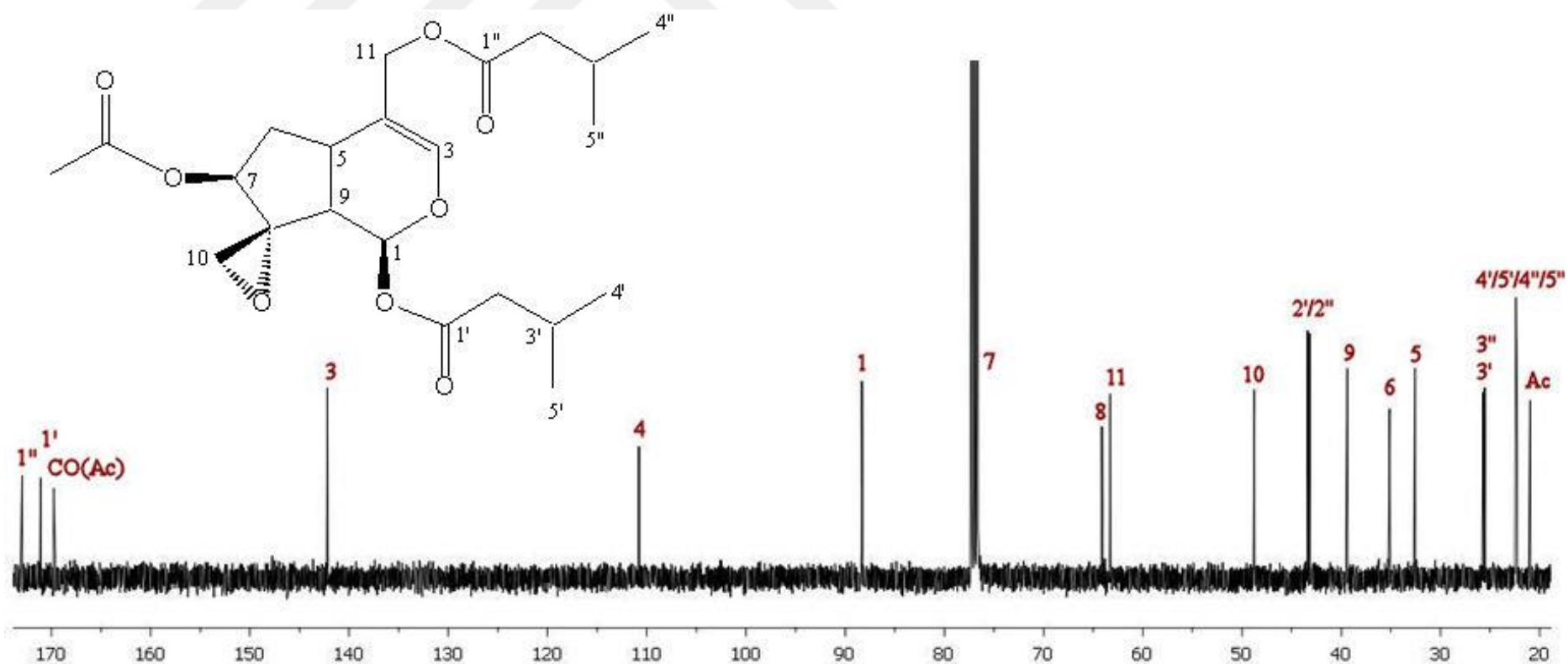
Table 73. ^{13}C NMR and ^1H data of didrovaltrate (**1**) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
1	CH	88.3	5.84 d (5.4)
3	CH	142.2	6.51 s
4	C	110.8	-
5	CH	32.6	2.97 m
6	CH ₂	35.1	2.26 m
			2.00 m
7	CH	76.8	4.95 t (5.7)
8	C	64.1	-
9	CH	39.4	2.71 dd (8.4, 5.4)
10	CH ₂	48.8	3.06 d (5.0)
			2.82 d (5.0)
11	CH ₂	63.3	4.68 d (12.3)
			4.46 d (12.3)
<i>Isovaleroyl (C-1)</i>			
1'	C	171.1	-
2'	CH ₂	43.4	2.22 d (7.0)
3'	CH	25.7	2.12 m
4'/5'	CH ₃	22.4	0.96 d (6.6)
7- <u>CO</u> CH ₃	C	169.8	-
7-CO <u>CH</u> ₃	CH ₃	21.0	2.05 s
<i>Isovaleroyl (C-11)</i>			
1''	C	173.0	-
2''	CH ₂	43.2	2.20 d (7.6)
3''	CH	25.5	2.07 m
4''/5''	CH ₃	22.3	0.96 d (6.6)

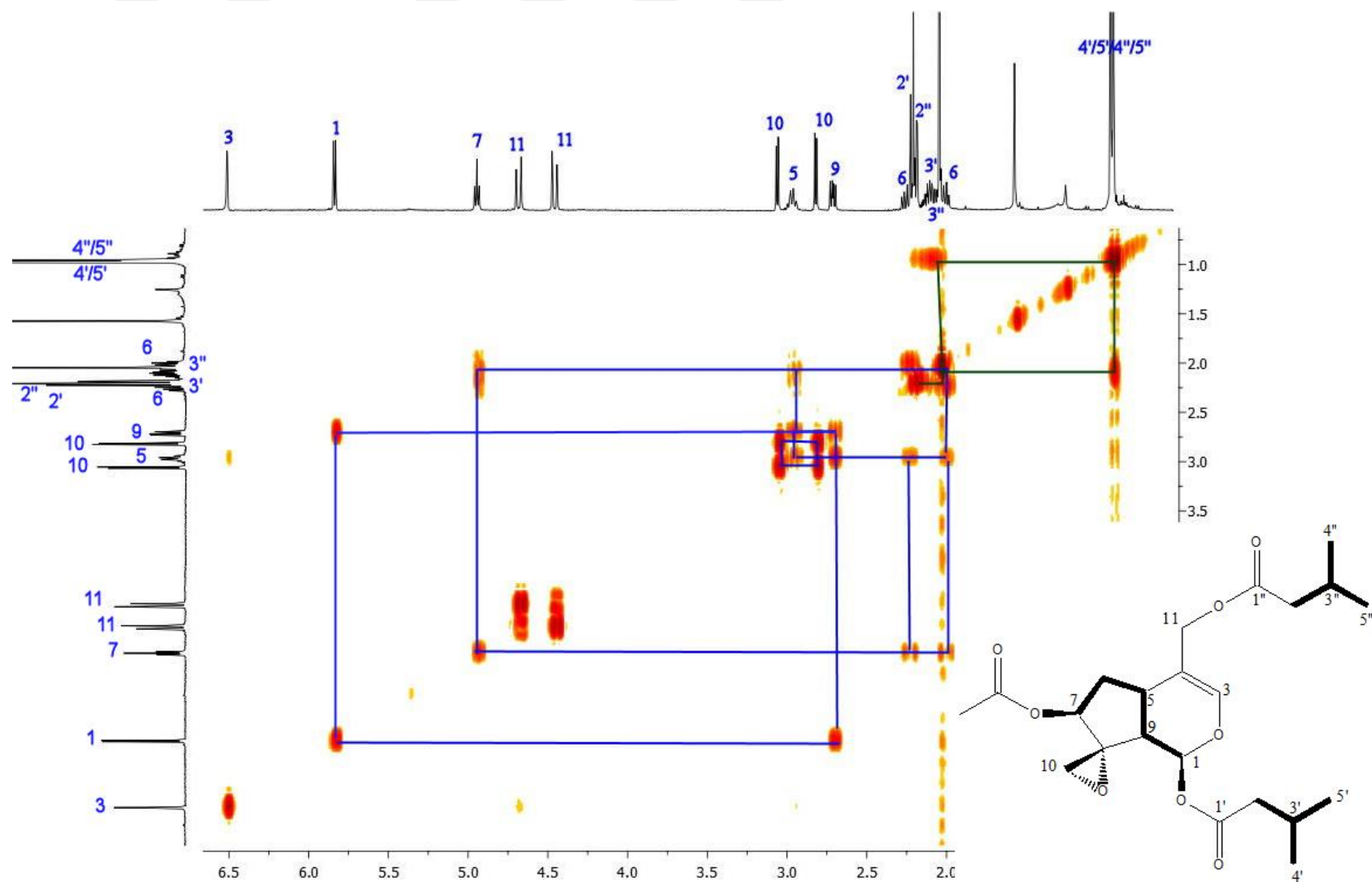
^aAssignments are based on COSY, HSQC, HMBC and NOESY experiments.



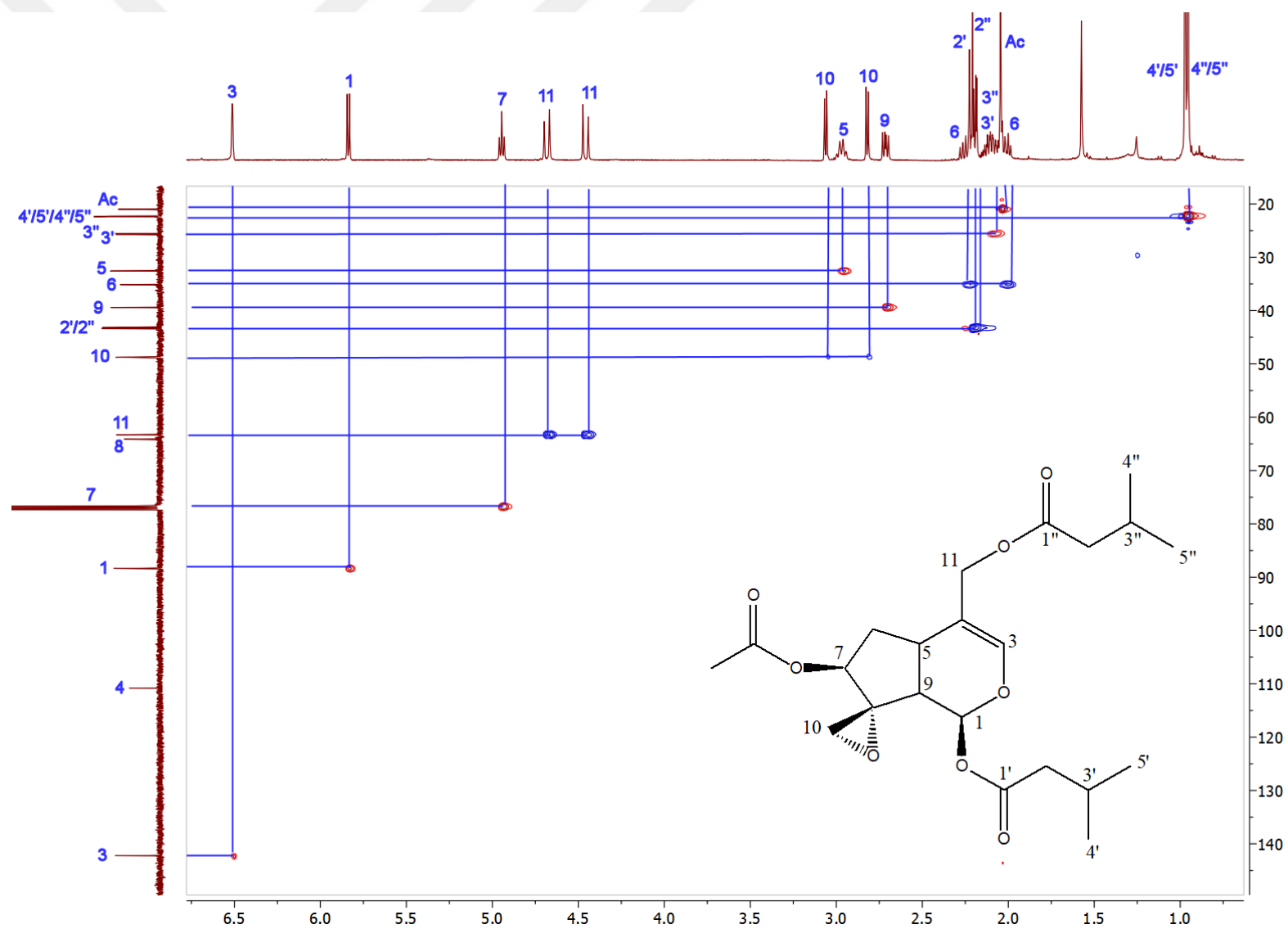
Spectrum 1. ¹H NMR Spectrum of Didrovaltrate (1) (CDCl₃, 400 MHz)



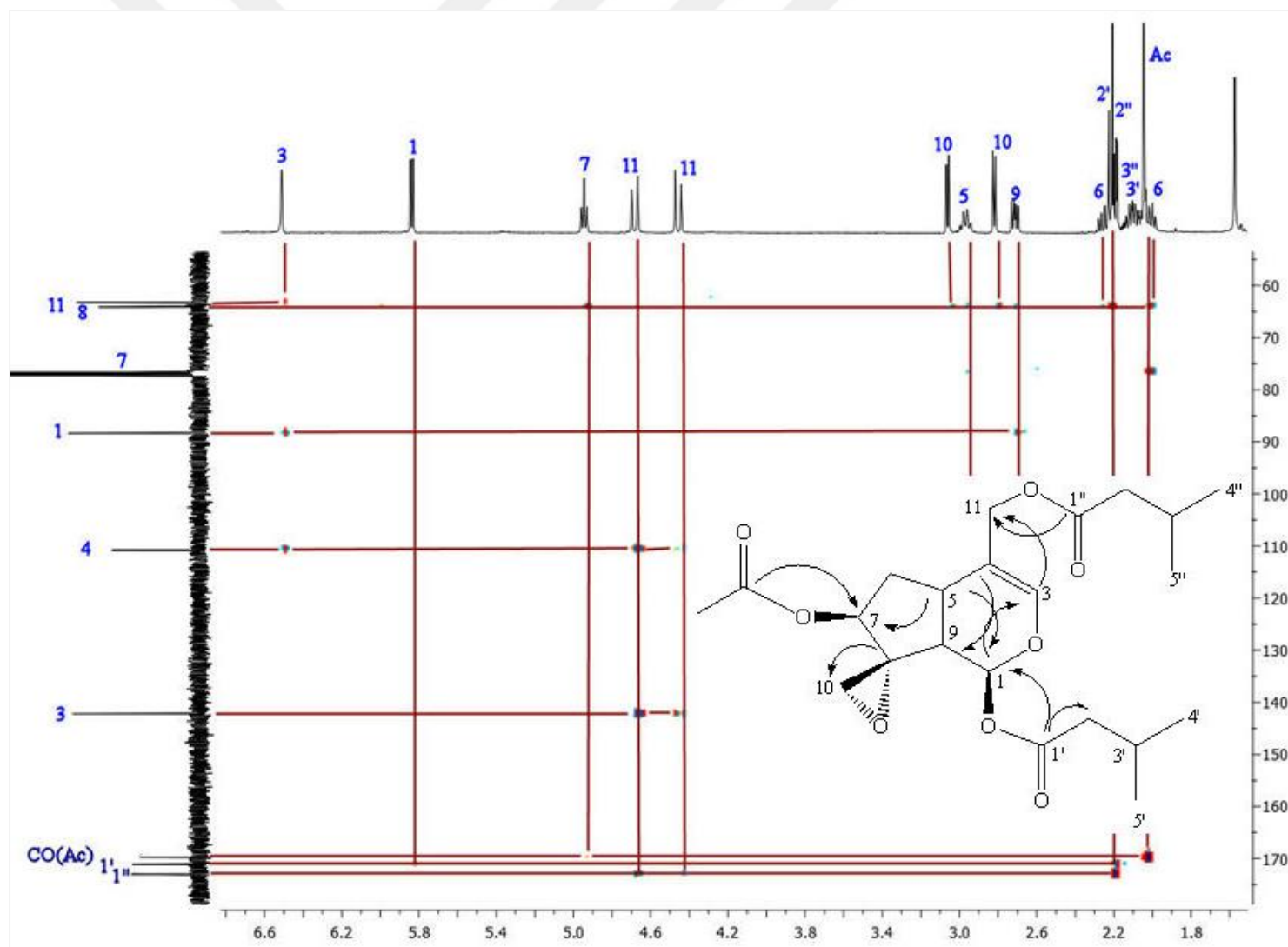
Spectrum 2. ^{13}C NMR Spectrum of Didrovaltrate (1) (CDCl_3 , 100 MHz)



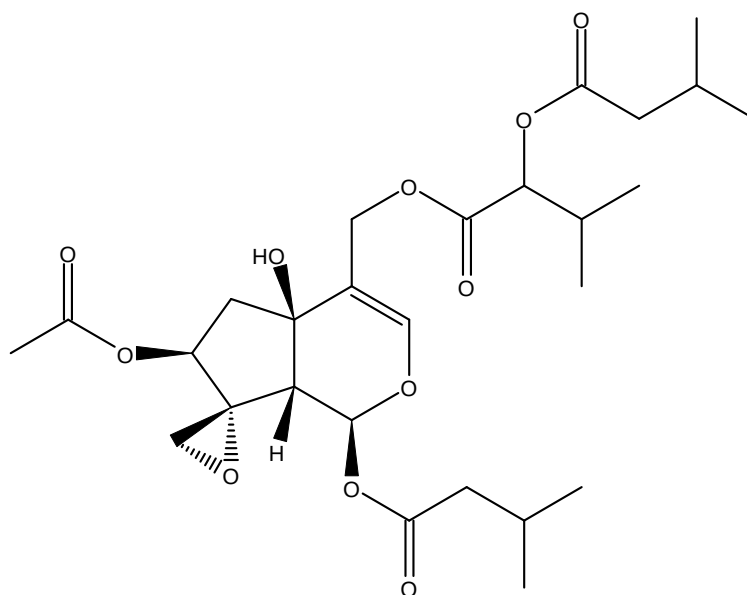
Spectrum 3. COSY Spectrum of Didrovaltrate (**1**)



Spectrum 4. HSQC Spectrum of Didrovaltrate (**1**)



Spectrum 5. HMBC Spectrum of Didrovaltrate (1)



ISOVALEROXYHYDROXYDIDRO(IVHD)-VALTRATE (2): C₂₇H₄₀O₁₁ (MW: 540.60)

UV λ_{max} (MeOH) nm:	208
IR ν_{max} (KBr) cm ⁻¹ :	3365 (OH), 1743 (C=O), 1662 (C=C)
ESI-MS m/z :	564.20 [M+Na] ⁺
¹ H NMR:	Table 74, Spectrum 6
¹³ C NMR:	Table 74, Spectrum 7
COSY:	Spectrum 8
HSQC:	Spectrum 9
HMBC:	Spectra 10 and 11

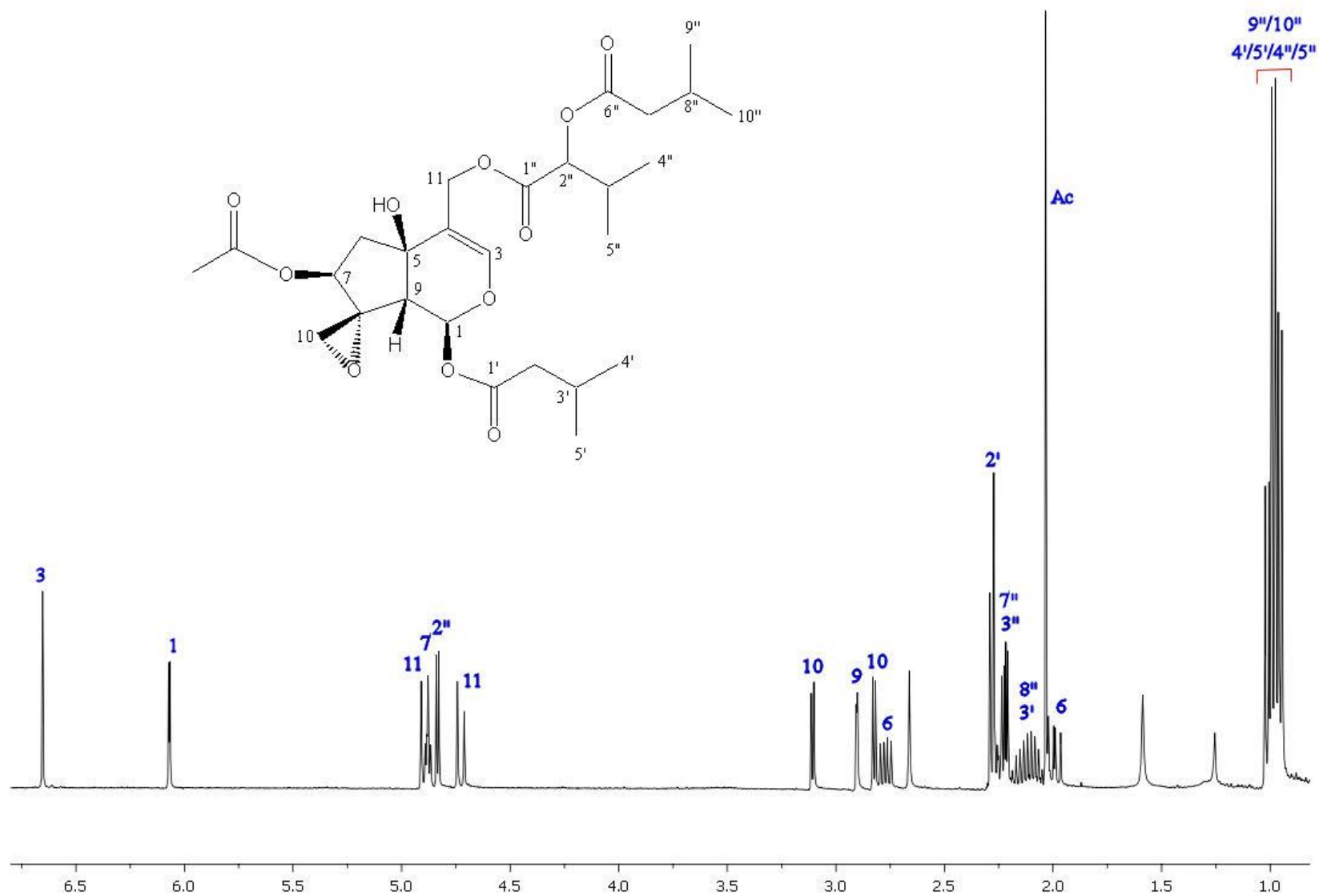
Compound **2** was isolated as colorless oil with the molecular formula C₂₇H₄₀O₁₁ by ESI-MS at m/z 564.20 [M+Na]⁺. The IR spectrum displayed absorption bands due to hydroxyl (3365 cm⁻¹), ester carbonyl (1743 cm⁻¹) and olefinic (1662 cm⁻¹) groups, whereas its UV spectrum showed absorption maximum at 208 nm. The ¹H and ¹³C data (Table 74) as well as the 2D (COSY, HSQC and HMBC) NMR spectra (Spectra 6-11) of **2** were found to be very identical to those of **1**, except for the presence of a hydroxy group at C-5 position in **2**, and presence of α -(isovaleroxy)-isovaleroxy groups instead of isovaleroxy group at C-11 position. Thus, the exact chemical structure of compound **2** was elucidated as IVHD-valtrate by comparing the NMR data with those published in the literature (161).

Table 74. ^{13}C NMR and ^1H data of IVHD-valtrate (**2**) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

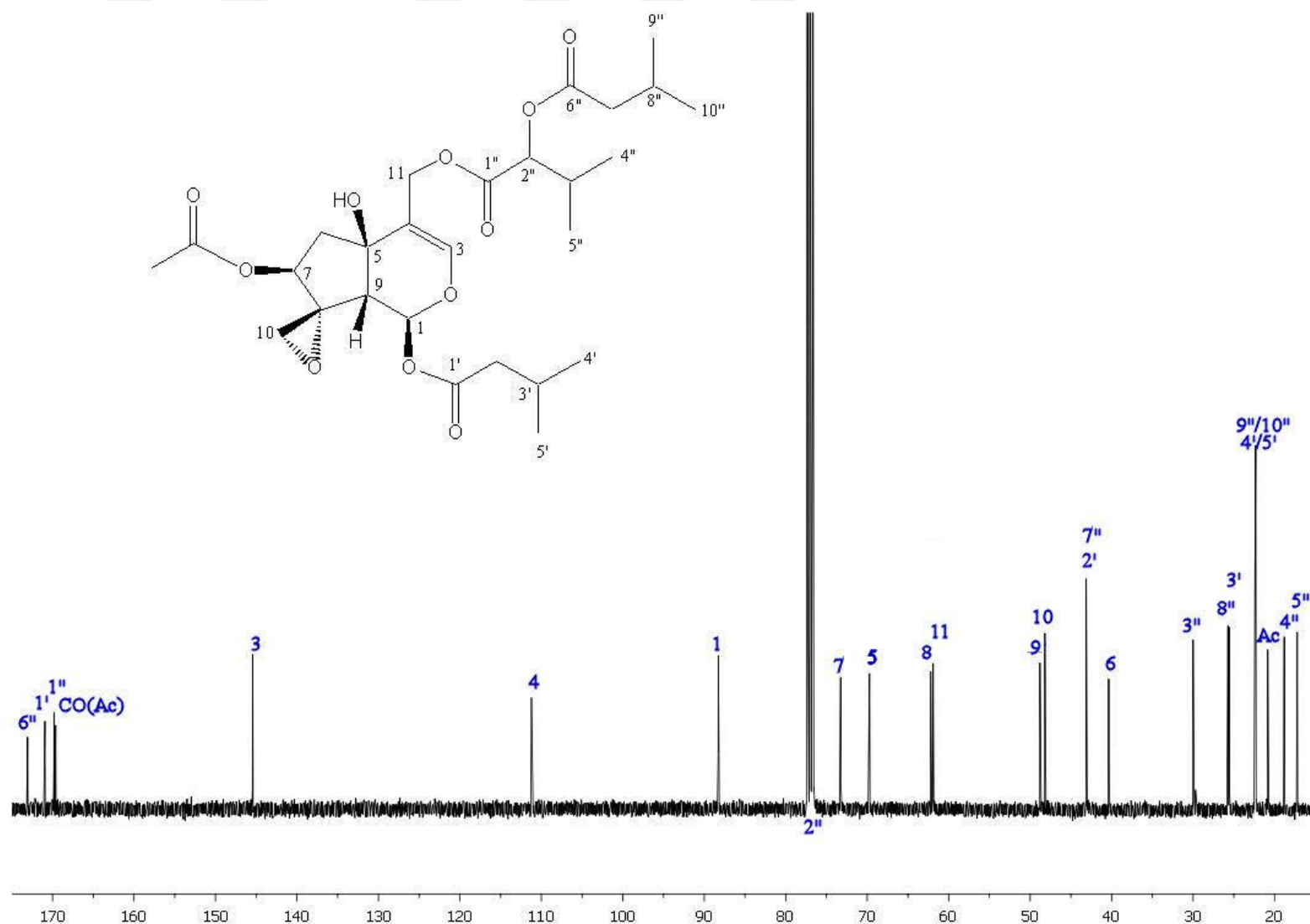
C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
1	CH	88.3	6.03 d (2.1)
3	CH	145.4	6.65 s
4	C	111.2	-
5	C	69.7	-
6	CH ₂	40.4	2.77 dd (13.0, 7.0) 1.99 dd (13.0, 9.9)
7	CH	73.7	4.88 †
8	C	62.2	-
9	CH	48.8	2.90 d (2.1)
10	CH ₂	48.2	3.11 d (4.8) 2.88 d (4.8)
11	CH ₂	61.9	4.90 d (12.4) 4.73 d (12.4)
<i>Isovaleroyl (C-1)</i>			
1'	C	171.1	-
2'	CH ₂	43.1	2.28 d (7.0)
3'	CH	25.7	2.11 m
4'/5'	CH ₃	22.3	0.96 d (6.6)
7-COCH ₃	C	169.6	-
7-COCH ₃	CH ₃	22.3	2.03 s
<i>α-(isovaleroxy)-isovaleroyl (C-7)</i>			
1''	C	169.8	-
2''	CH	76.8	4.83 d (4.5)
3''	CH	30.0	2.26 m
4''	CH ₃	18.8	1.02 d (6.9)
5''	CH ₃	17.2	0.99 d (6.9)
6''	C	173.1	-
7''	CH ₂	43.0	2.22 d (6.9)
8''	CH	25.6	2.11 m
9''/10''	CH ₃	22.4	0.99 d (6.6)

^aAssignments are based on COSY, HSQC, HMBC and NOESY experiments.

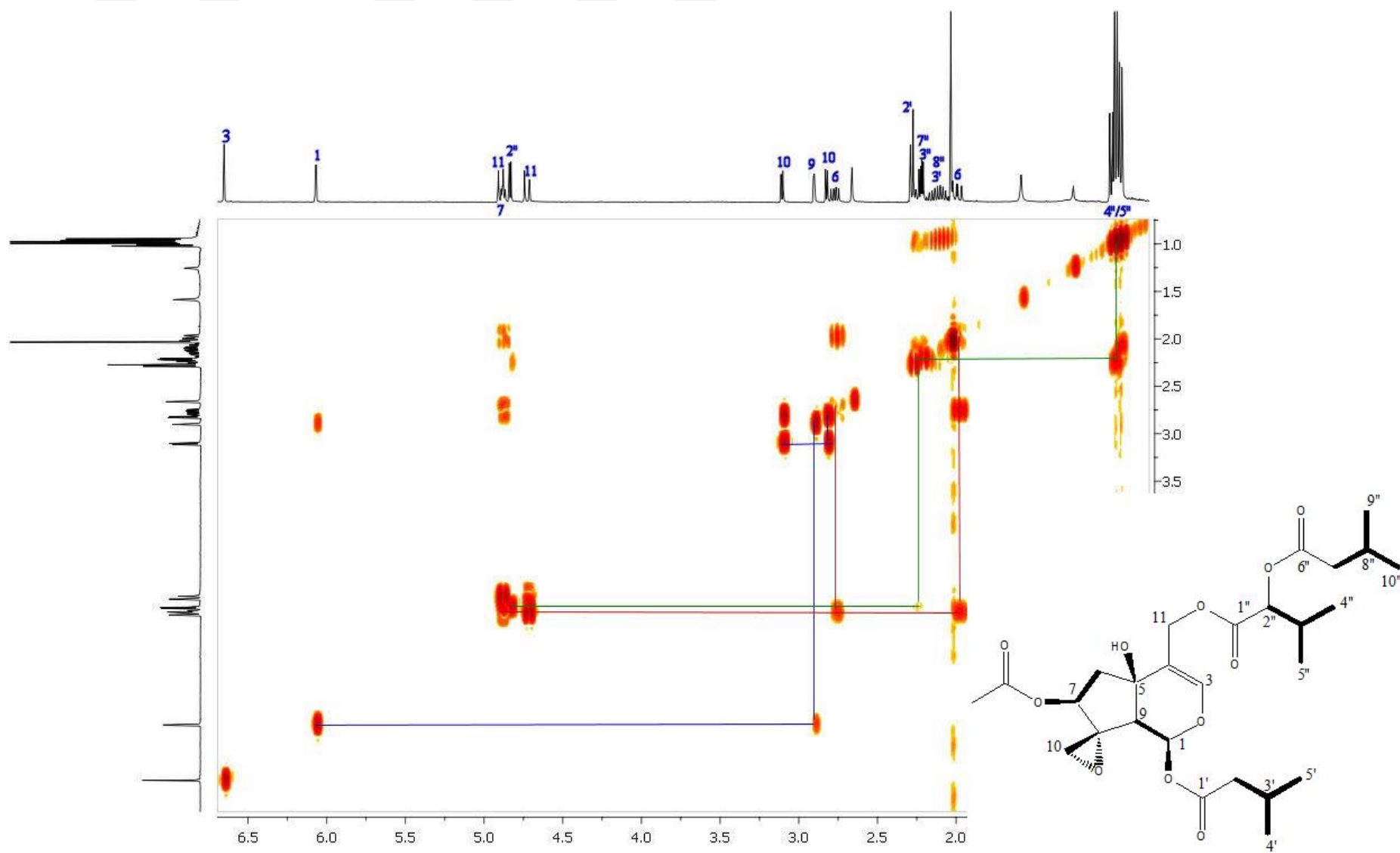
† Overlapped signals.



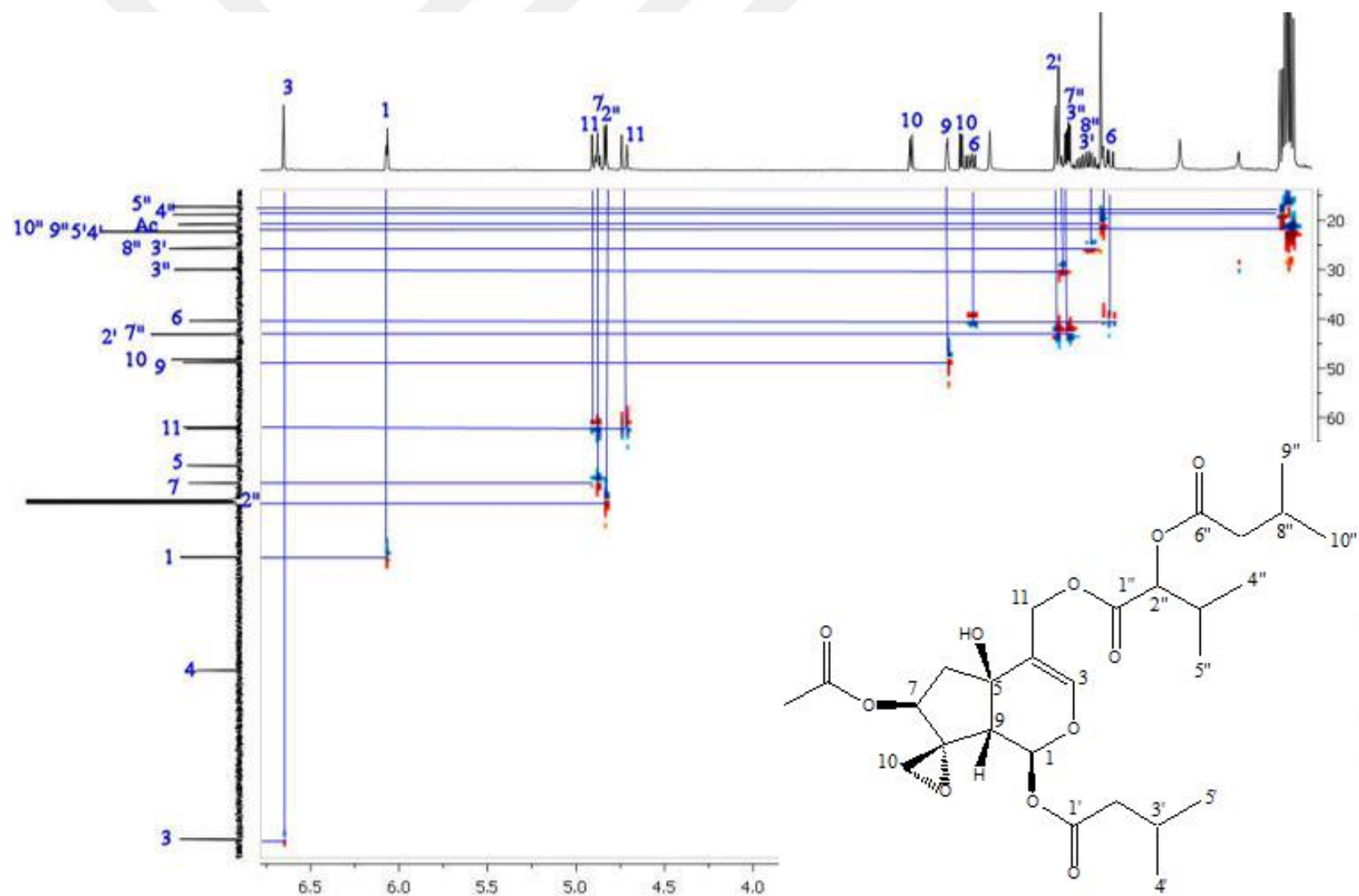
Spectrum 6. ^1H NMR Spectrum of IVHD-Valtrate (2) (CDCl_3 , 400 MHz)



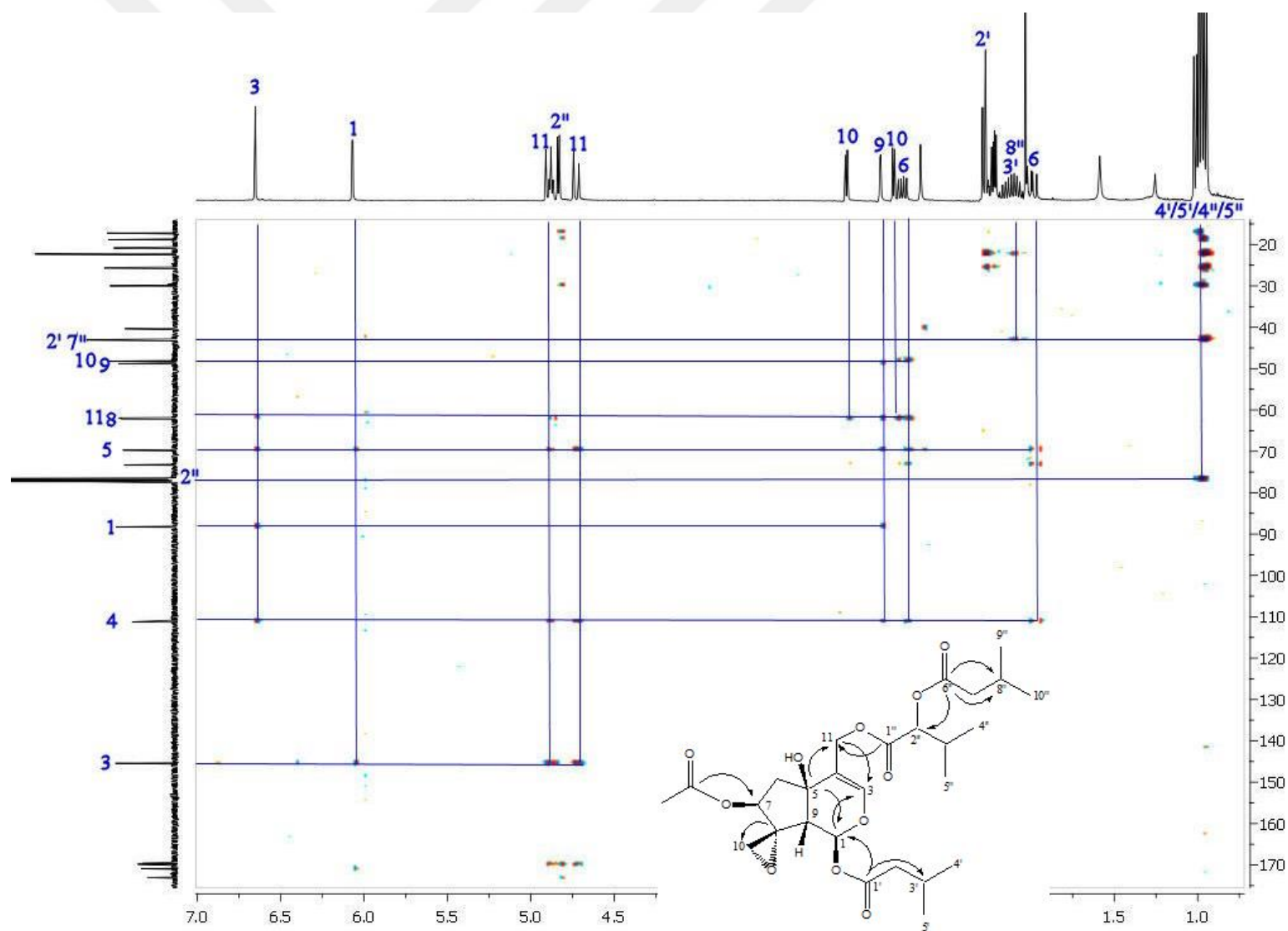
Spectrum 7. ^{13}C NMR Spectrum of IVHD-Valtrate (2) (CDCl_3 , 100 MHz)



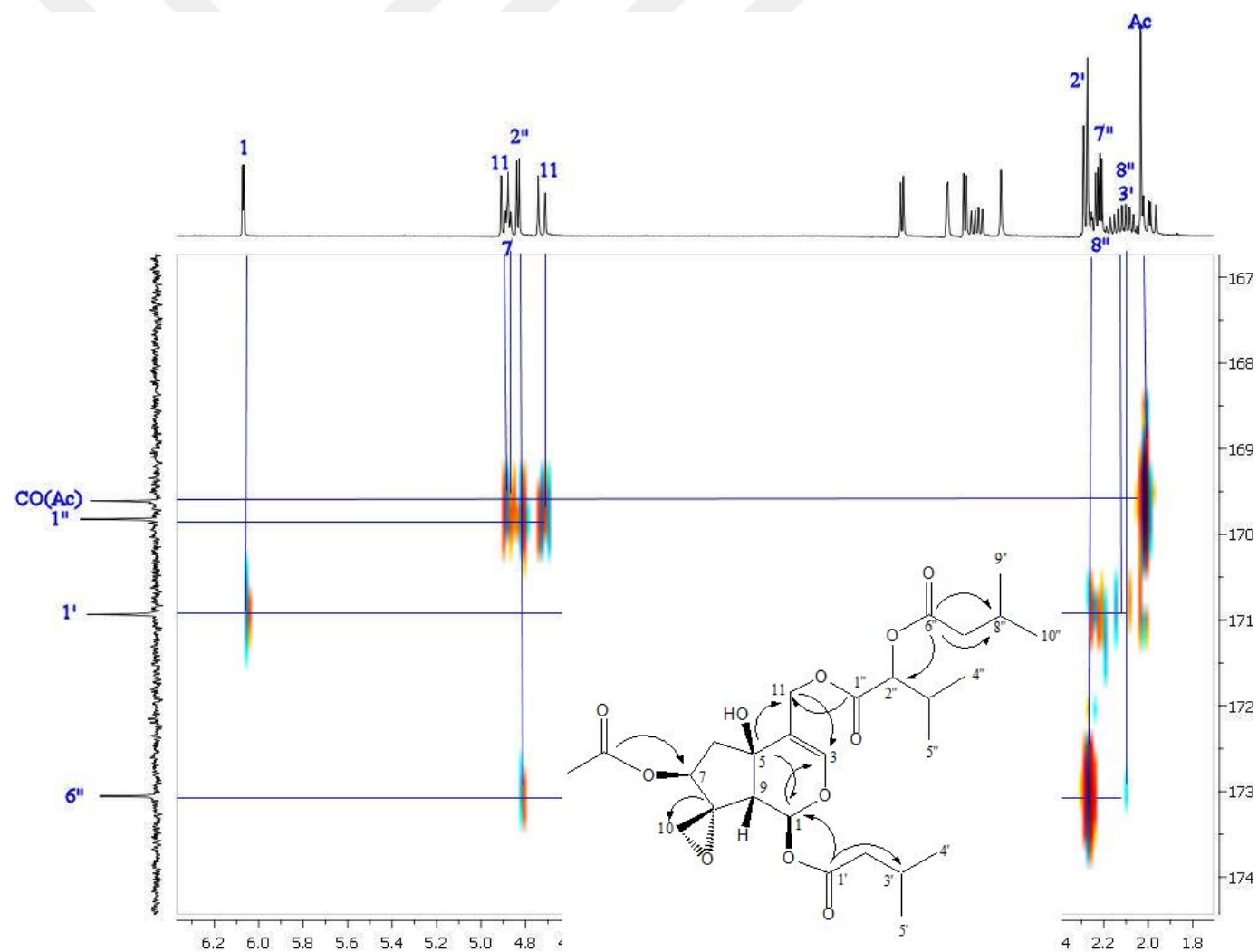
Spectrum 8. COSY Spectrum of IVHD-Valtrate (2)



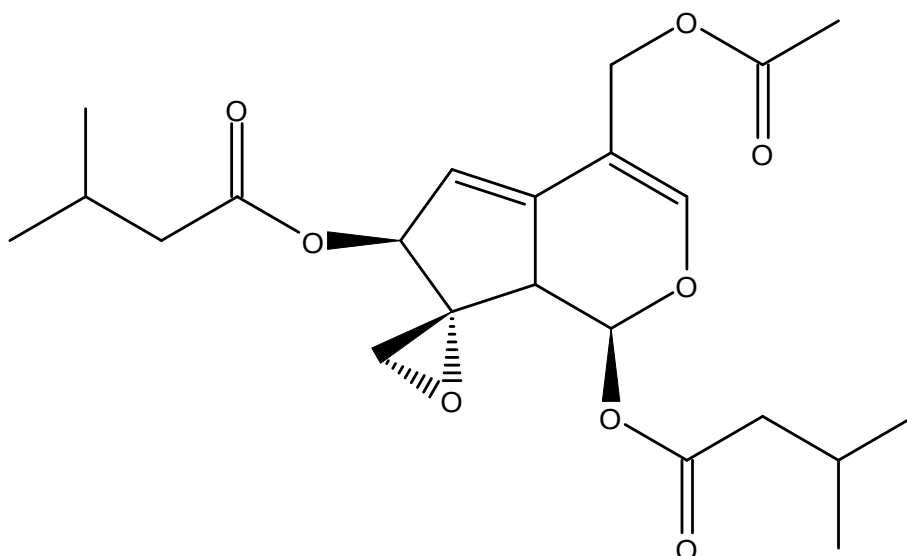
Spectrum 9. HSQC Spectrum of IVHD-Valtrate (2)



Spectrum 10. HMBC Spectrum of IVHD-Valtrate (2) (^{13}C :15-175 ppm, ^1H : 0.5-7.0 ppm)



Spectrum 11. HMBC Spectrum of IVHD-Valtrate (2) (^{13}C :167-174 ppm, ^1H : 1.7-6.4 ppm)



VALTRATE (3): C₂₂H₃₀O₈ (MW: 422.47)

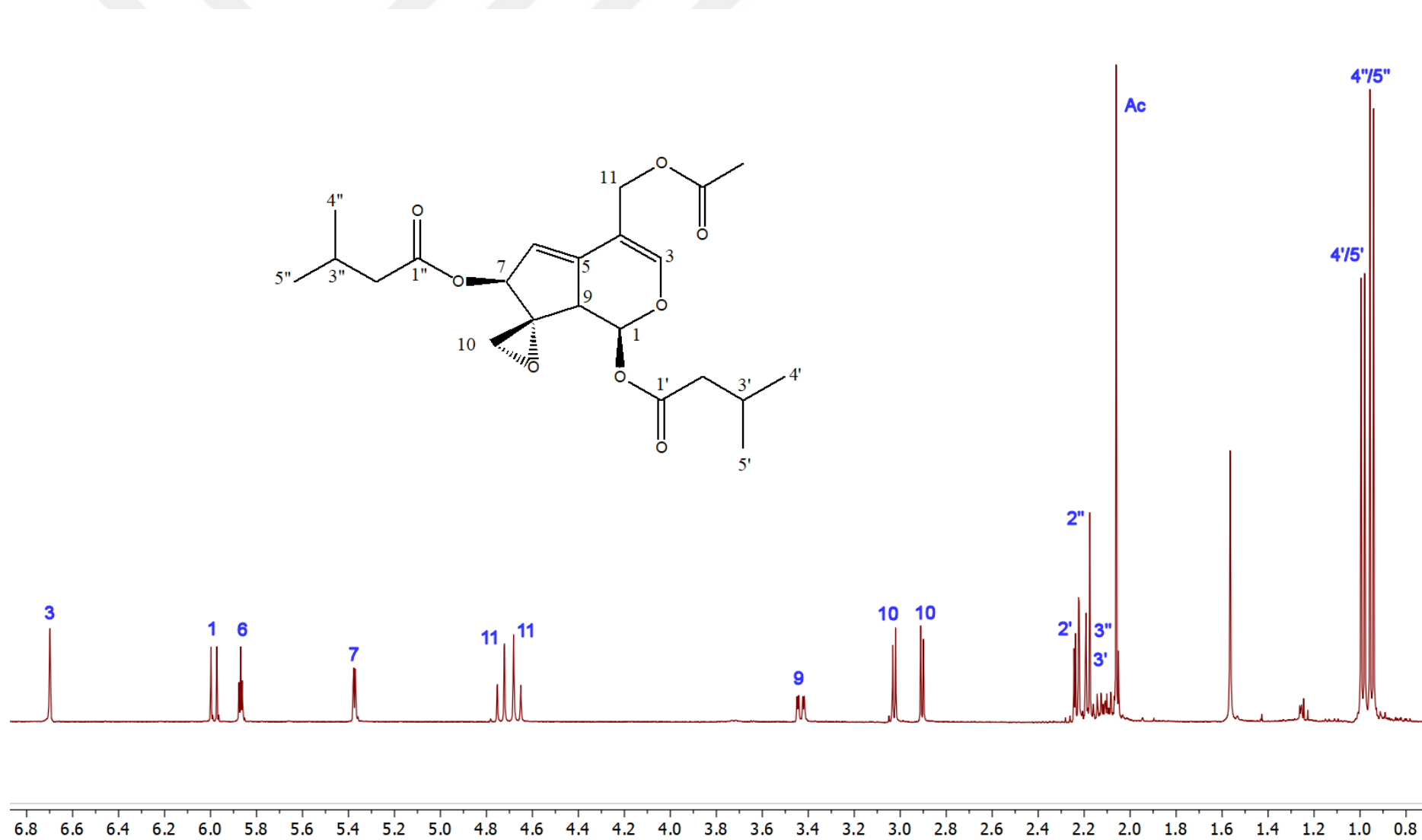
UV λ_{max} (MeOH) nm:	256
IR ν_{max} (KBr) cm ⁻¹ :	1741, 1738 (C=O), 1641,1611 (C=C)
ESI-MS m/z :	445.83 [M+Na] ⁺
¹ H NMR:	Table 75, Spectrum 12
¹³ C NMR:	Table 75, Spectrum 13
HMBC:	Spectrum 14

Compound **3** was isolated as colorless oil, whose molecular formula was found to be C₂₂H₃₀O₈ by ESI-MS (m/z 445.83 [M+Na]⁺) with an index of hydrogen deficiency (IHD) of eight. IR spectrum indicated absorption bands belonging to ester carbonyl (1741 and 1738 cm⁻¹) and olefinic (1641 and 1611 cm⁻¹) groups, while UV spectrum displayed absorption maximum at 256 nm. Its ¹H and ¹³C NMR findings (Table 75) suggested that compound **3** possessed diene-type valepotriate skeleton. Taken together, the 1D and HMBC NMR findings (Spectra 12-14) were superimposable with those reported for valtrate. Thus, compound **3** was assigned as valtrate (245).

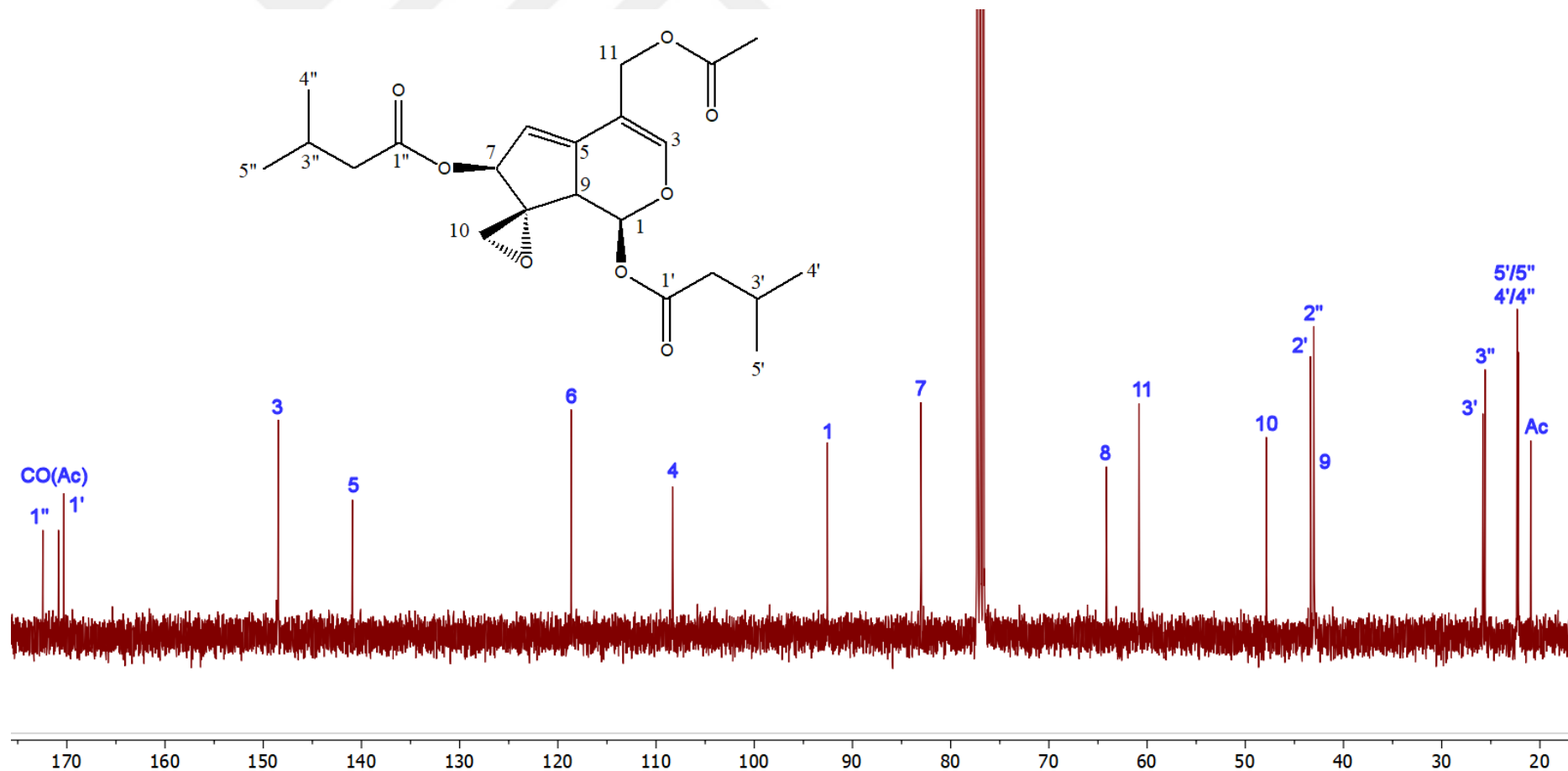
Table 75. ^{13}C NMR and ^1H data of valtrate (**3**) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
1	CH	92.6	5.98 d (10.2)
3	CH	148.4	6.70 s
4	C	108.3	-
5	C	140.9	-
6	CH	118.6	5.87 t (2.8)
7	CH	83.0	5.37 d (2.9)
8	C	64.1	-
9	CH	43.0	3.43 dd (10.0, 2.6)
10	CH ₂	47.9	3.03 d (4.9) 2.90 d (4.9)
11	CH ₂	60.8	4.74 d (12.4) 4.67 d (12.4)
<i>Isovaleroyl (C-1)</i>			
1'	C	170.3	-
2'	CH ₂	43.4	2.23 d (5.5)
3'	CH	25.8	2.14 m
4'/5'	CH ₃	22.3	0.99 d (6.6)
<i>Isovaleroyl (C-7)</i>			
1''	C	172.4	-
2''	CH ₂	43.0	2.18 d (6.7)
3''	CH	25.6	2.10 m
4''/5''	CH ₃	22.3	0.95 d (6.6)
11- <u>CO</u> CH ₃	C	170.8	-
11- <u>CO</u> CH ₃	CH ₃	20.9	2.06 s

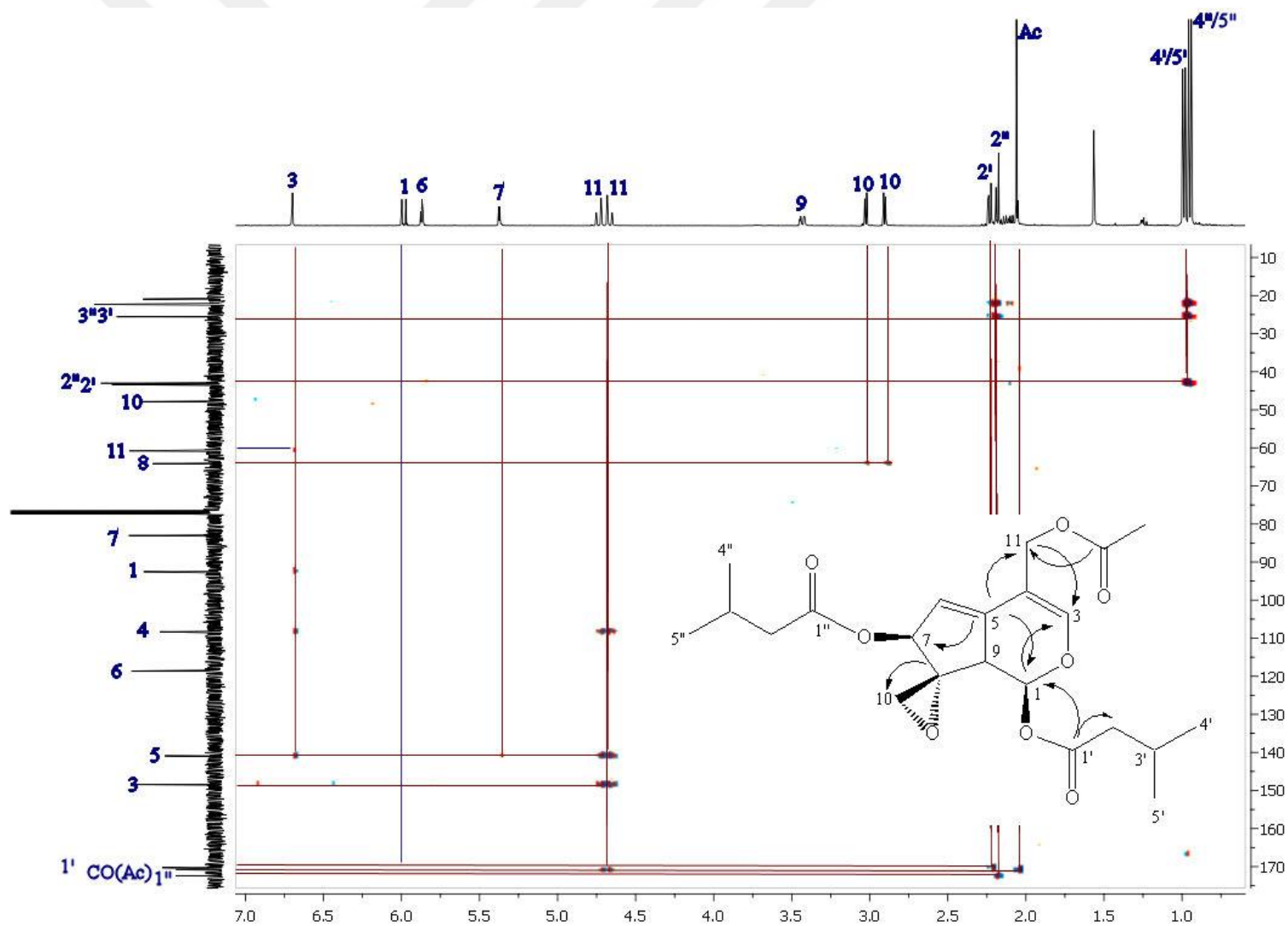
^aAssignments are based on HMBC experiment.



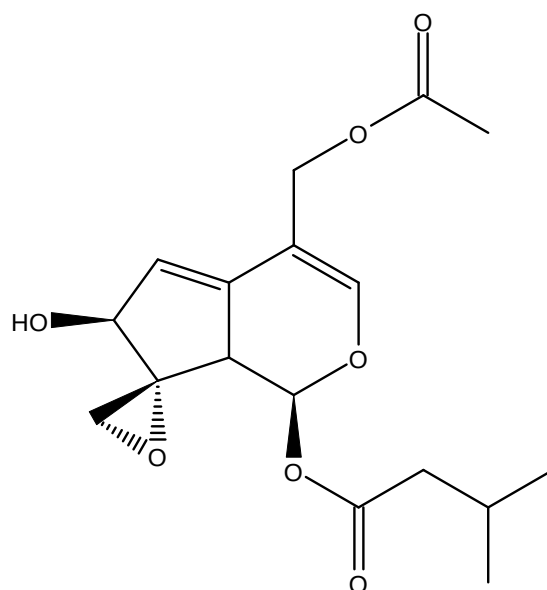
Spectrum 12. ¹H NMR Spectrum of Valtrate (3) (CDCl₃, 400 MHz)



Spectrum 13. ^{13}C NMR Spectrum of Valtrate (3) (CDCl_3 , 100 MHz)



Spectrum 14. HMBC Spectrum of Valtrate (3)



7-DEISOVALEROYVALTRATE (12): C₁₇H₂₂O₇ (MW: 338.35)

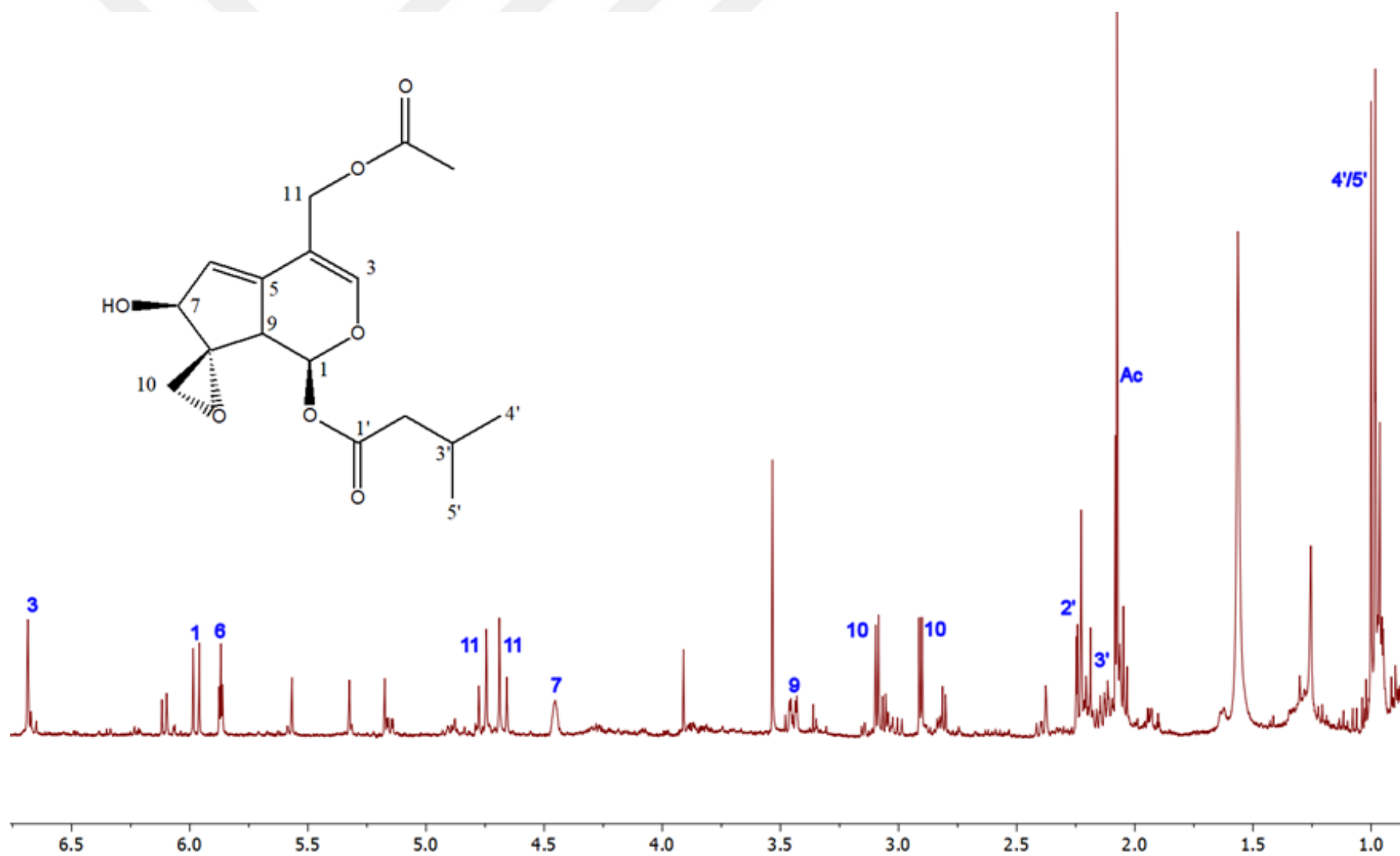
UV λ_{max} (MeOH) nm:	205, 259
IR ν_{max} (KBr) cm ⁻¹ :	3692 (OH), 1743 (C=O), 1559 (C=C)
HR-ESI-MS m/z :	361.1258 [M+Na] ⁺ (calcd. for C ₁₇ H ₂₂ O ₇ Na, 361.1263)
¹ H NMR:	Table 76, Spectrum 15
¹³ C NMR:	Table 76, Spectrum 16
COSY:	Spectrum 17

Compound **12** was purified as colorless oil, the molecular formula C₁₇H₂₂O₇, determined by the positive-ion HR-ESI-MS (m/z : 361.1258 [M+Na]⁺ (calcd. for C₁₇H₂₂O₇Na, 361.1263), indicating seven degrees of unsaturation. The IR spectrum contained absorbance bands at 3692, 1743 and 1559 cm⁻¹ revealing the presence of hydroxyl, ester carbonyl and olefinic functionalities, respectively. The UV spectrum displayed absorption maxima at 205 and 259 nm. The ¹H and ¹³C NMR data (Table 76, Spectra 15 and 16) of **12** were similar to those of **3** (valtrate), with the only difference being absence of an isovaleroxy group at C-7 in **12**. NMR findings of **12** were superimposed with that reported for synthetic compound in the literature (246). However, it is being reported from a natural source for the first time and named as 7-deisovaleroylvaltrate.

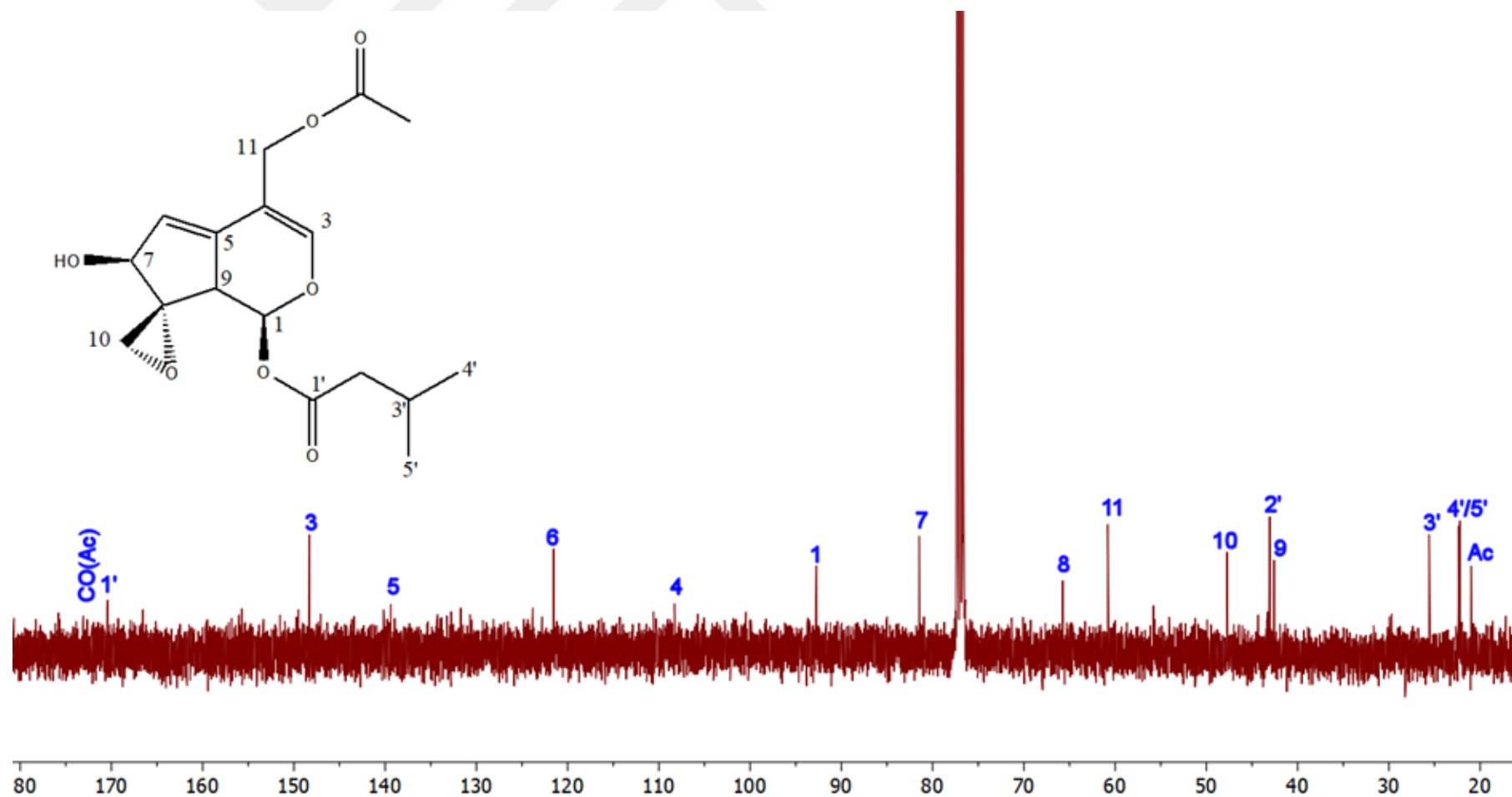
Table 76. ^{13}C NMR and ^1H data of 7-deisovaleroylvaltrate (**12**) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
1	CH	92.7	5.97 d (9.9)
3	CH	148.3	6.68 s
4	C	108.1	-
5	C	139.3	-
6	CH	121.3	5.87 t (2.7)
7	CH	81.6	4.45 gs
8	C	65.7	-
9	CH	42.5	3.44 dd (9.9, 2.7)
10	CH ₂	47.7	3.09 d (4.7) 2.92 d (4.7)
11	CH ₂	60.7	4.77 d (12.3) 4.67 d (12.3)
<i>Isovaleroyl (C-1)</i>			
1'	C	170.3	-
2'	CH ₂	43.0	2.23 d (6.0)
3'	CH	25.6	2.10 m
4'/5'	CH ₃	22.3	0.99 d (6.7)
11-COCH ₃	C	170.8	-
11-COCH ₃	CH ₃	21.0	2.07 s

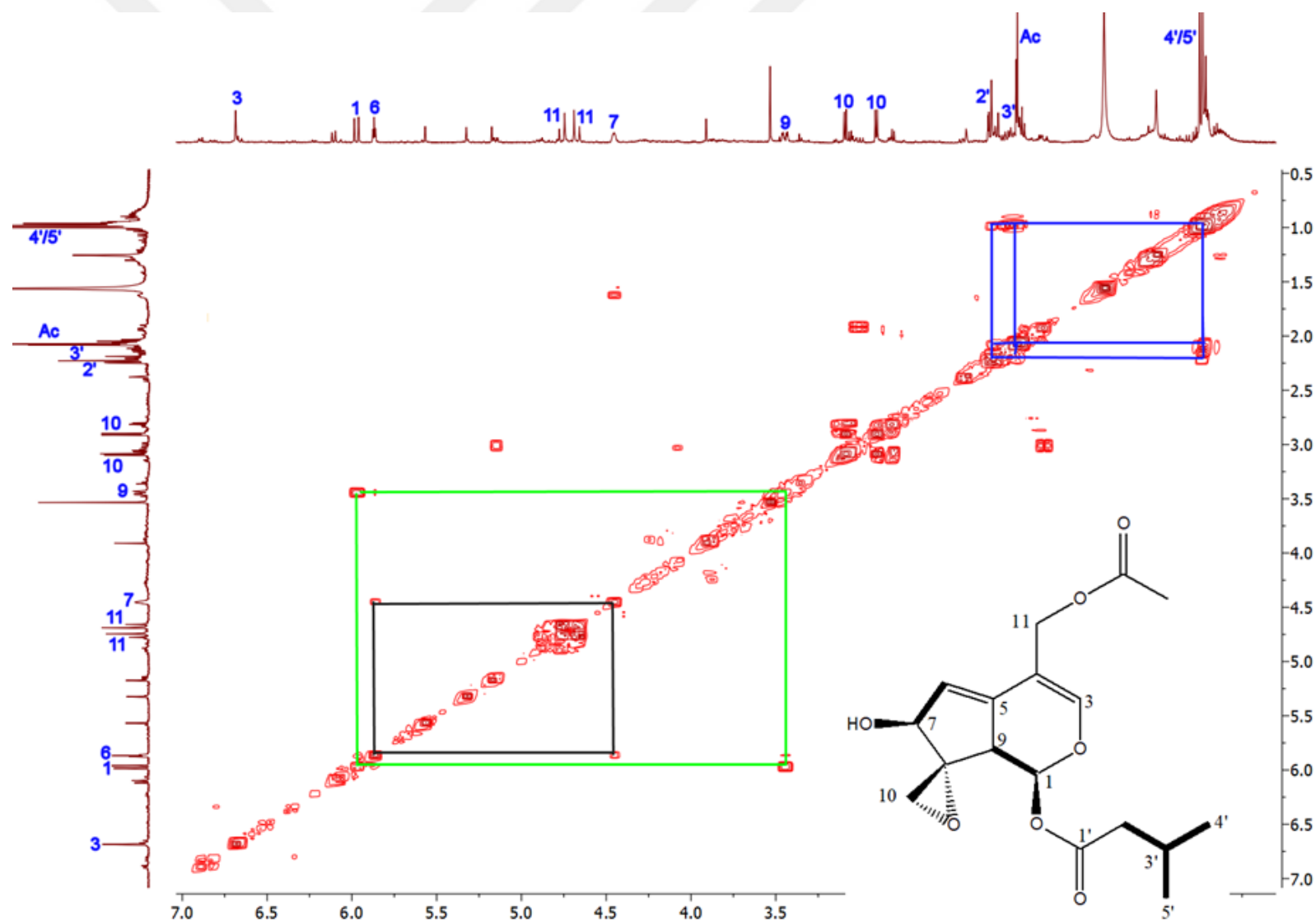
^aAssignments are based on COSY experiment.



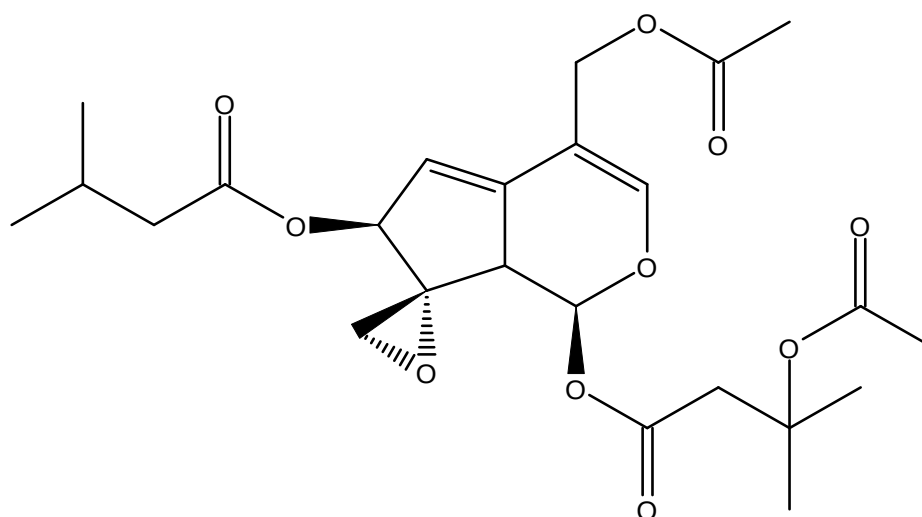
Spectrum 15. ¹H NMR Spectrum of 7-Deisovaleroylvaltrate (**12**) (CDCl₃, 400 MHz)



Spectrum 16. ¹³C NMR Spectrum of 7-Deisovaleroylvaltrate (12) (CDCl₃, 100 MHz)



Spectrum 17. COSY Spectrum of 7-Deisovaleroylvaltrate (12)



1-β-ACEVALTRATE (4): C₂₄H₃₂O₁₀ (MW:480.50)

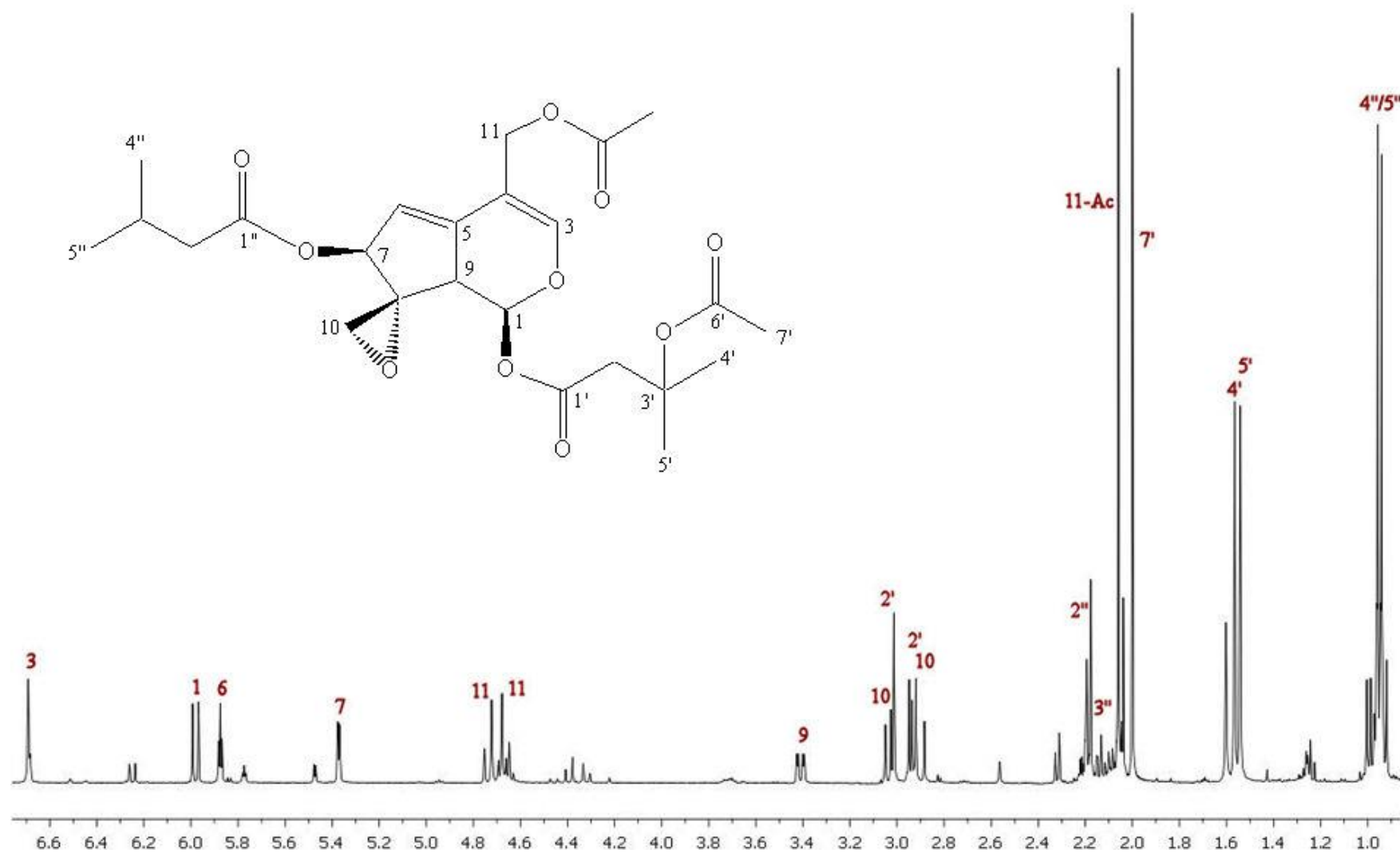
UV λ _{max} (MeOH) nm:	256
IR ν _{max} (KBr) cm ⁻¹ :	1775, 1738 (C=O), 1635,1612 (C=C)
ESI-MS <i>m/z</i> :	504.12 [M+Na] ⁺
¹ H NMR:	Table 77, Spectrum 18
¹³ C NMR:	Table 77, Spectrum 19
HMBC:	Spectrum 20

Compound **4**, a colorless oil, possessed the molecular formula C₂₄H₃₂O₁₀ based on its ESI-MS ion at *m/z* 504.12 [M+Na]⁺. Its UV spectrum displayed band at 256 nm, whereas IR spectrum showed absorption bands at 1775 and 1738 (carbonyl, C=O), 1635 and 1612 (olefinic, C=C) cm⁻¹. The NMR spectroscopic data (Table 77) of **4** closely resembled those of **3** (valtrate), except that **4** contained an β-(acetoxo)-isovaleroxy unit at C-1 instead of isovaleroyl group. Comparison of all the spectroscopic data with the published literature for 1-β-acevaltrate, led to the identification of compound **4** as 1-β-acevaltrate (179).

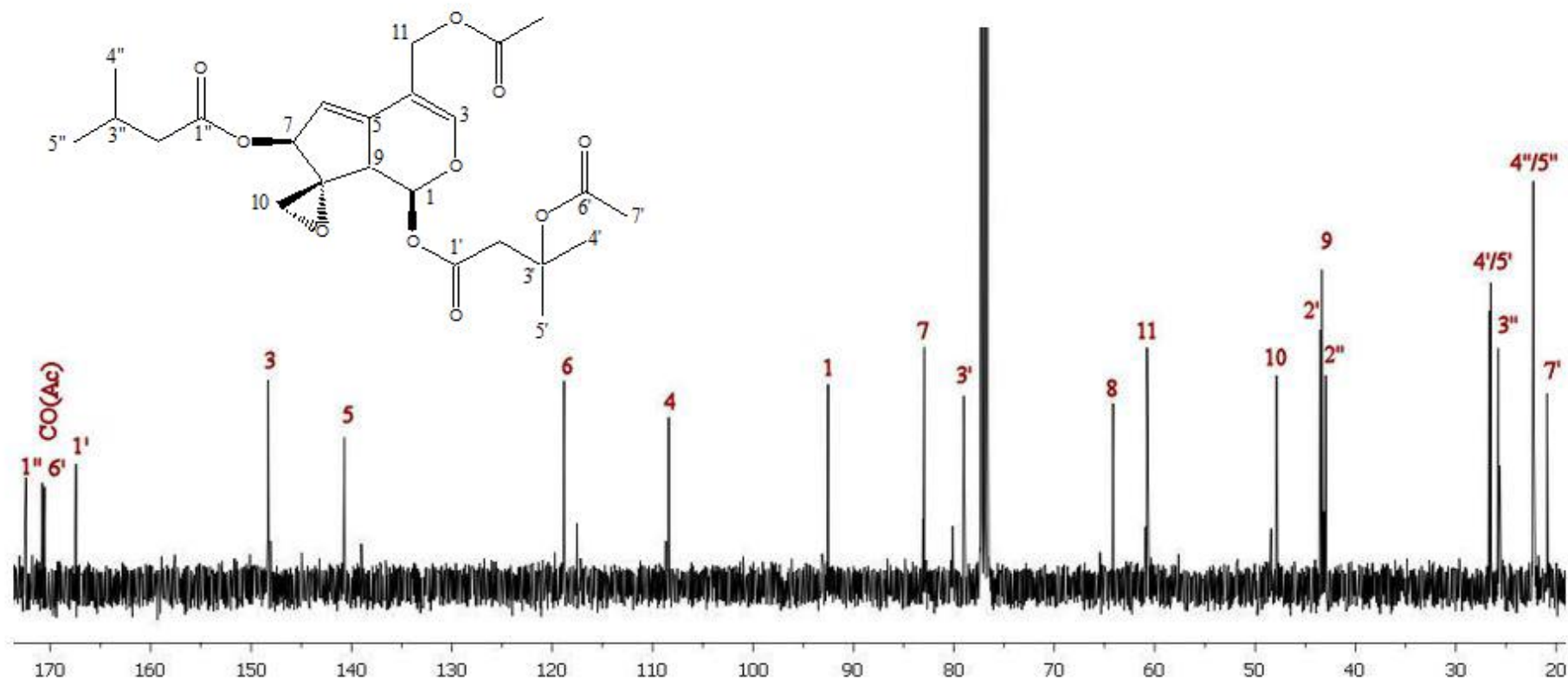
Table 77. ^{13}C and ^1H NMR data of 1- β -acevaltrate (**4**) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
1	CH	92.5	5.98 d (10.1)
3	CH	148.3	6.69 s
4	C	108.4	-
5	C	140.7	-
6	CH	118.8	5.88 t (2.8)
7	CH	83.0	5.37 d (2.8)
8	C	64.1	-
9	CH	43.0	3.41 dd (10.1, 2.6)
10	CH ₂	47.9	3.02 d (4.8) 2.94 d (4.8)
11	CH ₂	60.8	4.74 d (12.4) 4.66 d (12.4)
<i>β-(acetoxy)-isovaleroyl (C-1)</i>			
1'	C	167.5	-
2'	CH ₂	43.5	3.03 d (14.5) 2.90 d (14.5)
3'	C	79.0	-
4'	CH ₃	26.7	1.57 s
5'	CH ₃	26.5	1.54 s
6'	C	170.6	-
7'	CH ₃	20.9	2.0 s
<i>Isovaleroyl (C-7)</i>			
1''	C	172.4	-
2''	CH ₂	43.3	2.19 d (6.8)
3''	CH	25.8	2.08 m
4''/5''	CH ₃	22.3	0.95 d (6.6)
11-COCH ₃	C	170.8	-
11-COCH ₃	CH ₃	20.5	2.06 s

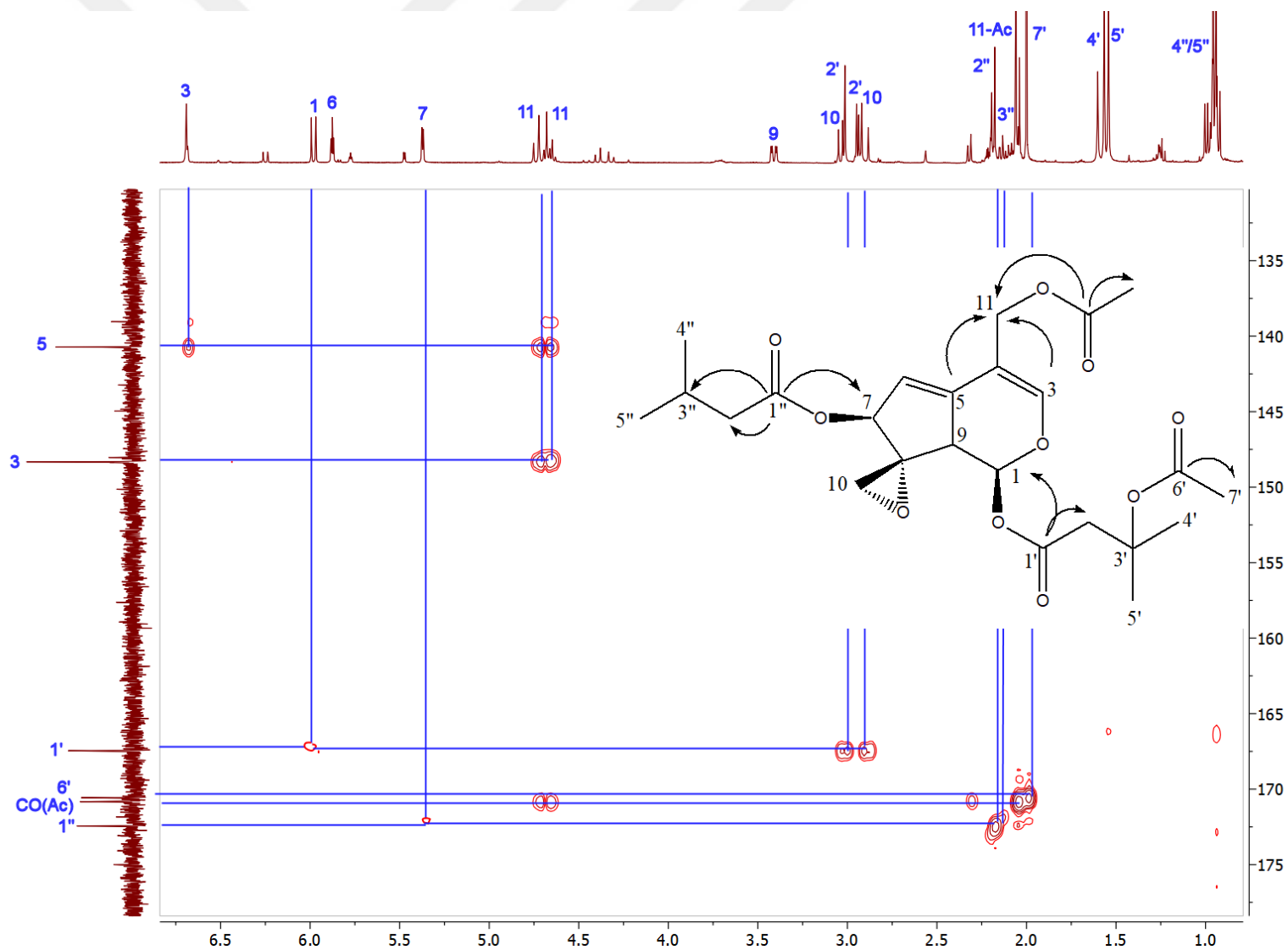
^aAssignments are based on HMBC experiment.



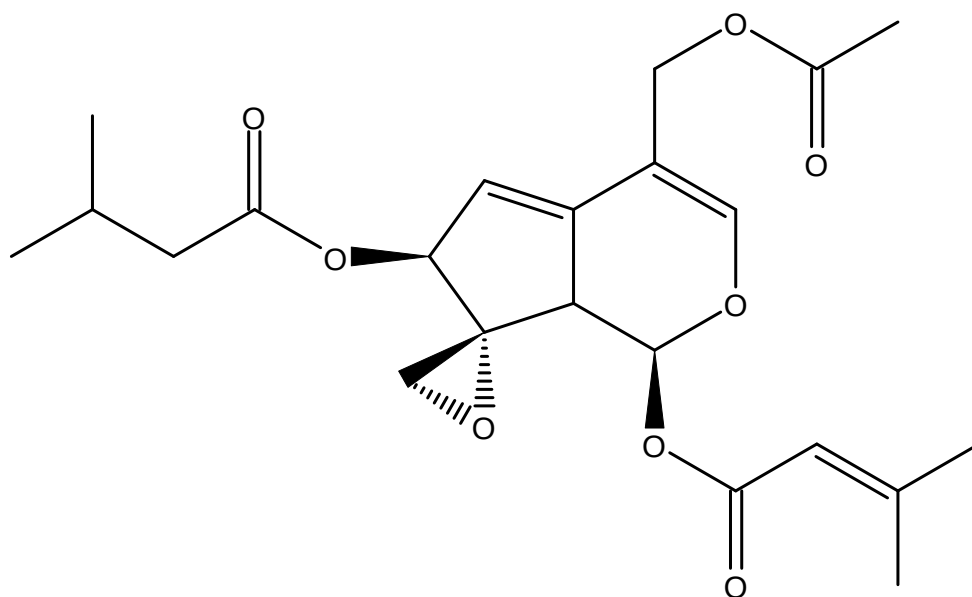
Spectrum 18. ¹H NMR Spectrum of 1-β-Acevaltrate (4) (CDCl₃, 400 MHz)



Spectrum 19. ¹³C NMR Spectrum of 1-β-Acevaltrate (**4**) (CDCl₃, 100 MHz)



Spectrum 20. HMBC Spectrum of 1-β-Acevaltrate (4)



SENECIOVALTRATE (5): C₂₂H₂₈O₈ (MW:420.45)

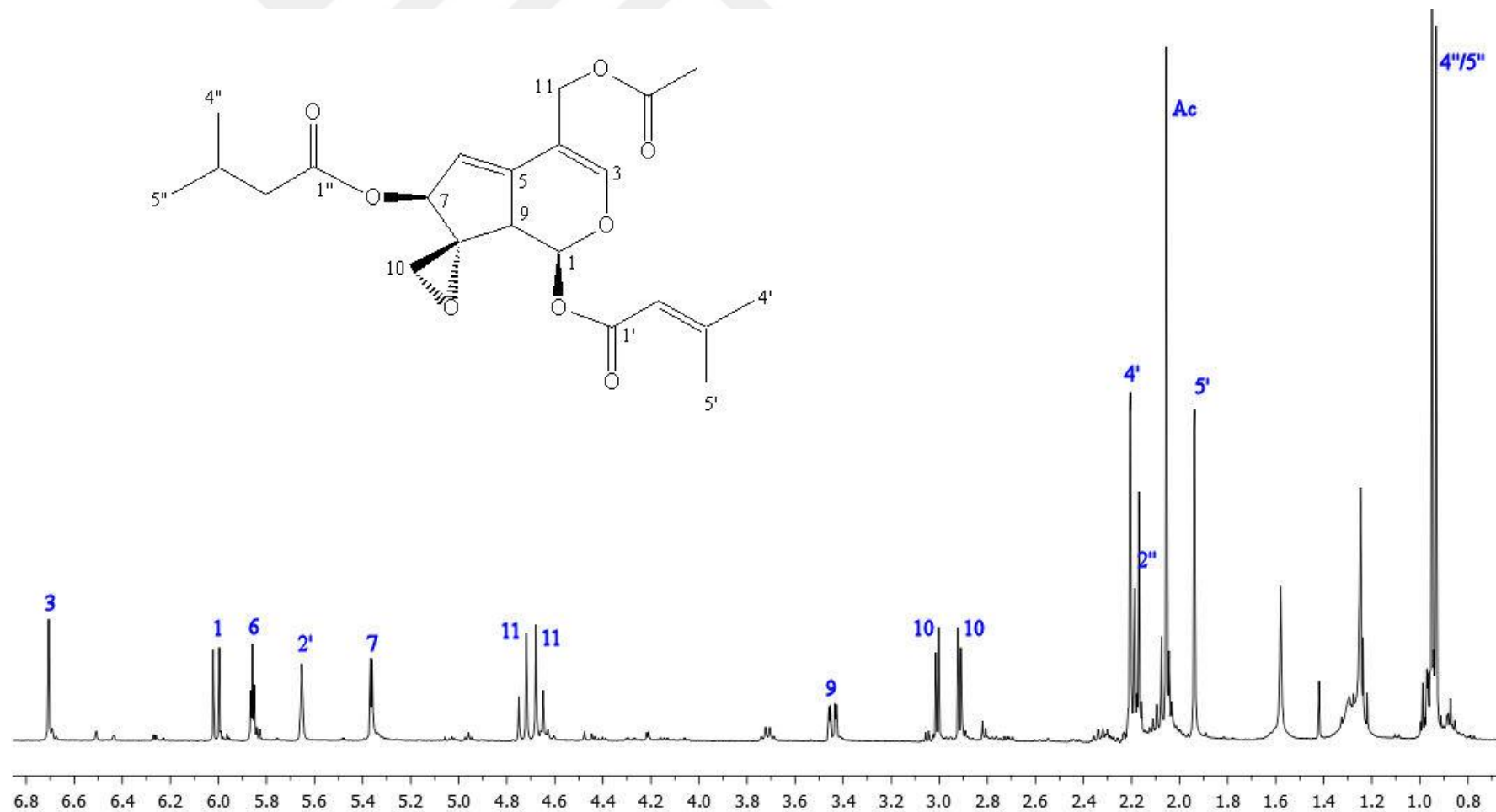
UV λ_{max} (MeOH) nm:	223, 256
IR ν_{max} (KBr) cm ⁻¹ :	1745 (C=O), 1605, 1558 (C=C)
ESI-MS m/z :	444.05 [M+Na] ⁺
¹ H NMR:	Table 78, Spectrum 21
¹³ C NMR:	Table 78, Spectrum 22
HMBC:	Spectrum 23

The molecular formula of compound **5** was assigned as C₂₂H₂₈O₈ by ESI-MS at m/z 444.05 [M+Na]⁺, showing nine degrees of unsaturation. The IR spectrum revealed bands at 1745 (carbonyl ester, C=O), 1605 and 1558 (olefinic, C=C) cm⁻¹. The UV spectrum showed absorption maxima at 223 and 256 nm. The ¹H and ¹³C NMR data (Table 78) of **5** were similar to those of **3** (valtrate), except for the replacement of the isovaleroxy group at C-1 in **3** with 3-methylcrotonoxy group in **5**. Also, the HMBC spectrum (Spectrum 23) of **5** provided information on the exact locations of acyloxy groups in the structure. Taken together, NMR findings were found to be compatible with those of seneciovaltrate (111).

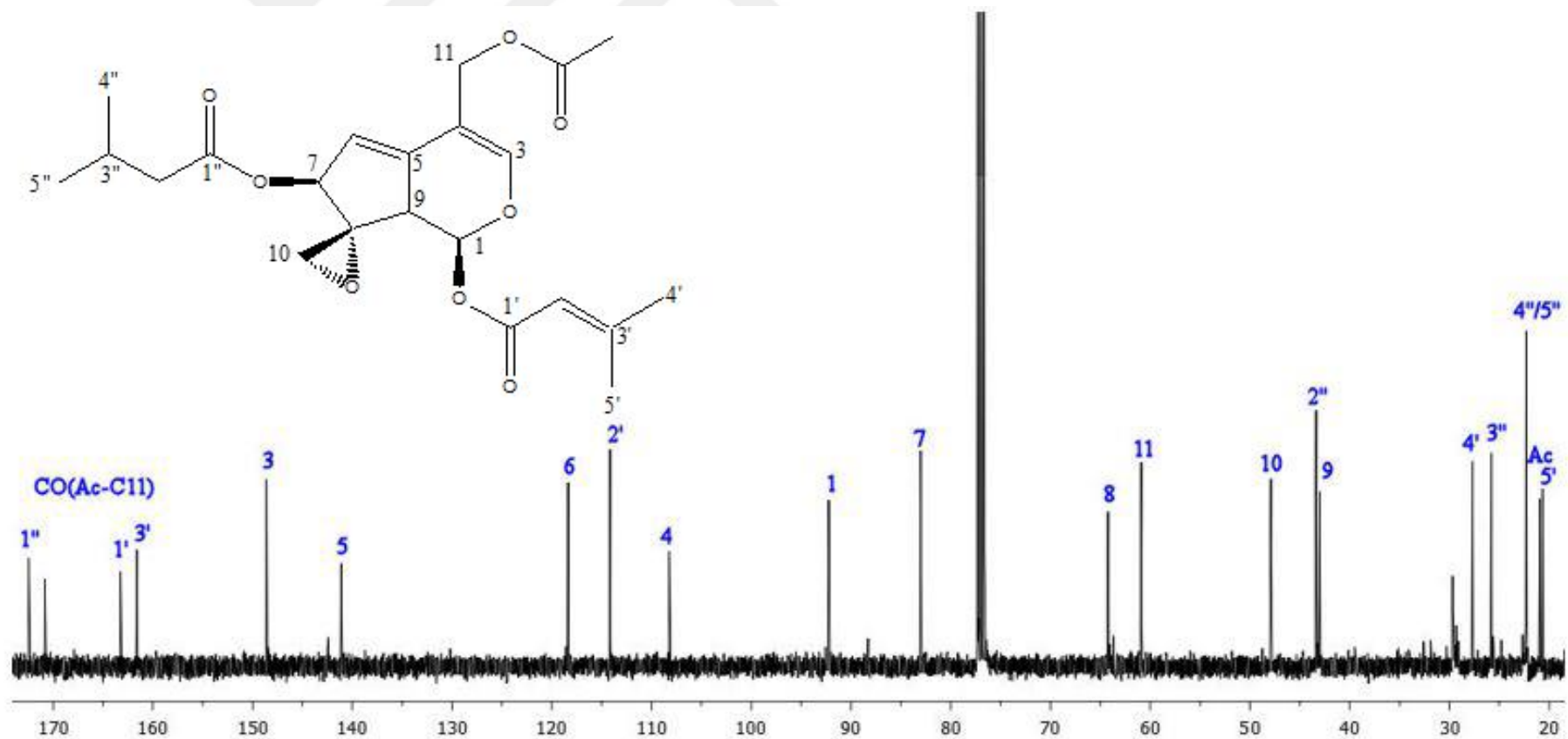
Table 78. ^{13}C and ^1H NMR data of seneciovaltrate (**5**) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

C/H atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
1	CH	92.3	6.01 d (10.1)
3	CH	148.6	6.71 s
4	C	108.2	-
5	C	141.5	-
6	CH	118.4	5.86 t (2.8)
7	CH	83.0	5.36 d (2.8)
8	C	64.2	-
9	CH	43.0	3.44 dd (10.1, 2.6)
10	CH ₂	47.9	3.01 d (4.8) 2.92 d (4.8)
11	CH ₂	60.9	4.73 d (12.4) 4.66 d (12.4)
<i>3-Methylcrotonoyl (C-1)</i>			
1'	C	163.3	-
2'	CH	114.2	5.66 s
3'	C	161.6	-
4'	CH ₃	27.7	2.21 s
5'	CH ₃	20.6	1.94 s
<i>Isovaleroyl (C-7)</i>			
1''	C	172.5	-
2''	CH ₂	43.4	2.18 d (6.8)
3''	CH	25.8	2.07 m
4''/5''	CH ₃	22.3	0.94 d (6.6)
11-COCH ₃	C	170.9	-
11-COCH ₃	CH ₃	20.9	2.06 s

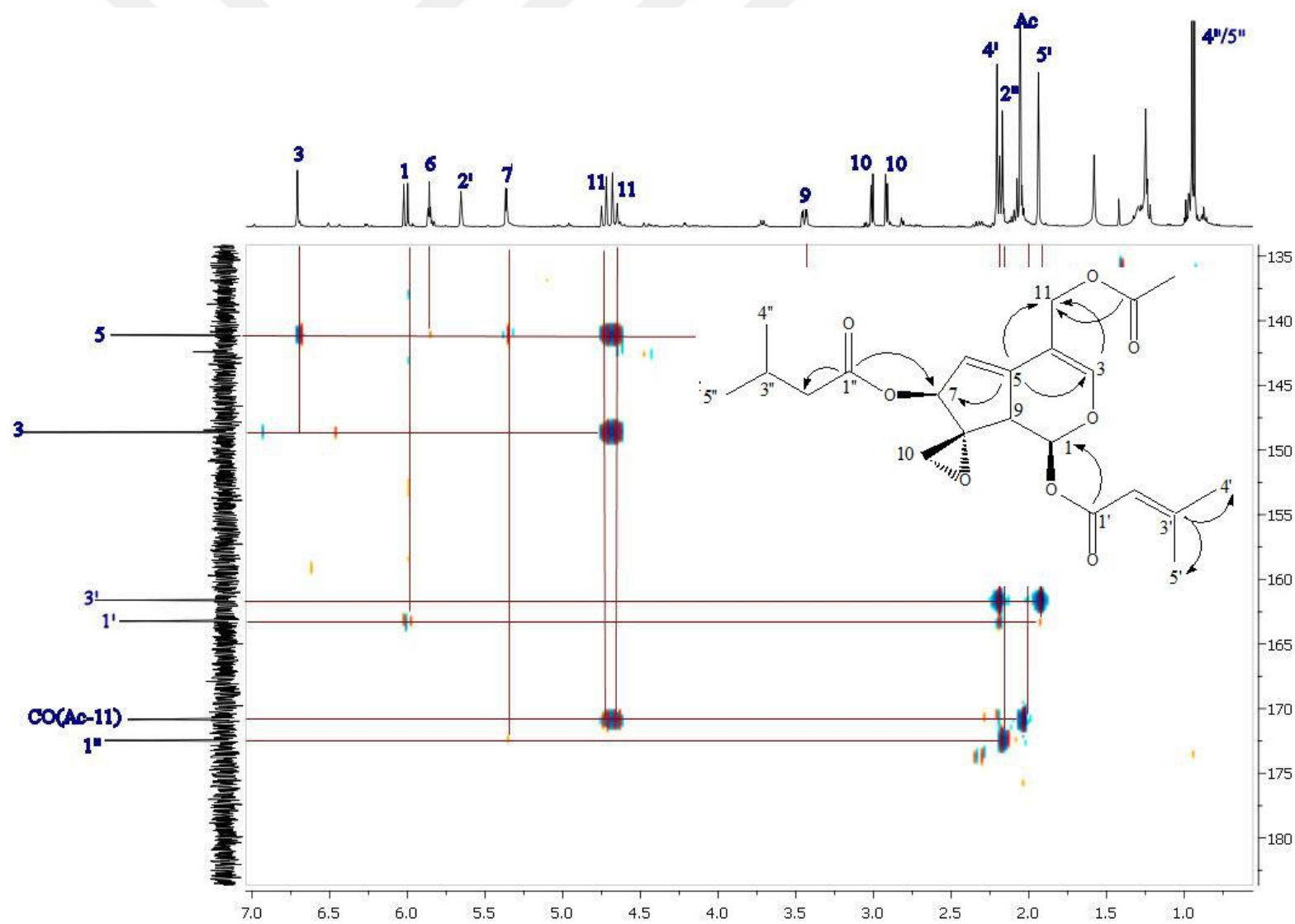
^aAssignments are based on HMBC experiment.



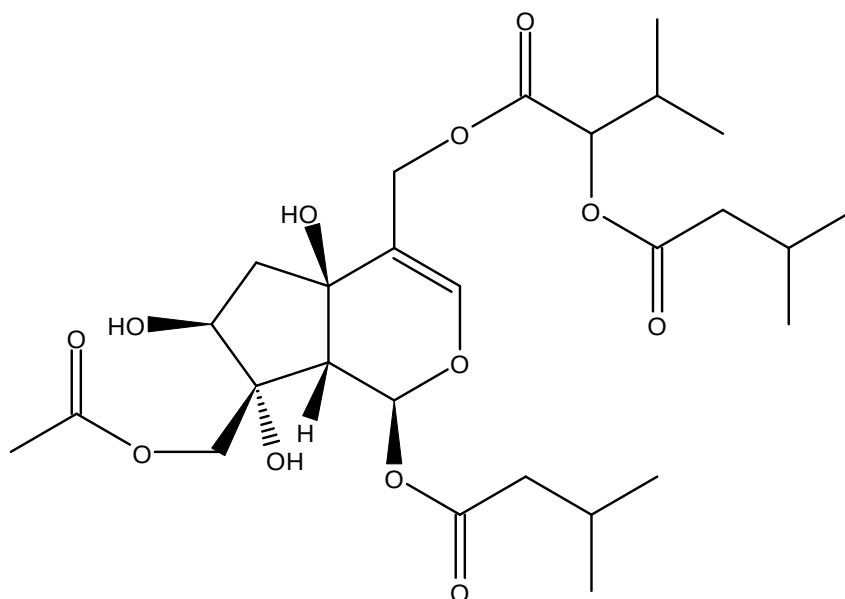
Spectrum 21. ^1H NMR Spectrum of Seneciovaltrate (5) (CDCl₃, 400 MHz)



Spectrum 22. ^{13}C NMR Spectrum of Seneciovaltrate (5) (CDCl_3 , 100 MHz)



Spectrum 23. HMBC Spectrum of Seneciovaltrate (5) (^{13}C :135-183 ppm, ^1H : 0.9-7.0 ppm)



VALERIOTRIATE B (13): C₂₇H₄₂O₁₂ (MW:558.62)

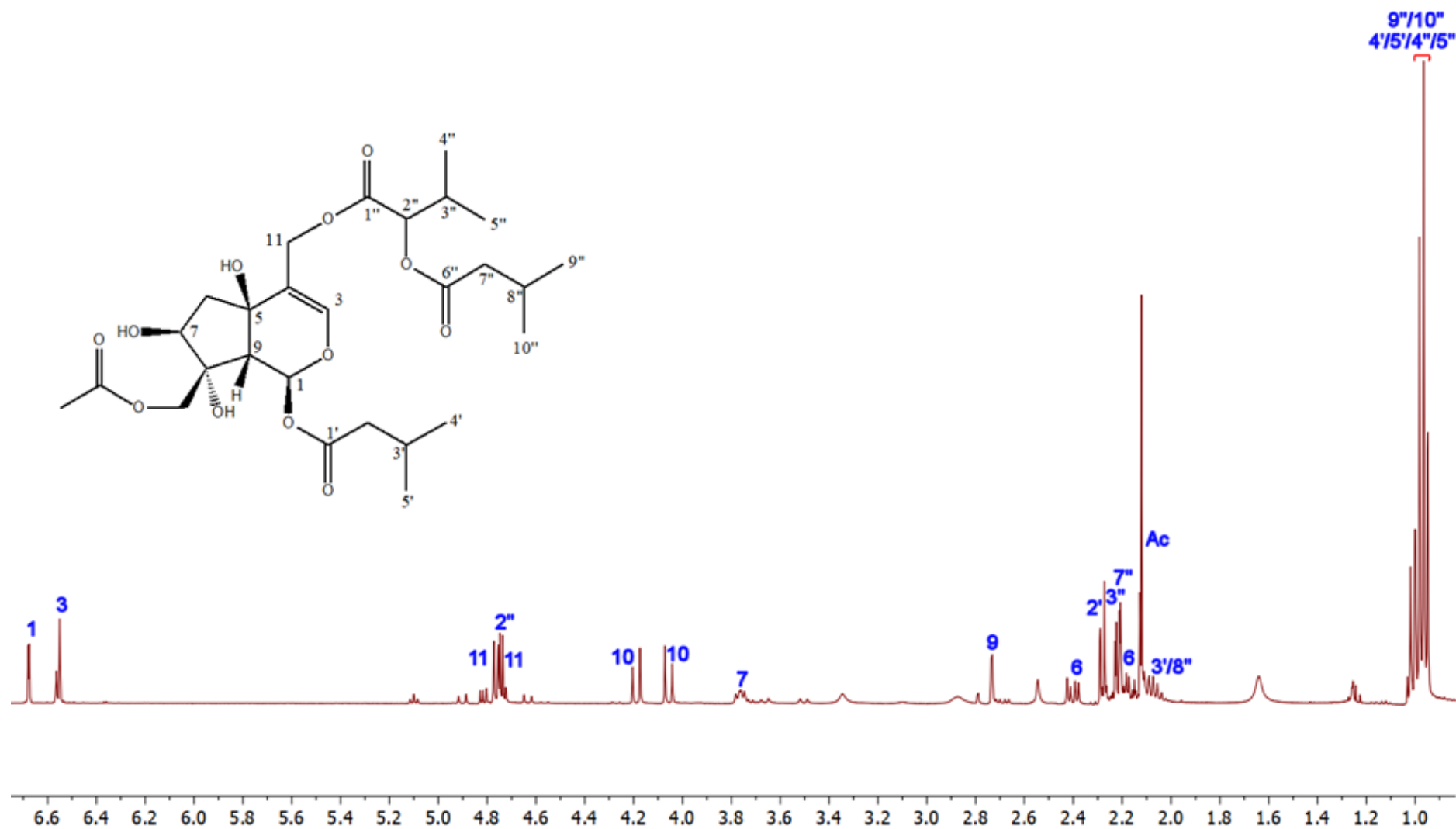
UV $\lambda_{\max}$ (MeOH) nm:	208
IR $\nu_{\max}$ (KBr) cm ⁻¹ :	3355 (OH), 1741 (C=O), 1645 (C=C)
ESI-MS m/z :	581.99 [M+Na] ⁺
¹ H NMR:	Table 79, Spectrum 24
¹³ C NMR:	Table 79, Spectrum 25
COSY:	Spectrum 26
HSQC:	Spectrum 27
HMBC:	Spectrum 28

Compound **13** was obtained as colorless oil. The molecular formula, C₂₇H₄₂O₁₂, was deduced by ESI-MS ([M+Na]⁺ at m/z 581.99). The IR spectrum of **13** showed absorption bands due to hydroxy (3355 cm⁻¹), ester carbonyl (1741 cm⁻¹) and olefinic (1645 cm⁻¹) groups. The UV spectrum exhibited absorption maximum at 208 nm. The ¹H and ¹³C NMR data (Table 79) of **13** revealed that it was a monoene-type valtrate hydrine. Taken together, 1D and 2D (COSY, HSQC and HMBC) NMR spectroscopic data (Table 79, Spectra 24-28) for **13** were consistent with which previously reported for valeriotriate B. Therefore, compound **13** was identified as valeriotriate B (247).

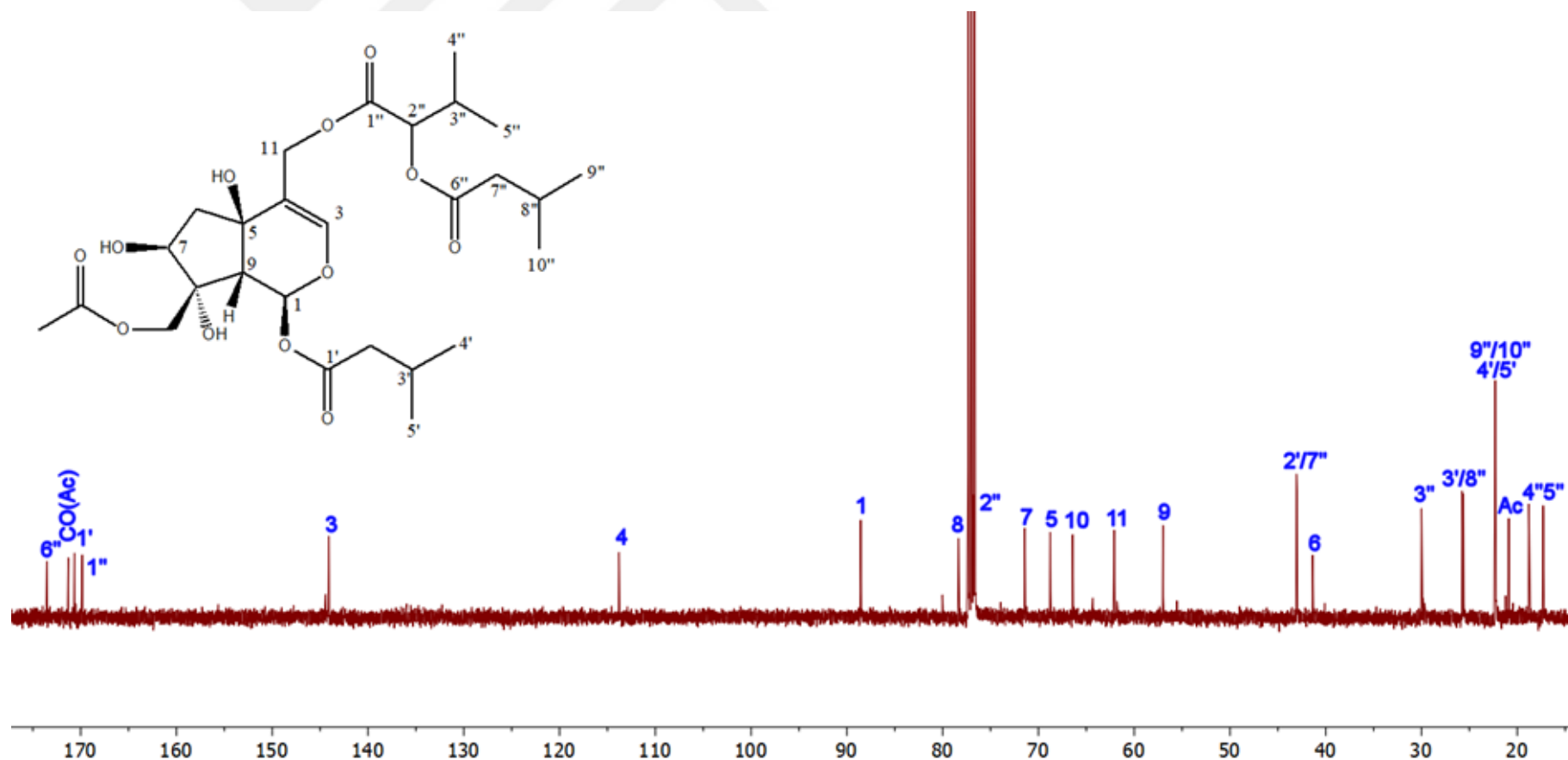
Table 79. ^{13}C and ^1H NMR data of valeriotriate B (**13**) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
1	CH	88.6	6.68 d (1.8)
3	CH	144.1	6.55 s
4	C	113.8	-
5	C	68.8	-
6	CH ₂	41.4	2.40 dd (13.0, 5.6) 2.17 m
7	CH	71.4	3.76 dd (8.4, 5.5)
8	C	78.4	-
9	CH	57.0	2.73 d (1.8)
10	CH ₂	66.4	4.19 d (12.1) 4.05 d (12.1)
11	CH ₂	62.1	4.79 d (12.4) 4.73 d (12.4)
<i>Isovaleroyl (C-1)</i>			
1'	C	170.7	-
2'	CH ₂	43.0	2.28 d (6.7)
3'	CH	25.8	2.08 m
4'/5'	CH ₃	22.3	0.96 d (6.6)
<i>α-(isovaleroxy)-isovaleroyl (C-11)</i>			
1''	CH	169.8	-
2''	CH	76.8	4.74 d (4.2)
3''	CH	30.0	2.24 m
4''	CH ₃	18.8	1.02 d (6.6)
5''	CH ₃	17.3	0.99 d (6.6)
6''	C	173.5	-
7''	CH ₂	43.0	2.22 d (6.8)
8''	CH	25.6	2.08 m
9''/10''	CH ₃	22.3	0.96 d (6.6)
10-COCH ₃	C	171.3	-
10-COCH ₃	CH ₃	20.9	2.12 s

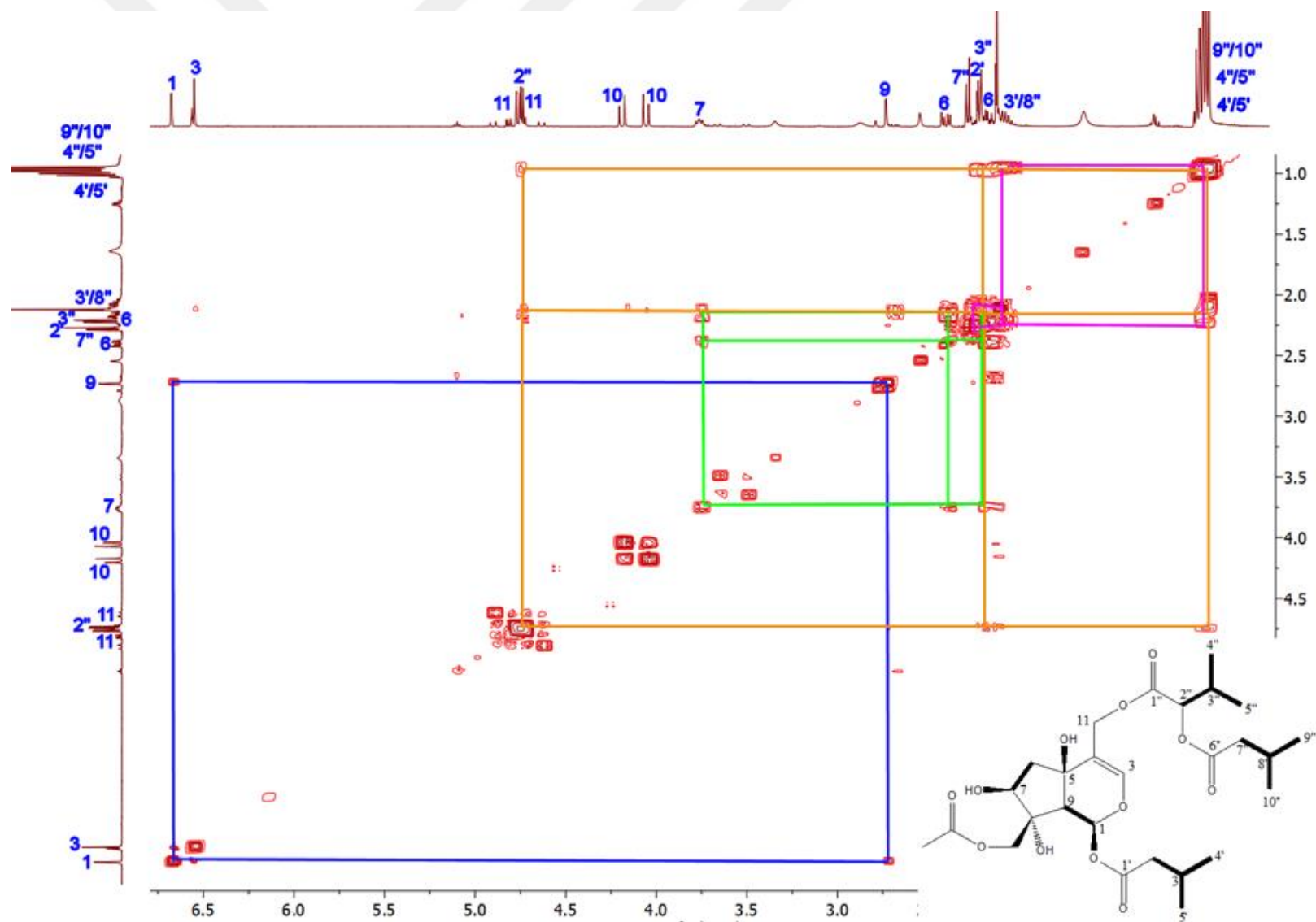
^aAssignments are based on COSY, HSQC and HMBC experiments.



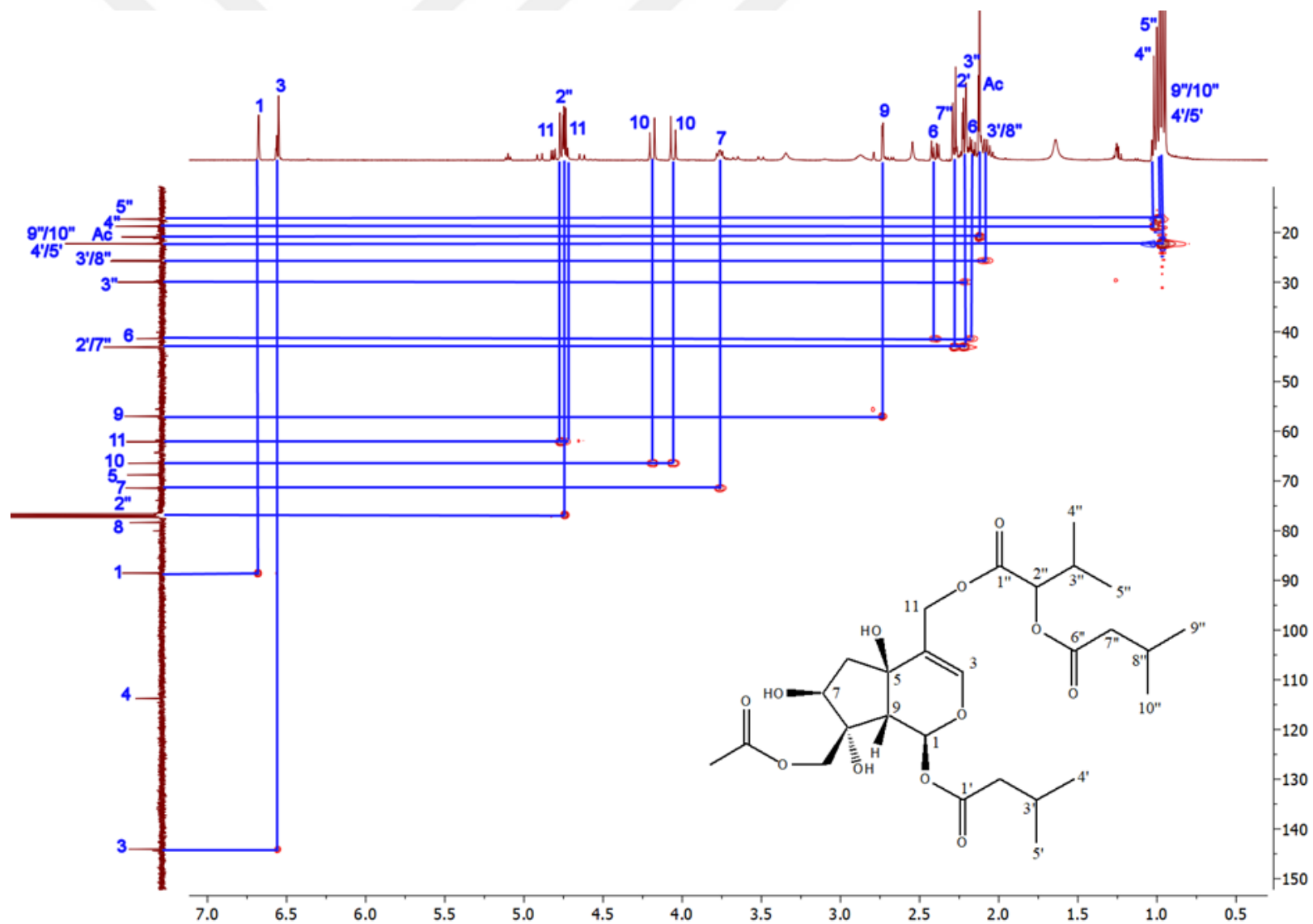
Spectrum 24. ^1H NMR Spectrum of Valeriotriate B (13) (CDCl_3 , 400 MHz)



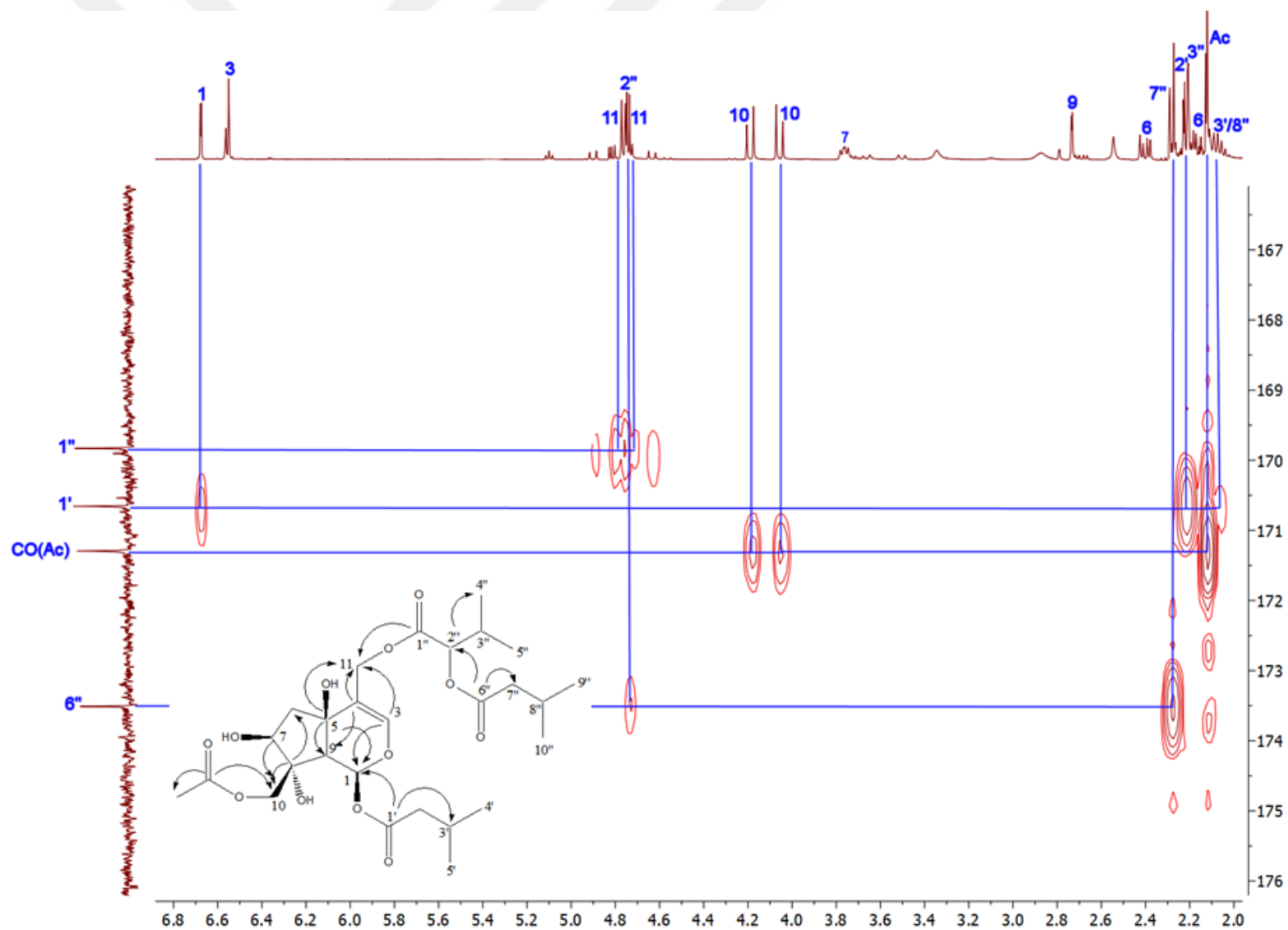
Spectrum 25. ¹³C NMR Spectrum of Valeriotriate B (13) (CDCl₃, 100 MHz)



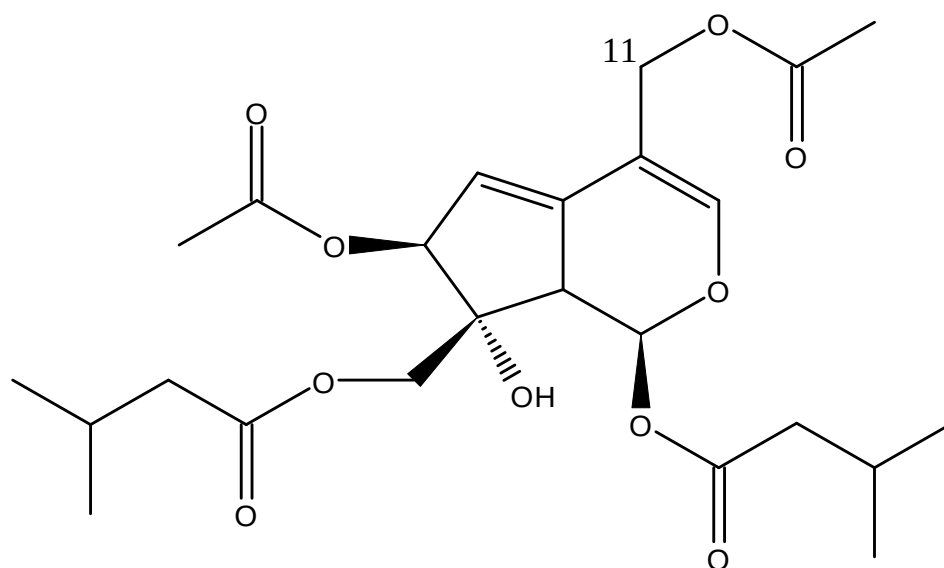
Spectrum 26. COSY Spectrum of Valeriotriate B (13)



Spectrum 27. HSQC Spectrum of Valeriotriate B (13)



Spectrum 28. HMBC Spectrum of Valeriotriate B (**13**) (^{13}C :165-176 ppm, ^1H : 2.0-6.8 ppm)



VALTRATE HYDRINE B2 (6): C₂₄H₃₄O₁₀ (MW:482.52)

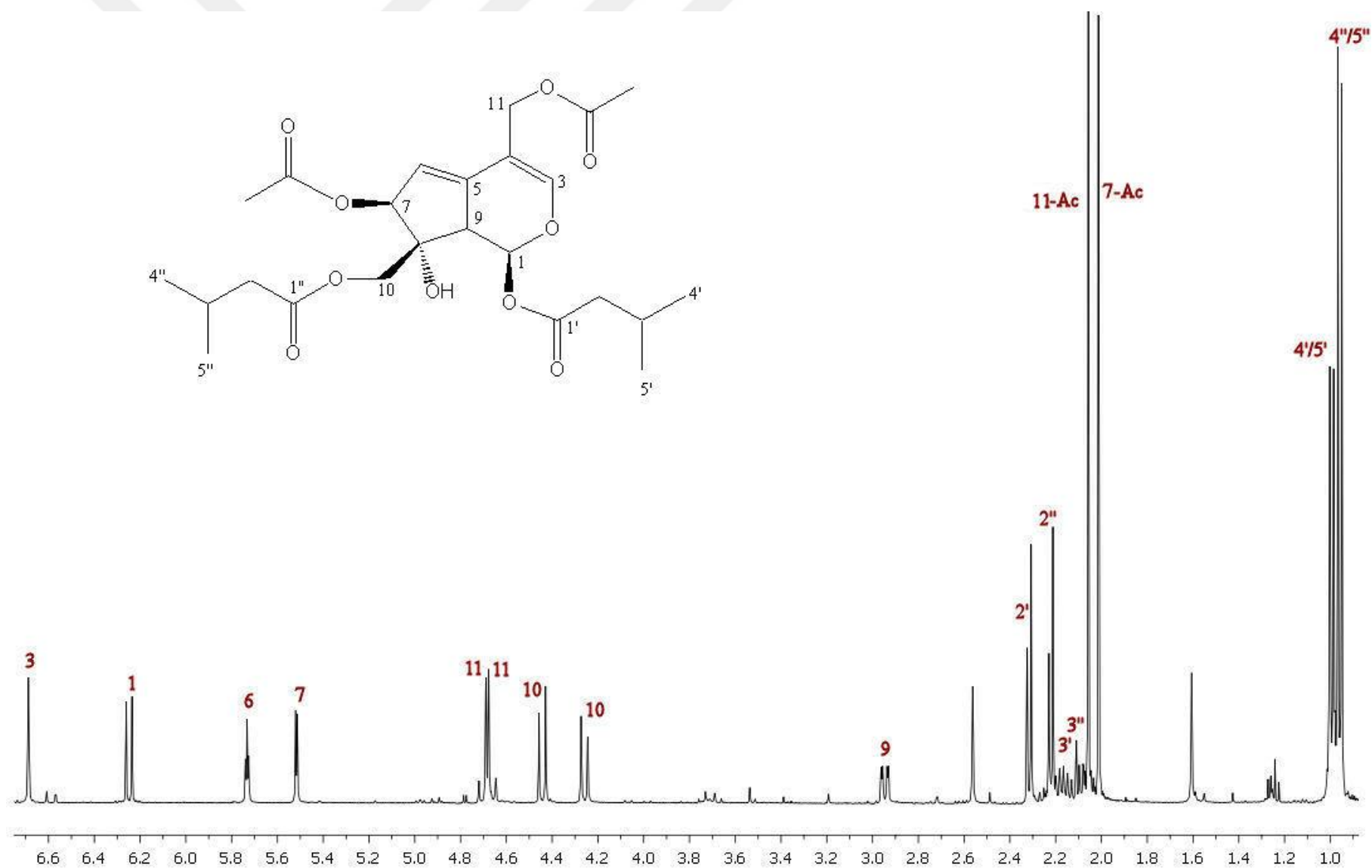
UV λ_{max} (MeOH) nm:	223, 255
IR ν_{max} (KBr) cm ⁻¹ :	3476 (OH), 1759, 1733, 1722 (C=O), 1644,1610 (C=C)
ESI-MS m/z :	506.13 [M+Na] ⁺
¹ H NMR:	Table 80, Spectrum 29
¹³ C NMR:	Table 80, Spectrum 30
HMBC:	Spectrum 31

Compound **6**, colorless oil, was deduced as C₂₄H₃₄O₁₀ from its positive-ion ESI-MS at m/z 506.13, indicating eight degrees of unsaturation. The IR spectrum of **6** showed the presence of hydroxyl (3476 cm⁻¹), ester (1759, 1733 and 1722 cm⁻¹) and olefinic (1644 and 1610 cm⁻¹) functionalities, whereas its UV spectrum displayed absorption maxima at 223 and 255 nm. The ¹H NMR (Table 80) demonstrated signals for two olefinic methines [δ_{H} 6.69 (s) and 5.73 (t, J = 2.8 Hz)], two oxymethylenes [δ_{H} 4.71 and 4.66 (each d, J = 12.4 Hz); δ_{H} 4.44 and 4.26 (each d, j = 11.6 Hz)], one oxymethine [δ_{H} 5.52 (d, J = 2.9 Hz)] and one hemiacetal [δ_{H} 6.25 (d, J = 10.0 Hz)], which were characteristic for a “valtrate hydrine” skeleton. The complete ¹H and ¹³C NMR data of **6** were identical to the those reported for valtrate hydrine B2 (248).

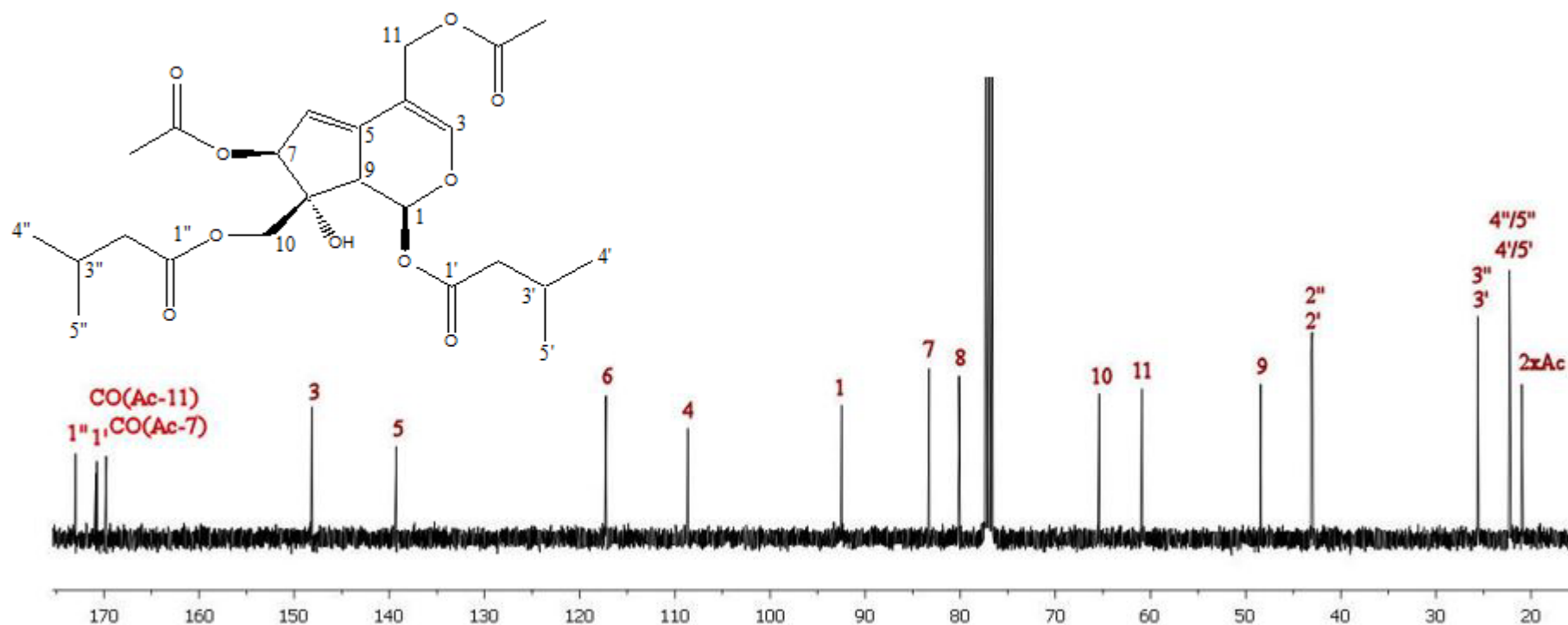
Table 80. ^{13}C and ^1H NMR data of valtrate hydrine B2 (**6**) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
1	CH	92.5	6.25 d (10.0)
3	C	148.2	6.69 s
4	C	108.7	-
5	C	139.3	-
6	CH	117.7	5.73 t (2.8)
7	CH	83.3	5.52 d (2.9)
8	C	80.1	-
9	CH	48.4	2.95 dd (10.0, 2.6)
10	CH ₂	65.4	4.44 d (11.6) 4.26 d (11.6)
11	CH ₂	60.9	4.71 d (12.4) 4.66 d (12.4)
<i>Isovaleroyl (C-1)</i>			
1'	C	170.8	-
2'	CH ₂	43.1	2.32 d (7.2)
3'	CH	25.6	2.17 m
4'/5'	CH ₃	22.3	1.00 d (6.6)
<i>Isovaleroyl (C-10)</i>			
1''	C	173.0	-
2''	CH ₂	43.0	2.22 d (7.2)
3''	CH	25.6	2.10 m
4''/5''	CH ₃	22.3	0.96 d (6.6)
7- <u>CO</u> CH ₃	C	169.8	-
7-CO <u>CH</u> ₃	CH ₃	21.0	2.01 s
11- <u>CO</u> CH ₃	C	170.9	-
11-CO <u>CH</u> ₃	CH ₃	20.9	2.06 s

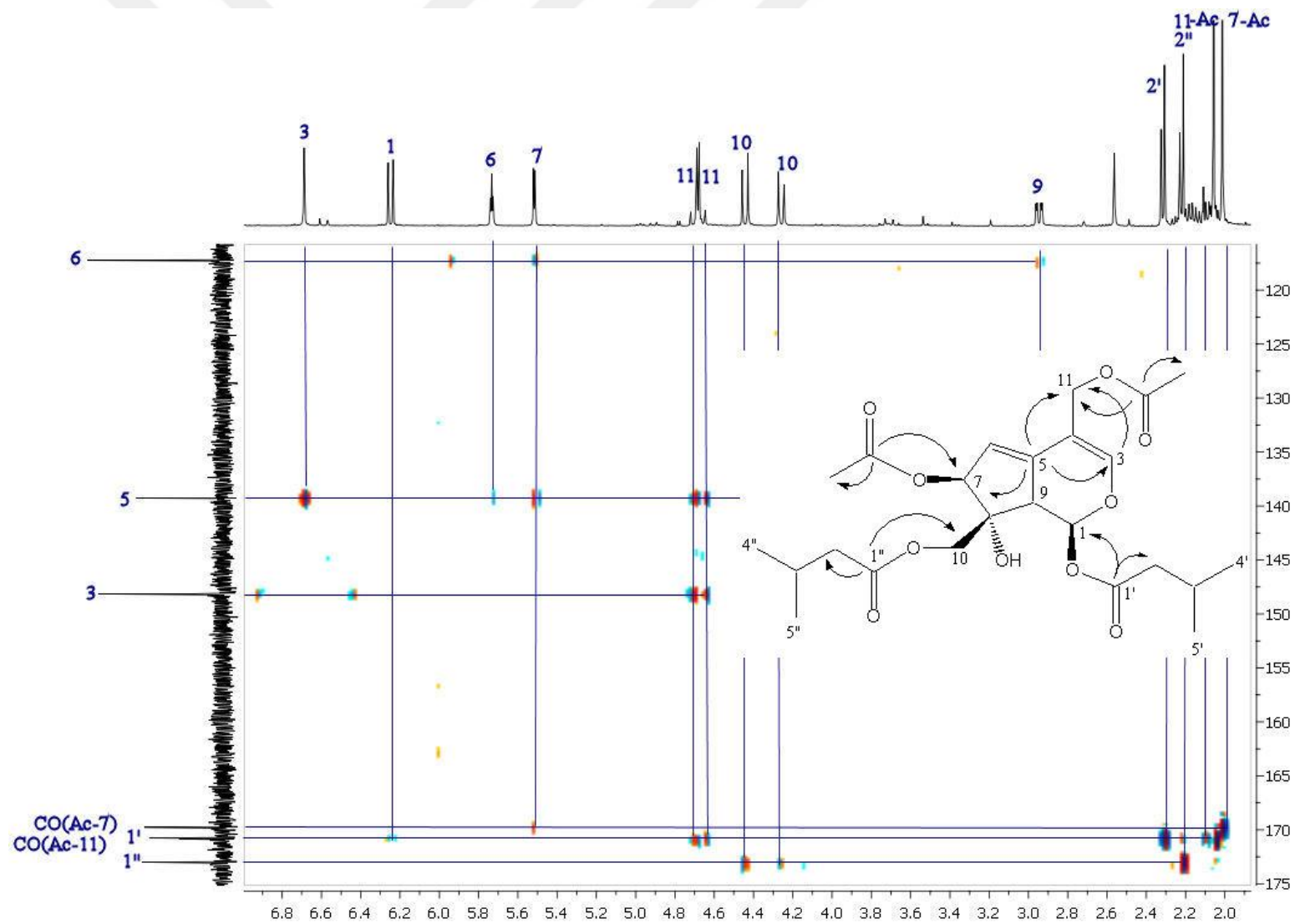
^aAssignments are based on HMBC experiment.



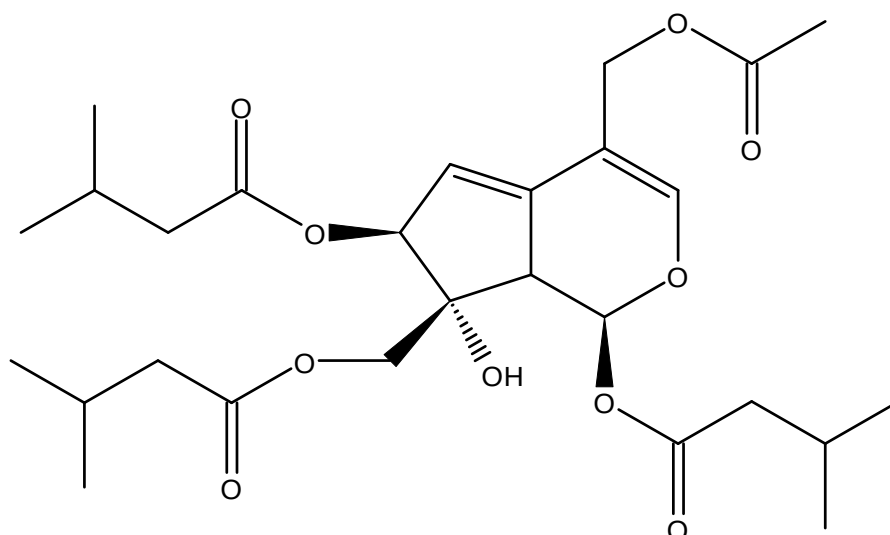
Spectrum 29. ¹H NMR Spectrum of Valtrate hydrine B2 (6) (CDCl₃, 400 MHz)



Spectrum 30. ¹³C NMR Spectrum of Valtrate hydrine B2 (**6**) (CDCl₃, 100 MHz)



Spectrum 31. HMBC Spectrum of Valtrate hydrine B2 (6)



ISOVALEROXYVALTRATE HYDRINE (7): C₂₇H₄₀O₁₀ (MW:524.60)

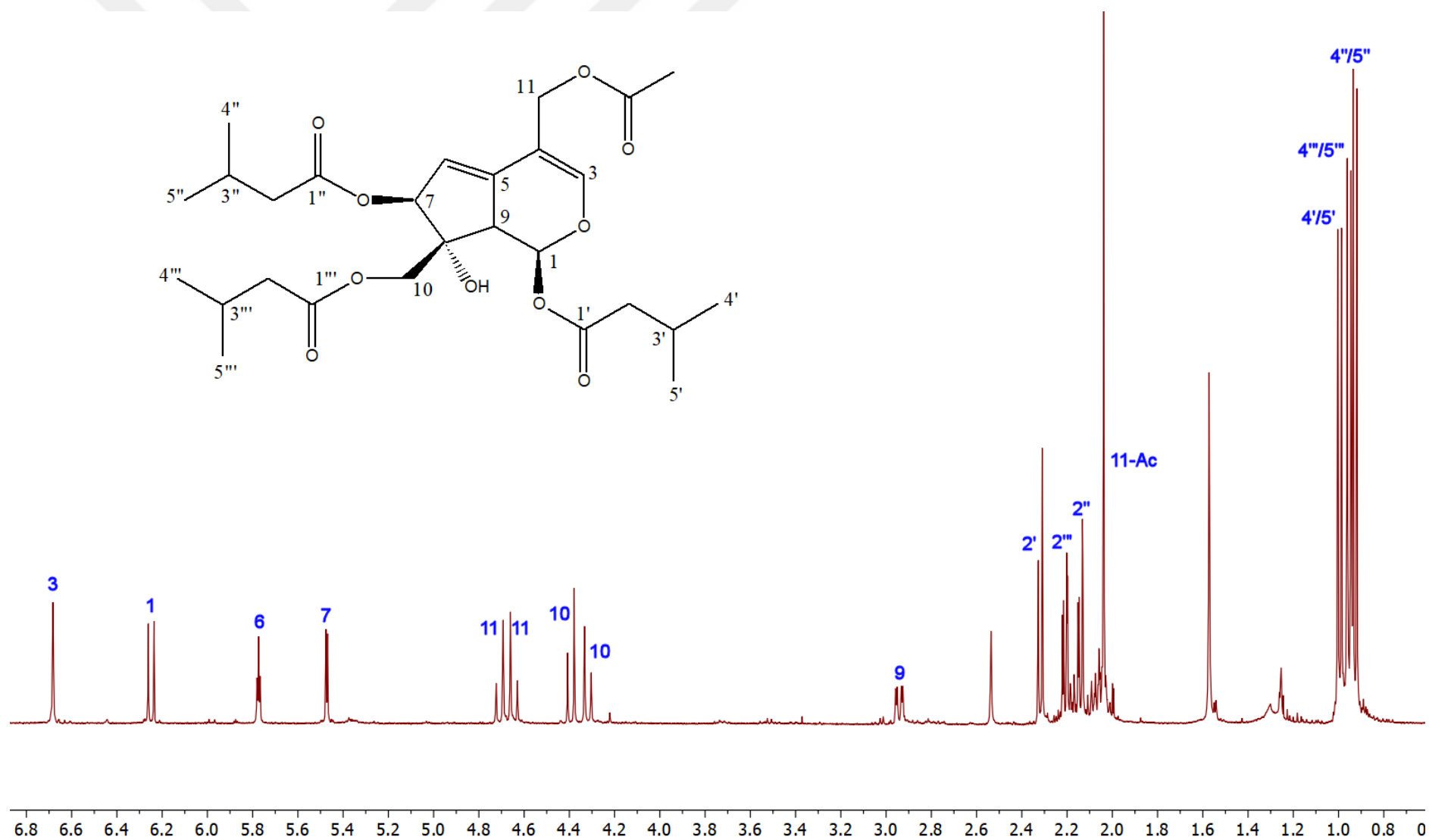
UV λ_{max} (MeOH) nm:	258
IR ν_{max} (KBr) cm ⁻¹ :	3680 (OH), 1743 (C=O), 1636,1558 (C=C)
ESI-MS m/z :	548.21 [M+Na] ⁺
¹ H NMR:	Table 81, Spectrum 32
¹³ C NMR:	Table 81, Spectrum 33
HMBC:	Spectrum 34

Compound **7** was isolated as colorless oil. The UV spectrum indicated the absorption maximum at 258 nm, while the IR spectrum showed presence of hydroxyl (3680 cm⁻¹), ester carbonyl (1743 cm⁻¹) and olefinic (1636 and 1558 cm⁻¹) groups. The ¹H and ¹³C NMR findings (Table 81) closely resembled those of **6** (valtrate hydrine B2), except that acetoxymethyl group at C-7 was replaced by an isovaleroxy unit in **7**. Taken together, ¹H and ¹³C NMR spectroscopic data (Spectra 32 and 33), secured by 2D NMR (spectrum 34) experiments were superimposed with those reported for isovaleroxyvaltrate hydrine in the literature. (248).

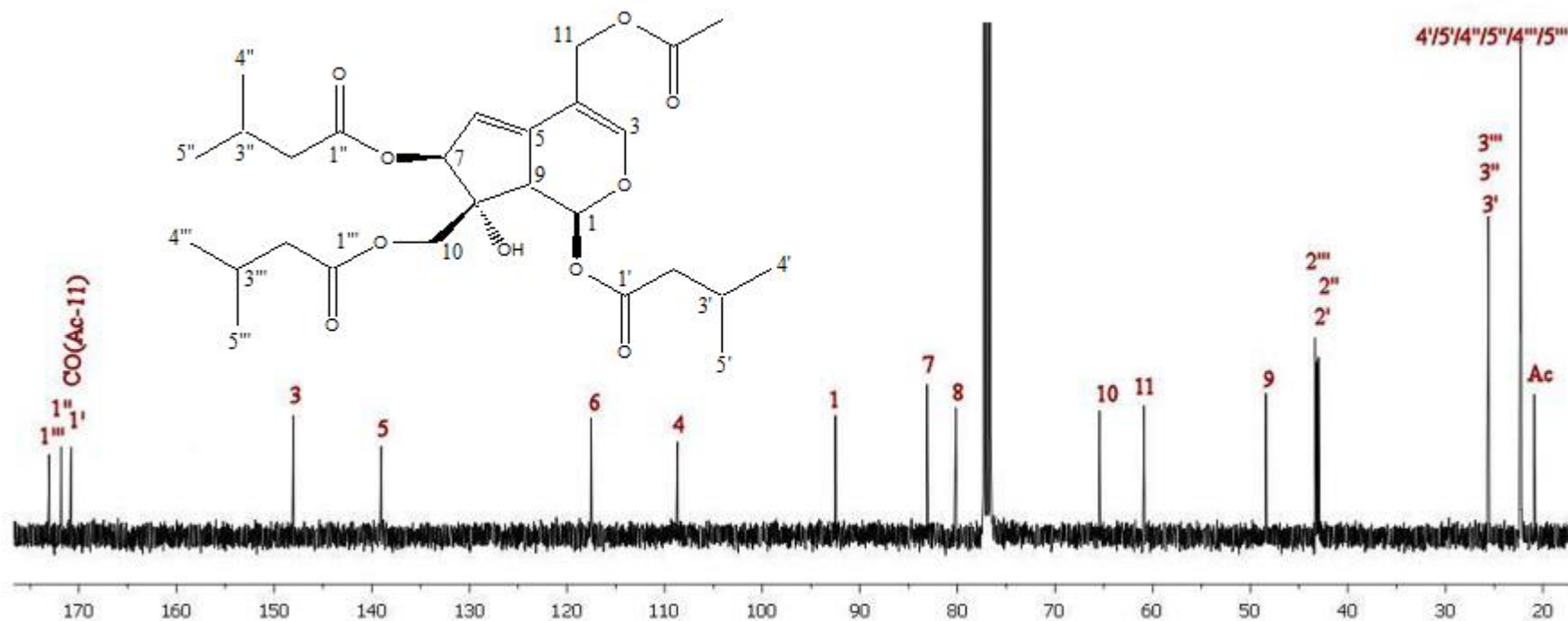
Table 81. ^{13}C and ^1H NMR data of isovaleroxyvaltrate hydrine (7) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), <i>J</i> (Hz)
1	CH	92.5	6.25 d (10.0)
3	CH	148.0	6.68 s
4	C	108.7	-
5	C	139.0	-
6	CH	117.5	5.77 t (2.7)
7	CH	83.1	5.47 d (2.8)
8	C	80.1	-
9	CH	48.4	2.94 dd (10.0, 2.6)
10	CH ₂	65.5	4.39 d (11.6)
			4.32 d (11.6)
11	CH ₂	60.9	4.71 d (12.4)
			4.64 d (12.4)
<i>Isovaleroyl (C-1)</i>			
1'	C	170.8	-
2'	CH ₂	43.2	2.32 d (7.2)
3'	CH	25.6	2.17 m
4'/5'	CH ₃	22.3	0.99 d (6.6)
<i>Isovaleroyl (C-7)</i>			
1''	C	171.8	-
2''	CH ₂	43.4	2.21 d (6.6)
3''	CH	25.7	2.06 m
4''/5''	CH ₃	22.3	0.93 d (6.6)
<i>Isovaleroyl (C-10)</i>			
1'''	C	173.1	-
2'''	CH ₂	43.0	2.14 d (6.1)
3'''	CH	25.6	2.09 m
4'''/5'''	CH ₃	22.2	0.95 d (6.6)
11-COCH ₃	C	170.8	-
11-COCH ₃	CH ₃	20.9	2.04 s

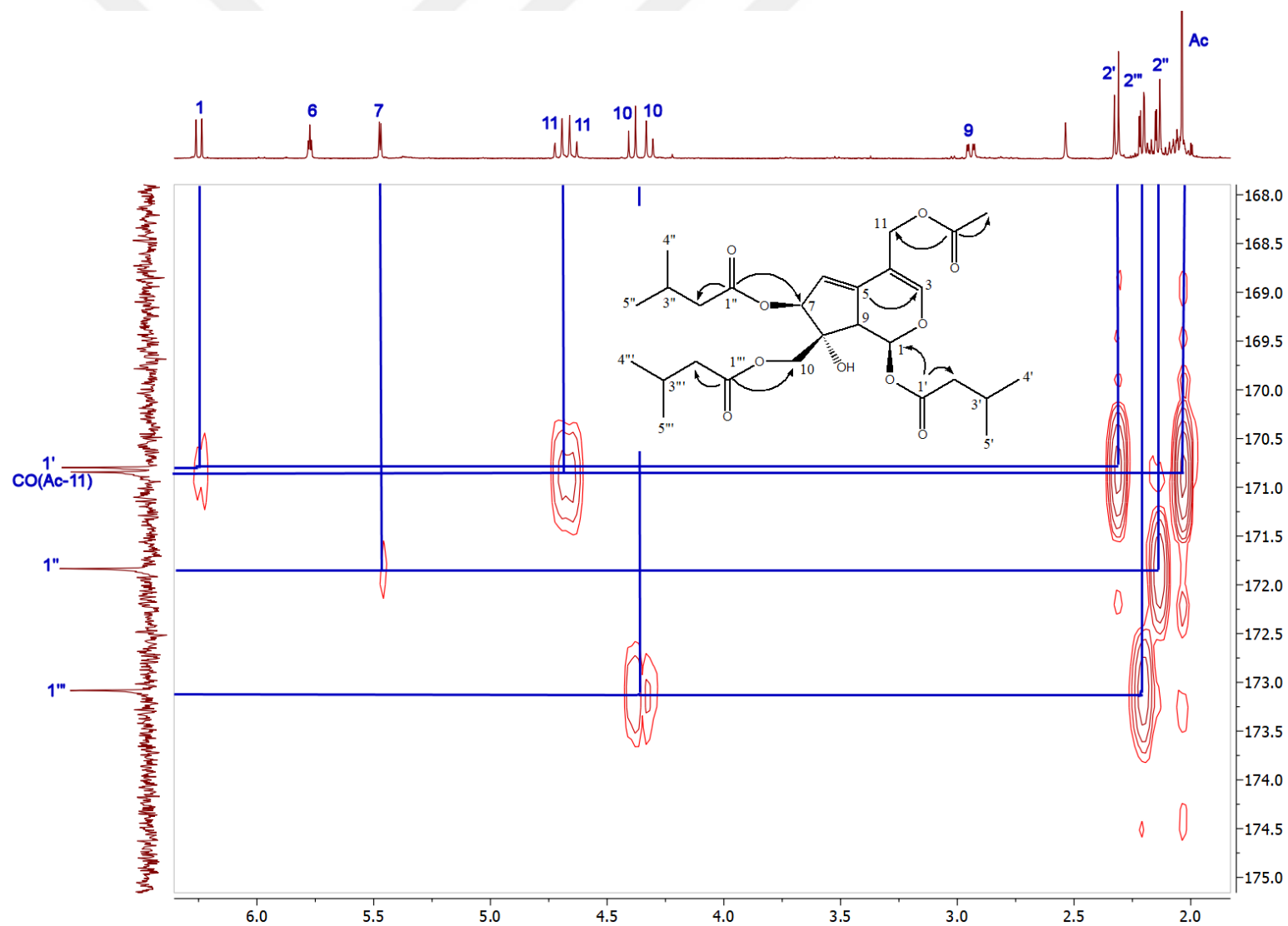
^aAssignments are based on HMBC experiment.



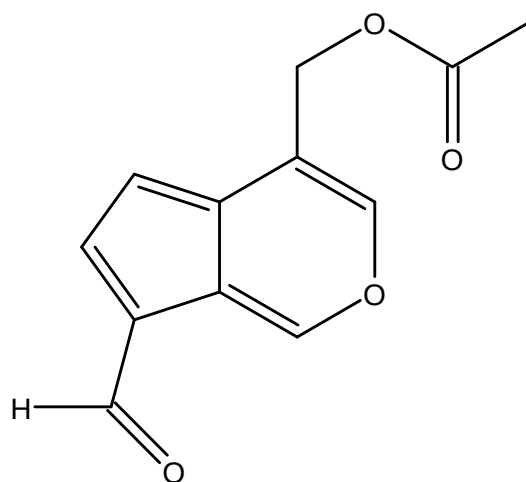
Spectrum 32. ^1H NMR Spectrum of Isovaleroxyvaltrate hydrine (7) (CDCl₃, 400 MHz)



Spectrum 33. ¹³C NMR Spectrum of Isovaleroxyvaltrate hydrate (7) (CDCl₃, 100 MHz)



Spectrum 34. HMBC Spectrum of Isovaleroxyvaltrate hydrate (7) (^{13}C :168-175 ppm, ^1H : 1.9-6.3 ppm)



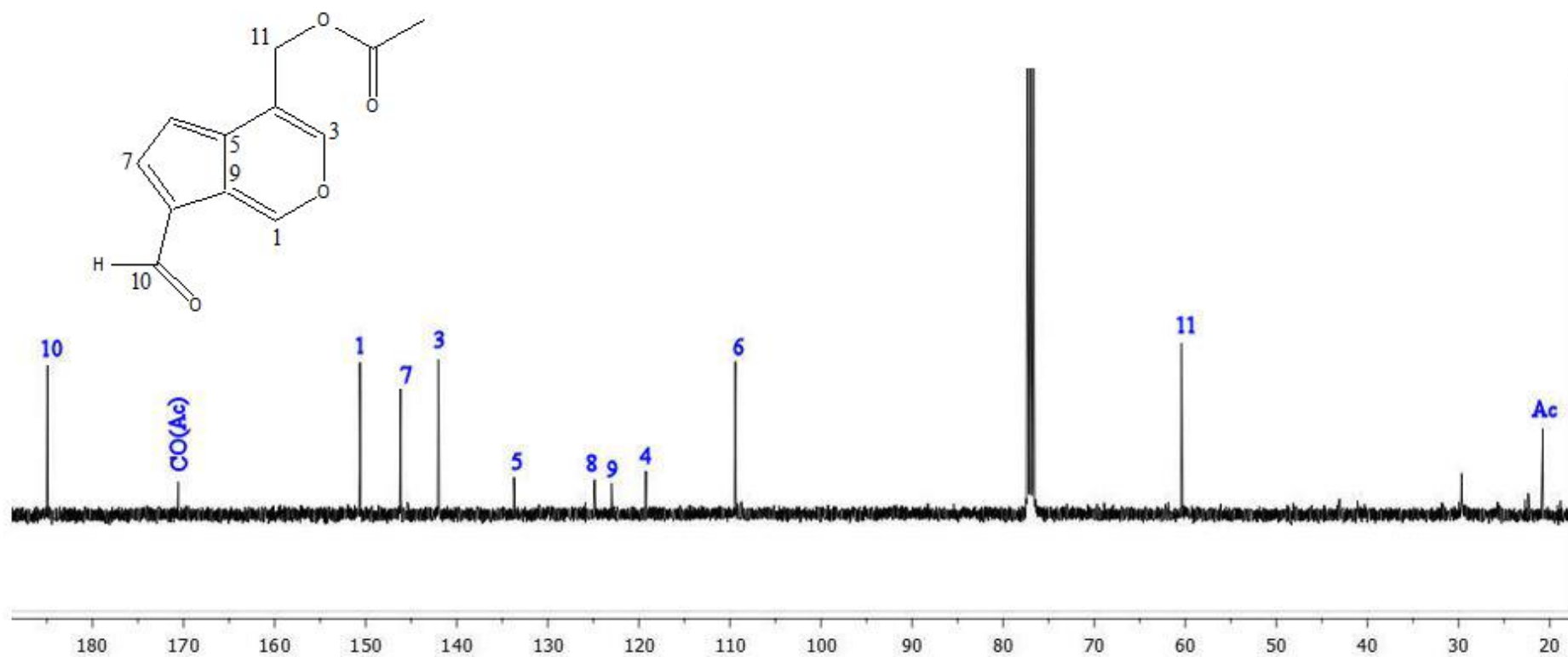
BALDRINAL (9): C₁₂H₁₀O₄ (MW:218.21)

UV λ_{max} (MeOH) nm:	203, 227, 240, 287
IR ν_{max} (KBr) cm ⁻¹ :	1746 (C=O), 1537 (conjugated, C=C)
ESI-MS m/z :	219.38 [M+H] ⁺
¹ H NMR:	Table 82, Spectrum 35
¹³ C NMR:	Table 82, Spectrum 36

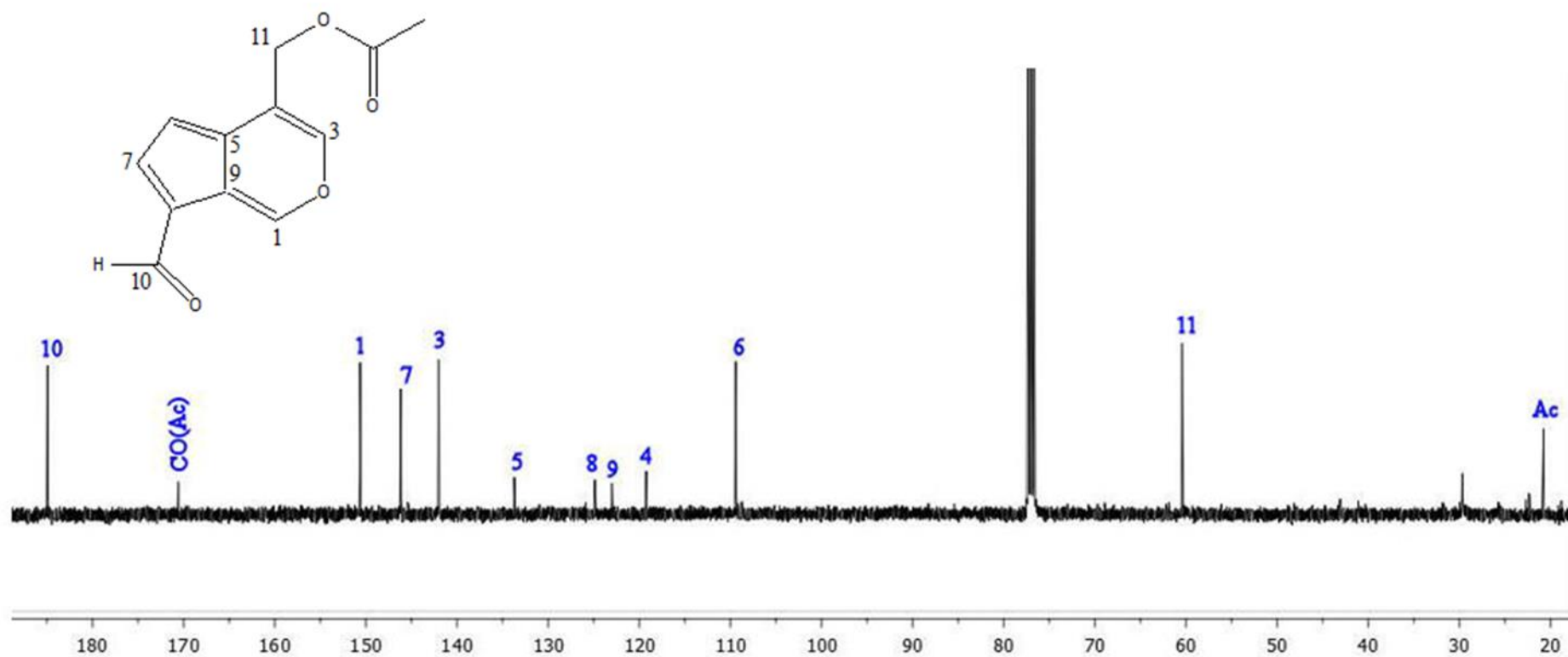
Compound **9** was obtained as yellowish oil. Its molecular formula was deduced to be C₁₂H₁₀O₄ based on the its quasi-molecular ion peak at m/z 219.38 [M+H]⁺ in positive ion ESI-MS, exhibiting eight degrees of unsaturation as well as NMR spectra. Its UV spectrum showed absorption maxima at 203, 227, 240 and 287 nm, whereas its IR spectrum indicated bands at 1746 and 1537 cm⁻¹ due to carbonyl and conjugated olefinic groups, respectively. The ¹H and ¹³C data (Table 82, Spectra 35 and 36) indicated that **9** was a baldrinal, an iridoid derivative by comparison of NMR data with those of reported. (186).

Table 82. ^{13}C and ^1H NMR data of baldrinal (**9**) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)

C/H atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
1	CH	150.7	9.18 s
3	CH	142.0	7.88 s
4	C	119.2	-
5	C	133.7	-
6	CH	109.4	6.61 d (3.3)
7	CH	146.2	7.87 d (3.3)
8	C	124.9	-
9	C	123.0	-
10	CH	185.0	9.94 s
11	CH_2	60.4	5.26 s
11- <u>CO</u> CH_3	C	170.6	-
11- CO <u>CH_3</u>	CH_3	20.8	2.12 s

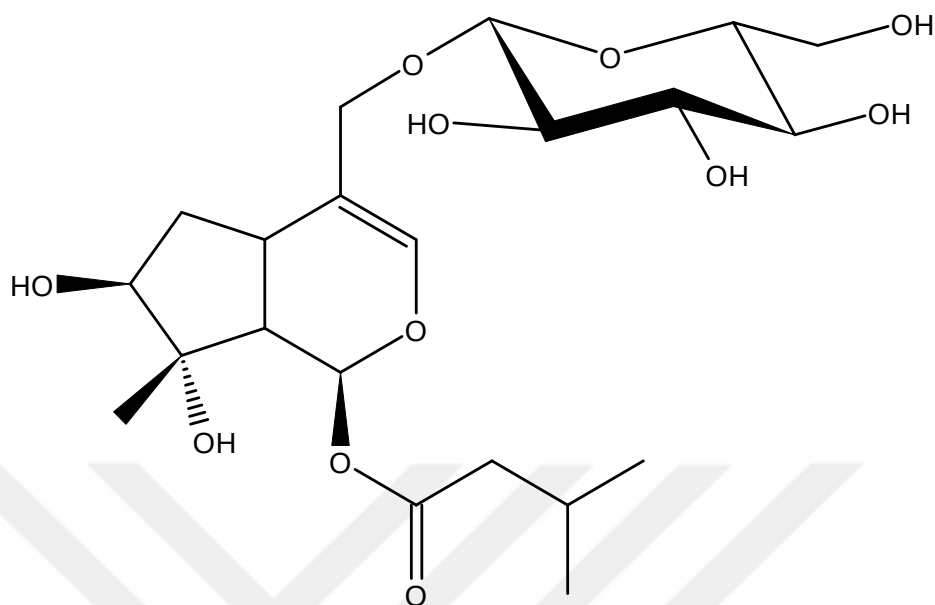


Spectrum 35. ¹H NMR Spectrum of Baldrial (9) (CDCl₃, 400 MHz)



Spectrum 36. ^{13}C NMR Spectrum of Baldrinal (9) (CDCl_3 , 100 MHz)

4.5.1.1.1.B. Iridoid Glycoside



VALEROSIDATE (8): C₂₁H₃₄O₁₁ (MW:462.49)

UV $\lambda_{\max}$ (MeOH) nm:	203
IR $\nu_{\max}$ (KBr) cm ⁻¹ :	3390 (OH), 1742 (C=O), 1652 (olefinic, C=C)
ESI-MS m/z :	485.85 [M+Na] ⁺
¹ H NMR:	Table 83, Spectrum 37
¹³ C NMR:	Table 83, Spectrum 38
COSY:	Spectrum 39
HMBC:	Spectrum 40

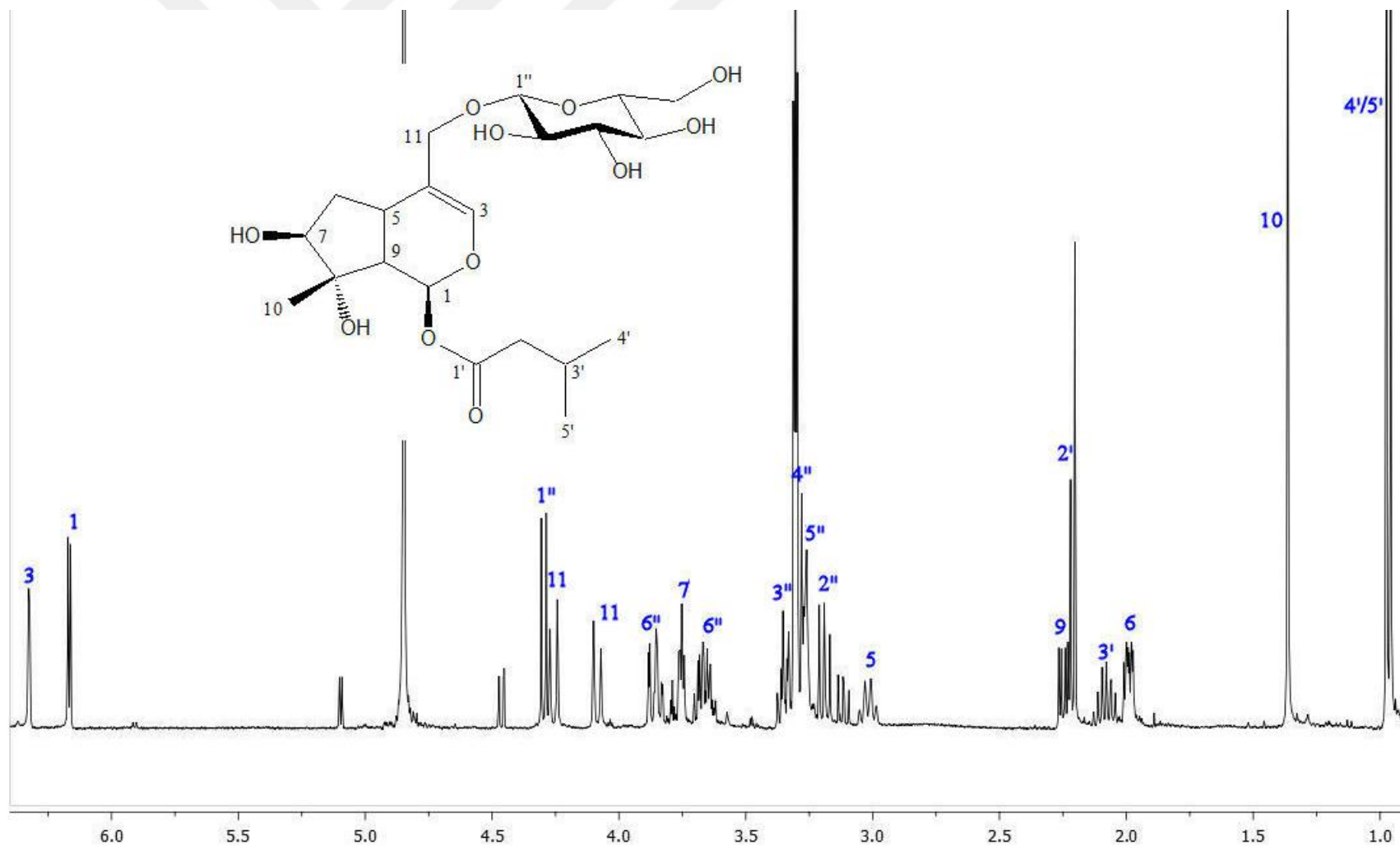
Compound **8** was isolated as an amorphous powder. The molecular formula, C₂₁H₃₄O₁₁, was established from the pseudomolecular ion at m/z 485.85 [M+Na]⁺ in positive ion ESI-MS and NMR data. Its UV spectrum gave maximum at 203 nm. The IR spectrum indicated absorption bands at 3390 (hydroxyl), 1742 (ester carbonyl) and 1652 (olefinic) cm⁻¹. Analysis of 1D (¹H and ¹³C) and 2D (COSY and HMBC) spectra (Table 83, Spectra 37-40) revealed that **8** possessed monoene type-iridoid glycoside structure. Spectroscopic findings were superimposed with those of valeroside reported in the literature (249).

Table 83. ^{13}C and ^1H NMR data of valerosidate (**8**) (CD_3OD ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

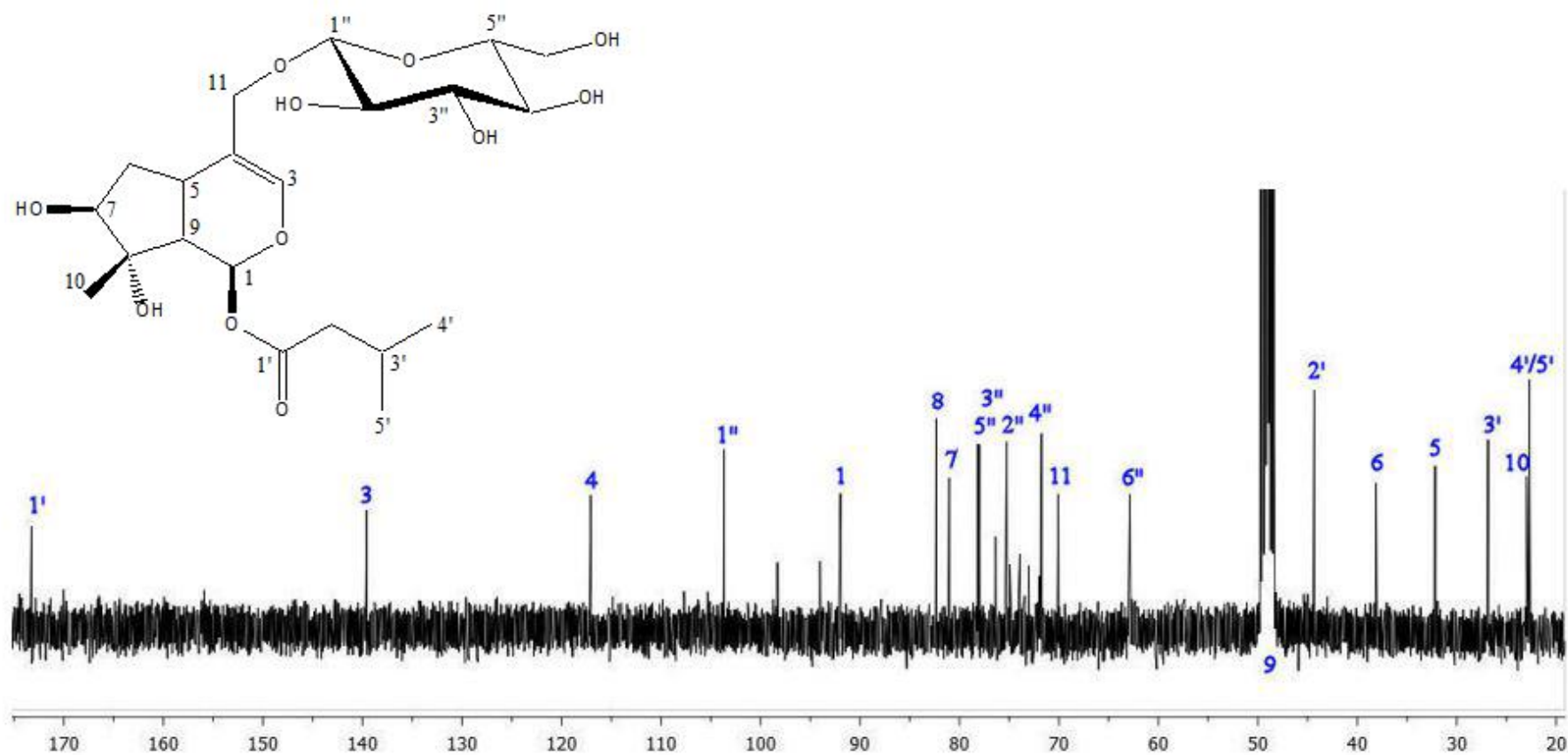
C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
<i>Aglycone</i>			
1	CH	92.0	6.17 d (3.9)
3	CH	139.6	6.33 s
4	C	117.1	-
5	CH	32.2	3.02 m
6	CH_2	38.1	1.99 m
7	CH	81.0	3.75 t (3.8)
8	C	82.3	-
9	CH	48.4	2.25 dd (10.2, 3.9)
10	CH_3	23.0	1.36 s
11	CH_2	70.1	4.26 d (11.5) 4.09 d (11.5)
<i>Isovaleroyl (C-1)</i>			
1'	C	173.3	-
2'	CH_2	44.3	2.21 d (6.7)
3'	CH	26.9	2.08 m
4'/5'	CH_3	22.7	0.97 d (6.6)
<i>Glucose (C-11)</i>			
1''	CH	103.7	4.29 d (7.8)
2''	CH	75.2	3.19 dd (9.0, 7.8)
3''	CH	78.0	3.35 t (9.0)
4''	CH	71.8	3.29 †
5''	CH	78.2	3.26 †
6''	CH_2	62.9	3.86 dd (12.0, 2.0) 3.66 dd (12.0, 5.4)

^aAssignments are based on COSY and HMBC experiments.

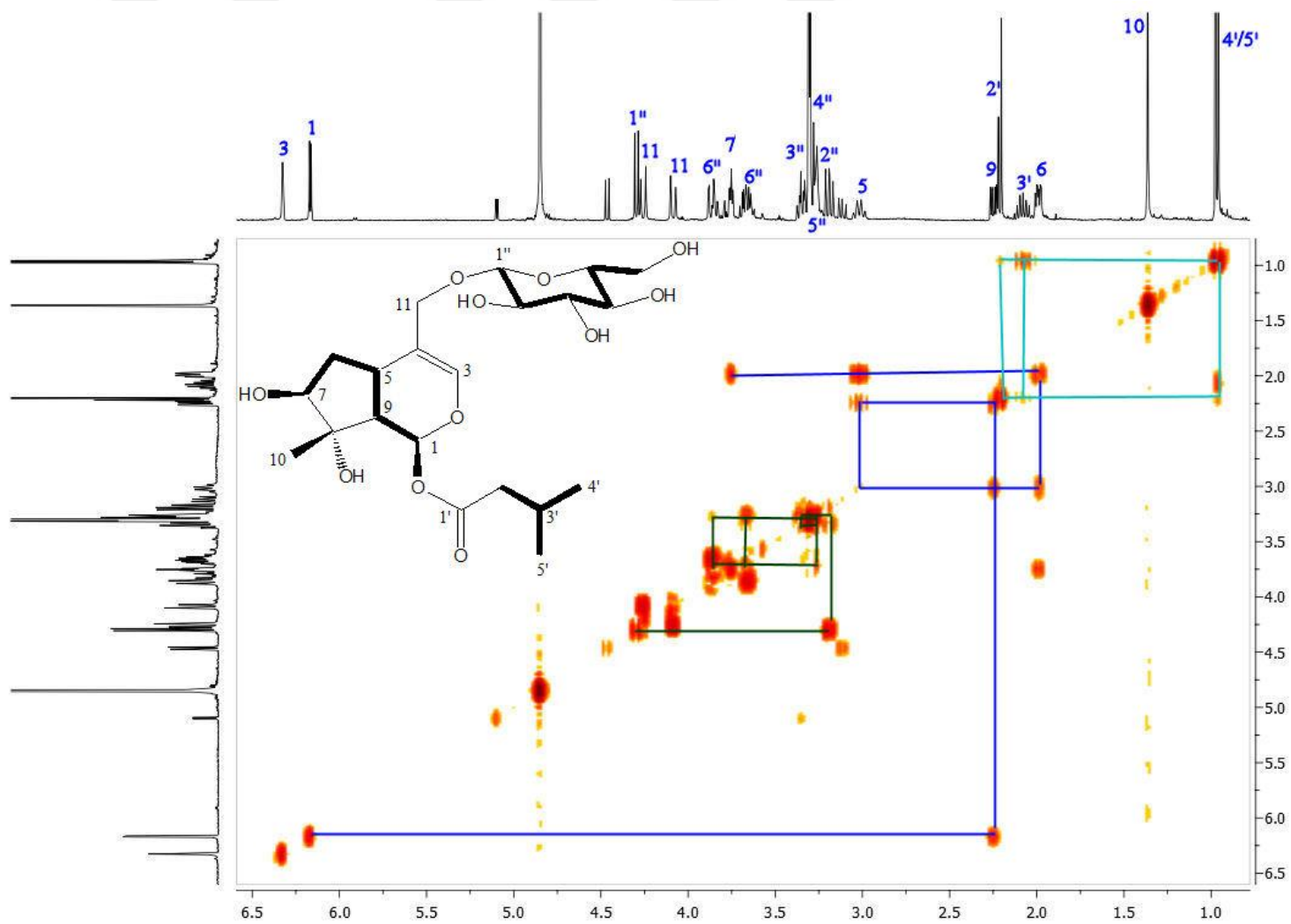
†: Overlapped signals.



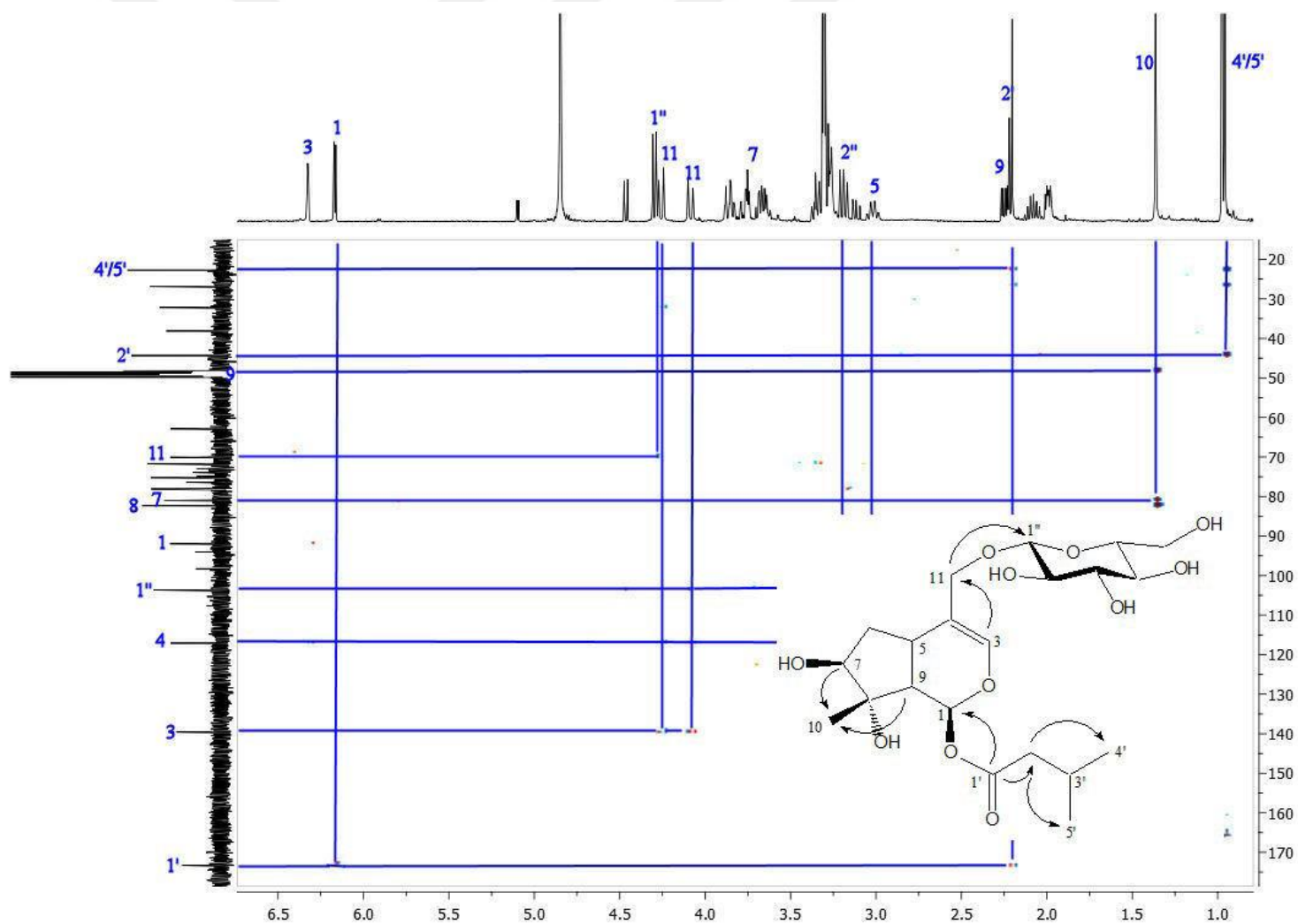
Spectrum 37. ^1H NMR Spectrum of Valerosidate (**8**) (CD_3OD , 400 MHz)



Spectrum 38. ^{13}C NMR Spectrum of Valeroside (**8**) (CD_3OD , 100 MHz)

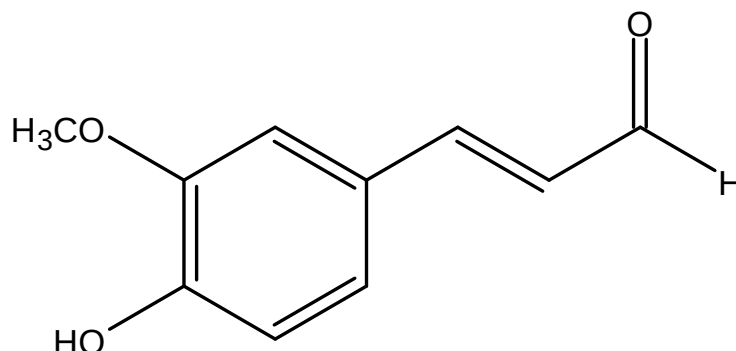


Spectrum 39. COSY Spectrum of Valerosidate (**8**)



Spectrum 40. HMBC Spectrum of Valeroside (**8**)

4.5.1.1.2. Phenylpropane Derivatives



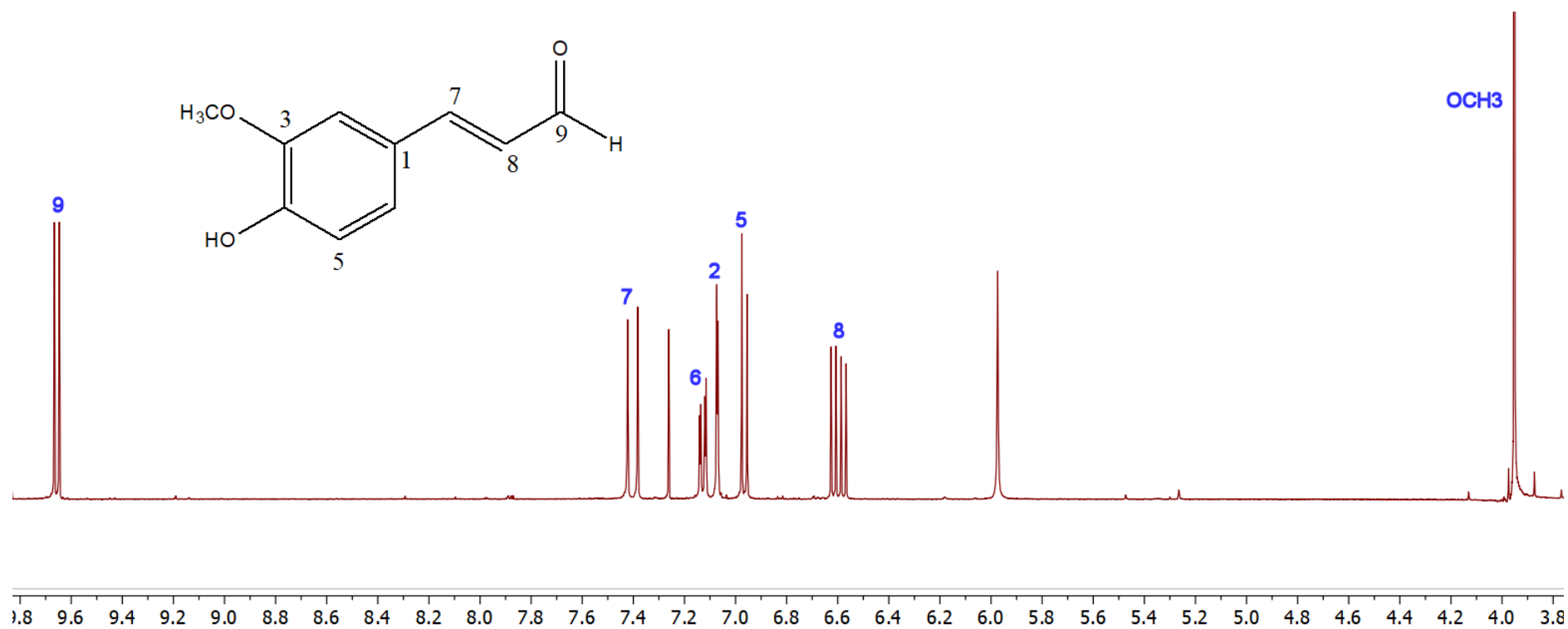
(E)-CONIFERYL ALDEHYDE (10): C₁₀H₁₀O₃ (MW:178.18)

UV λ_{max} (MeOH) nm:	222, 240, 339
IR ν_{max} (KBr) cm ⁻¹ :	3697 (OH), 1670 (C=O), 1597, 1586 (aromatic, C=C)
¹ H NMR:	Table 84, Spectrum 41
¹³ C NMR:	Table 84, Spectrum 42

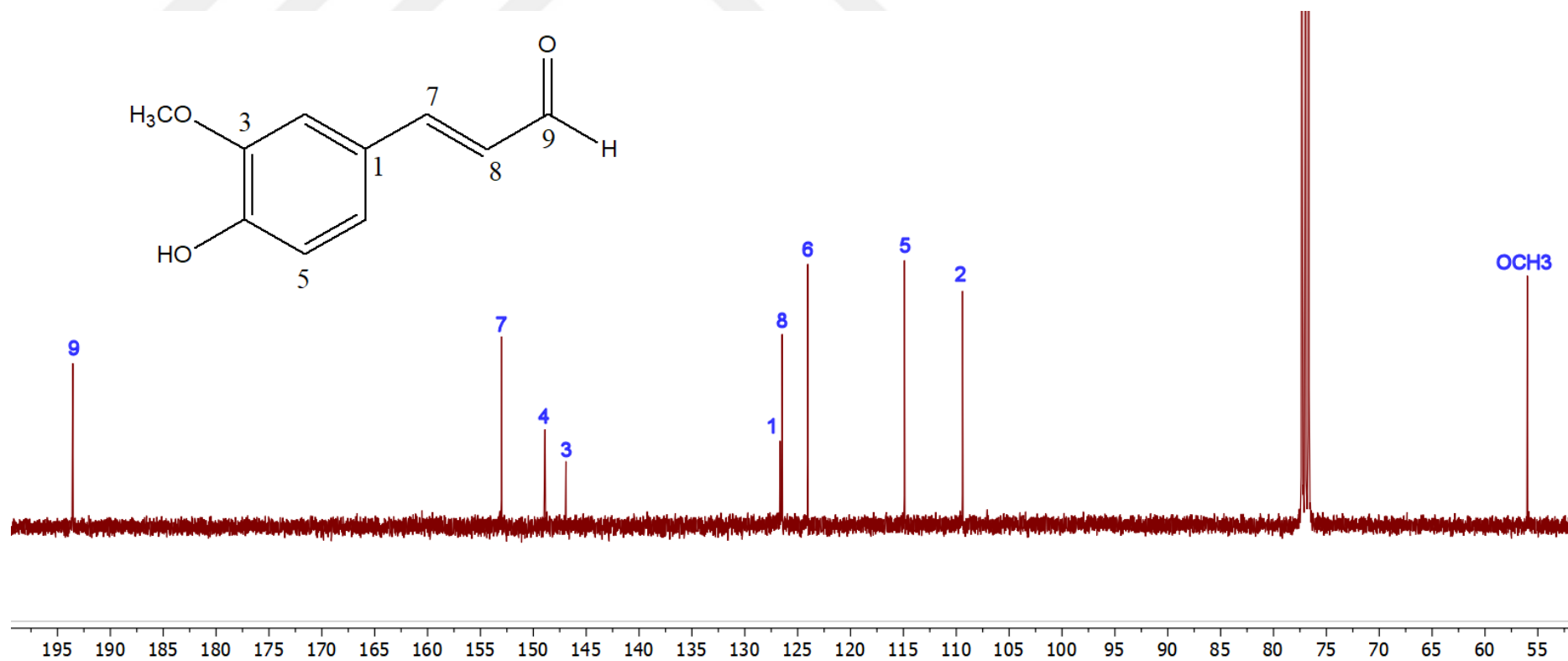
Compound **10** was obtained as white powder. In IR spectrum, absorption bands showing hydroxyl (3697 cm⁻¹), α , β -unsaturated carbonyl (1670 cm⁻¹) as well as aromatic ring (1597 and 1586 cm⁻¹) groups were observed. The UV spectrum demonstrated absorption maxima at 222, 240 and 339 nm, which was characteristic for phenylpropanes, particularly for ferulic acid derivatives. The ¹H and ¹³C NMR spectra (Table 84) indicated that compound **10** was a phenylpropane derivate and the spectroscopic findings were superimposed with the those reported for (*E*)-coniferyl aldehyde in the literature (250).

Table 84. ^{13}C and ^1H NMR data of (*E*)-coniferyl aldehyde (**10**) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)

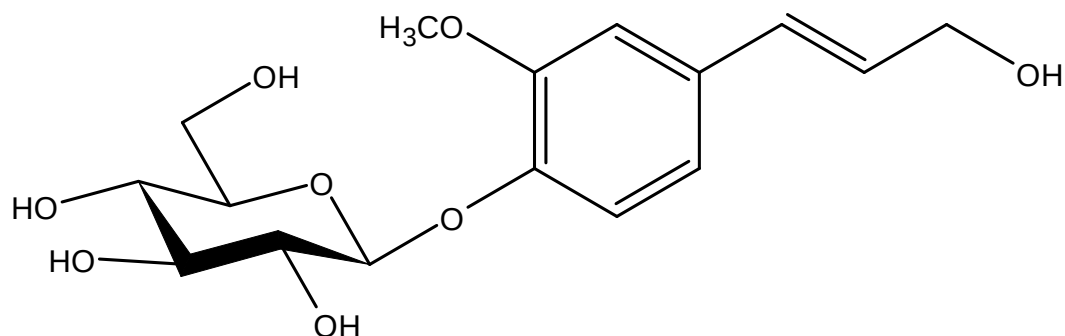
C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), <i>J</i> (Hz)
1	C	126.7	-
2	CH	109.4	7.07 d (1.9)
3	C	146.9	-
4	C	148.9	-
5	CH	114.9	6.97 d (8.2)
6	CH	124.0	7.13 dd (8.2, 1.9)
7	CH	153.0	7.40 d (15.8)
8	CH	126.5	6.60 dd (15.8, 7.7)
9	CH	193.6	9.66 d (7.7)
3-OCH ₃	CH ₃	56.0	3.95 s



Spectrum 41. ^1H NMR Spectrum of (E)-Coniferyl aldehyde (**10**) (CDCl_3 , 400 MHz)



Spectrum 42. ¹³C NMR Spectrum of *(E)*-Coniferyl aldehyde (**10**) (CDCl₃, 100 MHz)



CONIFERIN (11): C₁₆H₂₂O₈ (MW:342.34)

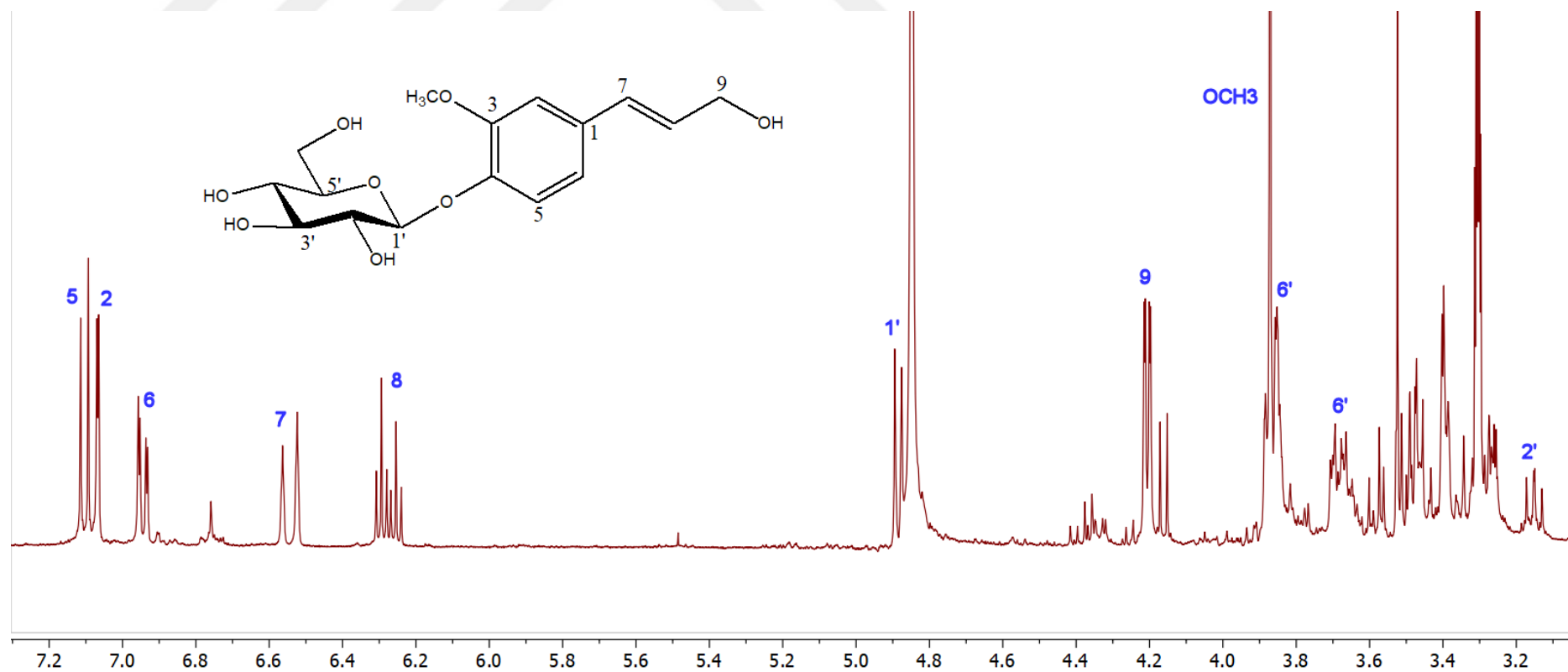
UV λ_{max} (MeOH) nm:	205, 259
IR ν_{max} (KBr) cm ⁻¹ :	3435 (OH), 1637 (olefinic, C=C), 1586 (aromatic, C=C)
¹ H NMR:	Table 85, Spectrum 43
¹³ C NMR:	Table 85, Spectrum 44

Compound **11** was purified as an amorphous powder. The IR spectrum showed absorption bands at 3435, 1637 and 1586 cm⁻¹ revealing the presence of hydroxyl, olefinic and aromatic ring functionalities, respectively. Its UV spectrum displayed absorption maxima at 205 and 259 nm. The ¹H and ¹³C NMR data (Table 85) of **11** were found to be similar to those of coniferin (251).

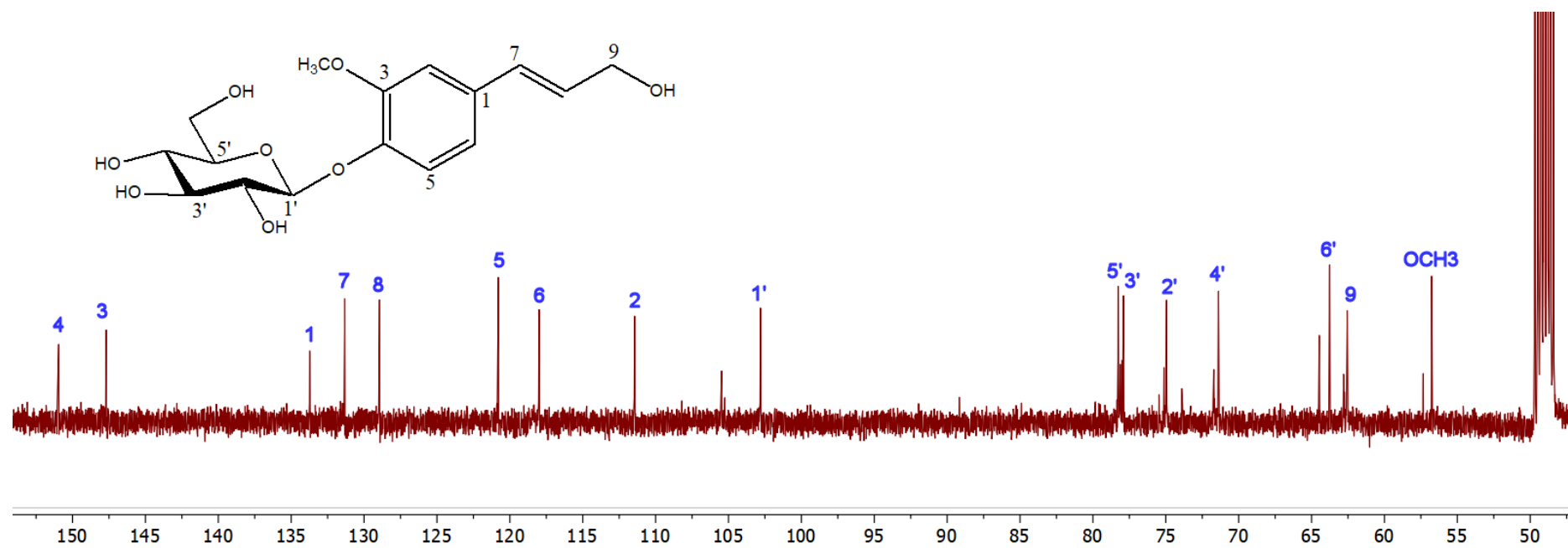
Table 85. ^{13}C and ^1H NMR data of coniferin (**11**) (CD_3OD ; ^{13}C : 100 MHz, ^1H : 400 MHz)

C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
<i>Aglycone</i>			
1	C	133.7	-
2	CH	114.4	7.07 d (2.0)
3	C	147.7	-
4	C	150.9	-
5	CH	120.8	7.10 d (8.4)
6	CH	118.0	6.94 dd (8.4, 2.0)
7	CH	131.3	6.54 d (15.9)
8	CH	128.9	6.27 dt (15.9, 5.7)
9	CH_2	62.6	4.21 dd (5.7, 1.5)
<i>Glucose (C-4)</i>			
1'	CH	102.8	4.88 d (7.5)
2'	CH	74.9	3.15 dd (9.0, 7.8)
3'	CH	77.9	3.49-3.26 †
4'	CH	71.4	3.49-3.26 †
5'	CH	78.3	3.49-3.26 †
6'	CH_2	63.8	3.87 †
			3.69 dd (12.0, 5.2)
3-OCH ₃	CH ₃	56.8	3.87 s

†: Overlapped signals.

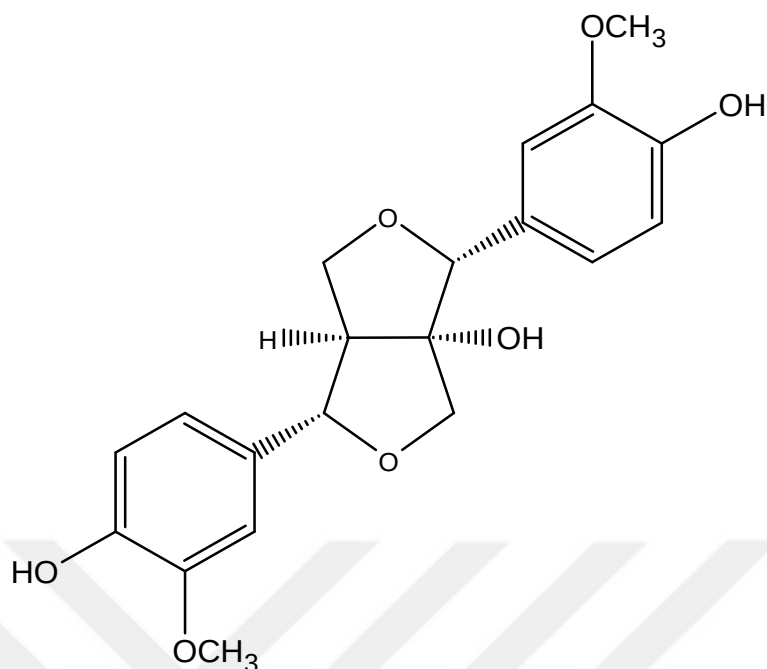


Spectrum 43. ^1H NMR Spectrum of Coniferin (**11**) (CD_3OD , 400 MHz)



Spectrum 44. ^{13}C NMR Spectrum of Coniferin (**11**) (CD₃OD, 100 MHz)

4.5.1.1.3. Lignan



8-HYDROXYPINORESINOL (14): C₂₀H₂₂O₇ (MW:374.38)

UV λ_{max} (MeOH) nm:	207, 232, 281
IR ν_{max} (KBr) cm ⁻¹ :	3692 (OH), 1518 (aromatic, C=C)
ESI-MS m/z :	373.87 [M-H] ⁻
¹ H NMR:	Table 86, Spectrum 45
¹³ C NMR:	Table 86, Spectrum 46
COSY:	Spectrum 47
HMBC:	Spectrum 48
NOESY:	Spectrum 49

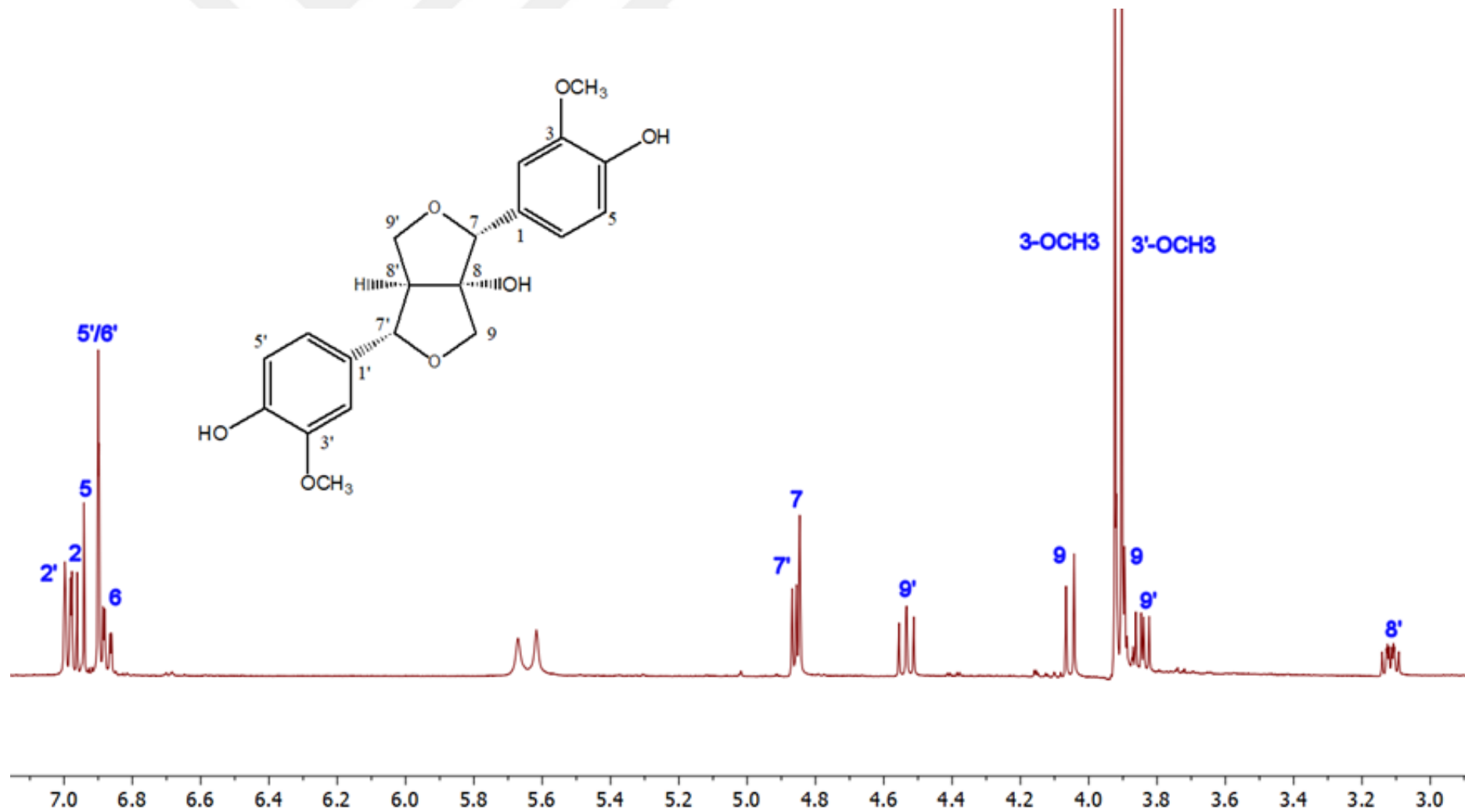
Compound **14** was isolated as a colorless solid. The molecular formula, C₂₀H₂₂O₇, was established from the pseudomolecular ion at m/z 373.87 [M-H]⁻ in negative ion ESI-MS and NMR data. Its UV spectrum gave maxima at 207, 232 and 281 nm. The IR spectrum indicated absorption bands at 3692 (hydroxyl) and 1518 (aromatic C=C) cm⁻¹. A careful comparison of the NMR spectroscopic data (Table 86, Spectra 45-49) of **14** with those of 8-hydroxypinoresinol reported in the literature revealed that **14** is 8-hydroxypinoresinol (252).

Table 86. ^{13}C and ^1H NMR data of 8-hydroxypinoresinol (**14**) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

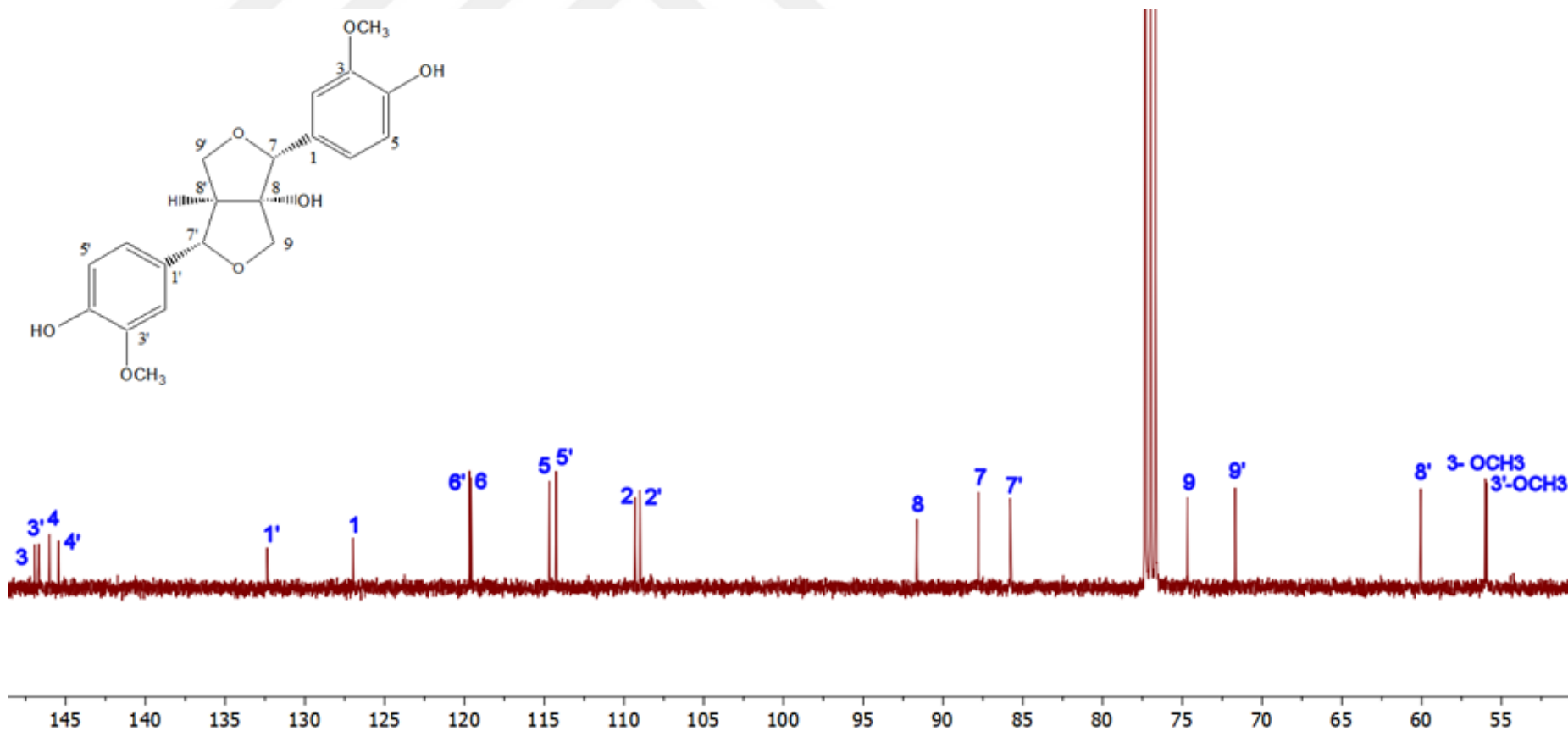
C/H Atom	Multiplicity	δ_{C} ppm	δ_{H} ppm, J (Hz)
1	C	127.0	-
2	CH	109.3	6.98 d (2.0)
3	C	146.9	-
4	C	146.0	-
5	CH	114.6	6.95 d (8.1)
6	CH	119.6	6.87 dd (8.1, 2.0)
7	CH	87.8	4.85 s
8	C	91.6	-
9	CH ₂	74.6	4.05 d (9.2) 3.91 d (9.2)
3-OCH ₃	CH ₃	56.0	3.92 s
1'	C	132.3	-
2'	CH	109.0	7.00 br.s
3'	C	146.6	-
4'	C	145.4	-
5'	CH	114.2	6.90 d (8.1)
6'	CH	119.7	6.90 †
7'	CH	85.7	4.86 d (4.90)
8'	CH	60.0	3.12 m
9'	CH ₂	71.7	4.53 dd (8.1, 9.4) 3.83 dd (6.3, 9.4)
3'-OCH ₃	CH ₃	55.9	3.90 s

^aAssignments are based on COSY, HSQC, HMBC and NOESY experiments.

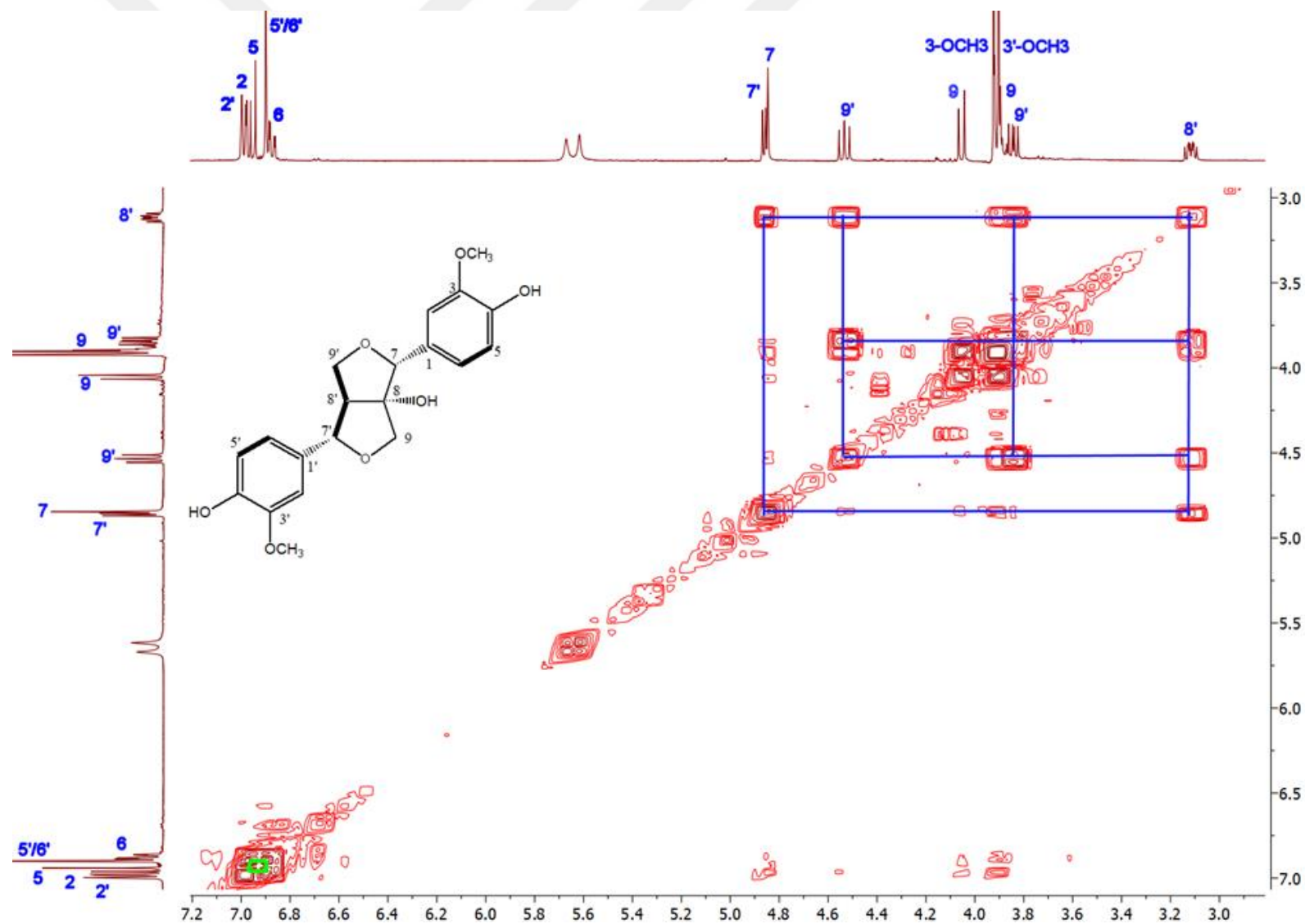
†: Overlapped signals.



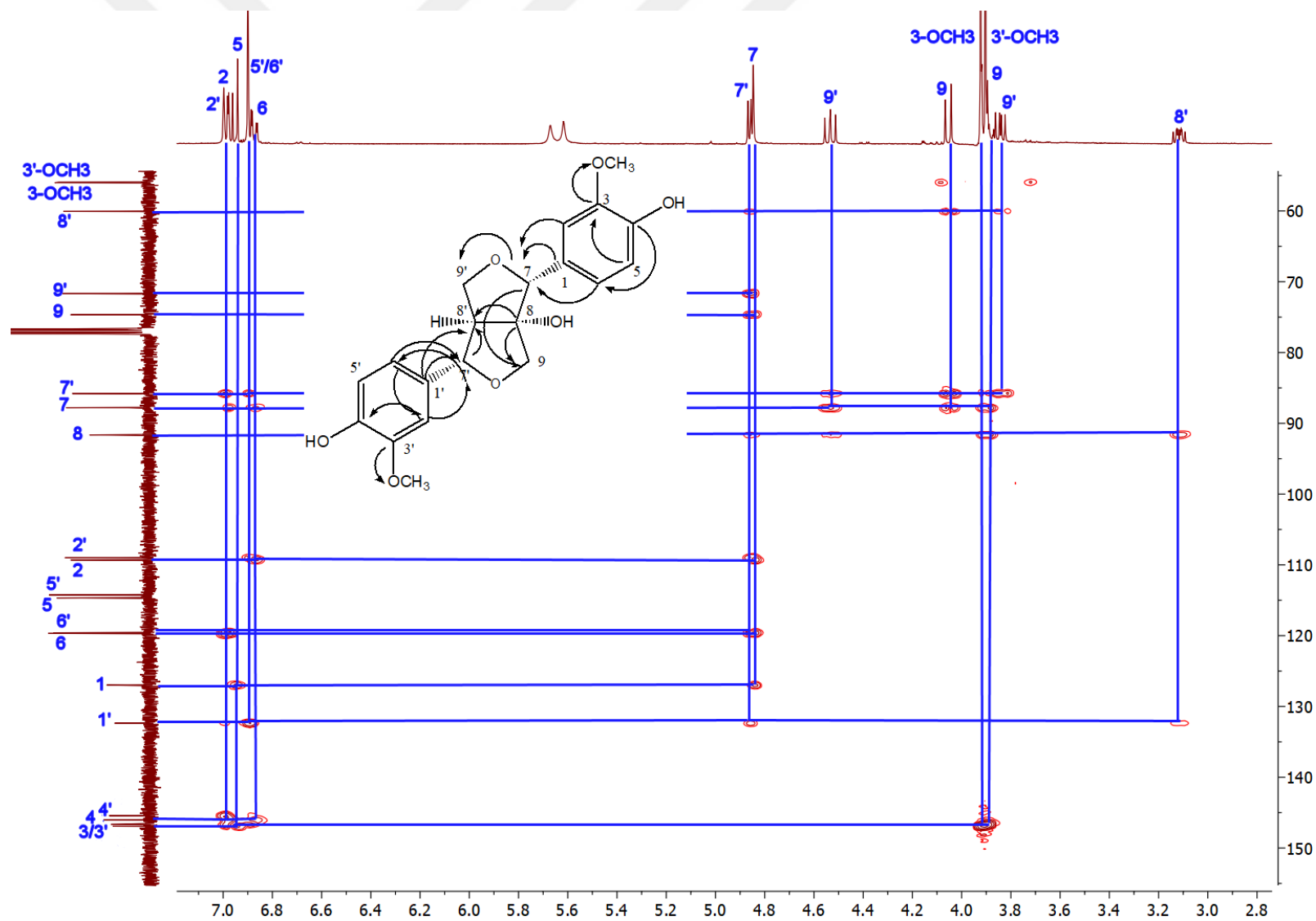
Spectrum 45. ^1H NMR Spectrum of 8-Hydroxypinoresinol (**14**) (CDCl_3 , 400 MHz)



Spectrum 46. ¹³C NMR Spectrum of 8-Hydroxypinoresinol (**14**) (CDCl₃, 100 MHz)



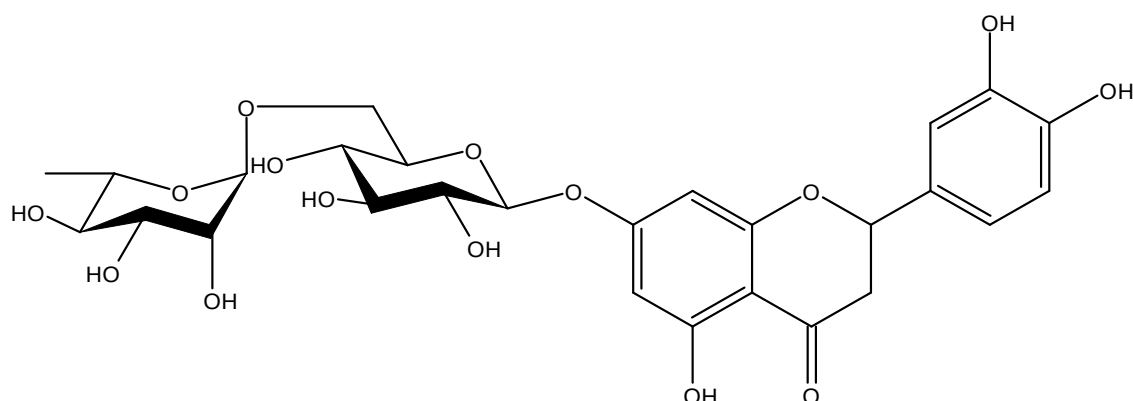
Spectrum 47. COSY Spectrum of 8-Hydroxypinoresinol (**14**)



Spectrum 48. HMBC Spectrum of 8-Hydroxypinoresinol (**14**)

4.5.1.2. Compounds Isolated from *n*-BuOH Subextract of *V. alliariifolia*

4.5.1.2.2. Flavonoid



(2R/2S)-ERIOCITRIN (33): C₂₇H₃₂O₁₅ (MW: 596.53)

UV $\lambda_{\max}$ (MeOH) nm:	228, 285, 333 (sh)
IR $\nu_{\max}$ (KBr) cm ⁻¹ :	3351 (OH), 1640 (ketone, C=O), 1512 (aromatic, C=C)
ESI-MS m/z :	595.94 [M-H] ⁻
¹ H NMR:	Table 87, Spectrum 50
¹³ C NMR:	Table 87, Spectrum 51
HMBC:	Spectrum 52

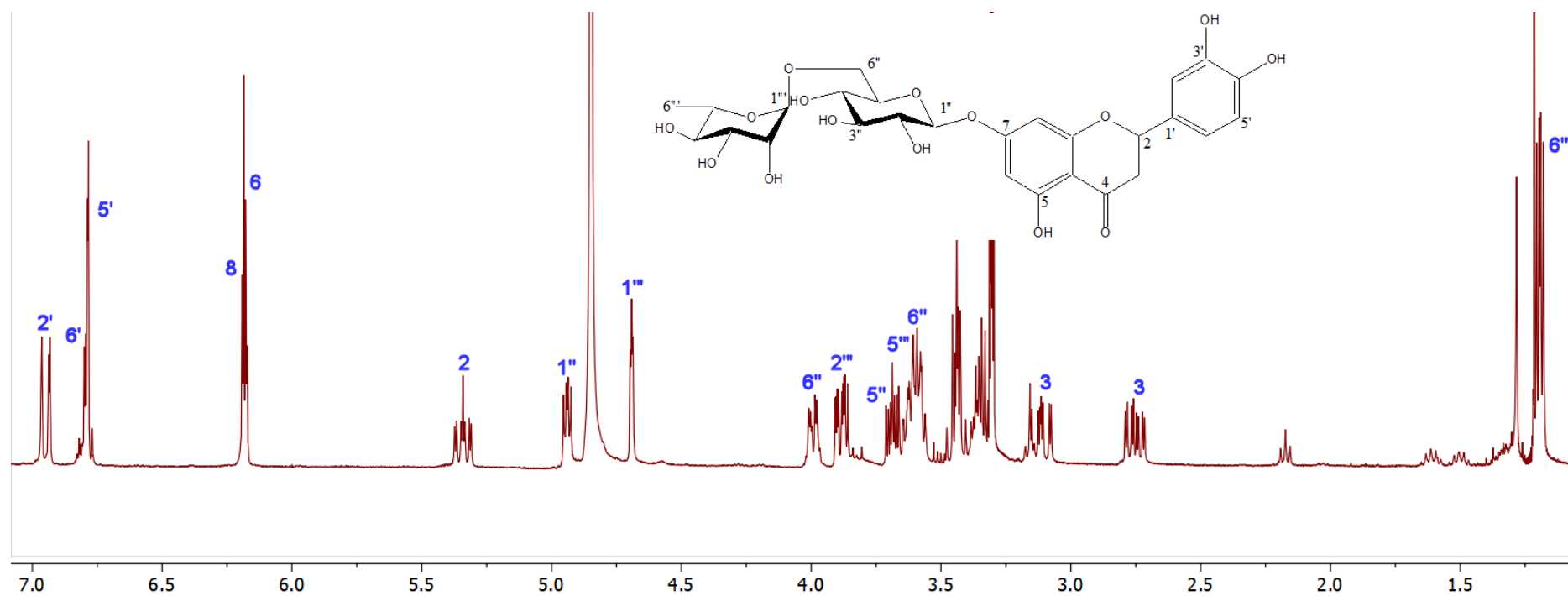
Compound **33** was isolated as yellowish amorphous solid. The IR spectrum displayed absorption bands at 3351 (OH), 1640 (ketone, C=O) and 1512 (aromatic, C=C) cm⁻¹. Its UV spectrum exhibited absorption maxima at 228, 285 and 333 (shoulder) nm for a characteristic flavanone structure. Analysis of its NMR (Table 87, Spectra 50-52) and MS data led to the characterization of **33** as (2R/2S)-Eriocitrin (253).

Table 87. ^{13}C and ^1H NMR data of (2*R*/2*S*)-Eriocitrin (**33**) (CD_3OD ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

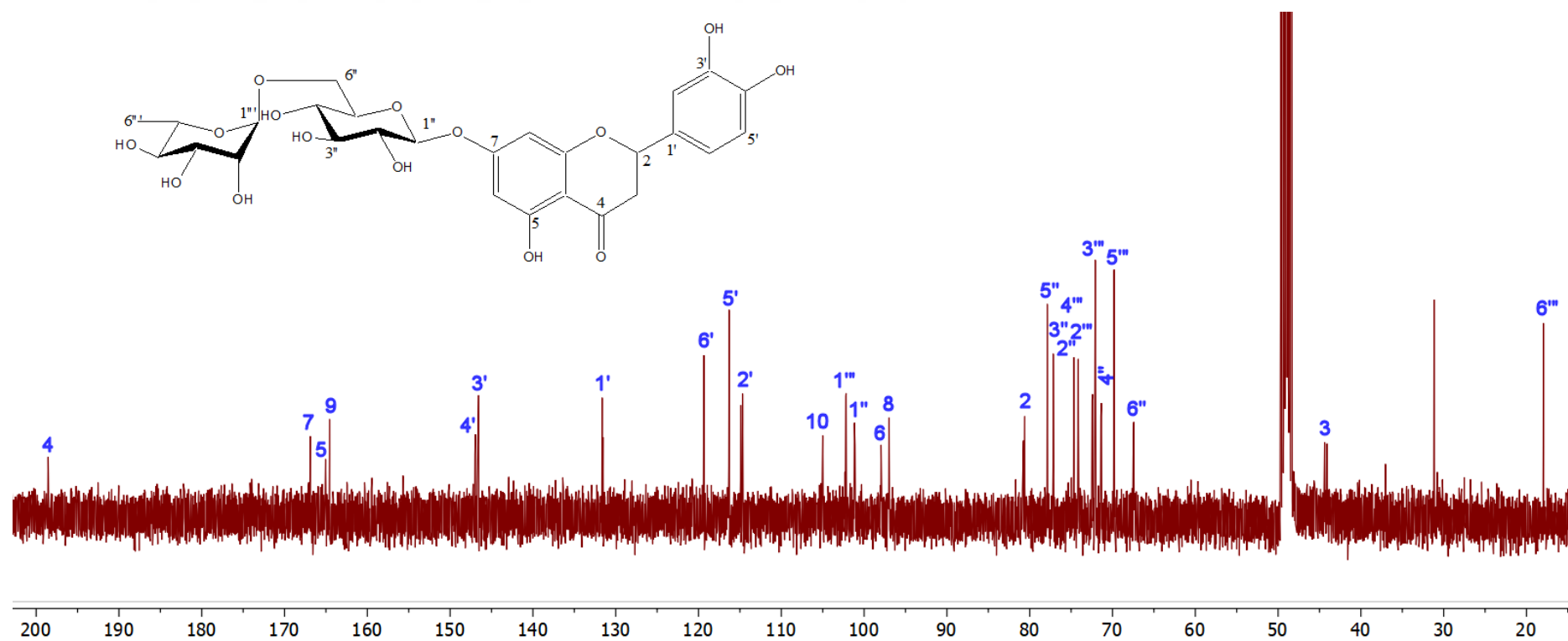
C/H Atom	Multiplicity	δ_{C} ppm	δ_{H} ppm, <i>J</i> (Hz)
<i>Aglycone</i>			
2	CH	80.6	5.33 dd (9.9, 2.9)
3	CH ₂	44.1	3.12 m 2.75 m
4	C	198.6	-
5	C	165.1	-
6	CH	97.0	6.18 d (2.3)
7	C	166.9	-
8	CH	98.0	6.19 d (2.3)
9	C	164.6	-
10	C	105.0	-
1'	C	131.6	-
2'	CH	114.7	6.92 d (1.8)
3'	C	146.6	-
4'	C	147.0	-
5'	CH	116.3	6.80 d (8.3)
6'	CH	119.6	6.81 dd (8.3, 1.8)
<i>Glucose (C-7)</i>			
1''	CH	101.2	4.94 d (7.4)
2''	CH	74.7	3.70-3.35 †
3''	CH	77.2	3.70-3.35 †
4''	CH	71.4	3.70-3.35 †
5''	CH	77.9	3.69 m
6''	CH ₂	67.4	3.99 † 3.59 †
<i>Rhamnose (C-6'')</i>			
1'''	CH	102.2	4.69 d (1.7)
2'''	CH	72.4	3.90 dd (1.7, 3.5)
3'''	CH	72.1	3.70-3.35 †
4'''	CH	74.1	3.70-3.35 †
5'''	CH	69.8	3.61 m
6'''	CH ₃	18.0	1.19 d (6.3)

^aAssignments are based on COSY, HSQC and HMBC experiments.

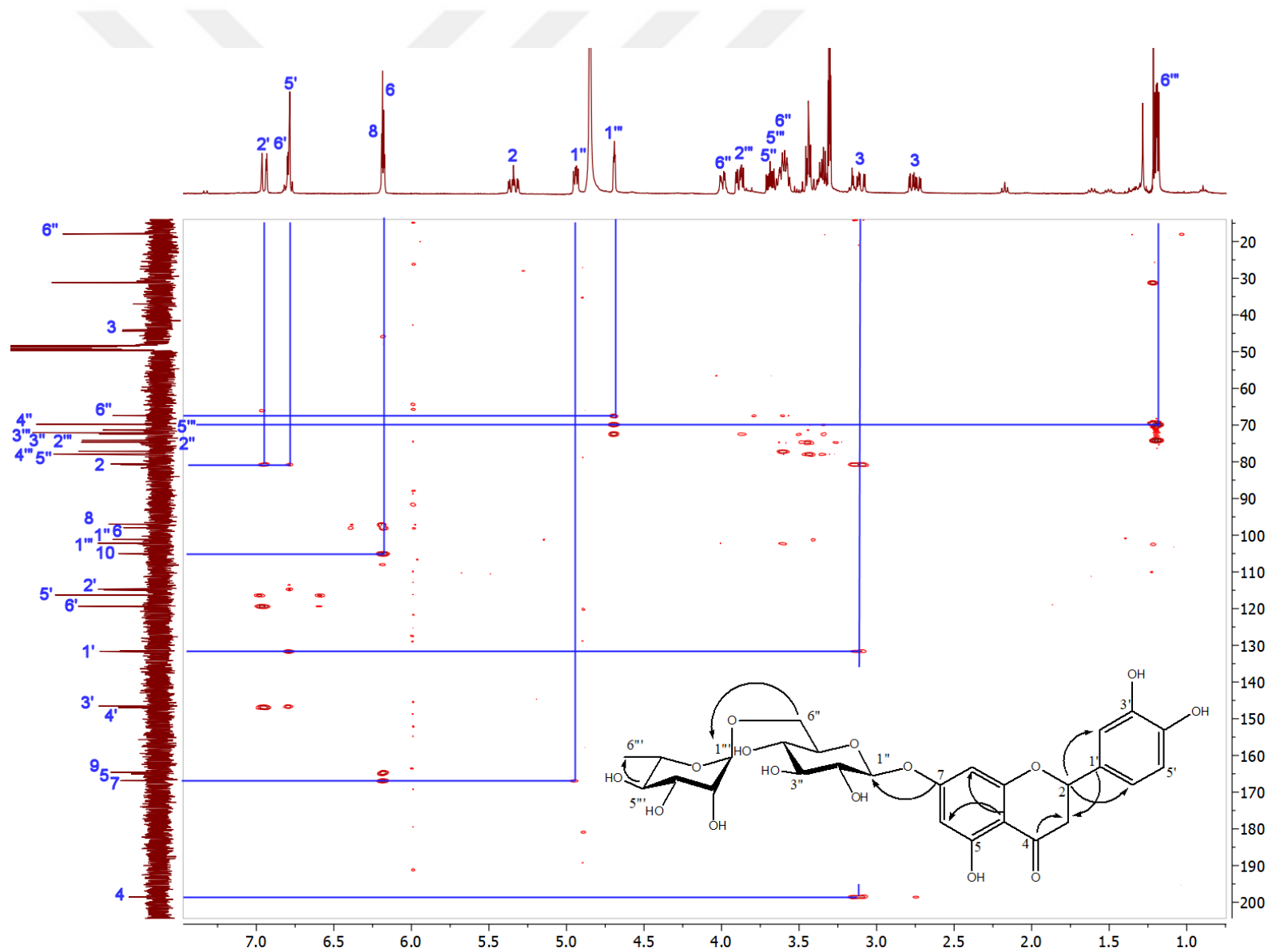
†: Overlapped signals.



Spectrum 50. ¹H NMR Spectrum of (2R/2S)-Eriocitrin (33) (CD₃OD, 400 MHz)

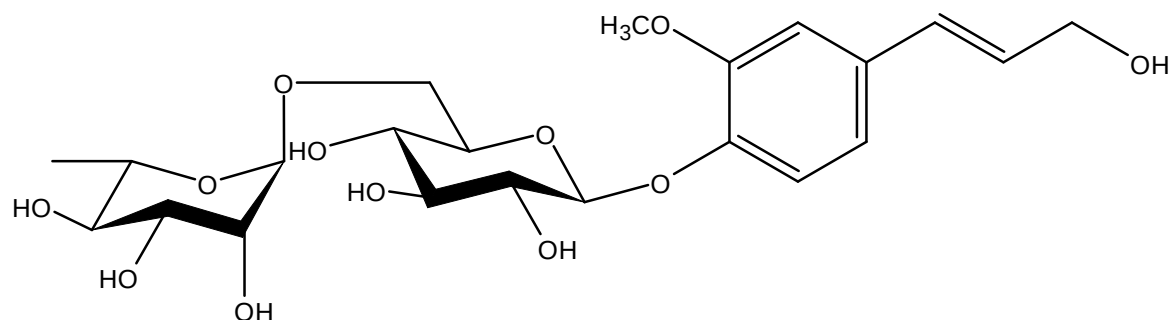


Spectrum 51. ^{13}C NMR Spectrum of (2*R*/2*S*)-Eriocitrin (**33**) (CD_3OD , 100 MHz)



Spectrum 52. HMBC Spectrum of (2R/2S)-Eriocitrin (33)

4.5.1.2.2. Phenylpropane Derivative



XIECAOSIDE E (34): C₂₂H₃₂O₁₂ (MW:488.48)

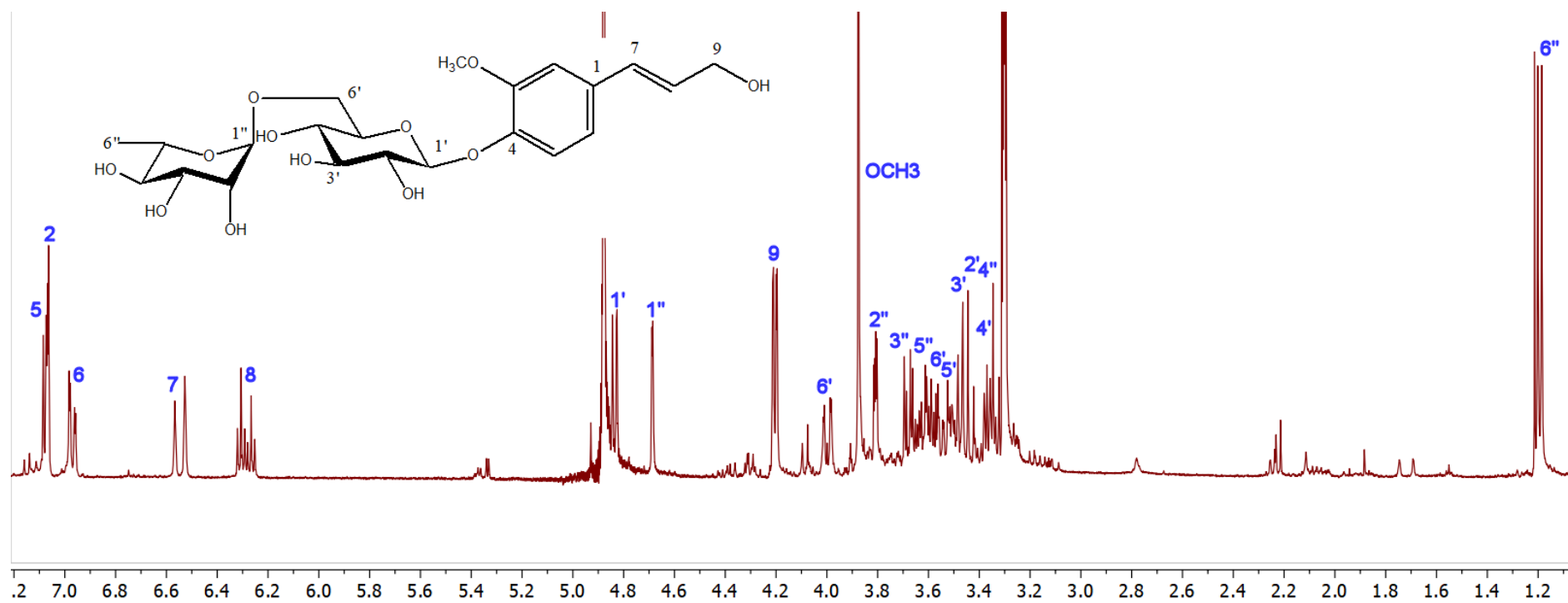
UV $\lambda_{\max}$ (MeOH) nm:	223, 258, 297 (sh)
IR $\nu_{\max}$ (KBr) cm ⁻¹ :	3430 (OH), 1645 (olefinic, C=C), 1512 (aromatic, C=C)
ESI-MS m/z :	512.14 [M+Na] ⁺
¹ H NMR:	Table 88, Spectrum 53
¹³ C NMR:	Table 88, Spectrum 54

Compound **34** was obtained as white amorphous powder. The UV spectrum demonstrated absorption maxima at 223, 258 and 297 (shoulder) nm, while the IR spectrum indicated absorptions for hydroxyl (3430 cm⁻¹), olefinic (1645 cm⁻¹) and aromatic rings (1512 cm⁻¹). The ¹H and ¹³C NMR spectra (Table 88, Spectra 53 and 54) of **34** indicated the presence of a phenylpropanol skeleton and the same sugar moiety with that of **33**. Detailed investigation of its NMR spectra led to the identification of **34** as xiecaoside E (83).

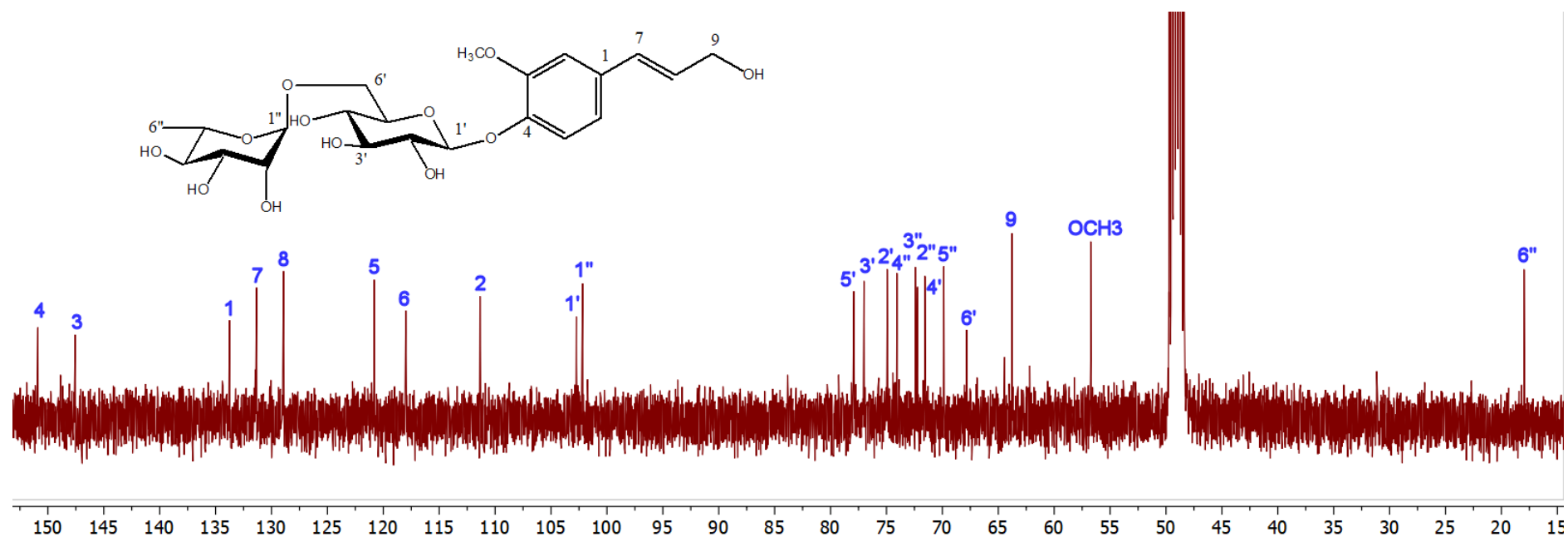
Table 88. ^{13}C and ^1H NMR data of Xiecaoside E (**34**) (CD_3OD ; ^{13}C : 100 MHz, ^1H : 400 MHz)

C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
<i>Aglycone</i>			
1	C	133.8	-
2	CH	111.3	7.07 d (2.0)
3	C	147.6	-
4	C	150.9	-
5	CH	120.8	7.07 d (8.4)
6	CH	118.0	6.97 dd (8.4,1.9)
7	CH	131.3	6.55 d (15.9)
8	CH	128.9	6.29 dt (15.9, 5.7)
9	CH ₂	63.8	4.20 dd (5.7,1.5)
3-OCH ₃	CH ₃	56.7	3.88 s
<i>Glucose (C-4)</i>			
1'	CH	102.7	4.84 d (7.4)
2'	CH	74.9	3.69-3.52 †
3'	CH	77.0	3.69-3.52 †
4'	CH	71.6	3.69-3.52 †
5'	CH	77.9	3.69-3.52 †
6'	CH ₂	67.8	4.00 †
<i>Rhamnose (C-6')</i>			
1''	CH	102.2	4.69 d (1.7)
2''	CH	72.2	3.81 dd (1.7, 3.4)
3''	CH	72.4	3.69-3.52 †
4''	CH	74.1	3.69-3.52 †
5''	CH	69.9	3.69-3.52 †
6''	CH ₃	18.0	1.19 d (6.2)

†: Overlapped signals.

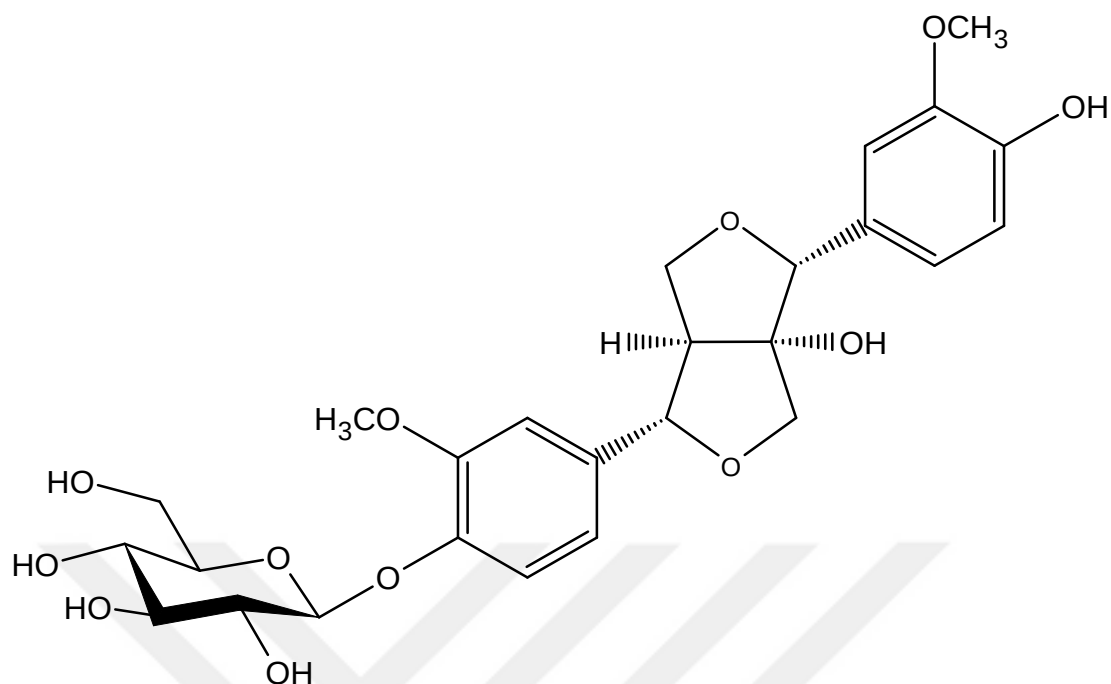


Spectrum 53. ^1H NMR Spectrum of Xiecaoside E (34) (CD_3OD , 400 MHz)



Spectrum 54. ¹³C NMR Spectrum of Xiecaoside E (**34**) (CD₃OD, 100 MHz)

4.5.1.2.3. Lignan glycoside



8-HYDROXYPINORESINOL 4'-O- β -GLUCOPYRANOSIDE (35):

C₂₆H₃₂O₁₂ (MW:536.13)

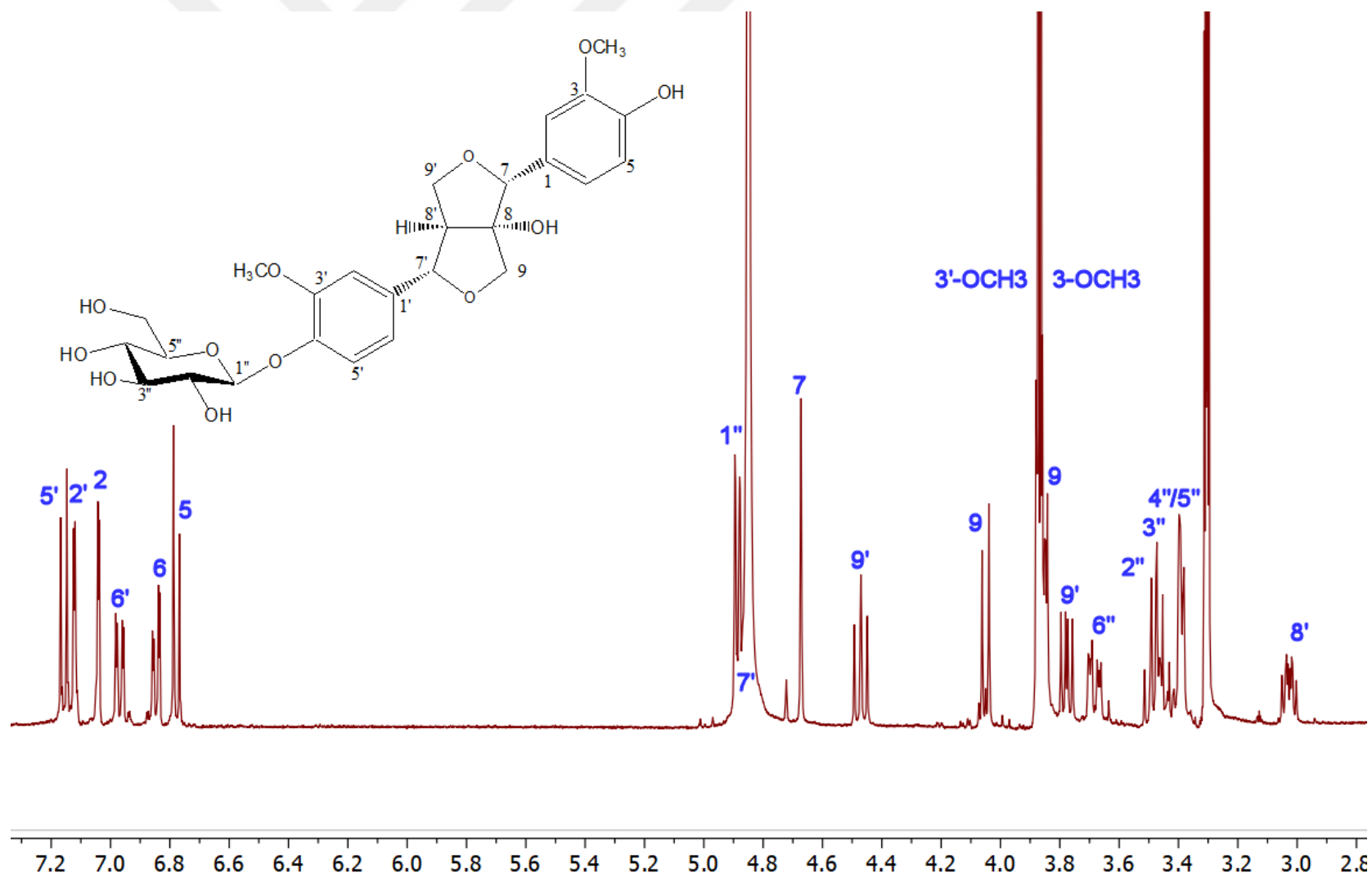
UV λ_{max} (MeOH) nm:	204, 230, 279
IR ν_{max} (KBr) cm ⁻¹ :	3390 (OH), 1652, 1515 (aromatic, C=C)
ESI-MS m/z :	559.95 [M+Na] ⁺
¹ H NMR:	Table 89, Spectrum 55
¹³ C NMR:	Table 89, Spectrum 56

Compound **35** was obtained as amorphous white solid. Its molecular formula, C₂₆H₃₂O₁₂, was deduced by ESI-MS ([M+Na]⁺ at m/z 559.95) and NMR experiments. The UV spectrum showed absorption maxima at 204, 230 and 279 nm, whereas IR spectrum indicated absorption bands for hydroxyl (3390 cm⁻¹) group and aromatic ring (1652 and 1515 cm⁻¹). Analysis of ¹H and ¹³C NMR data (Table 89, Spectra 55 and 56) suggested that compound **35** was similar to compound **14** (8-hydroxypinoresinol), except for the presence of additional signals arising from a β -glucopyranosyl unit. Based on spectroscopic analysis, it was identified as 8-hydroxypinoresinol 4'-O- β -glucopyranoside (118).

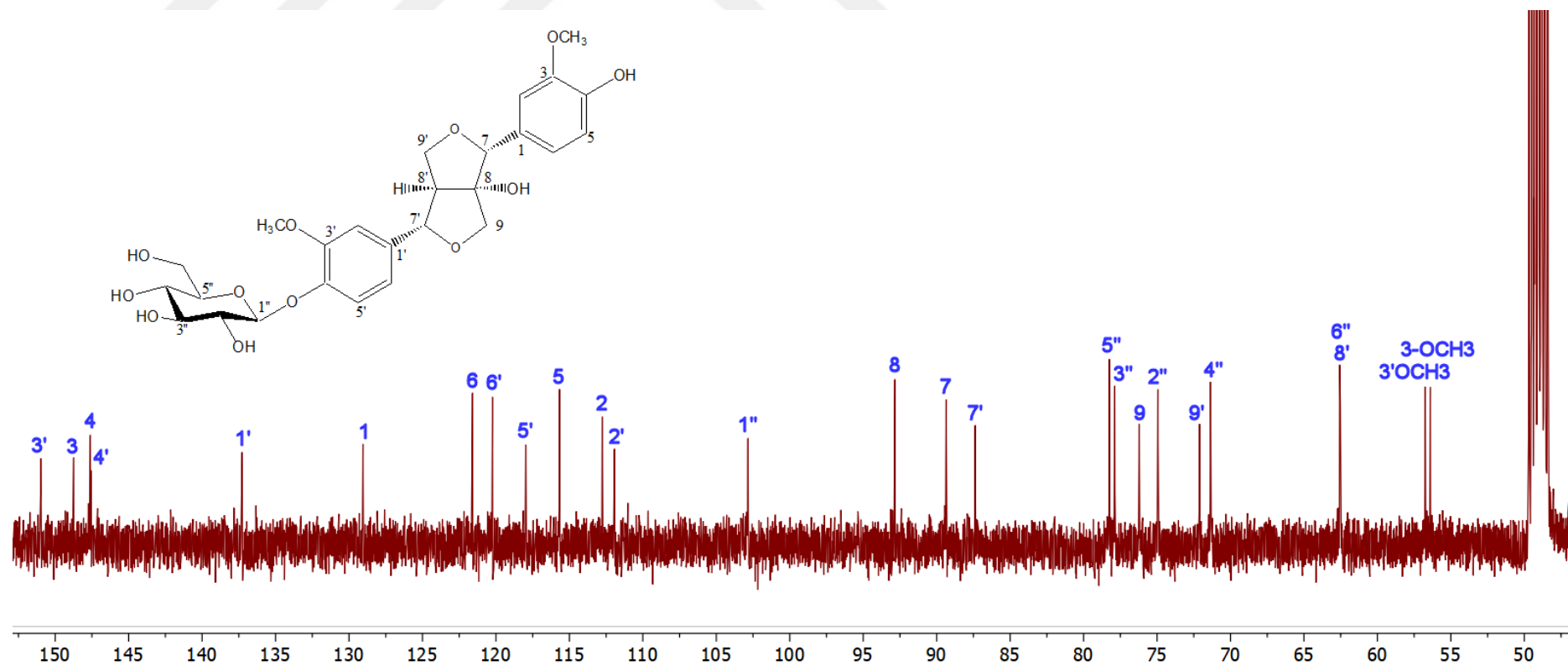
Table 89. ^{13}C and ^1H NMR data of 8-Hydroxypinoresinol 4'-O- β -glucopyranoside (**35**) (CD_3OD ; ^{13}C : 100 MHz, ^1H : 400 MHz)

C/H Atom	Multiplicity	δ_{C} ppm	δ_{H} ppm, J (Hz)
<i>Aglycone</i>			
1	C	128.9	-
2	CH	112.81	7.02 d (1.8)
3	C	148.8	-
4	C	147.8	-
5	CH	115.8	6.76 d (8.1)
6	CH	121.6	6.83 dd (8.1, 1.9)
7	CH	89.4	4.66 s
8	C	92.9	-
9	CH ₂	76.3	4.04 d (9.3) 3.85 d (9.3)
3-OCH ₃	CH ₃	56.4	3.85 s
1'	C	137.3	-
2'	CH	112.0	7.12 d (1.9)
3'	C	151.0	-
4'	C	147.6	-
5'	CH	118.0	7.16 d (8.4)
6'	CH	120.3	6.97 dd (8.4, 1.9)
7'	CH	87.4	4.88 d (5.0)
8'	CH	62.6	3.03 m
9'	CH ₂	72.3	4.47 t (8.7) 3.77 dd (6.3, 9.2)
3'-OCH ₃	CH ₃	56.8	3.87 s
<i>Glucose (C-4')</i>			
1''	CH	102.9	4.89 d (7.7)
2''	CH	75.0	3.48 †
3''	CH	77.9	3.39 m
4''	CH	71.4	3.31 †
5''	CH	78.3	3.30 †
6''	CH ₂	62.6	3.86 † 3.68 dd (12.1, 3.5)

†: Overlapped signals.



Spectrum 55. ^1H NMR Spectrum of 8-Hydroxypinoresinol 4'-O- β -glucopyranoside (35) (CD_3OD , 400 MHz)

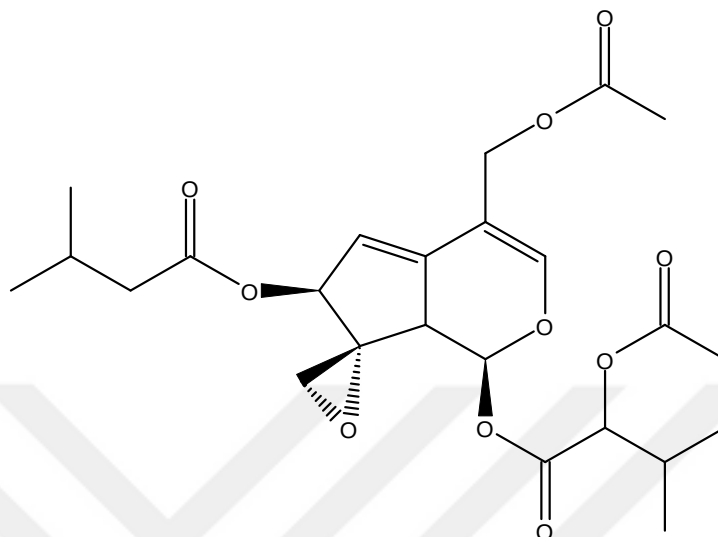


Spectrum 56. ^{13}C NMR Spectrum of 8-Hydroxypinoresinol 4'-O- β -glucopyranoside (35) (CD_3OD , 100 MHz)

4.5.1.3. Compounds Isolated from Cytotoxic Main Fractions of *V. sisymbriifolia*

4.5.1.3.1. Iridoids

4.5.1.3.1.A Non-glycosidic Iridoids



1- α -ACEVALTRATE (15): C₂₄H₃₂O₁₀ (MW:480.50)

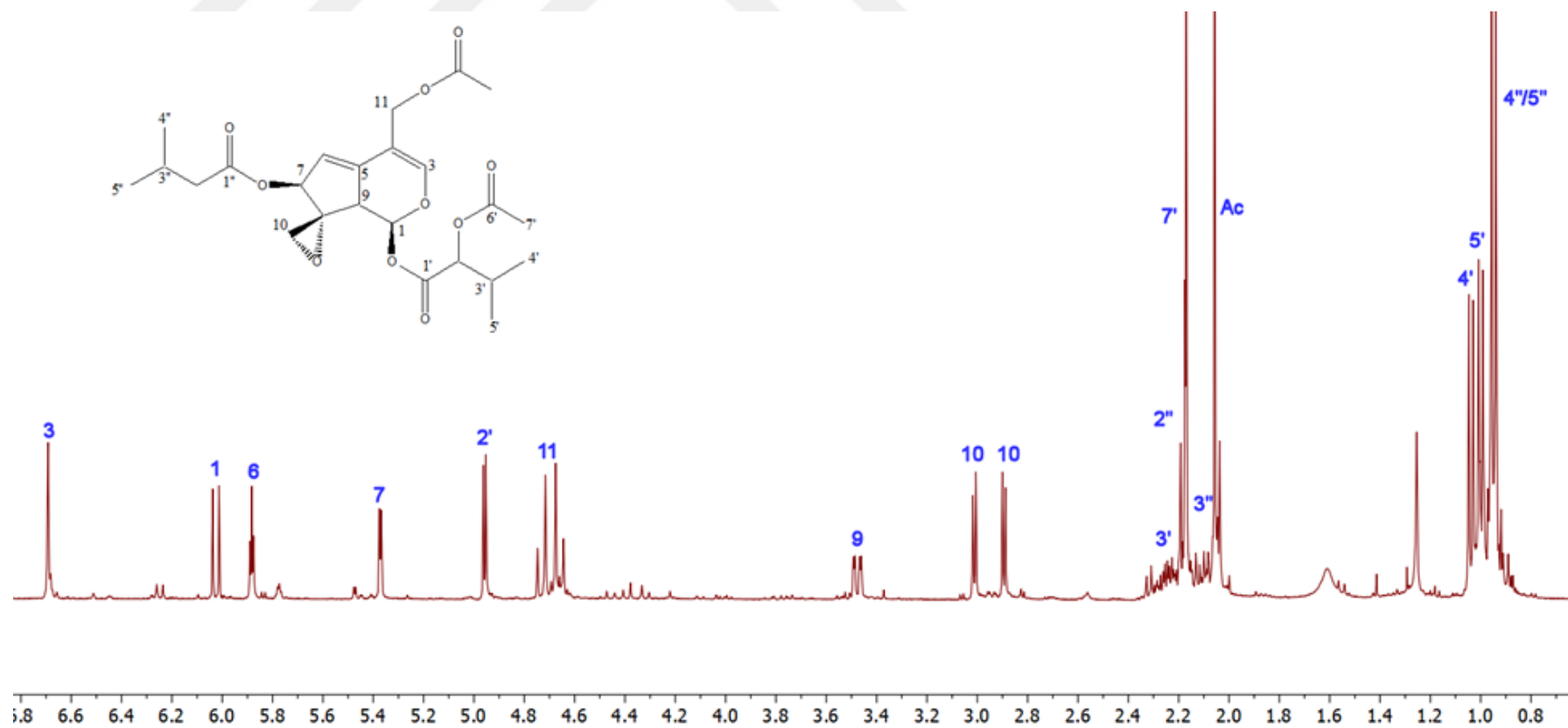
UV λ_{max} (MeOH) nm:	255
IR ν_{max} (KBr) cm ⁻¹ :	1777, 1740 (ester, C=O), 1639, 1611 (olefinic, C=C)
HR-ESI-MS m/z :	503.94 [M+Na] ⁺
¹ H NMR:	Table 90, Spectrum 57
¹³ C NMR:	Table 90, Spectrum 58
COSY:	Spectrum 59
HMBC:	Spectra 60 and 61

Compound **15** was isolated as colorless oil, whose molecular formula was assigned to be C₂₄H₃₂O₁₀ by ESI-MS (m/z 503.94 [M+Na]⁺), and NMR. The UV spectrum showed band at 255 nm. The IR spectrum exhibited absorption bands due to ester carbonyl (1777 and 1740 cm⁻¹) and olefinic (1639 and 1611 cm⁻¹) functionalities. The ¹H and ¹³C NMR data (Table 90) indicated that **15** possessed diene-type valepotriate structure. In addition, the similarities in the spectroscopic findings of **15** and **3** (valtrate) (Tables 90 and 75) revealed that they were structurally analogues, except that an isovaleroxy group at C-1 in **3** was replaced with an α -(acetoxy)-isovaleroxy moiety in **15**. The exact structure of **15** was determined as 1- α -acevaltrate by comparison of NMR data with those reported for 1- α -acevaltrate (161).

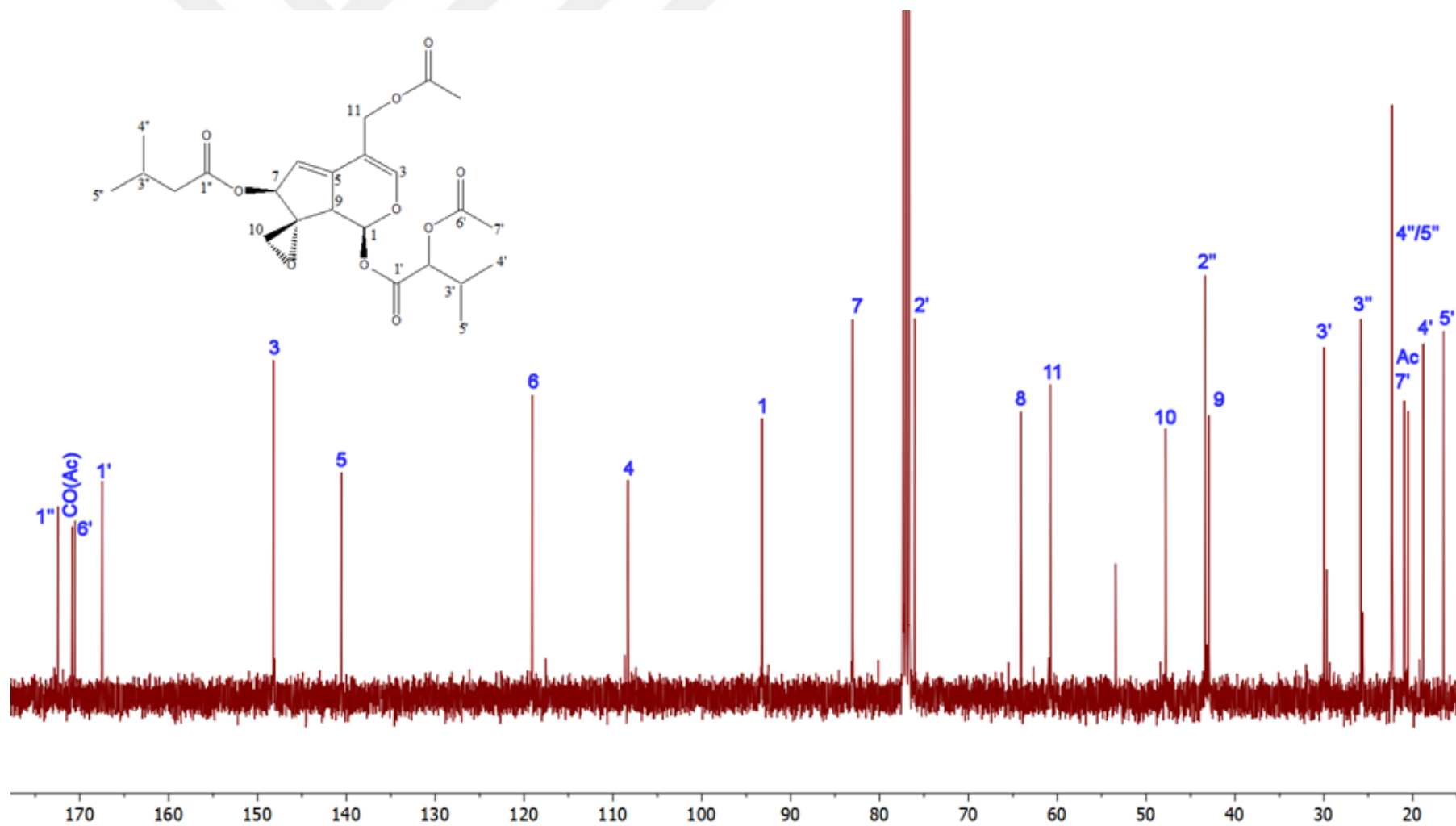
Table 90. ^{13}C and ^1H NMR data of 1- α -acevaltrate (**15**) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

C/H atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
1	CH	93.2	6.03 d (10.1)
3	CH	148.2	6.69 s
4	C	108.3	-
5	C	140.6	-
6	CH	119.1	5.88 t (2.8)
7	CH	83.0	5.37 d (2.8)
8	C	64.1	-
9	CH	42.9	3.48 dd (10.1, 2.5)
10	CH ₂	47.8	3.01 d (4.8) 2.89 d (4.8)
11	CH ₂	60.8	4.73 d (12.4) 4.66 d (12.4)
<i>α-(acetoxy)-isovaleroyl (C-1)</i>			
1'	C	167.5	-
2'	CH	76.0	4.96 d (4.1)
3'	CH	30.0	2.25 m
4'	CH ₃	18.8	1.04 d (6.9)
5'	CH ₃	16.5	1.00 d (6.9)
6'	C	170.6	-
7'	CH ₃	21.0	2.17 s
<i>Isovaleroyl (C-7)</i>			
1''	C	172.4	-
2''	CH ₂	43.3	2.18 d (7.0)
3''	CH	25.8	2.09 m
4''/5''	CH ₃	22.3	0.95 d (6.6)
11-COCH ₃	C	170.9	-
11-COCH ₃	CH ₃	20.5	2.06 s

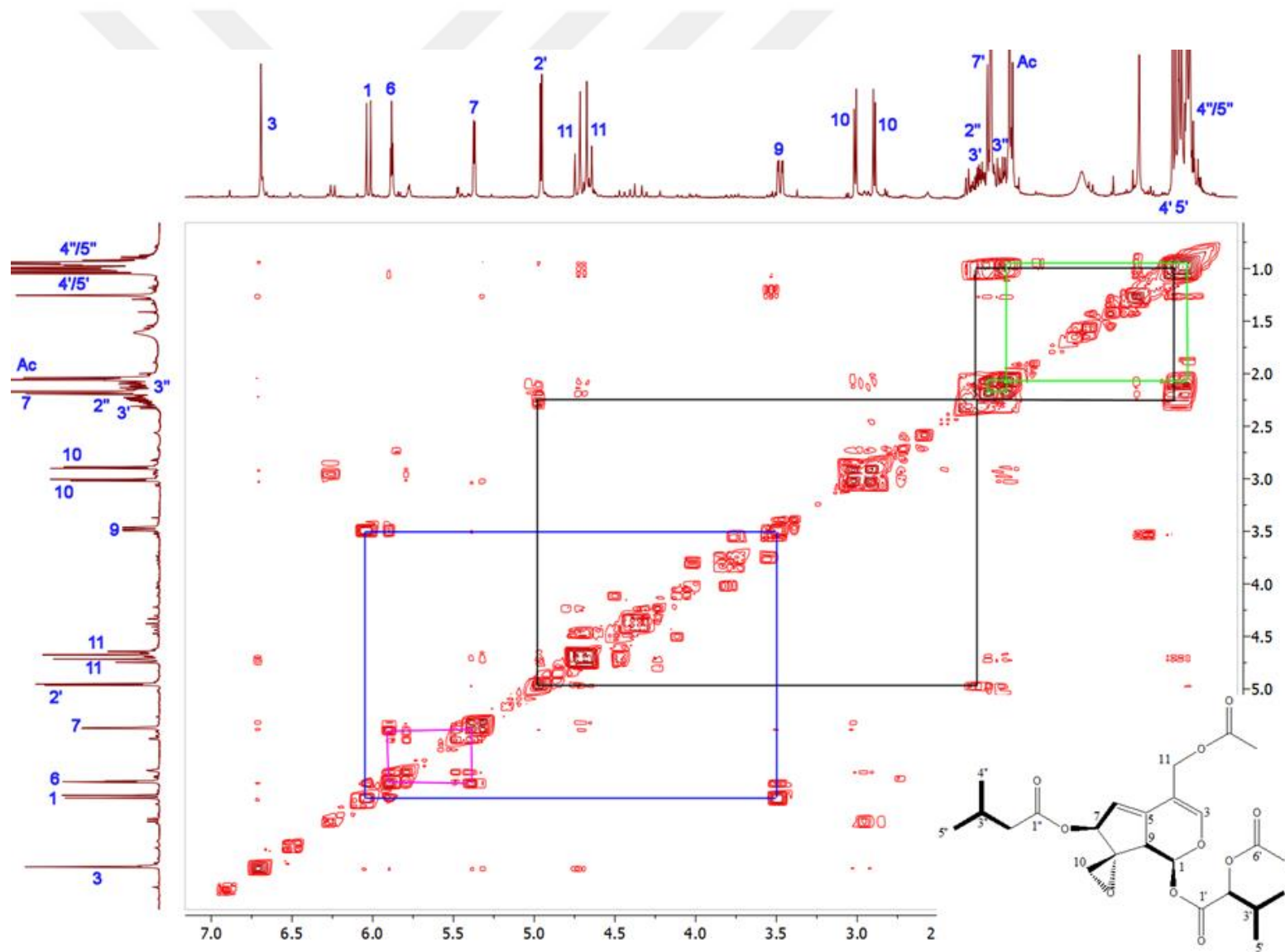
^aAssignments are based on COSY, HSQC and HMBC experiments.



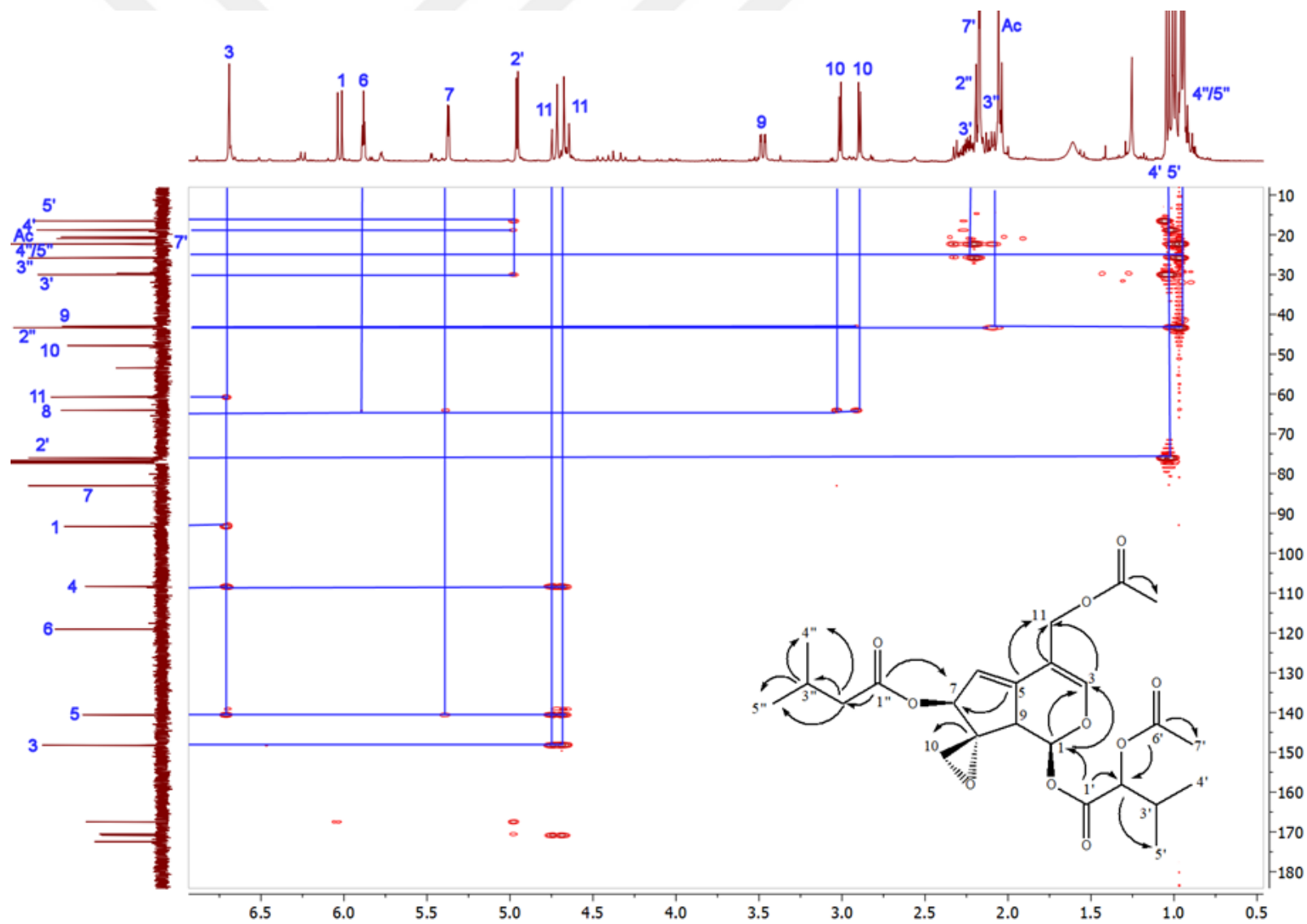
Spectrum 57. ^1H NMR Spectrum of 1- α -Acevaltrate (15) (CDCl_3 , 400 MHz)



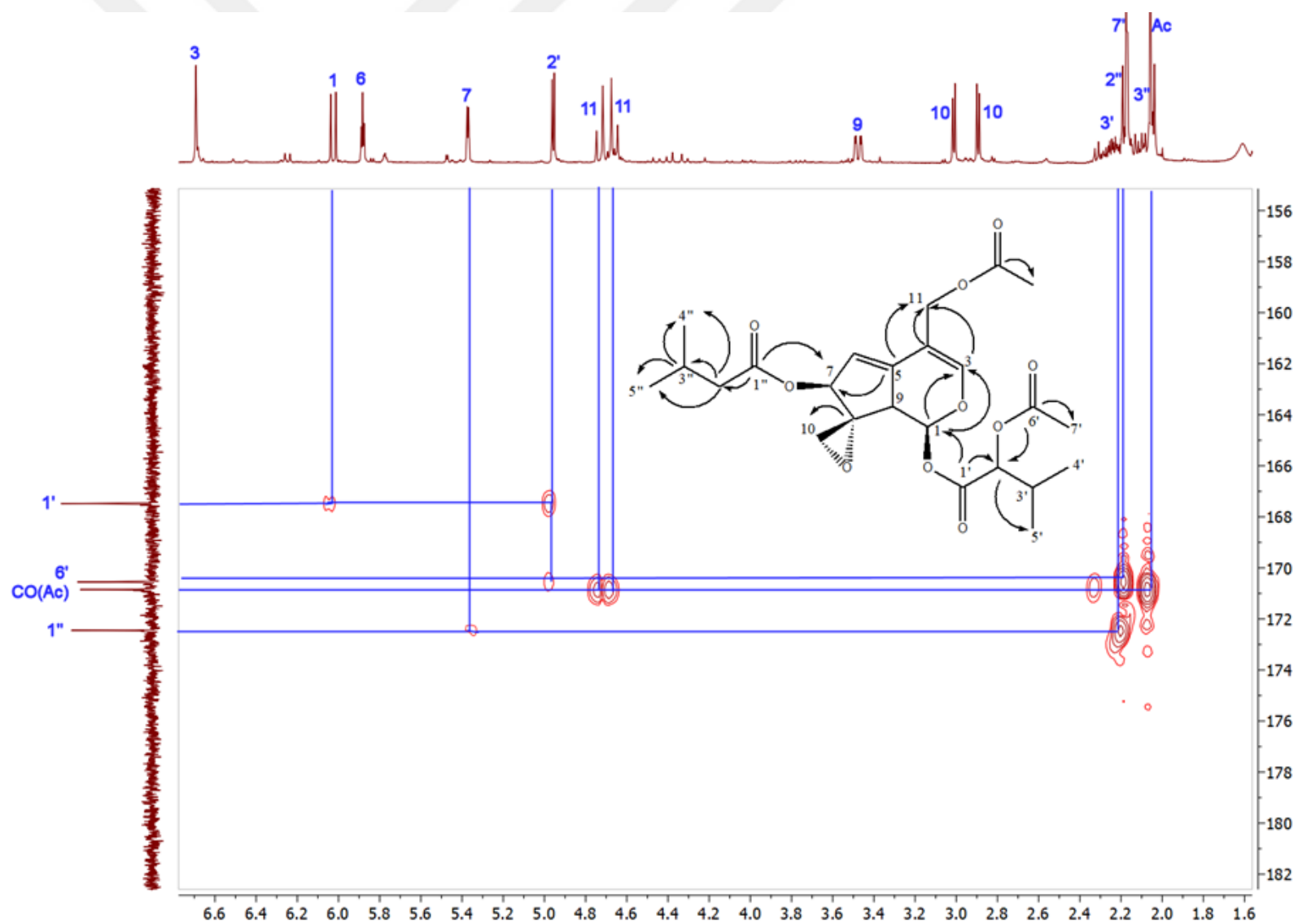
Spectrum 58. ¹³C NMR Spectrum of 1-α-Acevaltrate (**15**) (CDCl₃, 100 MHz)



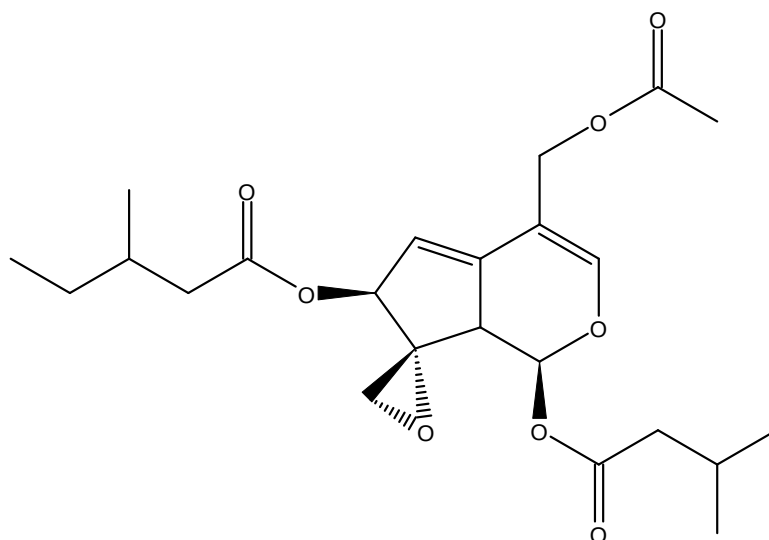
Spectrum 59. COSY Spectrum of 1- α -Acevaltrate (15)



Spectrum 60. HMBC Spectrum of 1- α -Acevaltrate (15) (^{13}C :10-80 ppm, ^1H : 0.5-6.8 ppm)



Spectrum 61. HMBC Spectrum of 1- α -acevaltrate (15) (^{13}C :156-182 ppm, ^1H : 1.6-6.75 ppm)



7-HOMOVALTRATE (16): C₂₃H₃₂O₈ (MW:436.50)

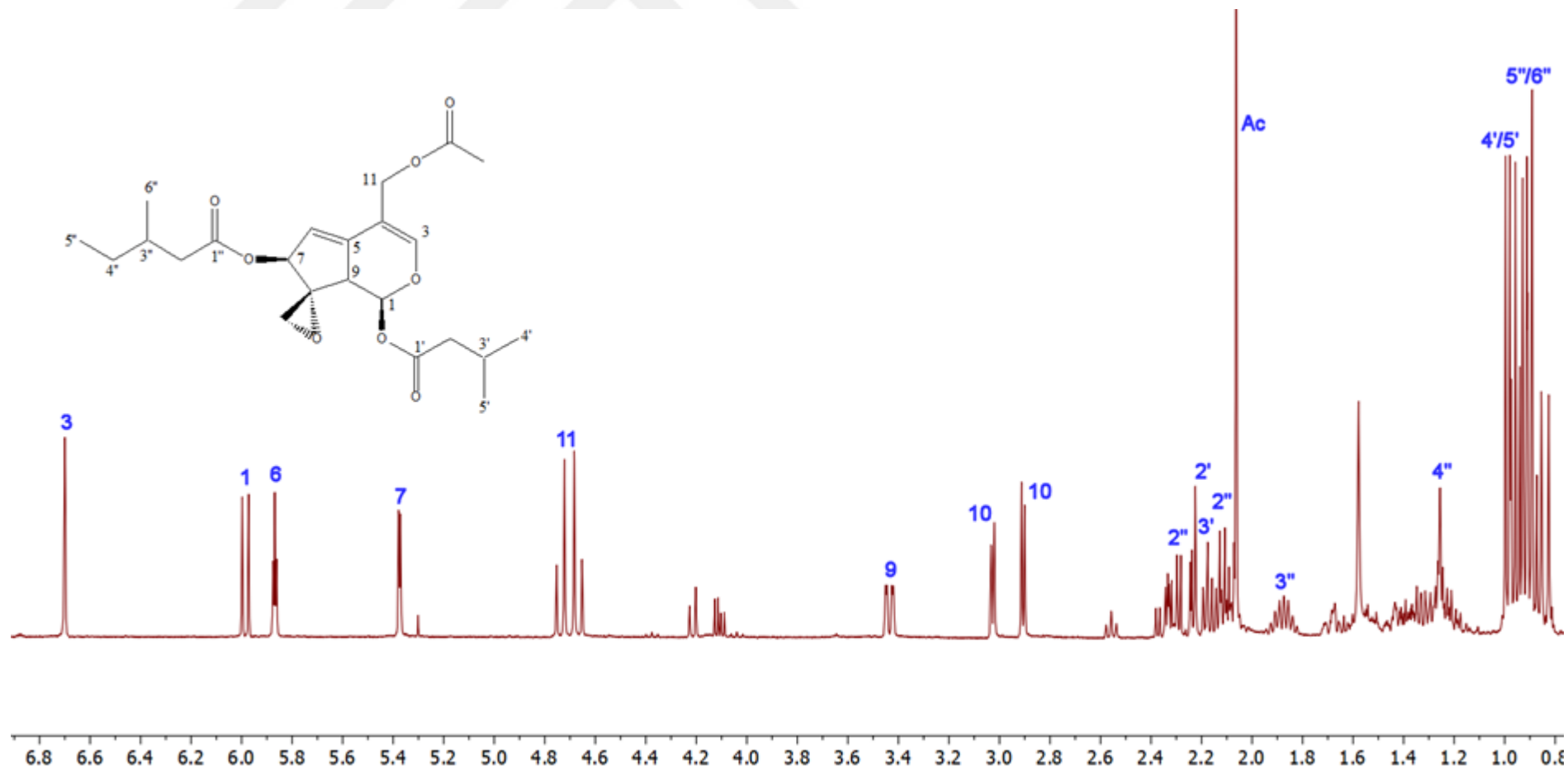
UV λ_{max} (MeOH) nm:	255
IR ν_{max} (KBr) cm ⁻¹ :	1768, 1740 (ester, C=O), 1637 (olefinic, C=C)
HR-ESI-MS m/z :	459.94 [M+Na] ⁺
¹ H NMR:	Table 91, Spectrum 62
¹³ C NMR:	Table 91, Spectrum 63
COSY:	Spectrum 64
HSQC:	Spectrum 65
HMBC:	Spectra 66 and 67

Compound **16** was obtained as colorless oil, and its molecular formula was determined to be C₂₃H₃₂O₈ by ESI-MS (m/z 459.94 [M+Na]⁺) and NMR experiments, suggesting eight degrees of unsaturation. The UV spectrum showed absorption maximum at 255 nm. The IR spectrum demonstrated absorption bands belonging to carbonyl ester (1768 and 1740 cm⁻¹) and olefinic (1637 cm⁻¹) groups. Comparison of the ¹H and ¹³C NMR findings of **16** (Tables 91 and 75) with those of **3** (valtrate) revealed that they were considerably similar to each other, except for the presence of 3-methylvaleroxy unit at 7th position in **16** instead of isovaleroxy group in **3**. Taken together, comparison of all the spectral data of **16** with the those reported for 7-homovaltrate, led to the elucidation of compound **16** as 7-homovaltrate (254).

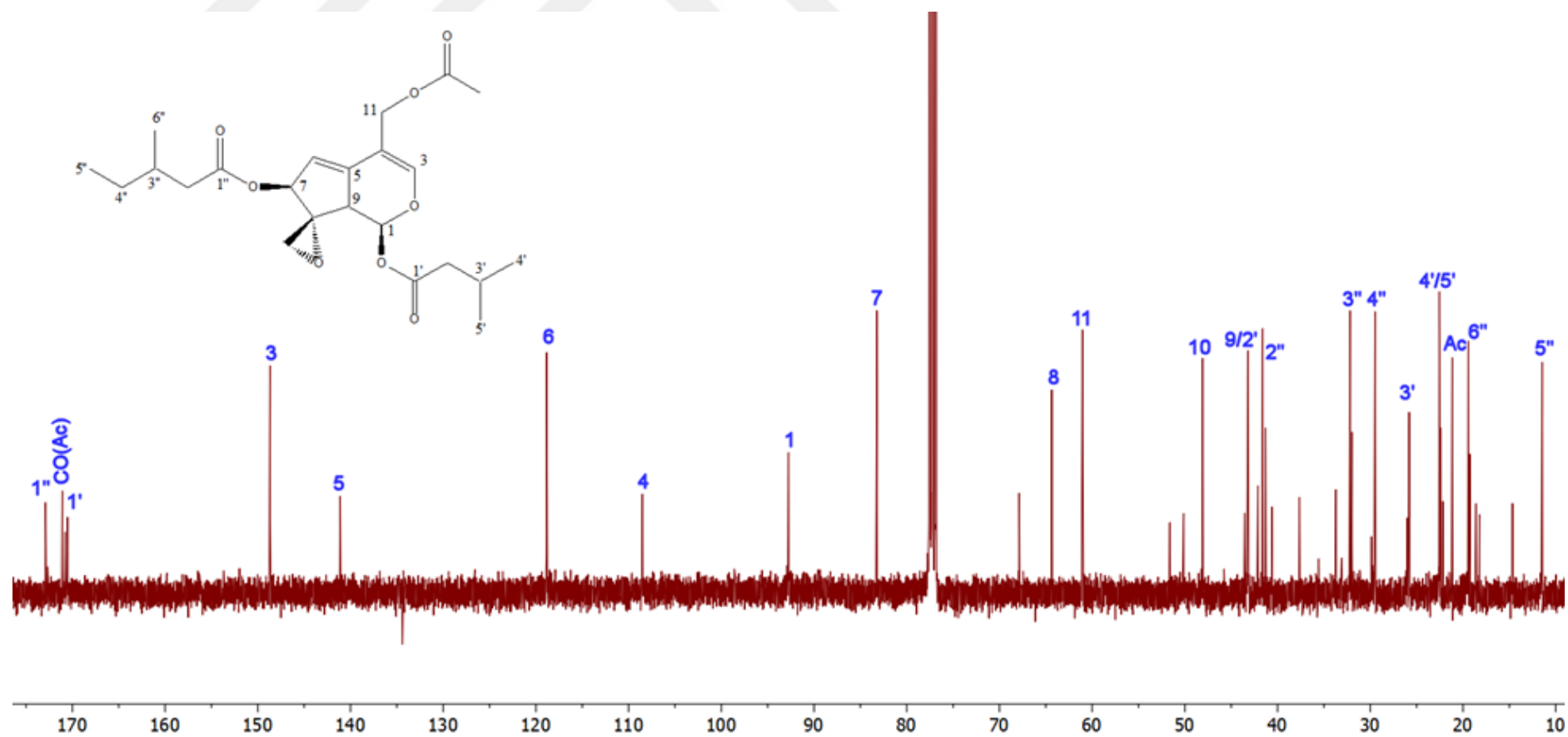
Table 91. ^{13}C and ^1H NMR data of 7-homovaltrate (**16**) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
1	CH	92.8	5.98 d (10.1)
3	CH	148.7	6.70 s
4	C	108.5	-
5	C	141.1	-
6	CH	118.8	5.87 t (2.8)
7	CH	83.2	5.37 d (2.9)
8	C	64.3	-
9	CH	43.2	3.43 dd (10.0, 2.5)
10	CH ₂	48.1	3.03 d (4.9) 2.91 d (4.9)
11	CH ₃	61.0	4.74 d (12.4) 4.67 d (12.4)
<i>Isovaleroyl (C-1)</i>			
1'	C	170.5	-
2'	CH ₂	43.2	2.23 d (5.9)
3'	CH	25.8	2.13 m
4'/5'	CH ₃	22.5	0.99 d (6.6)
<i>3-Methylvaleroyl (C-7)</i>			
1''	C	172.9	-
2''	CH ₂	41.6	2.33 d (6.0) 2.12 d (5.4)
3''	CH	32.2	1.88 m
4''	CH ₂	29.4	1.26 m
5''	CH ₃	11.5	0.89 t (8.0)
6''	CH ₃	19.4	0.93 d (6.7)
11- <u>CO</u> CH ₃	C	171.1	-
11-CO <u>CH</u> ₃	CH ₃	21.2	2.06 s

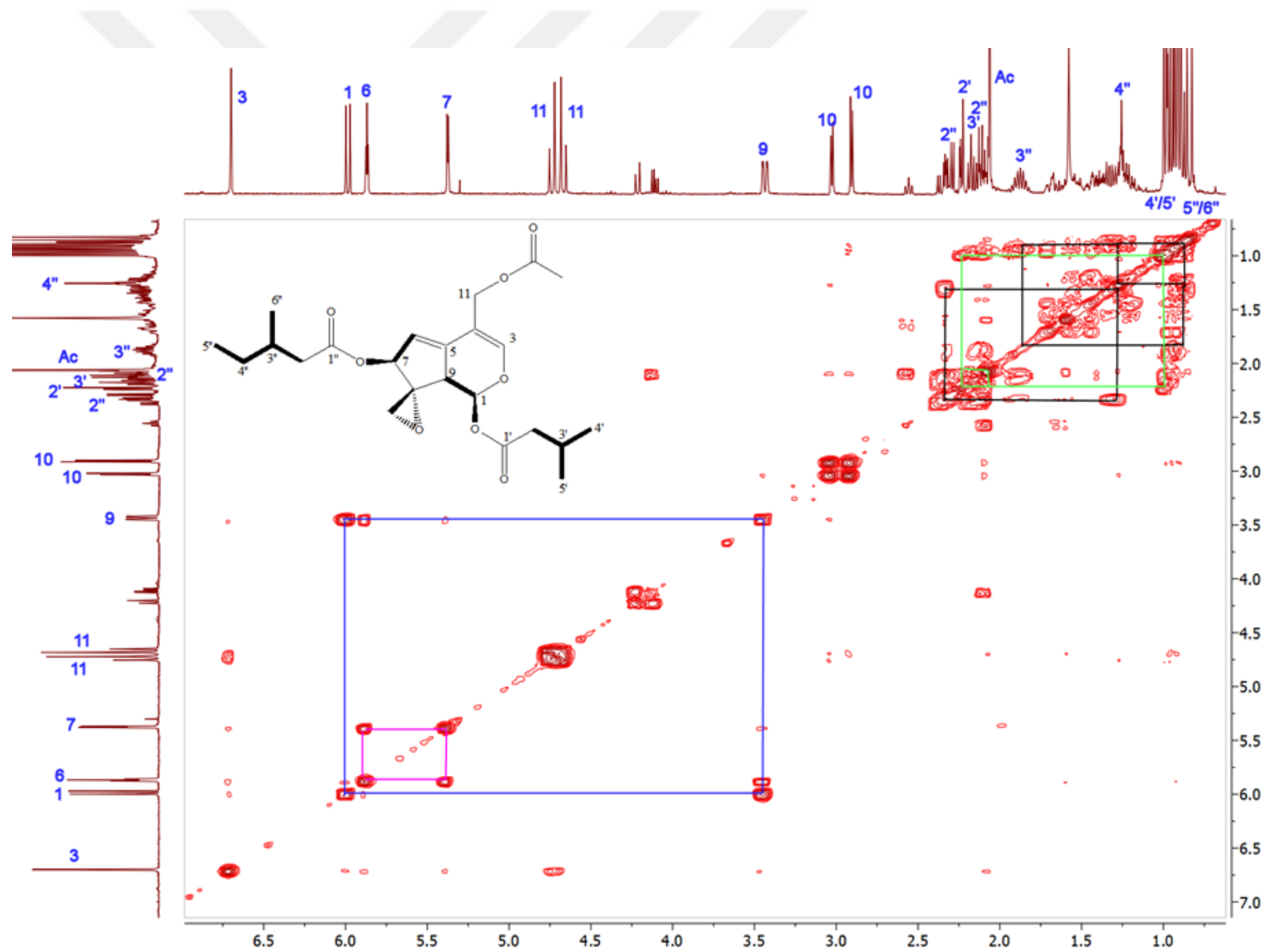
^aAssignments are based on COSY, HSQC and HMBC experiments.



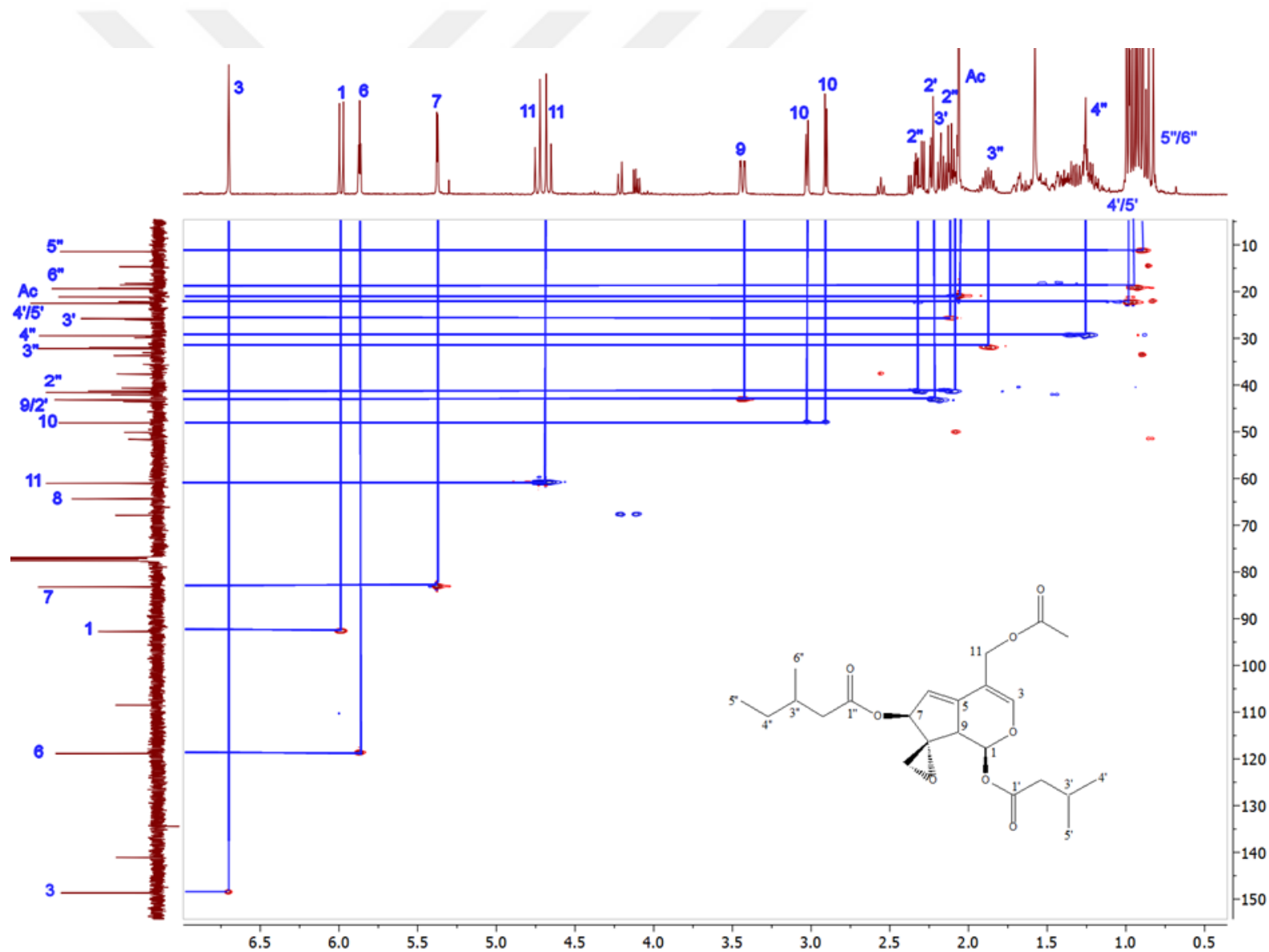
Spectrum 62. ^1H NMR Spectrum of 7-Homovaltrate (**16**) (CDCl_3 , 400 MHz)



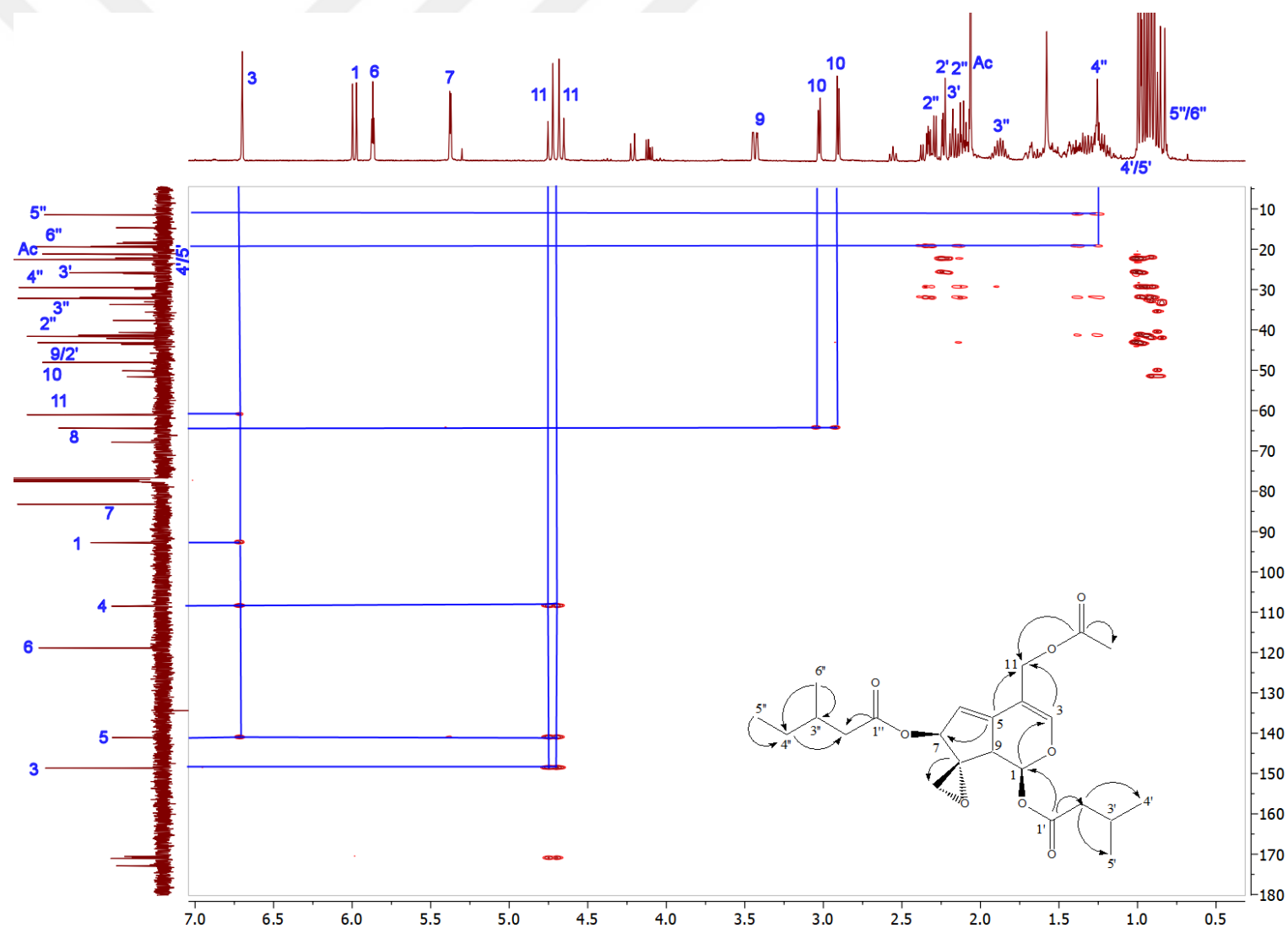
Spectrum 63. ^{13}C NMR Spectrum of 7-Homovaltrate (**16**) (CDCl₃, 100 MHz)



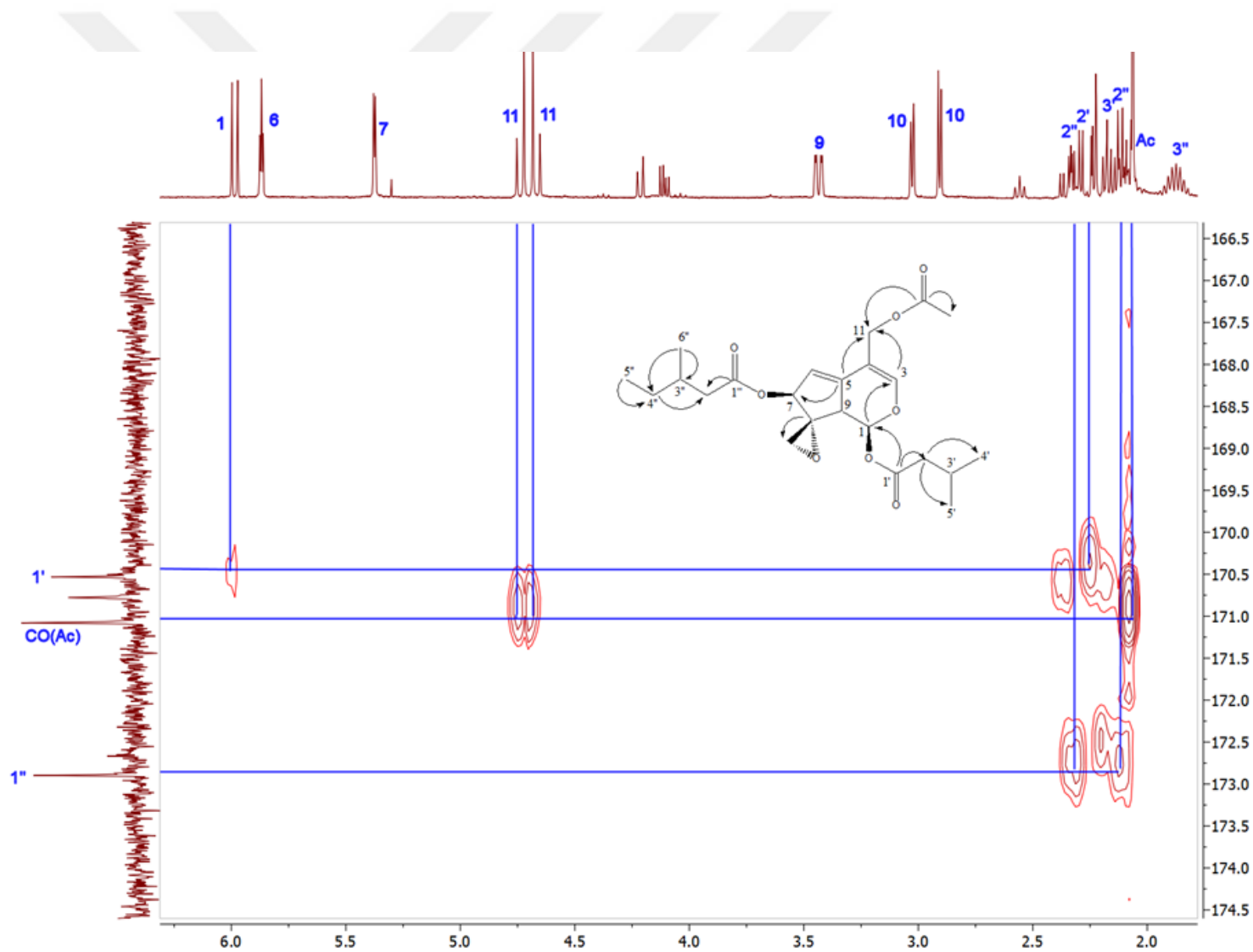
Spectrum 64. COSY Spectrum of 7-Homovaltrate (**16**)



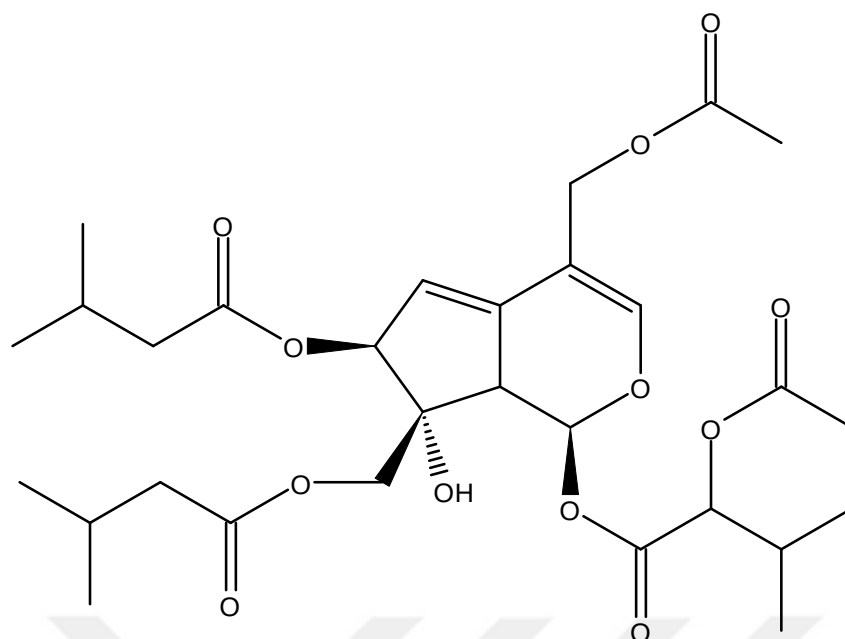
Spectrum 65. HSQC Spectrum of 7-Homovaltrate (**16**)



Spectrum 66. HMBC Spectrum of 7-Homovaltrate (**16**) (^{13}C : 5-180 ppm, ^1H : 0.3-7.0 ppm)



Spectrum 67. HMBC Spectrum of 7-Homovaltrate (**16**) (¹³C: 166–174.5 ppm, ¹H: 1.75–6.8 ppm)



SISYMBRIIFOLIVALTRATE (17): C₂₃H₃₂O₈ (MW:582.64)

UV λ_{max} (MeOH) nm:	254
IR ν_{max} (KBr) cm ⁻¹ :	3460 (OH), 1741 (ester, C=O), 1641, 1613 (olefinic, C=C)
HR-ESI-MS m/z :	605.2576 [M+Na] ⁺ (calcd for C ₂₉ H ₄₂ O ₁₂ Na, 605.2574)
¹ H NMR:	Table 92, Spectrum 68
¹³ C NMR:	Table 92, Spectrum 69
COSY:	Spectrum 70
HSQC:	Spectrum 71
HMBC:	Spectra 72 and 73
NOESY:	Spectrum 74

SISYMBRIIFOLIVALTRATE (17)

Compound **17** was obtained as colorless oil. The molecular formula of $C_{29}H_{42}O_{12}$ was determined from the pseudomolecular ion peak for $[M + Na]^+$ at m/z 605.2576 (calcd for $C_{29}H_{42}O_{12}Na$, 605.2574) in its HRESIMS. Its UV spectrum showed absorption maximum at 254 nm. The IR spectrum of **17** exhibited the presence of ester (1741 cm^{-1}) and olefinic (1641 and 1613 cm^{-1}) functionalities.

The ^1H NMR (Table 92) spectrum of **17** demonstrated signals for two olefinic methines [δ_{H} 6.68 (s) and 5.77 (t, $J = 2.6\text{ Hz}$)], one oxymethylene [δ_{H} 4.38 and 4.23 (each d, $J = 11.5\text{ Hz}$)], one oxymethine [δ_{H} 5.48 (d, $J = 2.8\text{ Hz}$)] and one hemiacetal [δ_{H} 6.28 (d, $J = 9.8\text{ Hz}$)] which were characteristic for a “valtrate hydrine” skeleton (248). According to previous studies on *Valeriana* species, it was observed that compounds bearing valtrate hydrine structure were generally esterified with various groups at four different positions including C-1, -7, -10 and -11 (166). The methyl signals at δ_{H} 1.04 and 0.99 (each 3H, d, $J = 6.9\text{ Hz}$) and δ_{H} 0.95 and 0.92 (each 6H, d, $J = 6.5$ and 6.6 Hz , respectively) in the ^1H NMR spectrum indicated six methyl groups arising from three isovaleroyl derivatives. Also, the signals at δ_{H} 2.18 and 2.03 (each 3H, s) indicated the presence of two acetyl moieties in the molecule. The ^{13}C NMR (Table 92) spectrum of **17** contained 29 resonances including five carbonyls (δ_{C} 172.9, 171.8, 170.8, 170.7 and 167.8), four olefinic (δ_{C} 147.8, 138.8, 117.8 and 108.6), two methylenes (δ_{C} 43.3 and 43.0) arising from isovaleroyl derivatives. However, the chemical shift of the C-2' (δ_{C} 76.4) signal for one of the isovaleroyl derivatives was found to be shifted downfield (δ_{C} 76.4) due to the hydroxylation. The remaining carbon signals were typical for a C_{10} -iridoid skeleton. The COSY spectrum (Spectrum 70) of **17** displayed two spin systems for the iridoid core between H-1/H-9 and H-6/H-7 indicating the presence of quaternary carbons of C-5 and C-8. The acylation sites of the substituents on the iridoid skeleton were determined with key long-range coupling in the HMBC spectrum (Spectrum 73). Thus, one of the acetyl groups was located at C-11(OH) inferred from the cross-peak between C=O (δ_{C} 170.8) and $H_{1,2-11}$ (δ_{H} 4.70 and 4.63) in its HMBC spectrum. Moreover, binding site of the other acetyl group was unambiguously identified to be C-2'(OH) by the cross-peak between C-6' (δ_{C} 170.7) of the α -hydroxyisovaleroyl unit and H-2' (δ_{H} 4.95). Thus, one of the ester groups in the molecule was determined to be α -(acetoxy)-isovaleroyl (162) which was located at C-1(OH) deduced by the long-range interaction between C-1' (δ_{C} 167.8) and hemiacetal proton (δ_{H} 6.28, H-1). Positions of the other two isovaleroyl groups were elucidated by the following strong HMBC interactions; C-1'' $\rightarrow$ H-7 and C-1''' $\rightarrow$ $H_{1,2-10}$ (Spectrum 73).

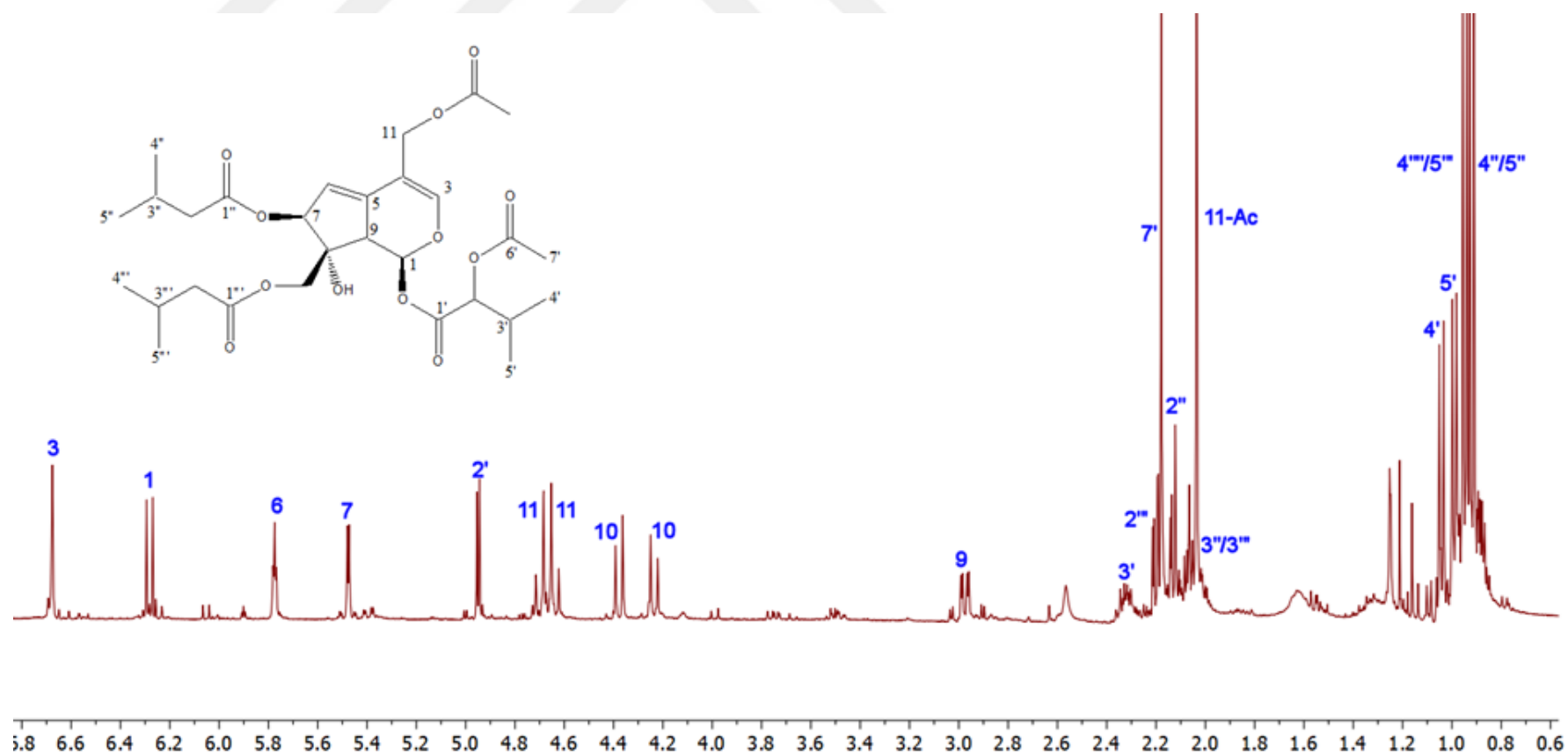
The relative stereochemistry of **17** was determined based on the NOESY experiment (Spectrum 74) and biosynthetic considerations. As all the naturally occurring valepotriates possess an α -orientation for H-1 and β -orientation for H-9 (193), the NOE correlation between H-9 and H₂-10 showed that the hydroxyl group at C-8 was in the α -orientation. Furthermore, the absence of NOE correlation between H-9 and H-7 confirmed that the isovaleroyloxy group at C-7 position was in β orientation. Based on these findings, compound **17** was identified as a new non-glycosidic ester iridoid and named **sisymbriifolivaltrate**.



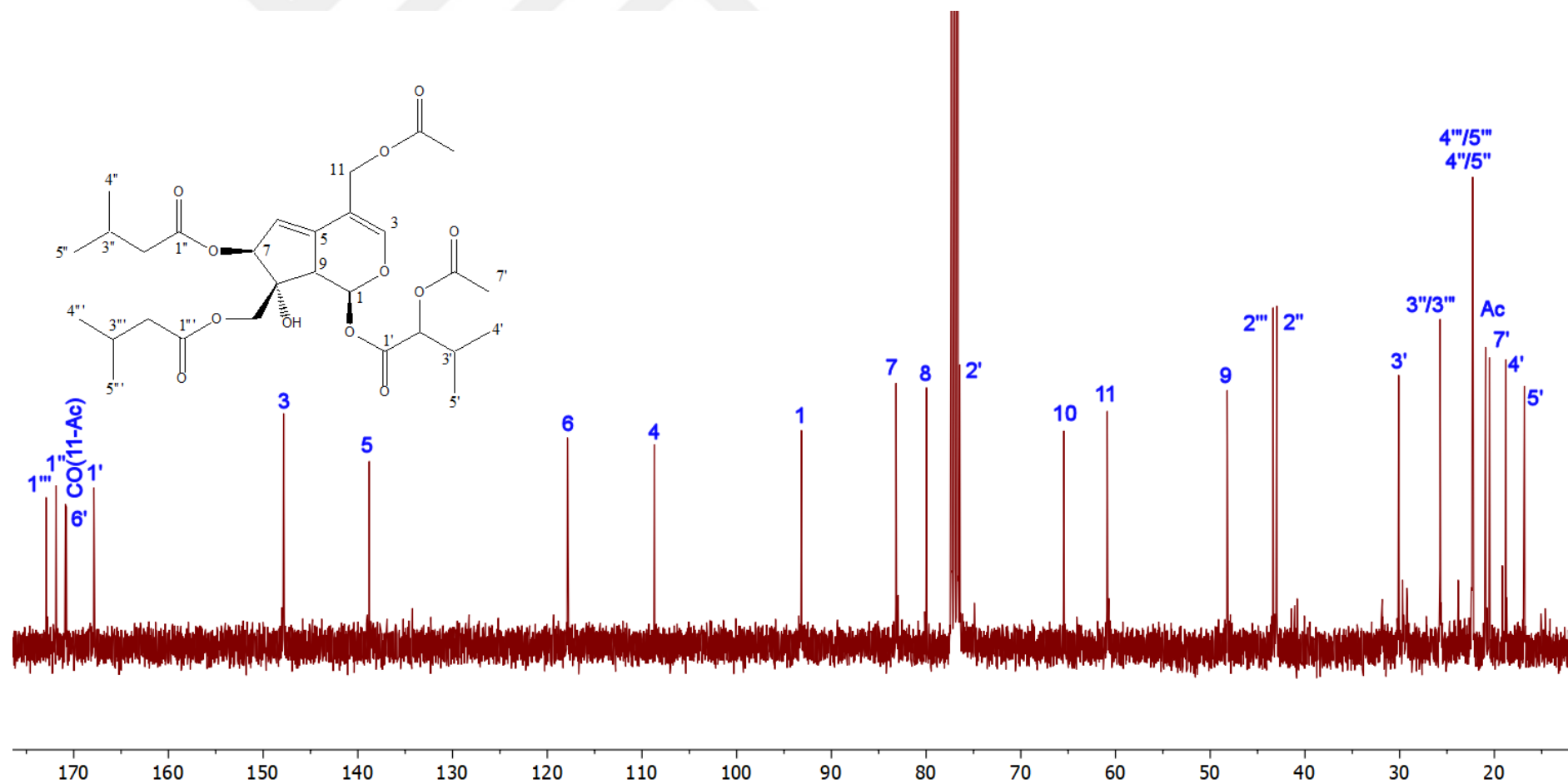
Table 92. ^{13}C and ^1H NMR data of sisymbriifolivaltrate (**17**) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
1	CH	93.1	6.28 d (9.9)
3	CH	147.8	6.68 s
4	C	108.6	-
5	C	138.8	-
6	CH	117.8	5.77 t (2.6)
7	CH	83.2	5.48 d (2.8)
8	C	79.9	-
9	CH	48.2	2.97 dd (9.9, 2.6)
10	CH ₂	65.5	4.38 d (11.5)
			4.23 d (11.5)
11	CH ₂	60.8	4.70 d (12.4)
			4.63 d (12.4)
<i>α-(acetoxy)-isovaleroyl (C-1)</i>			
1'	C	167.8	-
2'	CH	76.4	4.95 d (4.6)
3'	CH	30.0	2.32 m
4'	CH ₃	18.7	1.04 d (6.9)
5'	CH ₃	16.8	0.99 d (6.9)
6'	C	170.7	-
7'	CH ₃	20.5	2.18 s
<i>Isovaleroyl (C-7)</i>			
1''	C	171.8	-
2''	CH ₂	43.0	2.13 d (6.0)
3''	CH	25.6	2.04 m
4''/5''	CH ₃	22.3	0.92 d (6.6)
<i>Isovaleroyl (C-10)</i>			
1'''	C	172.9	-
2'''	CH ₂	43.3	2.20 d (6.7)
3'''	CH	25.7	2.04 m
4'''/5'''	CH ₃	22.3	0.95 d (6.5)
11-COCH ₃	C	170.8	-
11-COCH ₃	CH ₃	20.9	2.03 s

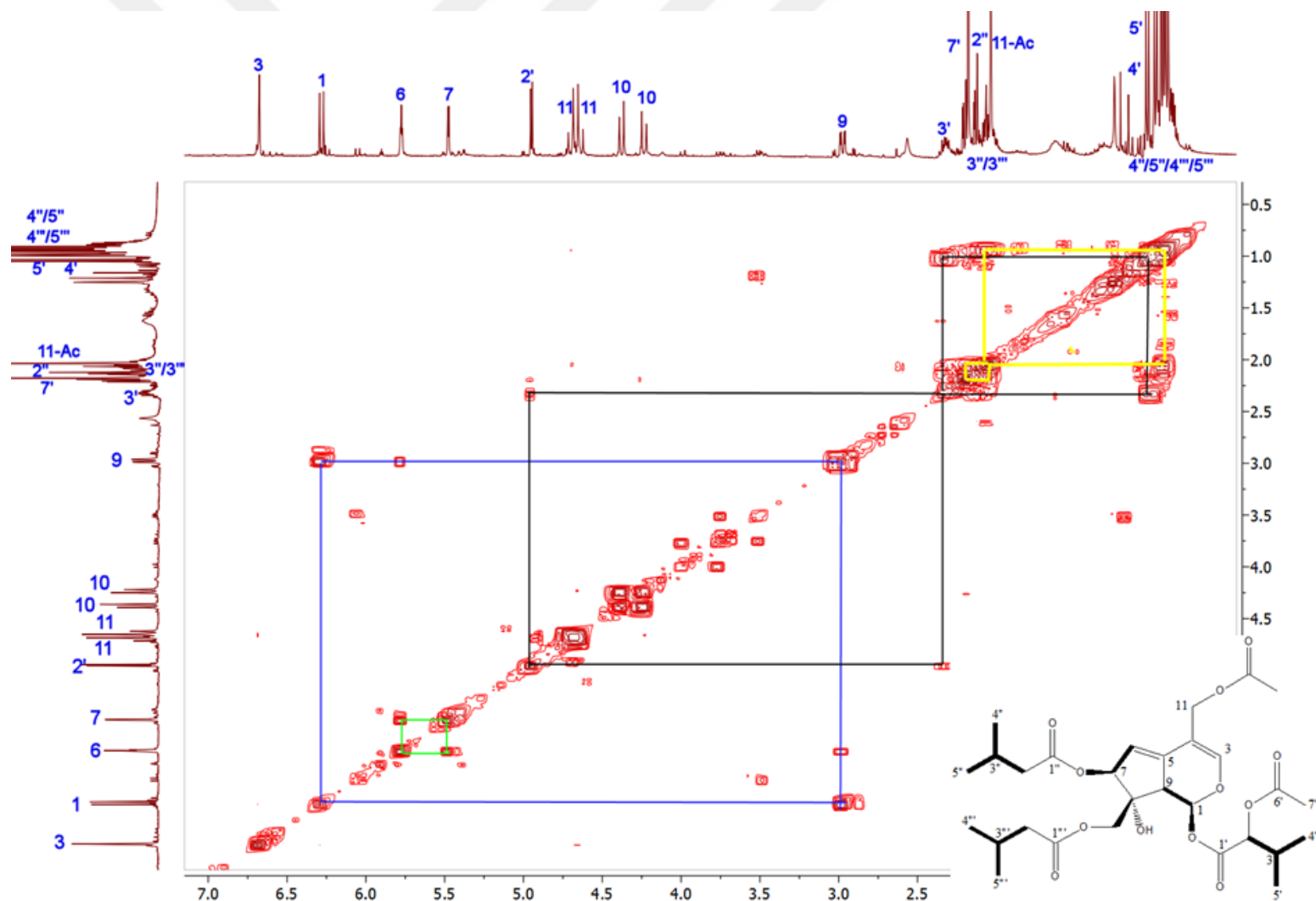
^aAssignments are based on COSY, HSQC HMBC and NOESY experiments.



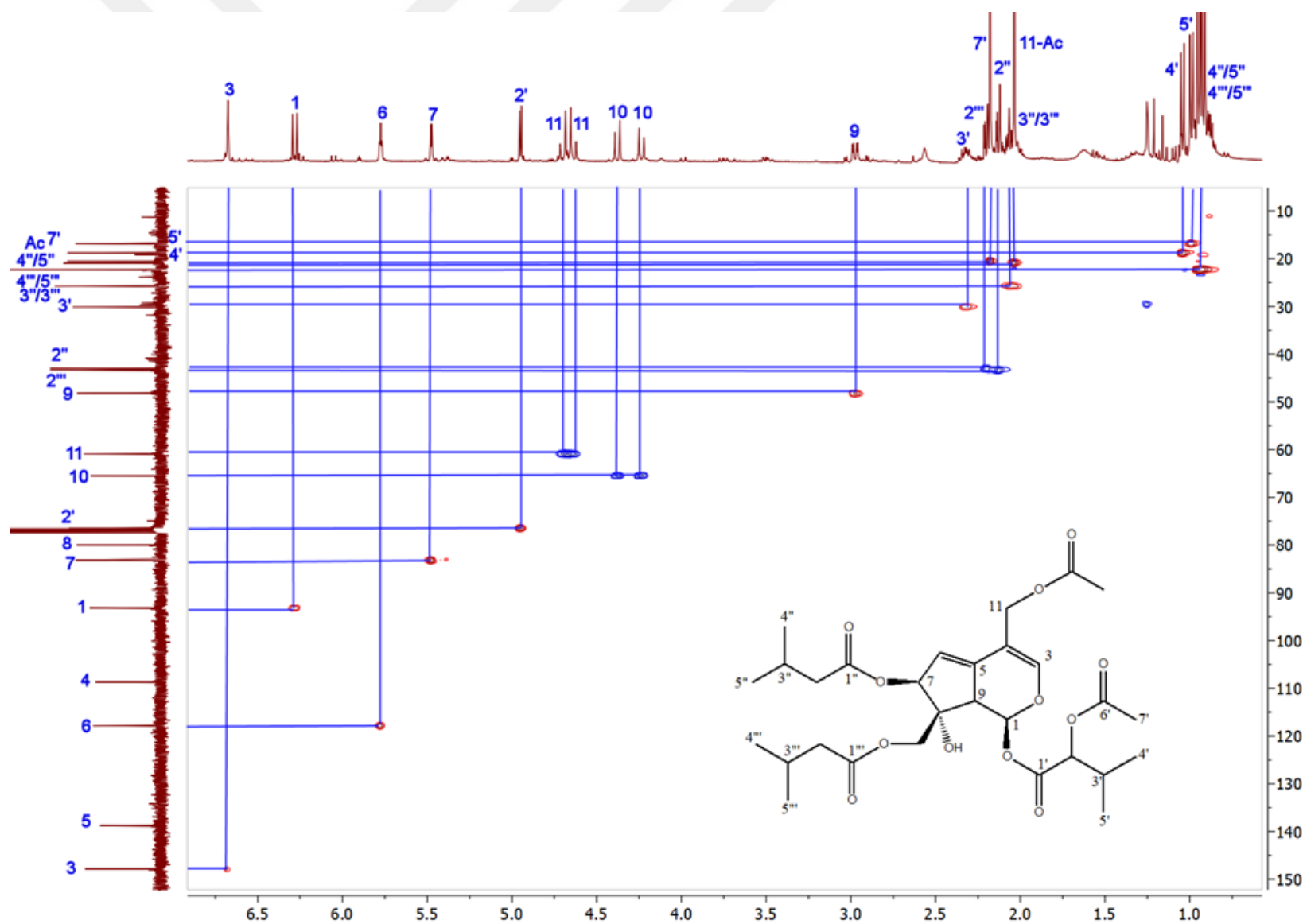
Spectrum 68. ¹H NMR Spectrum of Sisymbriifolivaltrate (17) (CDCl₃, 400 MHz)



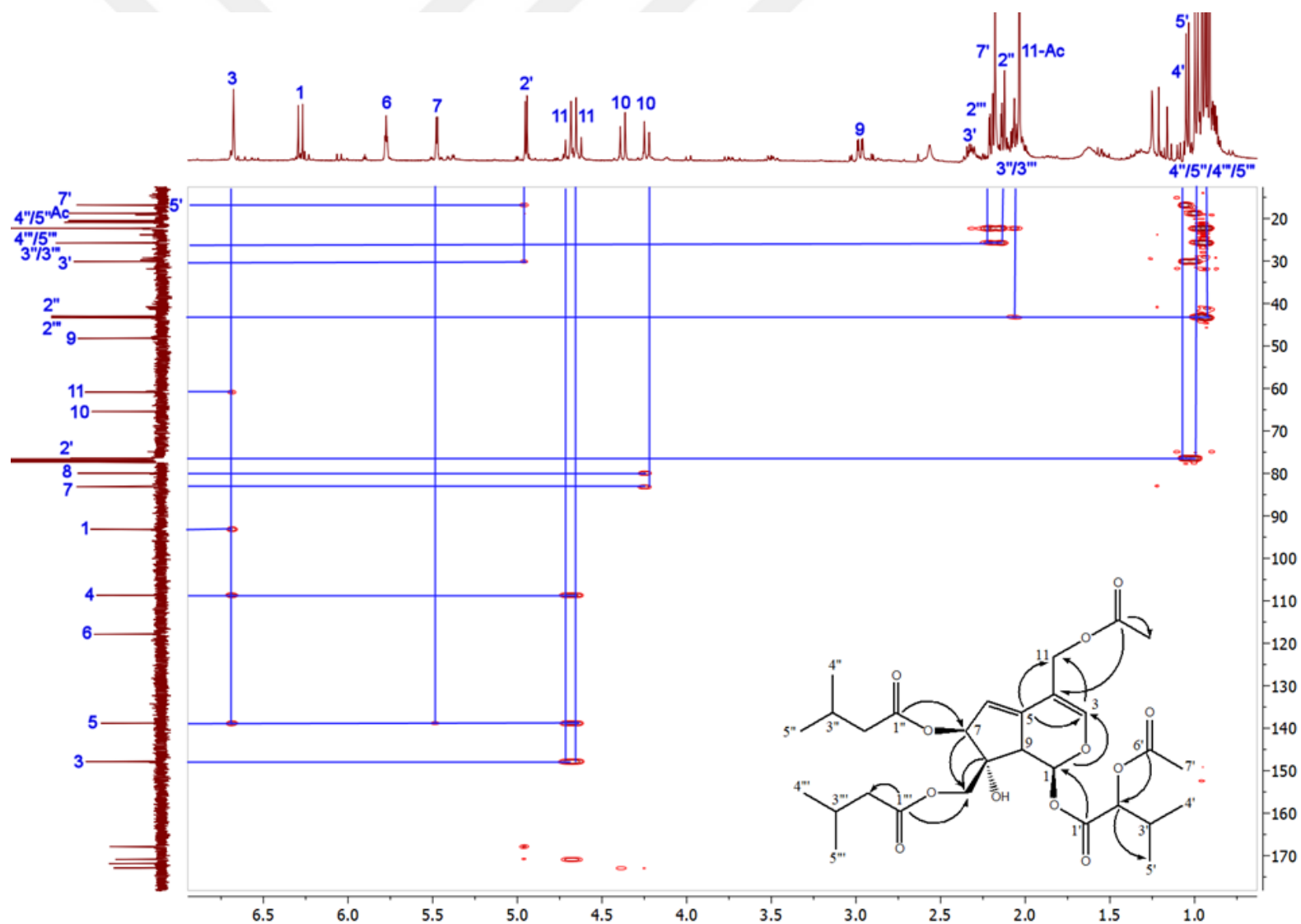
Spectrum 69. ¹³C NMR Spectrum of Sisymbriifolivaltrate (**17**) (CDCl₃, 100 MHz)



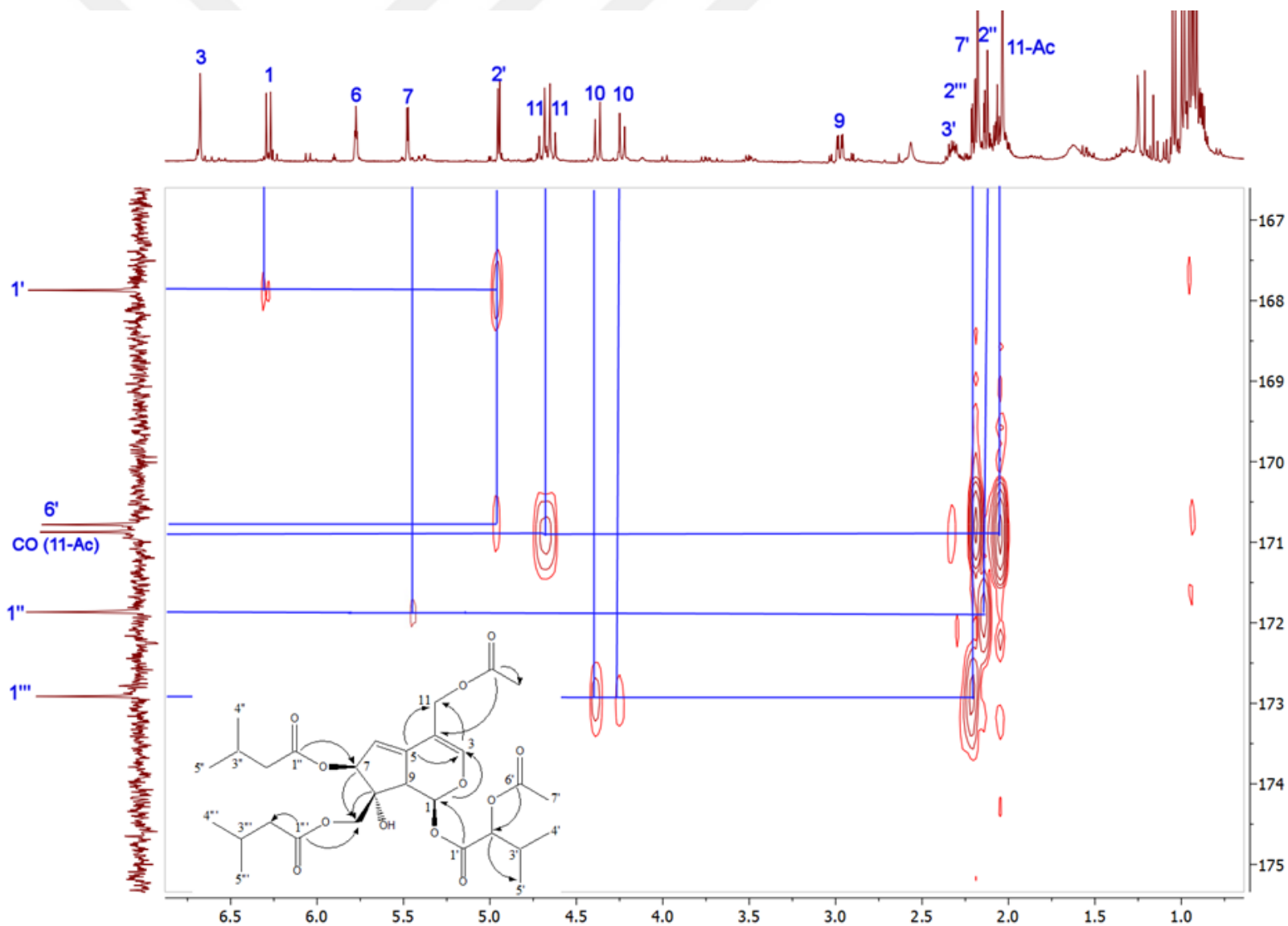
Spectrum 70. COSY Spectrum of Sisymbriifolivaltrate (17)



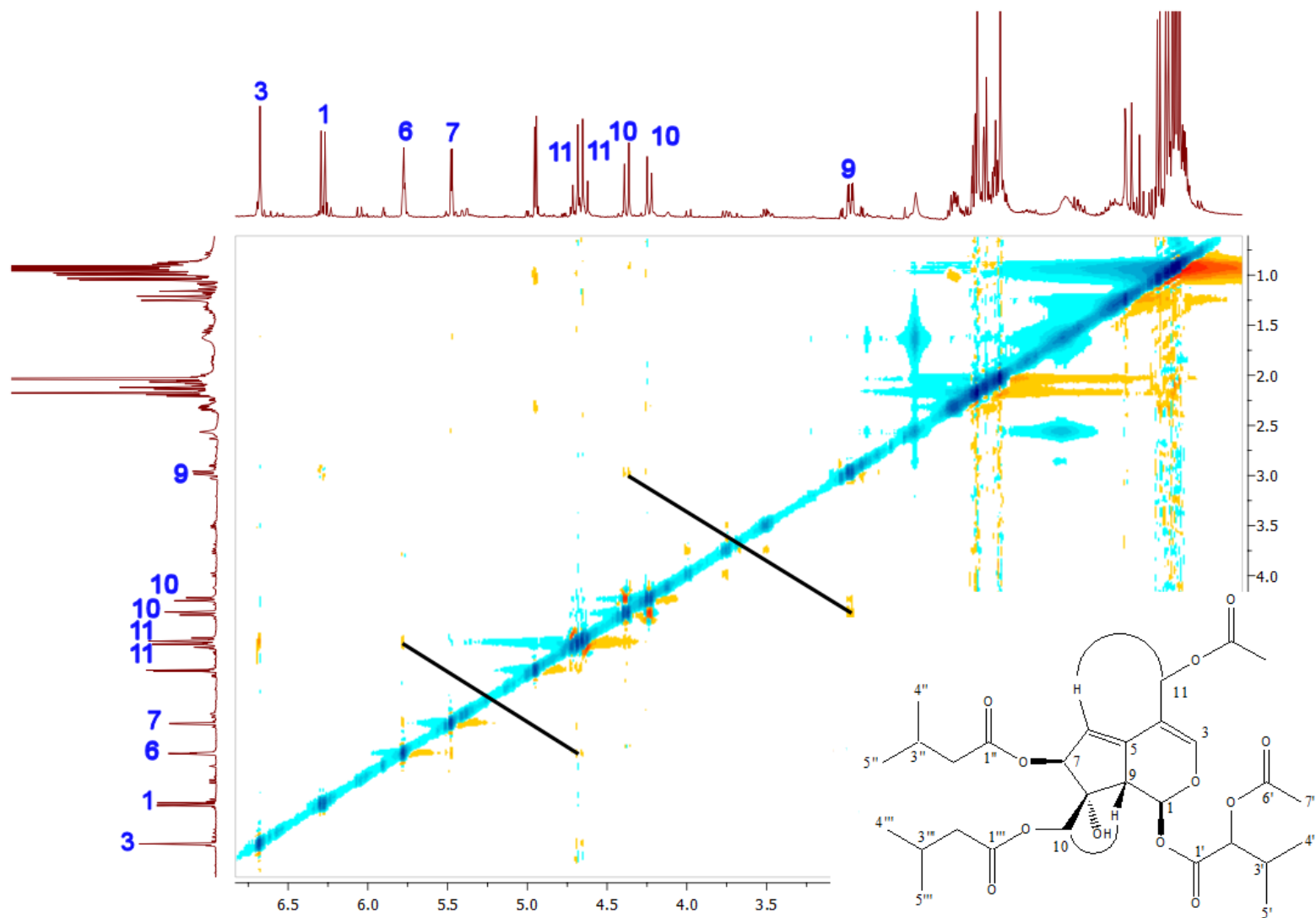
Spectrum 71. HSQC Spectrum of Sisymbriifolivaltrate (17)



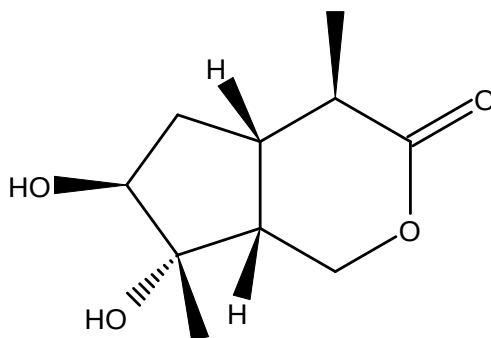
Spectrum 72. HMBC Spectrum of Sisymbriifolivaltrate (17) (^{13}C :14-176 ppm, ^1H : 0.5-6.8 ppm)



Spectrum 73. HMBC Spectrum of Sisymbriifolivaltrate (**17**) (^{13}C :166.5-175.5 ppm, ^1H : 0.6-6.8 ppm)



Spectrum 74. NOESY Spectrum of Sisymbriifolivaltrate (17)



PATRISCABROL (19): C₁₀H₁₆O₄ (MW:200.23)

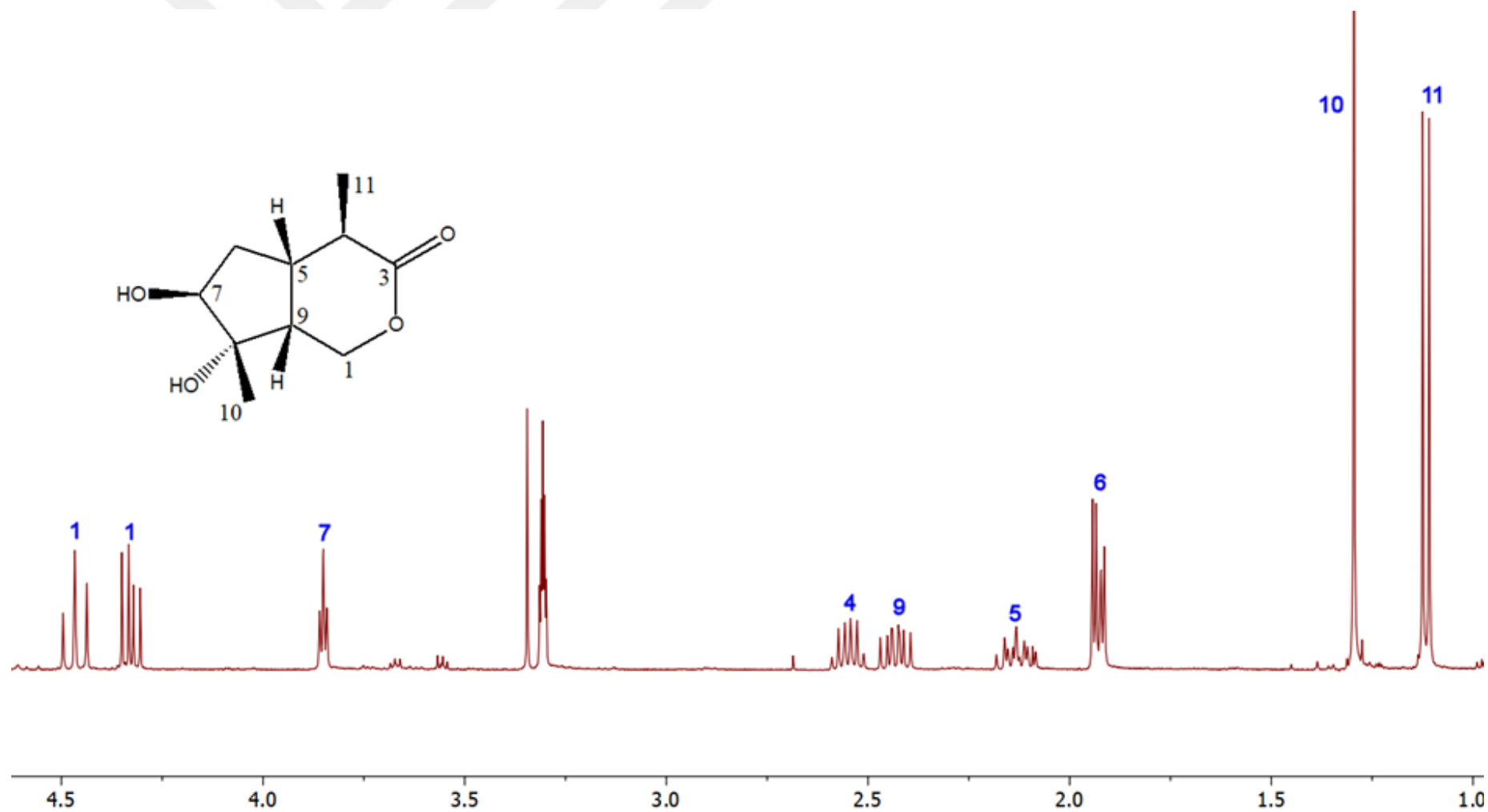
IR $\nu_{\max}$ (KBr) cm ⁻¹ :	3439 (OH) 1720 (C=O)
ESI-MS m/z :	223.74 [M+Na] ⁺ , 201.61 [M+H] ⁺
¹ H NMR:	Table 93, Spectrum 75
¹³ C NMR:	Table 93, Spectrum 76
COSY:	Spectrum 77
HMBC:	Spectrum 78

Compound **19** was isolated as colorless oil, possessed the molecular formula C₁₀H₁₆O₄ with an IHD of three, as inferred from the quasi-molecular ion peaks at m/z 223.74 [M+Na]⁺ and 201.61 [M+H]⁺ in ESI-MS. The IR spectrum indicated absorption bands belonging to hydroxyl (3439 cm⁻¹) and lactone carbonyl (1720 cm⁻¹) groups. The ¹H and ¹³C NMR spectra (Table 93, Spectra 75 and 76) revealed that compound **19** possessed iridolactone structure, particularly iridomyrmecin-type iridoid skeleton. Detailed analysis of 1D and 2D (COSY and HMBC) NMR spectra (Spectra 75-78) suggested compound **19** to be patriscabrol (255).

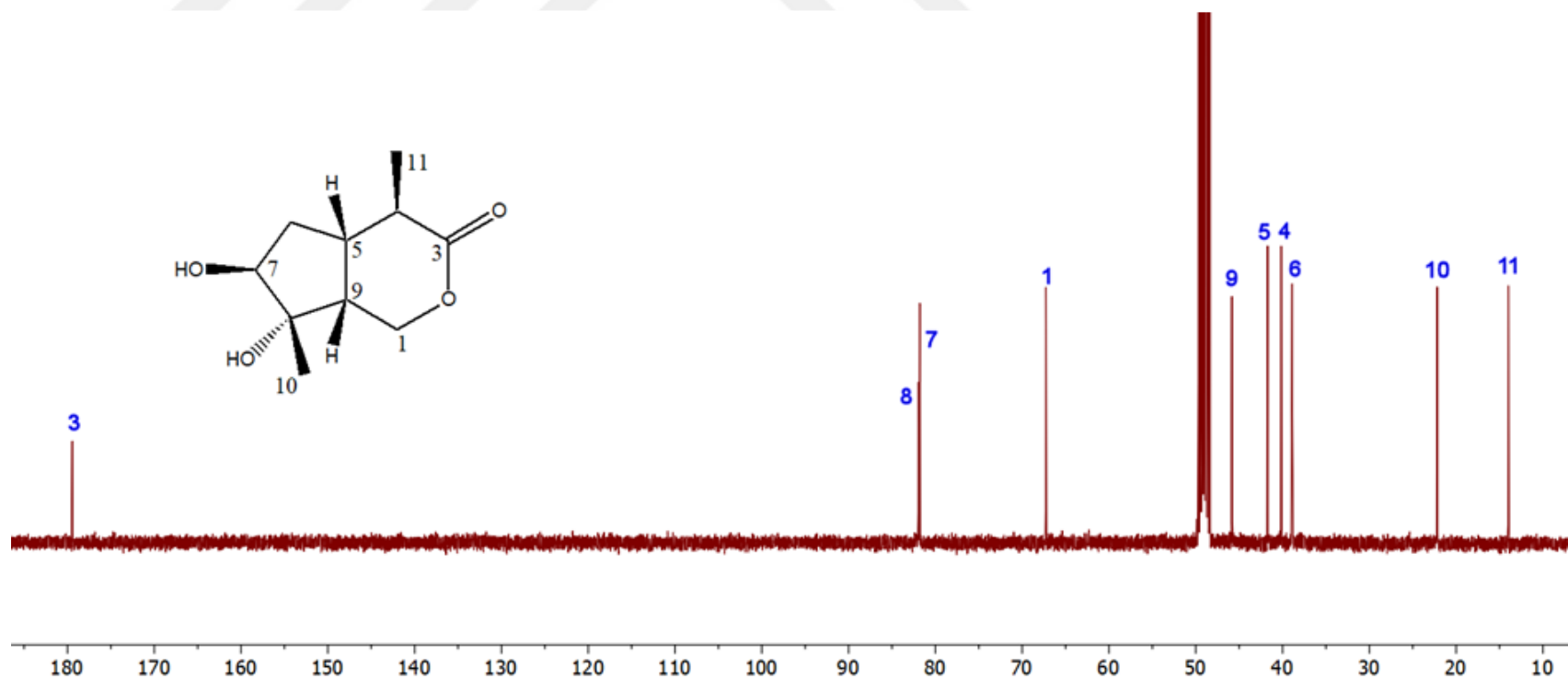
Table 93. ^{13}C and ^1H NMR data of patriscabrol (**19**) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
1	CH_2	67.3	4.47 t (11.7) 4.33 dd (11.4, 6.8)
3	C	179.4	-
4	CH	40.2	2.55 m
5	CH	41.7	2.13 m
6	CH_2	38.9	1.93 dd (8.1, 3.6)
7	CH	81.8	3.85 t (3.6)
8	C	82.0	-
9	CH	45.8	2.43 m
10	CH_3	22.2	1.29 s
11	CH_3	14.0	1.12 d (6.6)

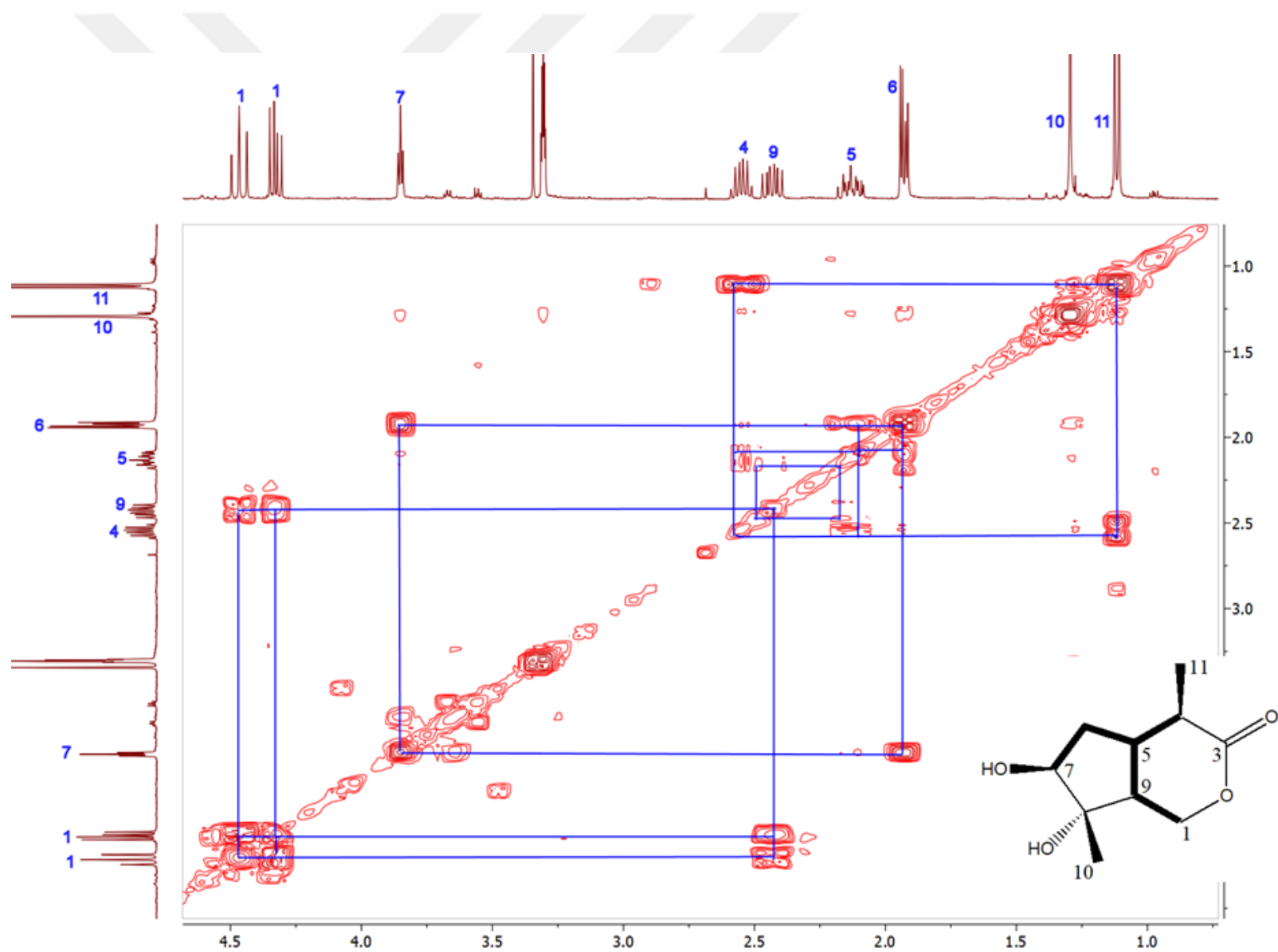
^aAssignments are based on COSY, HSQC and HMBC experiments.



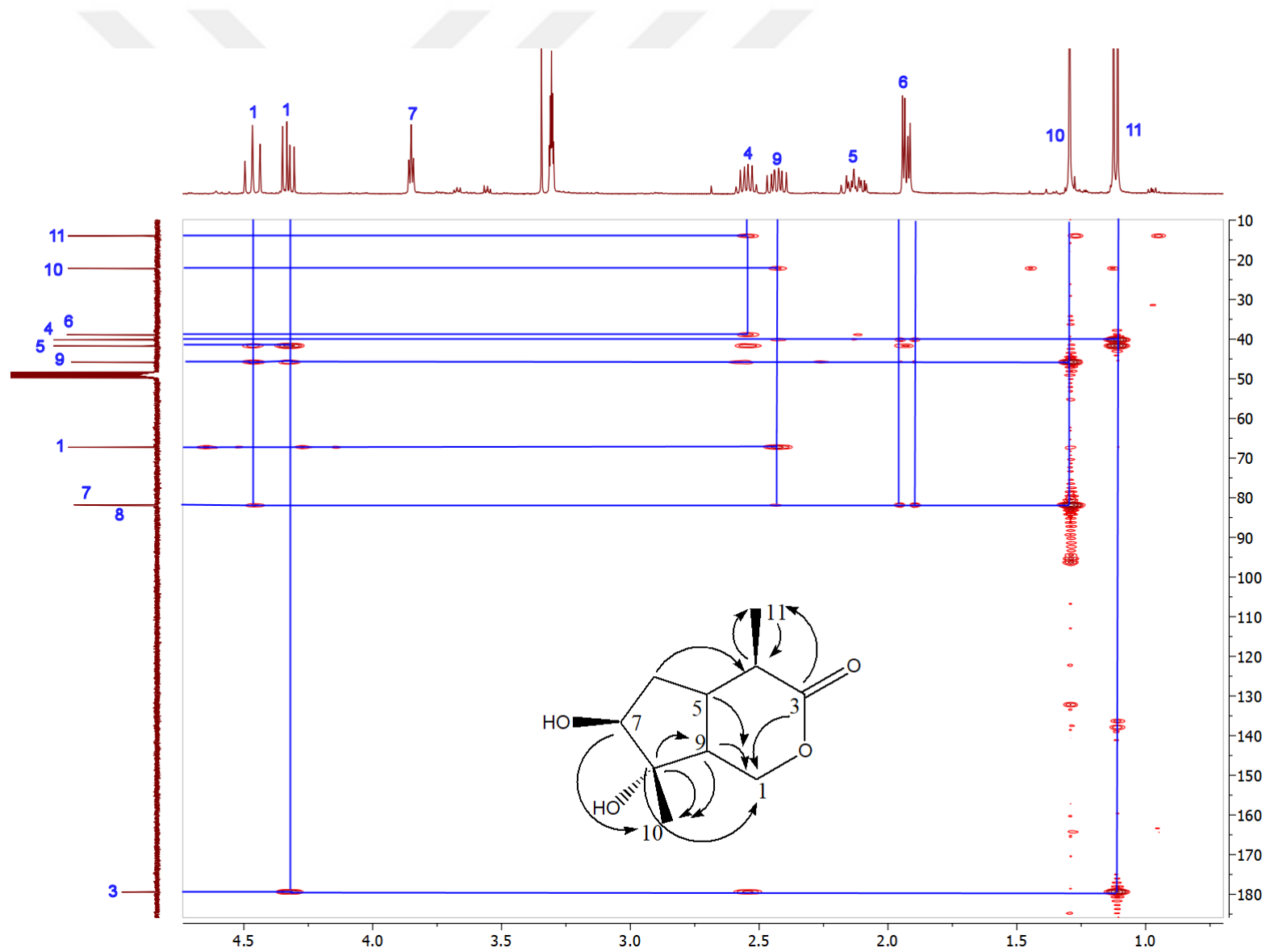
Spectrum 75. ¹H NMR Spectrum of Patriscabrol (**19**) (CDCl₃, 400 MHz)



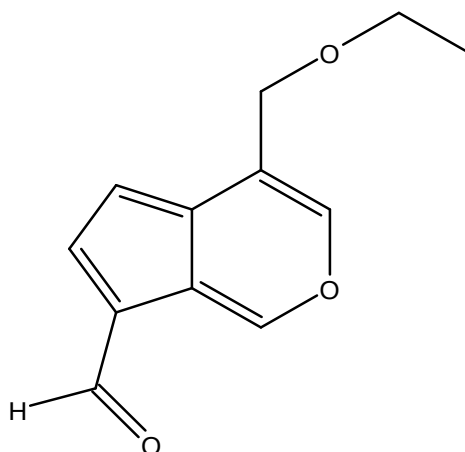
Spectrum 76. ¹³C NMR Spectrum of Patriscabrol (**19**) (CDCl₃, 100 MHz)



Spectrum 77. COSY Spectrum of Patriscabrol (**19**)



Spectrum 78. HMBC Spectrum of Patriscabrol (19)



11-ETHOXYVIBURTINAL (20): C₁₂H₁₂O₃ (MW:204.22)

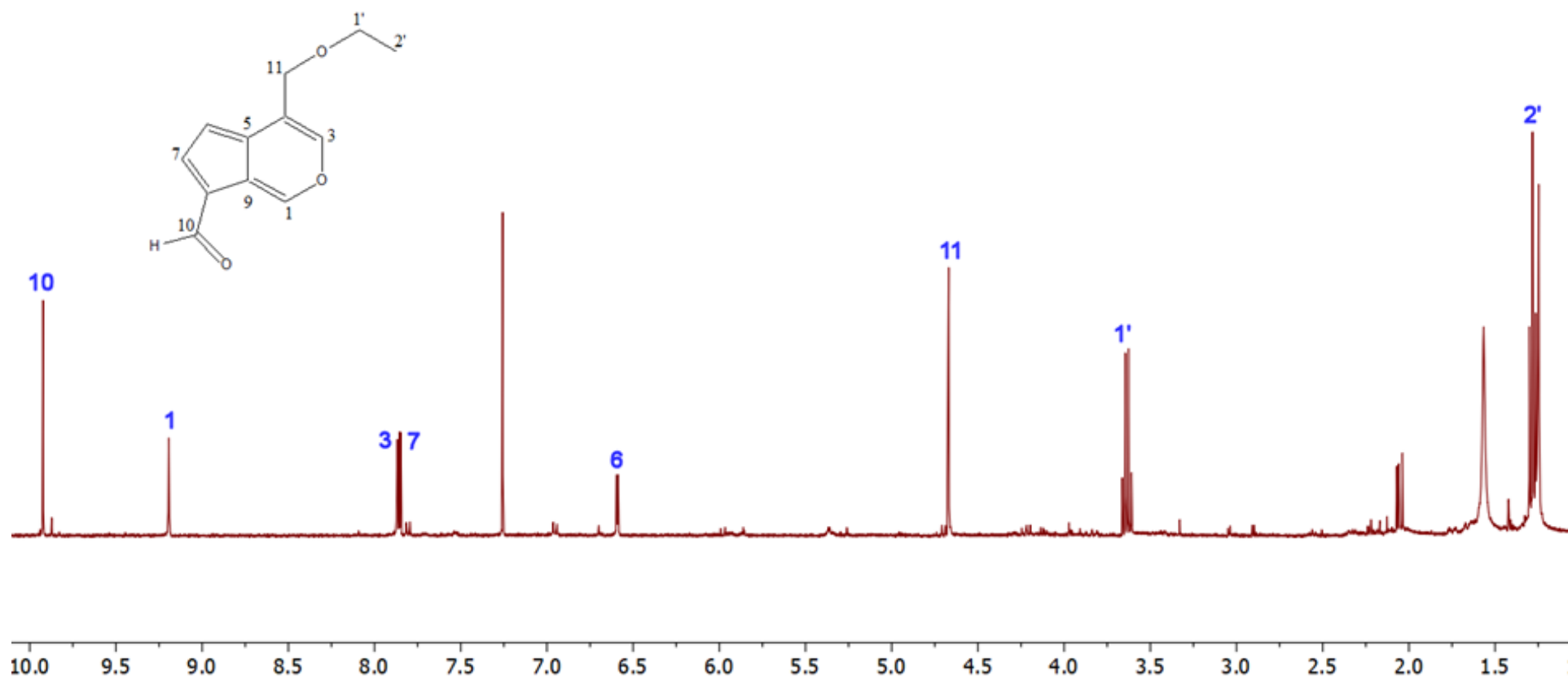
UV λ_{max} (MeOH) nm:	228, 245, 286, 417
IR ν_{max} (KBr) cm ⁻¹ :	1636 (aldehyde, C=O)
ESI-MS m/z :	205.52 [M+H] ⁺
¹ H NMR:	Table 94, Spectrum 79
¹³ C NMR:	Table 94, Spectrum 80
COSY:	Spectrum 81
HMBC:	Spectrum 82

Compound **20** was isolated as yellowish oil that analyzed for the molecular formula C₁₂H₁₂O₃ by ESI-MS at m/z 205.52 [M+H]⁺. The UV spectrum showed absorption maxima at 228, 245, 286 and 417 nm, which was characteristic for baldrinal-type compounds. The IR spectrum demonstrated an absorption band due to a conjugated aldehyde carbonyl (1636 cm⁻¹) group. The ¹H and ¹³C NMR findings (Table 94) closely resembled to those of **9** (baldrinal), except for presence of ethoxy group at 11th position in **20**, instead of acetoxy group. NMR findings of **20** were superimposed with those reported for 11-ethoxyviburtinal (194).

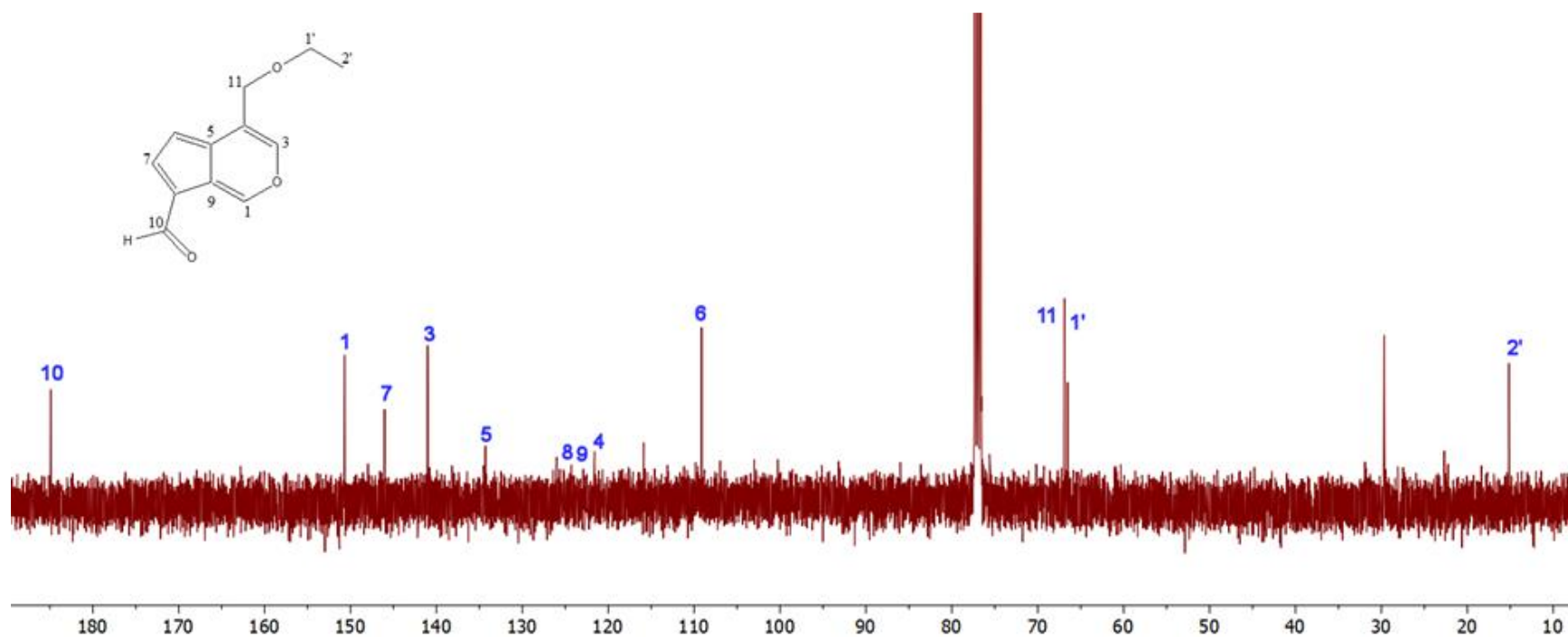
Table 94. ^{13}C and ^1H NMR data of 11-ethoxyviburtinal (**20**) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), <i>J</i> (Hz)
1	CH	150.7	9.19 s
3	CH	141.0	7.87 s
4	C	121.6	-
5	C	134.3	-
6	CH	109.1	6.59 dd (3.3, 0.9)
7	CH	146.1	7.85 d (3.3)
8	C	126.0	-
9	C	124.3	-
10	CH	184.9	9.92 s
11	CH ₂	66.9	4.67 s
<i>Ethyl (C-11)</i>			-
1'	CH ₂	66.5	3.64 q (7.0)
2'	CH ₃	15.1	1.28 t (7.0)

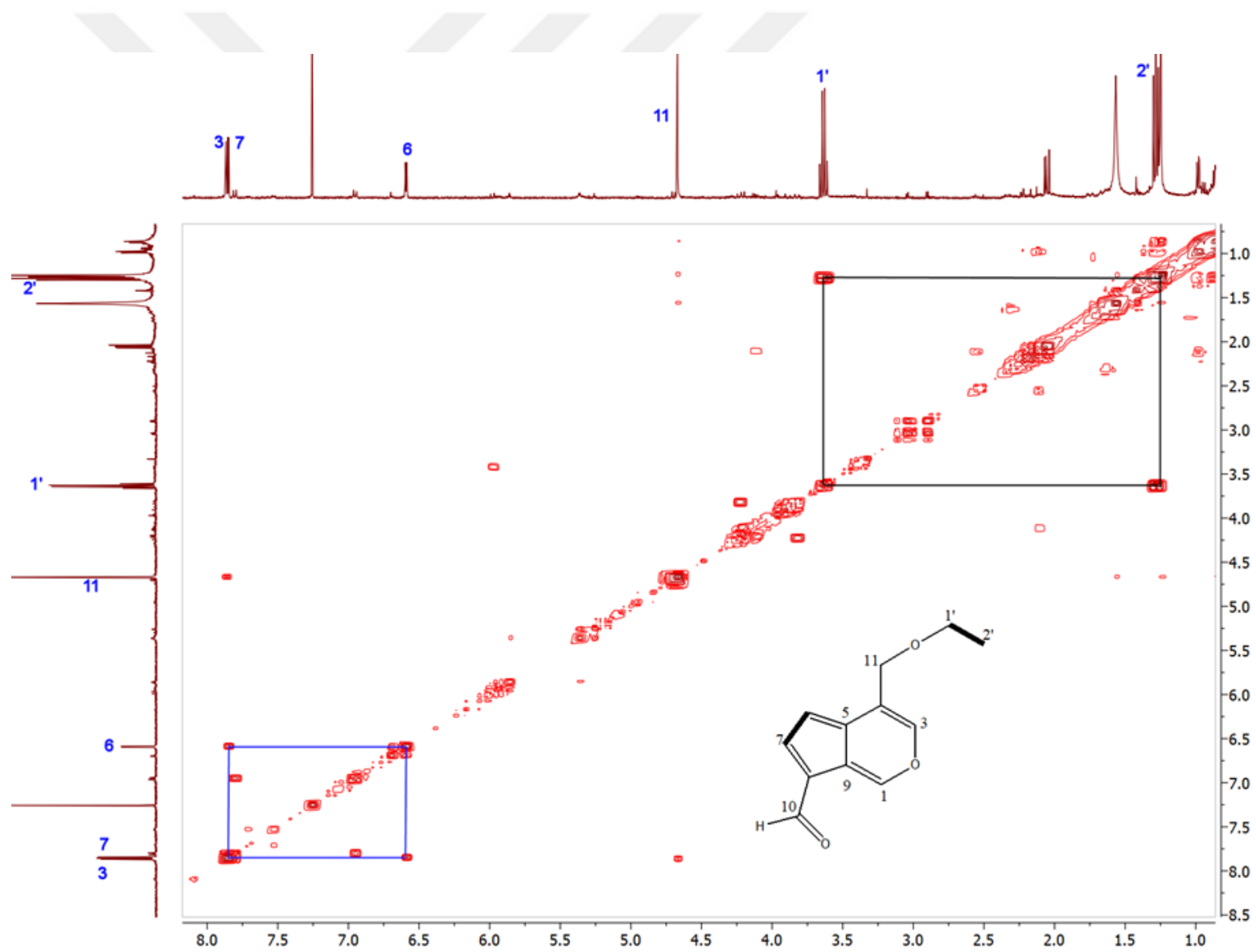
^aAssignments are based on COSY, HSQC and HMBC experiments.



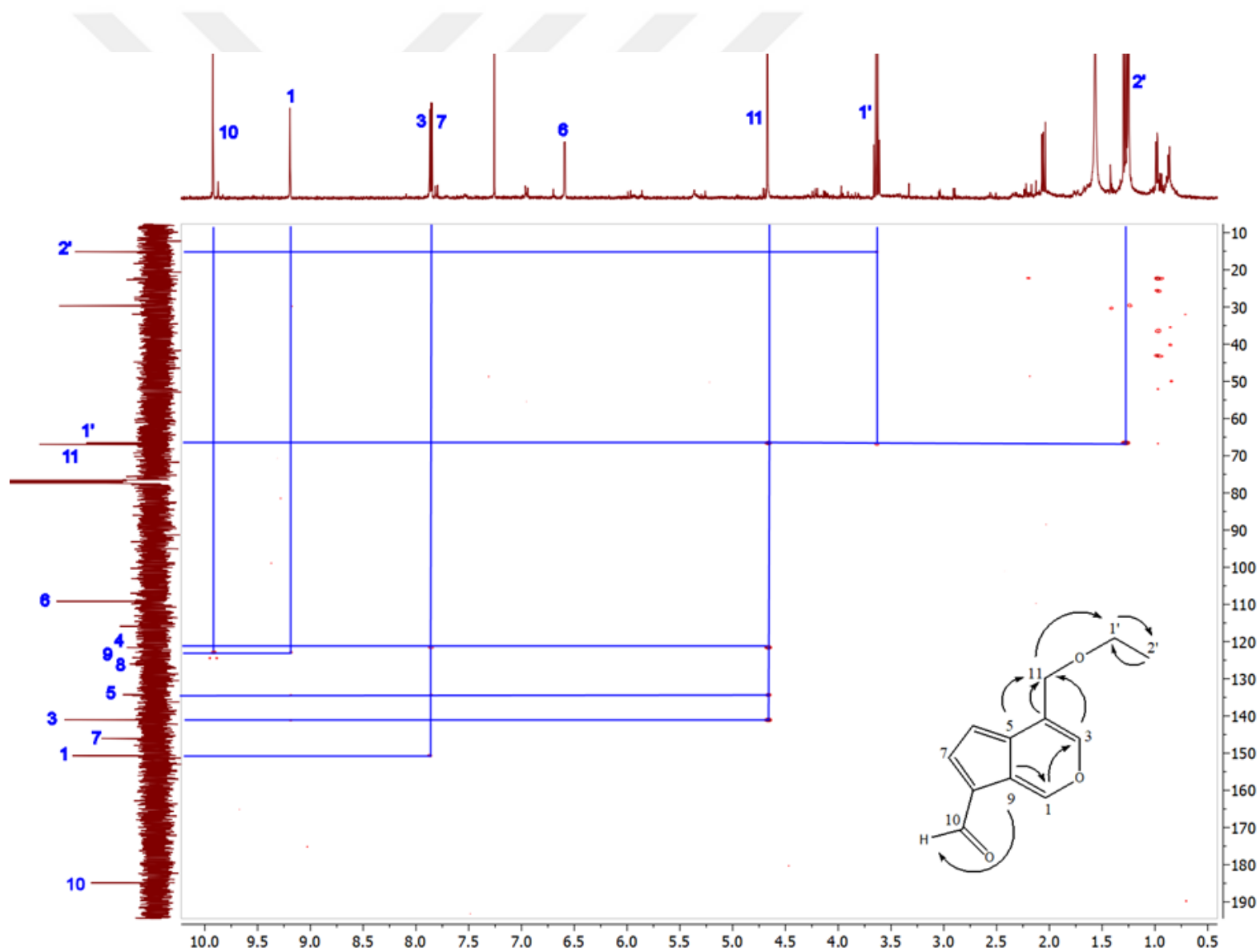
Spectrum 79. ^1H NMR Spectrum of 11-Ethoxyviburtinal (**20**) (CDCl_3 , 400 MHz)



Spectrum 80. ^{13}C NMR Spectrum of 11-Ethoxyviburtinal (**20**) (CDCl_3 , 100 MHz)

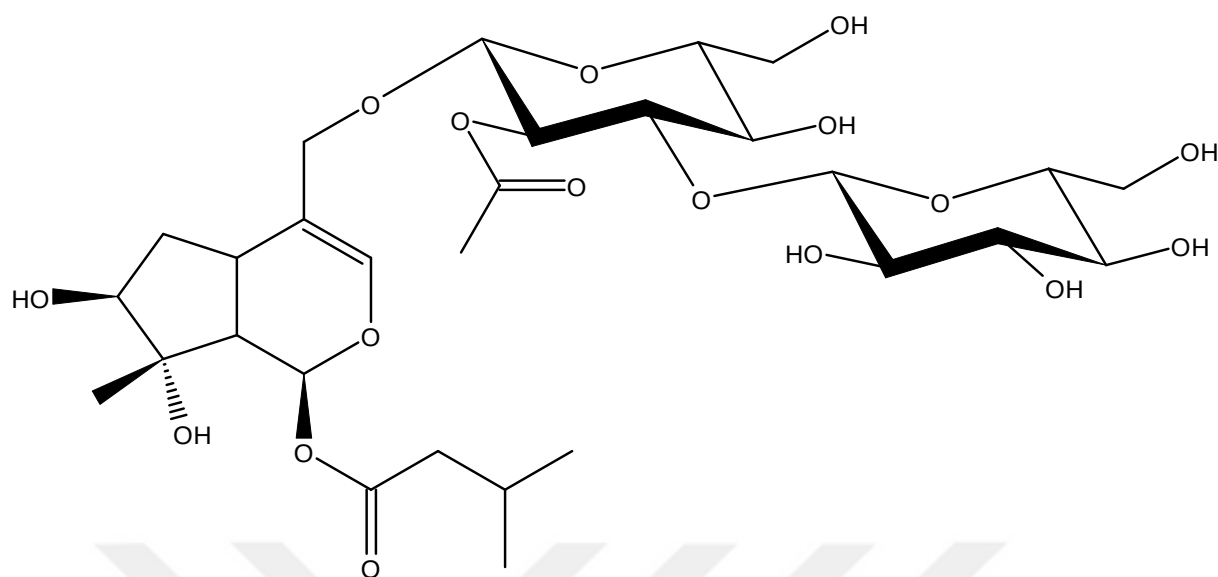


Spectrum 81. COSY Spectrum of 11-Ethoxyviburtinal (20)



Spectrum 82. HMBC Spectrum of 11-Ethoxyviburtinal (20)

4.5.1.3.1.B. Iridoids Glycoside



SISYMBRIIFOLIOSIDE (18): C₂₉H₄₆O₁₇ (MW:666.67)

UV $\lambda_{\max}$ (MeOH) nm:	207
IR $\nu_{\max}$ (KBr) cm ⁻¹ :	3230 (OH), 1735 (C=O), 1634, 1583 (olefinic, C=C)
HR-ESI-MS m/z :	689.2626 [M+Na] ⁺ (calcd for C ₂₉ H ₄₆ O ₁₇ Na, 689.2627), 667.2800 [M + H] ⁺ (calcd for C ₂₉ H ₄₇ O ₁₇ , 667.2808)
¹ H NMR:	Table 95, Spectrum 83
¹³ C NMR:	Table 95, Spectrum 84
COSY:	Spectrum 85
HSQC:	Spectrum 86
HMBC:	Spectrum 87
NOESY:	Spectrum 88

SISYMBRIIFOLIOSIDE (18)

Compound **18** was obtained as an amorphous powder. Its molecular formula was deduced to be $C_{29}H_{46}O_{17}$, on the basis of pseudomolecular ion peaks at m/z 689.2626 $[M + Na]^+$ (calcd for $C_{29}H_{46}O_{17}Na$, 689.2627) and m/z 667.2800 $[M + H]^+$ (calcd for $C_{29}H_{47}O_{17}$, 667.2808) in the HR-ESI-MS spectrum, indicating seven degrees of unsaturation. Its IR spectrum showed absorption bands due to the hydroxyl (3230 cm^{-1}), ester carbonyl (1735 cm^{-1}) and olefinic (1634 and 1583 cm^{-1}) groups.

The 1H NMR spectrum (Table 95) of **18** displayed signals for an olefinic methine (δ_H 6.27, s), a hemiacetal hydrogen (δ_H 6.16, d, $J = 3.9\text{ Hz}$), an oxymethine (δ_H 3.74) and an oxymethylene [δ_H 4.20 and 4.08 (each d, $J = 11.5\text{ Hz}$)] arising from an iridoid core (249). The presence of two anomeric signals at δ_H 4.54 (d, $J = 8.0\text{ Hz}$) and 4.35 (d, $J = 7.8\text{ Hz}$) in its 1H NMR spectrum indicated its diglycosidic nature. The spectrum also contained an acetyl methyl (δ_H 2.10, s) signal as well as the characteristic signals for isovaleroyl unit [one methylene (δ_H 2.22, d, $J = 6.5\text{ Hz}$, H-2'), one methine (δ_H 2.07, m, H-3') and two equivalent methyl (δ_H 0.97, d, $J = 6.6\text{ Hz}$, H-4'/5') signals (12). The ^{13}C NMR and HSQC spectra (Table 95, Spectra 84 and 86) of **18** revealed 29 carbon resonances, 12 of them were ascribed to two β -glucopyranose units, five signals were ascribed to an isovaleroyl moiety, and two carbons were assigned to an acetyl unit. Thus, the remaining carbon signals were characteristic of a C_{10} iridoid aglycone. The comprehensive analysis of 2D NMR experiments (COSY, HSQC and HMBC) suggested that NMR data of **18** were identical to those of compound **8** (valerosidate) except for the presence of additional signals due to second β -glucopyranose and an acetyl unit in **18**. The downfield shift for C-3'' (δ_C 85.5) of the inner glucose around 8 ppm and the key HMBC correlation (Spectrum 87) between C-3'' and H-1''' (δ_H 4.35) clearly indicated that the second β -glucopyranosyl unit was attached to C-3''(OH). Thus, the disaccharide unit was identified as β -glucopyranosyl (1 $\rightarrow$ 3)- β -glucopyranoside. The binding site of this disaccharide unit to the aglycone was assigned from the long-range interactions between C-1'' (δ_C 100.8) and H_{1,2}-11 (δ_H 4.20 and 4.09) in the HMBC experiment. The sites of acylations were determined by downfield shifts for H-1 (δ_H 6.16) and H-2'' (δ_H 4.88) as well as HMBC experiment. The acetyl carbonyl at δ_C 172.0 displayed long-range coupling with H-2'' (δ_H 4.88) of inner glucose whereas isovaleroyl carbonyl at δ_C 173.3 coupled with H-1 (δ_H 6.16) of aglycone in the HMBC spectrum.

The NOESY correlation (Spectrum 88) between H-5 and H-9, and between H-9/Me-10 suggested that H-5, H-9 and Me-10 were β -oriented. The coupling constants of $J_{1-9}=3.8$ Hz and $J_{5-9}=10.2$ Hz confirmed the β -orientations of H-5 and H-9 as well as the α -orientation of H-1 (249). The absence of NOE correlations between Me-10 and H-7 revealed that they lie on the opposite side of the plane. To the best of our knowledge compound **18** is reported for the first time and named as **sisymbriifolioside**.

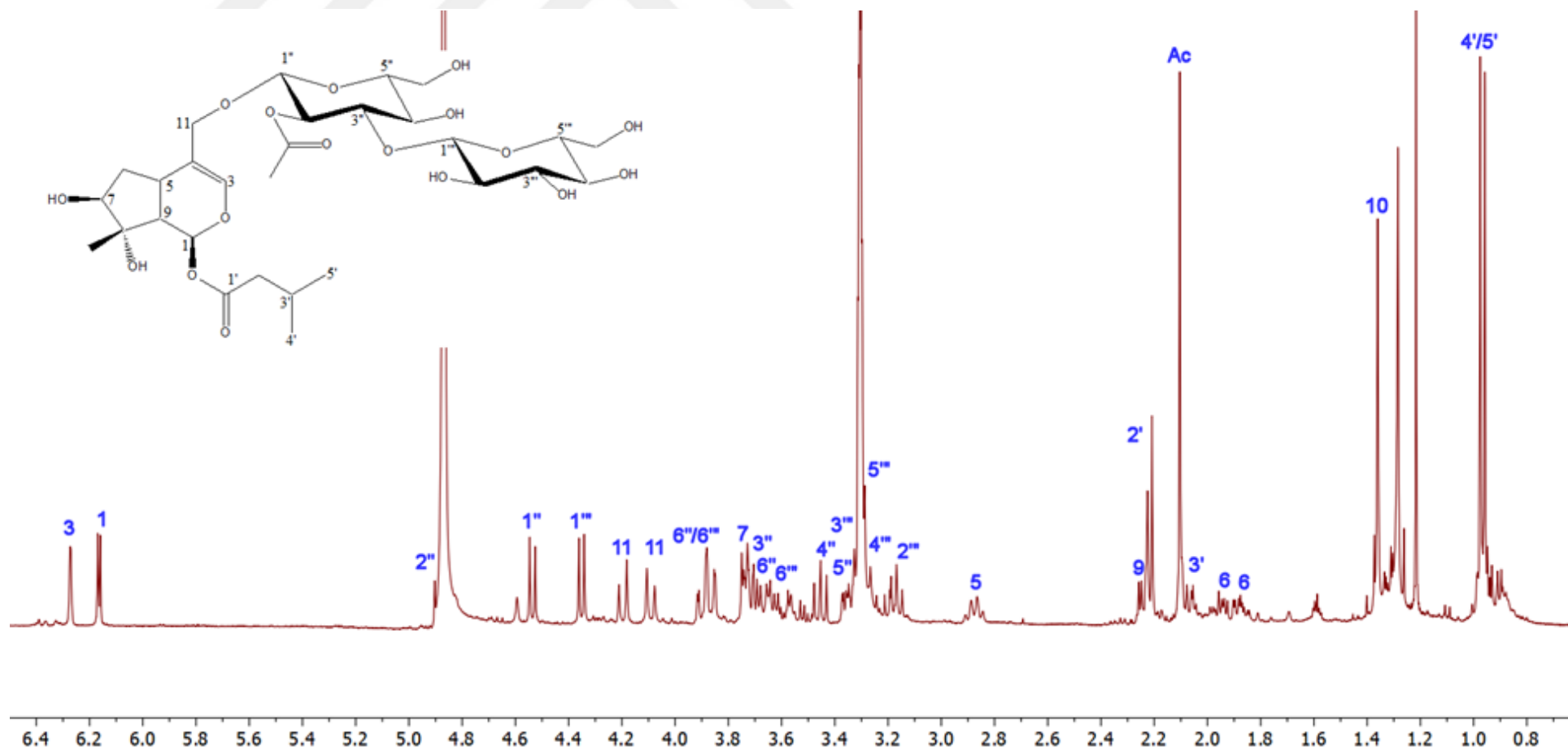


Table 95. ^{13}C and ^1H NMR data of sisymbriifolivaltrate (**18**) (CD_3OD ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

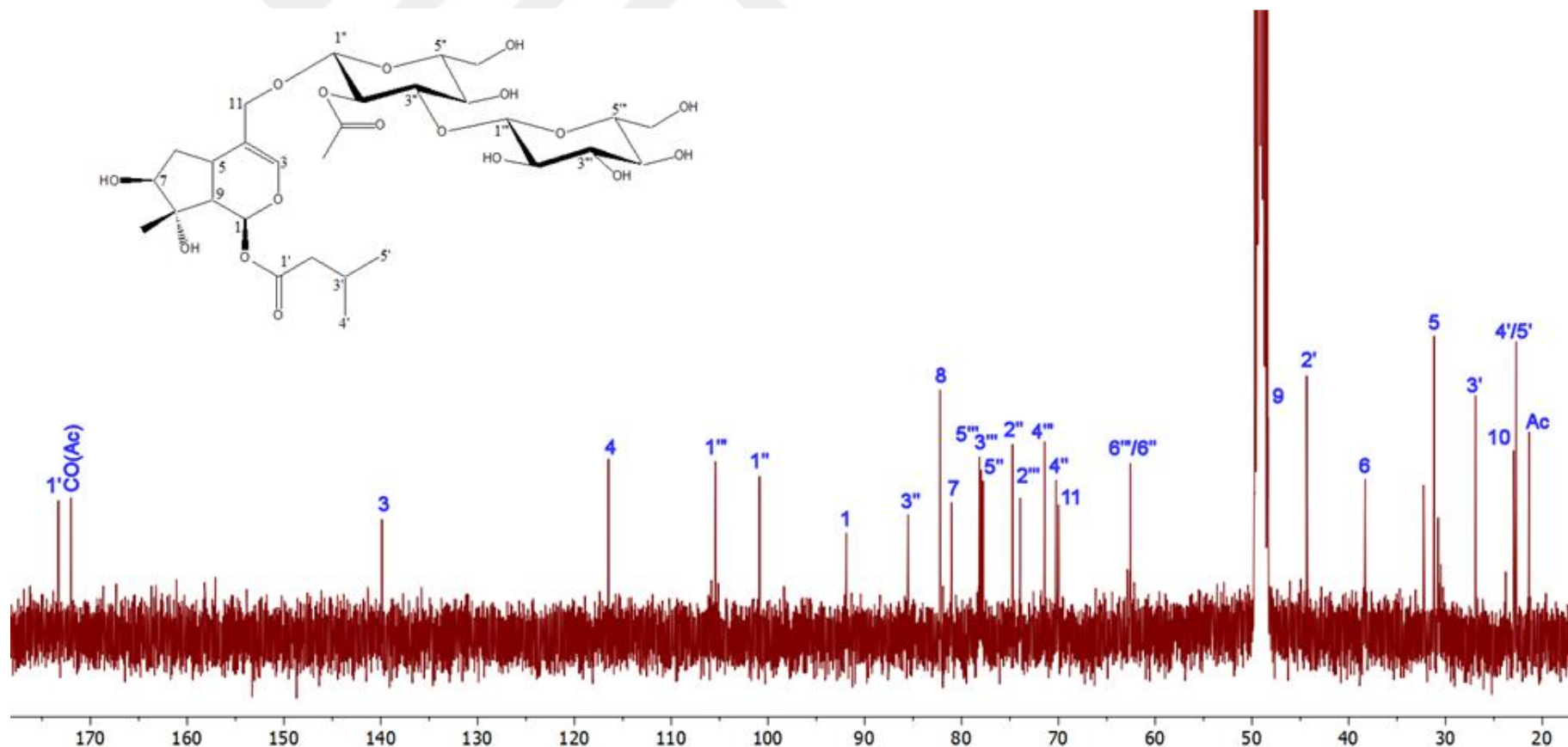
C/H Atom	Multiplicity	$\delta_{\text{C}}(\text{ppm})$	$\delta_{\text{H}}(\text{ppm}), J (\text{Hz})$
<i>Aglycone</i>			
1	CH	91.9	6.16 d (3.9)
3	CH	139.9	6.27 s
4	C	116.5	-
5	CH	31.1	2.87 m
6	CH ₂	38.3	1.95 m 1.87 m
7	CH	81.0	3.74 †
8	C	82.2	-
9	CH	48.2	2.24 dd (10.2, 3.8)
10	CH ₃	22.9	1.36 s
11	CH ₂	70.0	4.20 d (11.5) 4.09 d (11.5)
<i>Isovaleroyl (C-1)</i>			
1'	C	173.3	-
2'	CH ₂	44.3	2.22 d (6.5)
3'	CH	26.8	2.07 m
4'/5'	CH ₃	22.7	0.97 d (6.6)
<i>Glucose (C-11)</i>			
1''	CH	100.8	4.54 d (8.0)
2''	CH	74.7	4.88 †
3''	CH	85.5	3.71 †
4''	CH	70.2	3.45 t (9.5)
5''	CH	77.8	3.36 m
6''	CH ₂	62.5	3.89 dd (11.9, 2.1) 3.70 dd (11.9, 5.5)
2''-COCH ₃	C	172.0	-
2''-COCH ₃	CH ₃	21.4	2.10 s
<i>Glucose (C-3'')</i>			
1'''	CH	105.4	4.35 d (7.8)
2'''	CH	73.9	3.17 t (8.7)
3'''	CH	78.0	3.32 †
4'''	CH	71.4	3.27 t (9.3)
5'''	CH	78.1	3.30 m
6'''	CH ₂	62.6	3.87 dd (12.1, 1.8) 3.63 dd (12.1, 5.7)

^aAssignments are based on COSY, HSQC, HMBC and NOESY experiments.

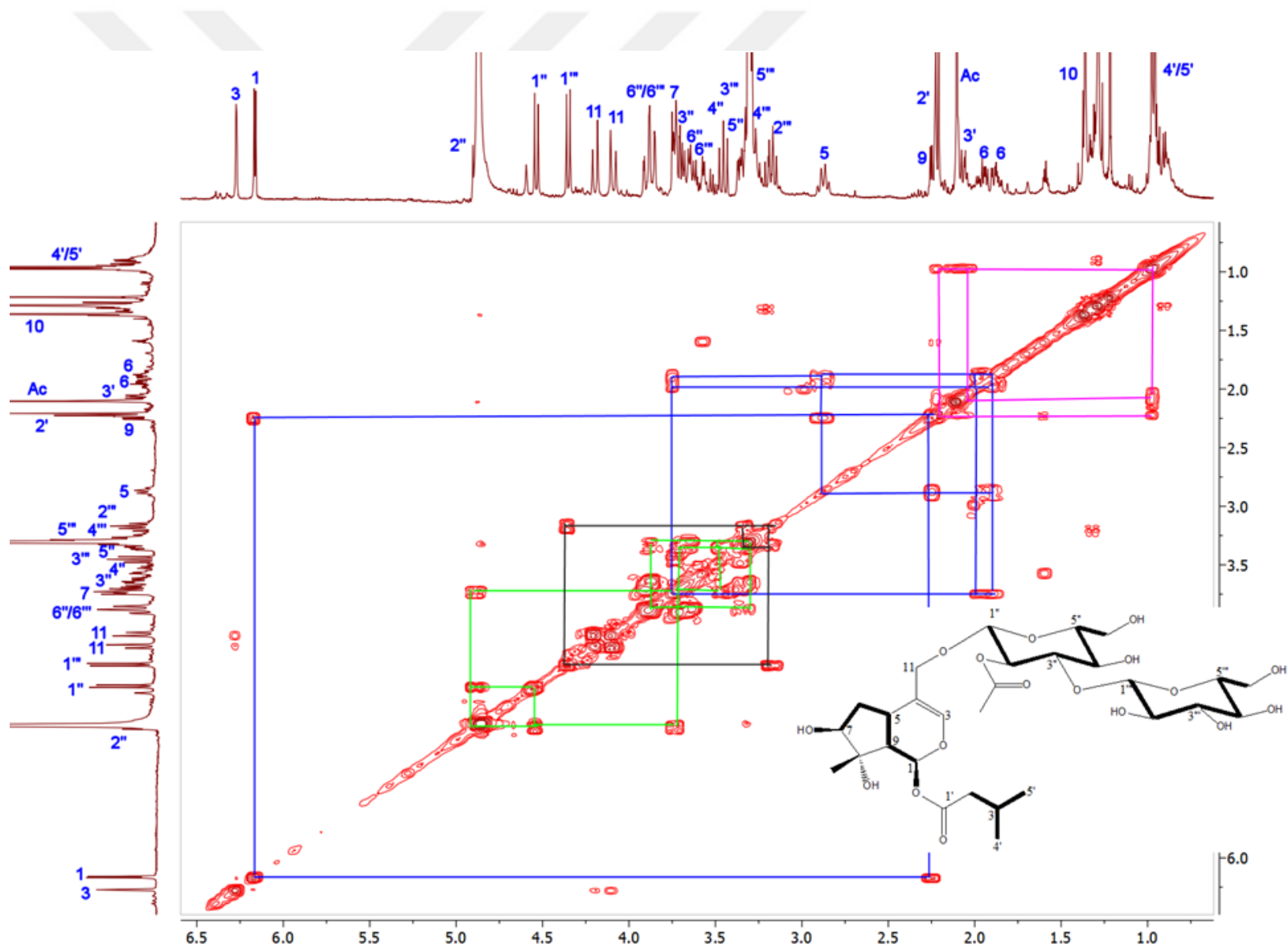
†: Overlapped signals.



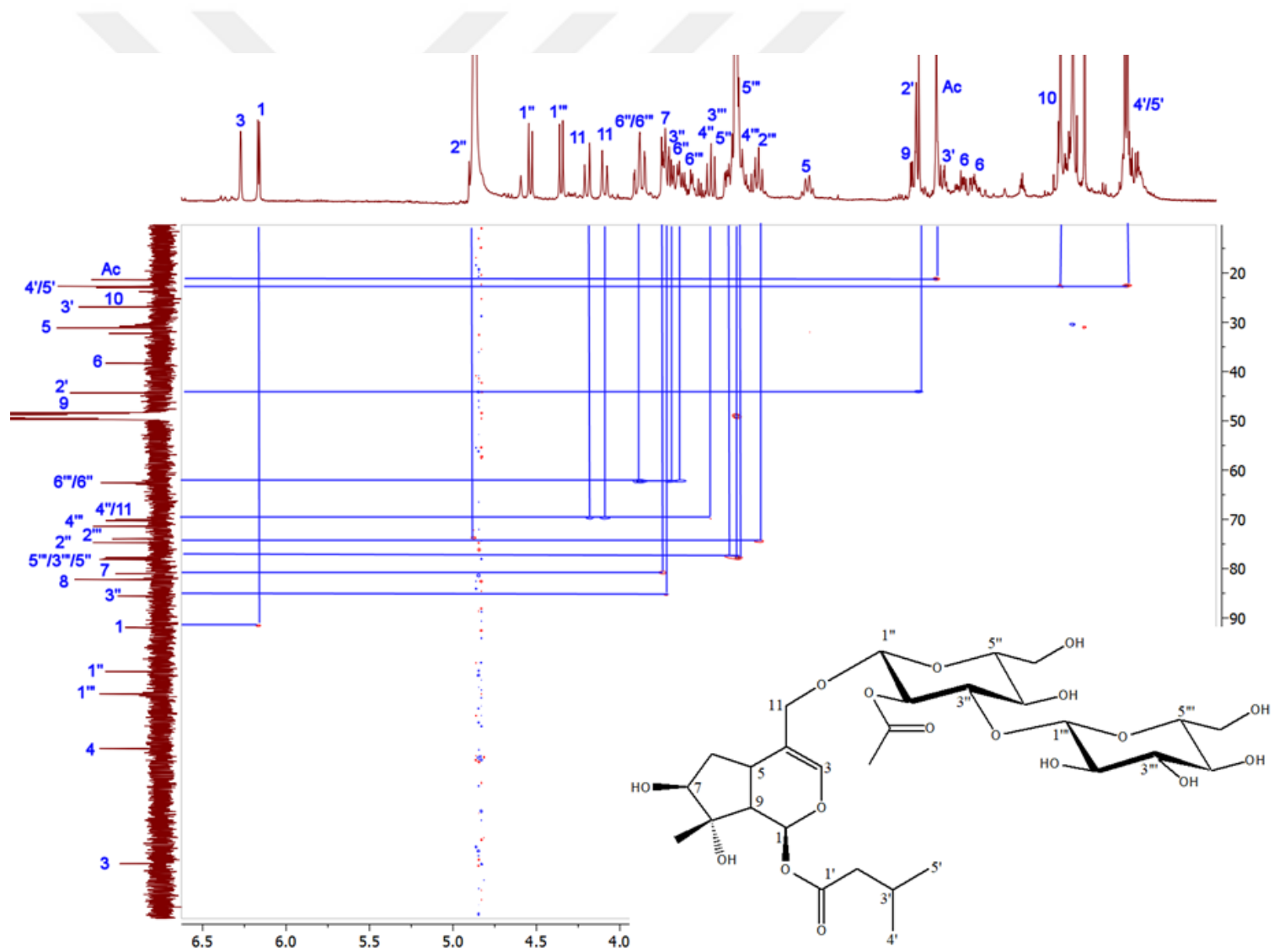
Spectrum 83. ^1H NMR Spectrum of Sisymbriifolioside (**18**) (CD₃OD, 400 MHz)



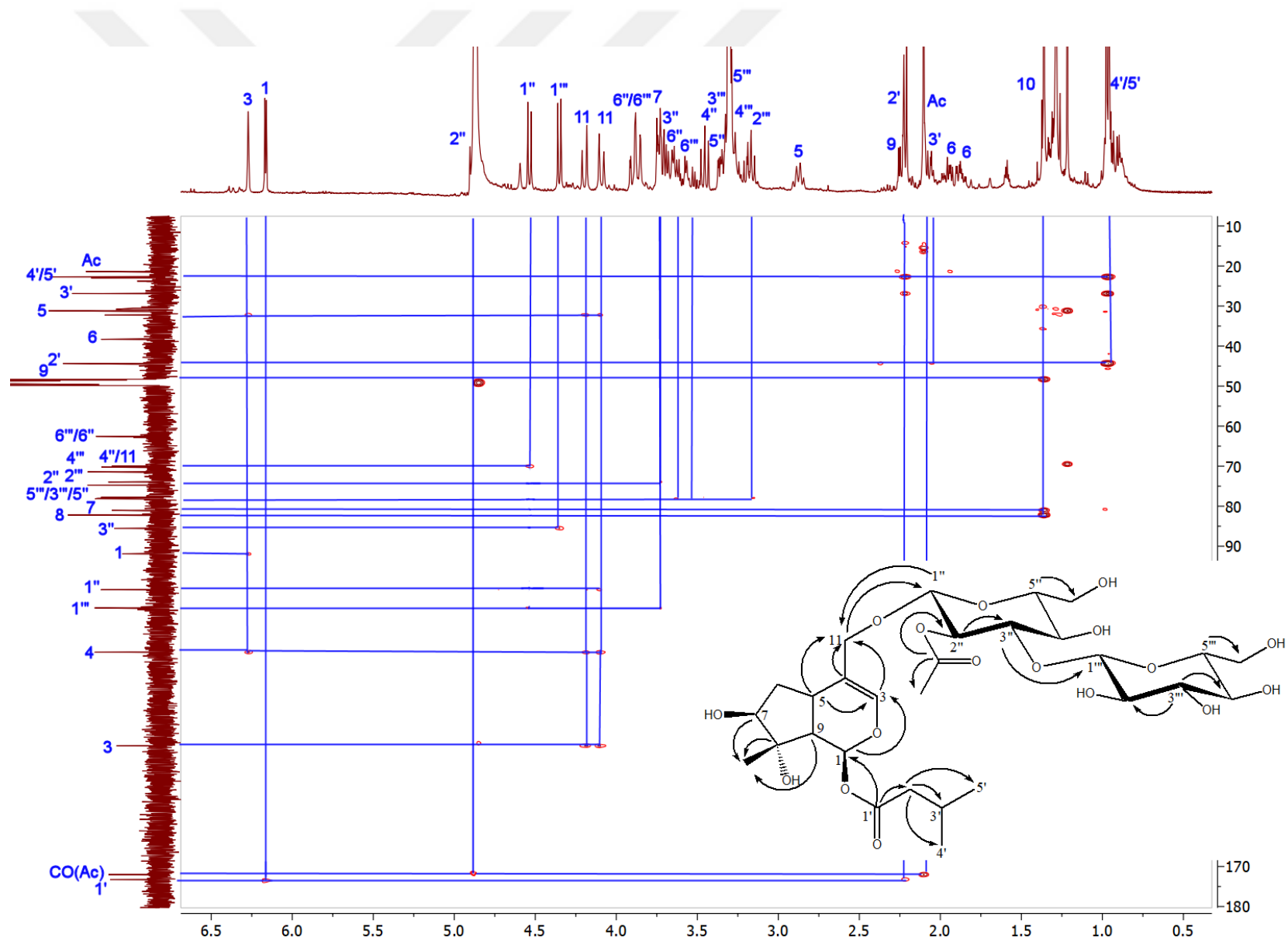
Spectrum 84. ¹³C NMR Spectrum of Sisymbriifolioside (**18**) (CD₃OD, 100 MHz)



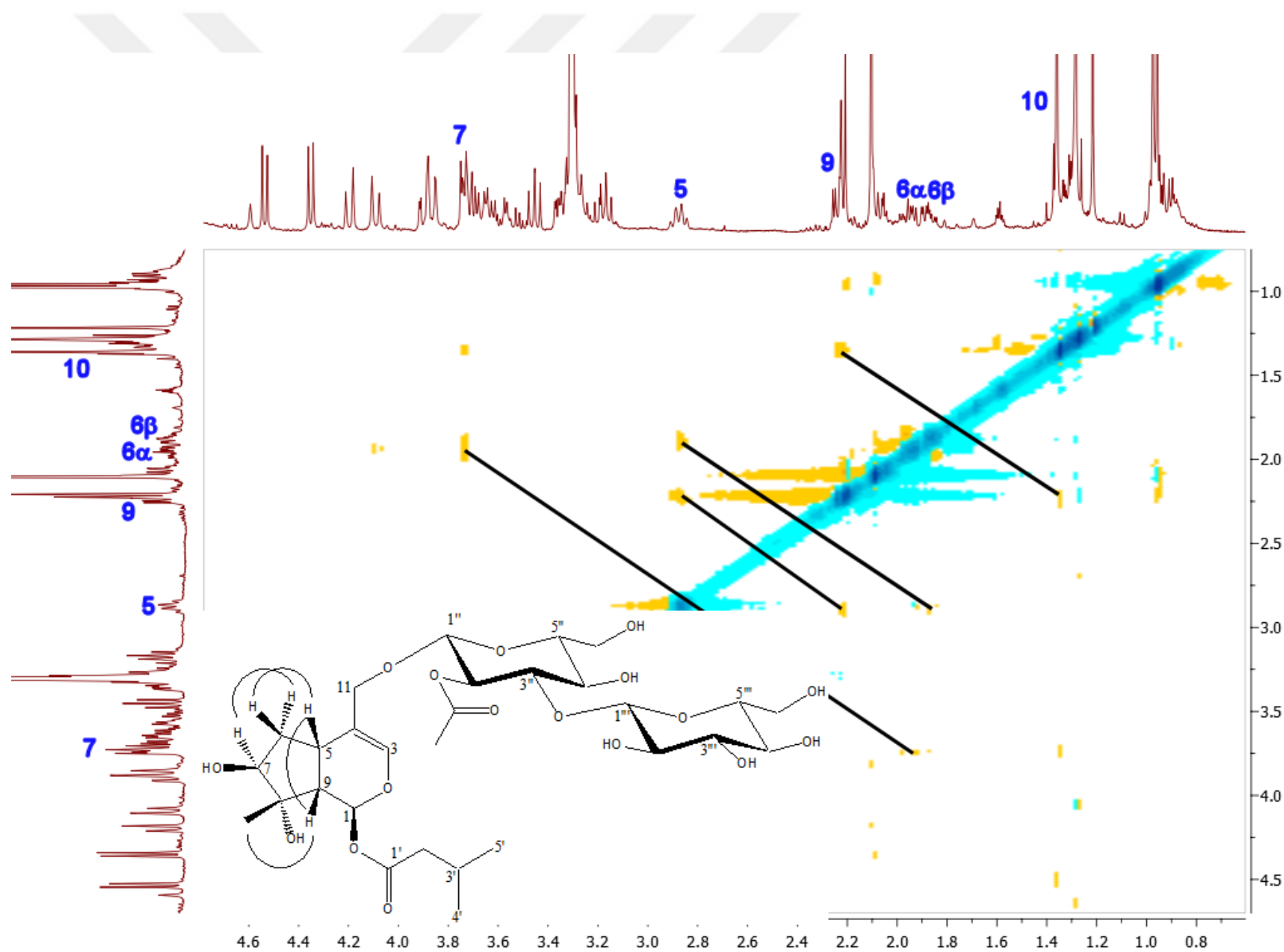
Spectrum 85. COSY Spectrum of Sisymbriifolioside (**18**)



Spectrum 86. HSQC Spectrum of Sisymbriifolioside (**18**)

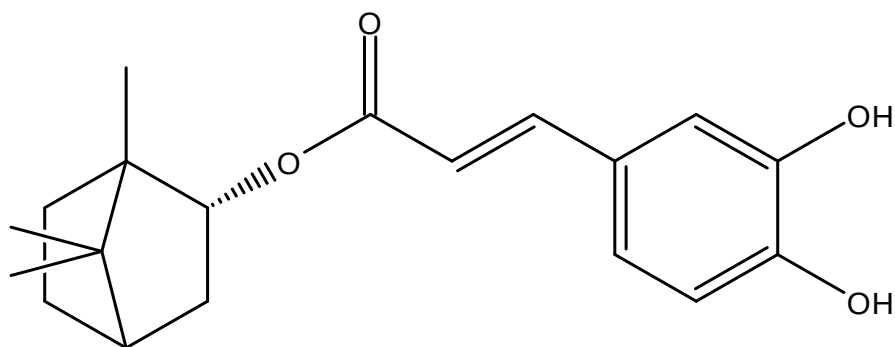


Spectrum 87. HMBC Spectrum of Sisymbriifolioside (**18**)



Spectrum 88. NOESY Spectrum of Sisymbriifolioside (18)

4.5.1.3.2. Monoterpene Ester



BORNYL CAFFEATE (21): C₁₉H₂₄O₄ (MW:316.39)

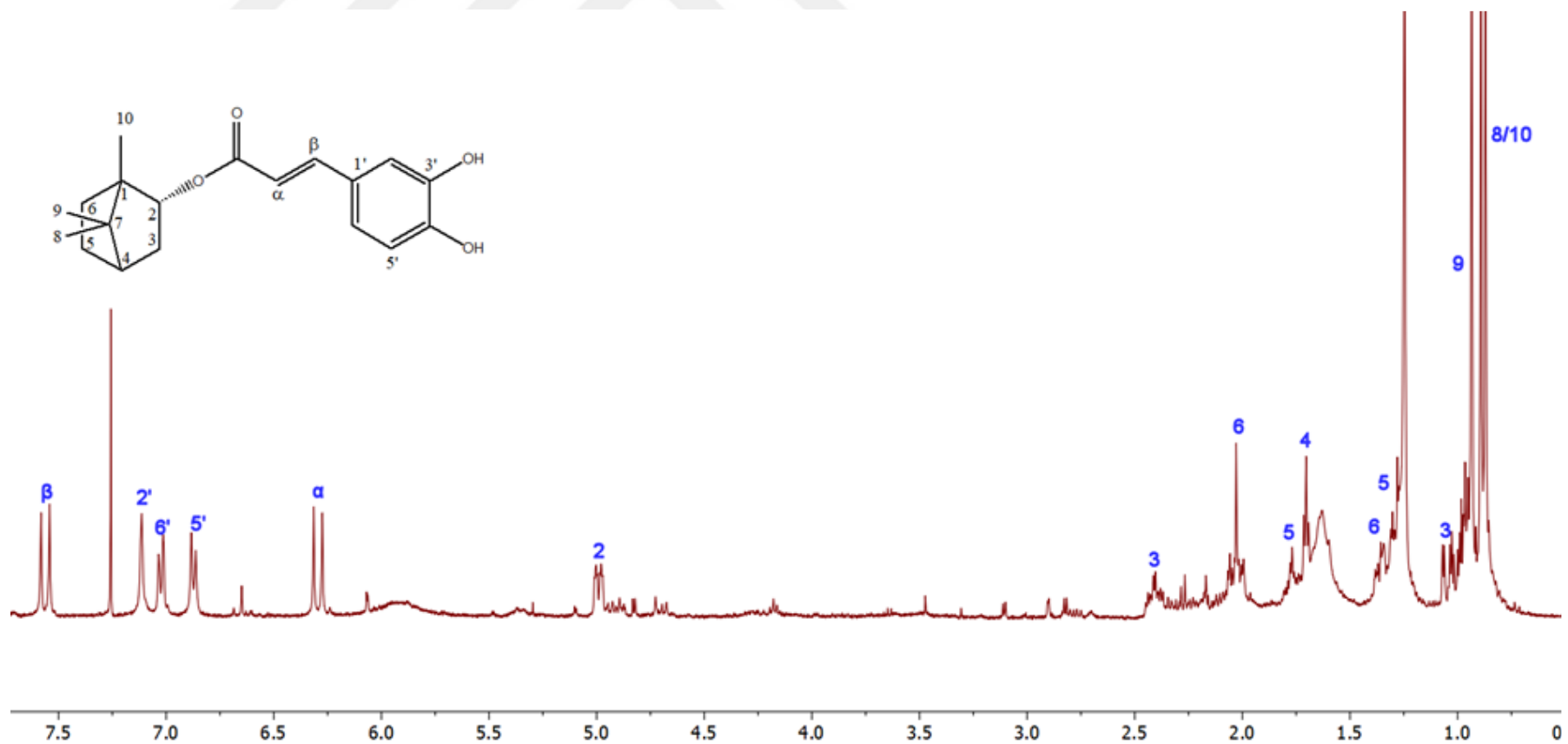
UV λ_{max} (MeOH) nm:	241, 307, 330
IR ν_{max} (KBr) cm ⁻¹ :	3354 (OH), 1707 (C=O), 1633, 1605 (olefinic, C=C), 1516 (aromatic, C=C)
ESI-MS m/z :	315.80 [M-H] ⁻
¹ H NMR:	Table 96, Spectrum 89
¹³ C NMR:	Table 96, Spectrum 90

Compound **21** was isolated as white amorphous powder with the molecular formula C₁₉H₂₄O₄ as determined from negative ESI-MS (m/z 315.80 [M-H]⁻) and NMR spectra. The IR showed absorption bands for hydroxyl (3354 cm⁻¹), ester carbonyl (1707 cm⁻¹), olefinic (1633 and 1605 cm⁻¹) and aromatic ring (1516 cm⁻¹) groups. The UV spectrum displayed absorption maxima at 241, 307 and 330 nm. The ¹H and ¹³C spectroscopic findings (Table 96) of **21** were found to be superimposable with bornyl caffeate (256).

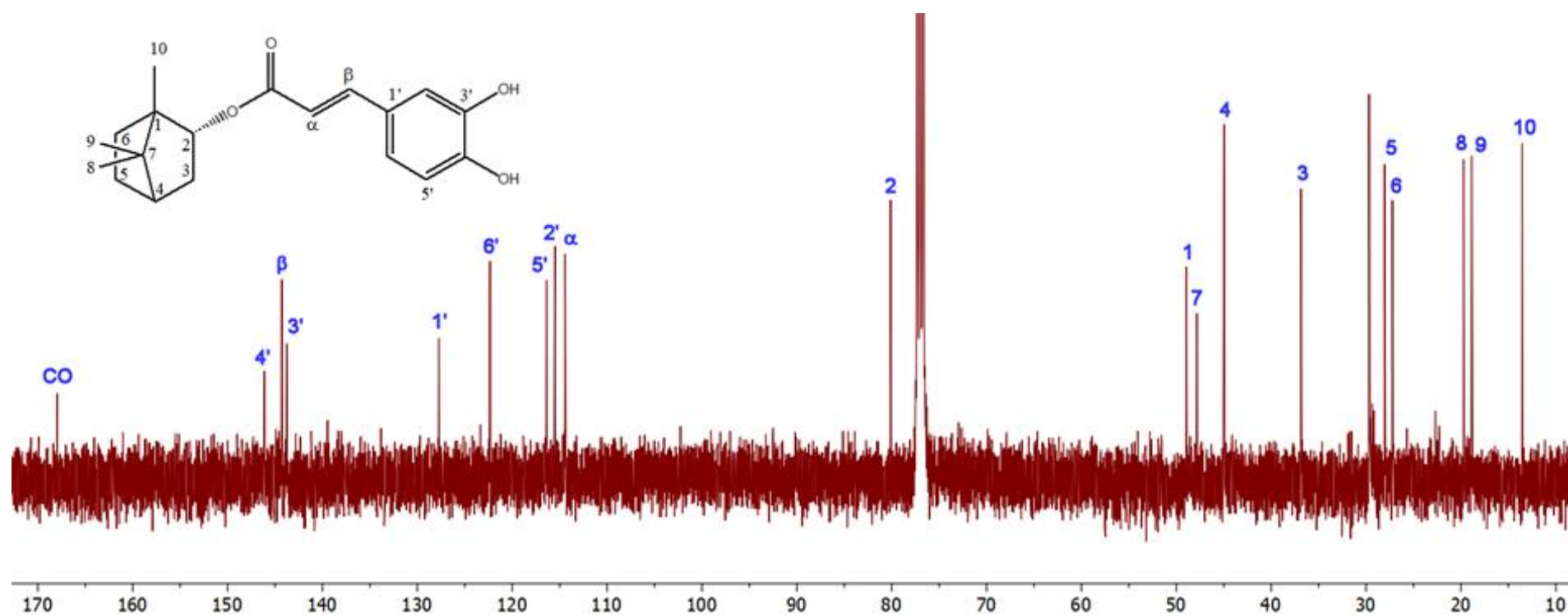
Table 96. ^{13}C and ^1H NMR data of bornyl caffeate (**21**) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)

C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
1	C	48.9	-
2	CH	80.1	4.99 †
3	CH ₂	36.8	2.41 † 1.05 dd (13.8, 3.5)
4	CH	44.9	1.70 m
5	CH ₂	28.0	1.77 m 1.28 m
6	CH ₂	27.2	2.03 m 1.35 m
7	C	47.8	-
8	CH ₃	19.7	0.89 s
9	CH ₃	18.9	0.93 s
10	CH ₃	13.6	0.87 s
<i>Caffeoyl (C-2)</i>			
1'	C	127.7	-
2'	CH ₂	115.5	7.11 d (2.0)
3'	C	143.7	-
4'	C	146.1	-
5'	CH	116.4	6.87 d (8.2)
6'	CH	122.3	7.02 dd (8.2, 2.0)
α	CH	114.4	6.30 d (15.9)
β	CH	144.3	7.56 d (15.9)
CO	C	168.0	-

† Overlapped signals.

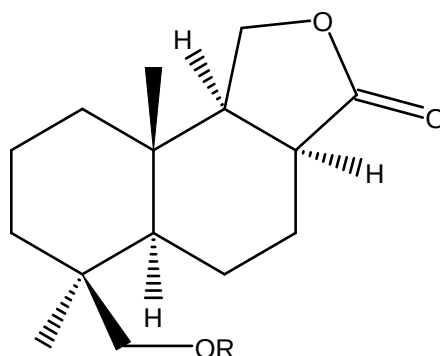


Spectrum 89. ¹H NMR Spectrum of Bornyl caffeate (21) (CDCl₃, 400 MHz)



Spectrum 90. ^{13}C NMR Spectrum of Bornyl caffeate (**21**) (CDCl_3 , 100 MHz)

4.5.1.3.2. Sesquiterpene Lactones



R
 22: H
 23: (*E*)-Caff.

SISYMBRIIFOLIN A (22): C₁₅H₂₄O₃ (MW:252.3493)

SISYMBRIIFOLIN B (23): C₂₄H₃₀O₆ (MW: 414.4914)

	Sisymbriifolin A (22)	Sisymbriifolin B (23)
UV $\lambda_{\max}$ (MeOH) nm:	205	206, 245, 297, 329
IR $\nu_{\max}$ (KBr) cm ⁻¹ :	3341 (OH), 1757 (C=O)	3366 (OH), 1754 (C=O), 1655 (aromatic, C=C)
HR-ESI-MS m/z :	253.1814 [M + H] ⁺ (calcd for C ₁₅ H ₂₅ O ₃ , 253.1804)	415.2077 [M + H] ⁺ (calcd for C ₂₄ H ₃₁ O ₆ , 415.2115)
¹ H NMR:	Table 97, Spectrum 91	Table 97, Spectrum 97
¹³ C NMR:	Table 97, Spectrum 92	Table 97, Spectrum 98
COSY:	Spectrum 93	Spectrum 99
HSQC:	Spectrum 94	Spectrum 100
HMBC:	Spectrum 95	Spectrum 101
NOESY:	Spectrum 96	Spectrum 102

SISYMBRIIFOLIN A (22)

Compound **22** was obtained as a colorless solid. The HRESIMS of **22** exhibited a quasimolecular ion peak at m/z 253.1814 ($[M + H]^+$, calcd 253.1804 for $C_{15}H_{25}O_3$), describing its molecular formula as $C_{15}H_{24}O_3$. The IR absorption bands demonstrated at 3341 and 1757 cm^{-1} revealed the presence of hydroxyl and δ -lactone carbonyl groups, respectively (257). The 1H NMR spectrum of **22** (Table 97) contained signals due to two oxymethylene [δ_H 4.26 (d, $J = 9.7$ Hz), 4.23 (dd, $J = 9.7, 5.4$ Hz; δ_H 3.70 and 3.27 (each d, $J = 11.1$ Hz)] and two methyl (δ_H 0.95 s and 0.80 s) groups. The ^{13}C NMR and HSQC spectra (Table 97 and Spectrum 94) suggested the presence of 15 carbon signals including one carbonyl (δ_C 181.7), two oxymethylene (δ_C 69.3 and 64.8), two quaternary carbon (δ_C 36.5 and 36.4) and two methyl (δ_C 27.6 and 15.9) groups signals. The complete 1H and ^{13}C NMR signal assignments and linkages between fragments were described from a combination of extensive HSQC, 1H - 1H COSY, and HMBC analyses. The spectroscopic findings showed that **22** possessed a drimane-type sesquiterpene lactone core (258). According to HSQC spectrum, C-4 (δ_C 39.4) and C-10 (δ_C 36.5) were found as quaternary carbons by lack of correlations which was also confirmed by the 1H - 1H COSY experiment (Spectrum 93) exhibiting two spin systems [$(H_2-1 \rightarrow H_2-2 \rightarrow H_2-3)$ and $(H-5 \rightarrow H_2-6 \rightarrow H_2-7 \rightarrow H-8 \rightarrow H-9 \rightarrow H_2-11)$]. The exact location of lactone carbonyl (δ_C 181.7) was deduced from its long-range correlation with H_2-7 (δ_H 2.21) and H_2-11 (δ_H 4.26) in the HMBC spectrum (Spectrum 95) as well as the downfield shifts for H_2-11 (δ_H 4.26 and 4.16) signals than expected (259). The location of methyl group (H_3-15) at δ_H 0.80 was found to be C-10 on the basis of HMBC correlations of C-10 (δ_C 36.5), C-5 (δ_C 53.3), C-9 (δ_C 51.5) and C-1 (δ_C 41.3) with H_3-15 . Likewise, the long-range couplings of C-3 (δ_C 36.4) and C-14 (δ_C 64.8) with H_3-13 (δ_H 0.95) indicated that the H_3-13 is attached at C-4. The above NMR findings were found to be similar to those of kongiiline A (258), except for the absence of acyloxy group at the 6th position in **22**, and opposite configurations of the methyl and hydroxymethylene substituents at C-4.

The relative configuration of the **22** was described by a NOESY experiment (Spectrum 96) combined with considering 1H - 1H coupling constants. The NOE cross-peak between H_3-15 and H_2-14 indicated that they lie on the β side of the molecule. On the contrary, the NOE coupling of $H-5\alpha$ with H_3-13 and $H-8$ with $H-9$ showed that they have α -configuration. Accordingly, the structure of compound **22** was identified and named as **sisymbriifolin A**.

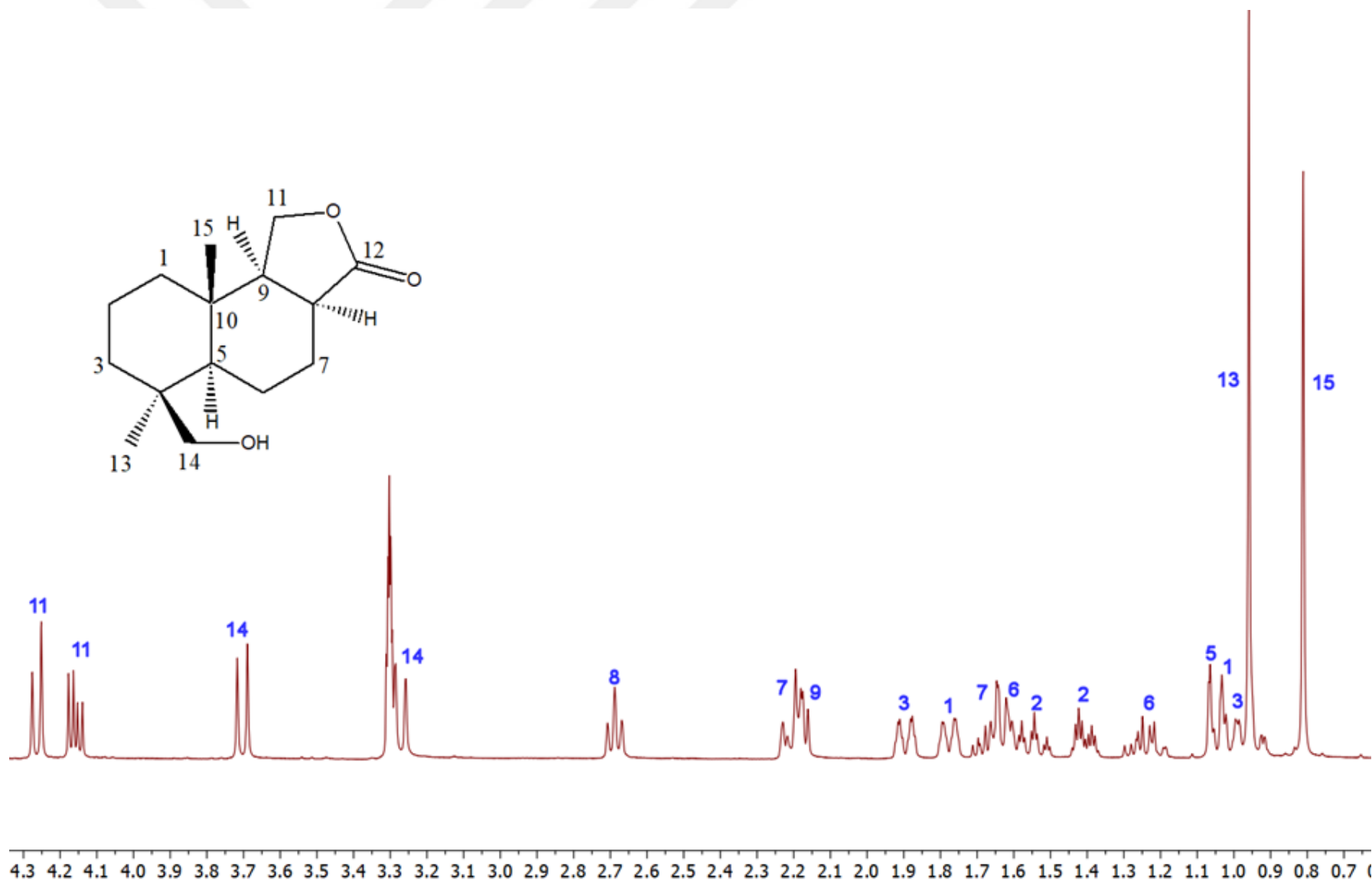
SISYMBRIIFOLIN B (23)

Compound **23** was obtained as a colorless solid. Its molecular formula was assigned to be $C_{24}H_{30}O_6$ by the HRESIMS at m/z 415.2077 $[M + H]^+$ (calcd for $C_{24}H_{31}O_6$, 415.2115), exhibiting ten degrees of unsaturation. Its UV (λ_{max} 245, 297 and 329 nm) and IR spectra (1655 cm^{-1}) indicated the presence of an aromatic ring in **23**. The 1H and ^{13}C NMR data of **23** (Table 97) were very similar to those of **22**, suggesting that they had the same skeleton, except for the presence of signals corresponding to (*E*)-caffeoyl moiety in **23** which was deduced from characteristic signals of three aromatic protons assignable to an *ABX* system [δ_H 7.03, (d, $J = 2.0$ Hz), 6.94, (dd, $J = 8.2, 2.0$ Hz) and 6.77 (d, $J = 8.2$ Hz)] as well as two olefinic signals as an *AX* system [δ_H 7.52 and 6.24 (each d, $J_{AX}=15.9$ Hz)]. Furthermore, additional nine carbon resonances belonging to the *trans*-caffeoyl group were observed in ^{13}C NMR spectrum of **23**. The exact location of caffeoyl unit was identified by the downfield shifts of oxymethylene [δ_H 4.43 and 3.95 (each d, $J = 11.1$ Hz), H₂-14] signals around 0.7 ppm when compared to those in compound **22**. This downfield shift could be described as an ester effect in consequence of the esterification of (*E*)-caffeoyl unit at C-14. Also, the key HMBC correlation (Spectrum 101) of carbonyl carbon (δ_C 169.6) to oxymethylene proton (δ_H 4.43, H₁-14) confirmed the esterification point. The relative configuration of **23** was found to be the same as with **22** based on the NOESY experiment (Spectrum 102). Consequently, compound **23** was identified as (*E*)-caffeoyl ester of sisymbriifolin A and named as **sisymbriifolin B**.

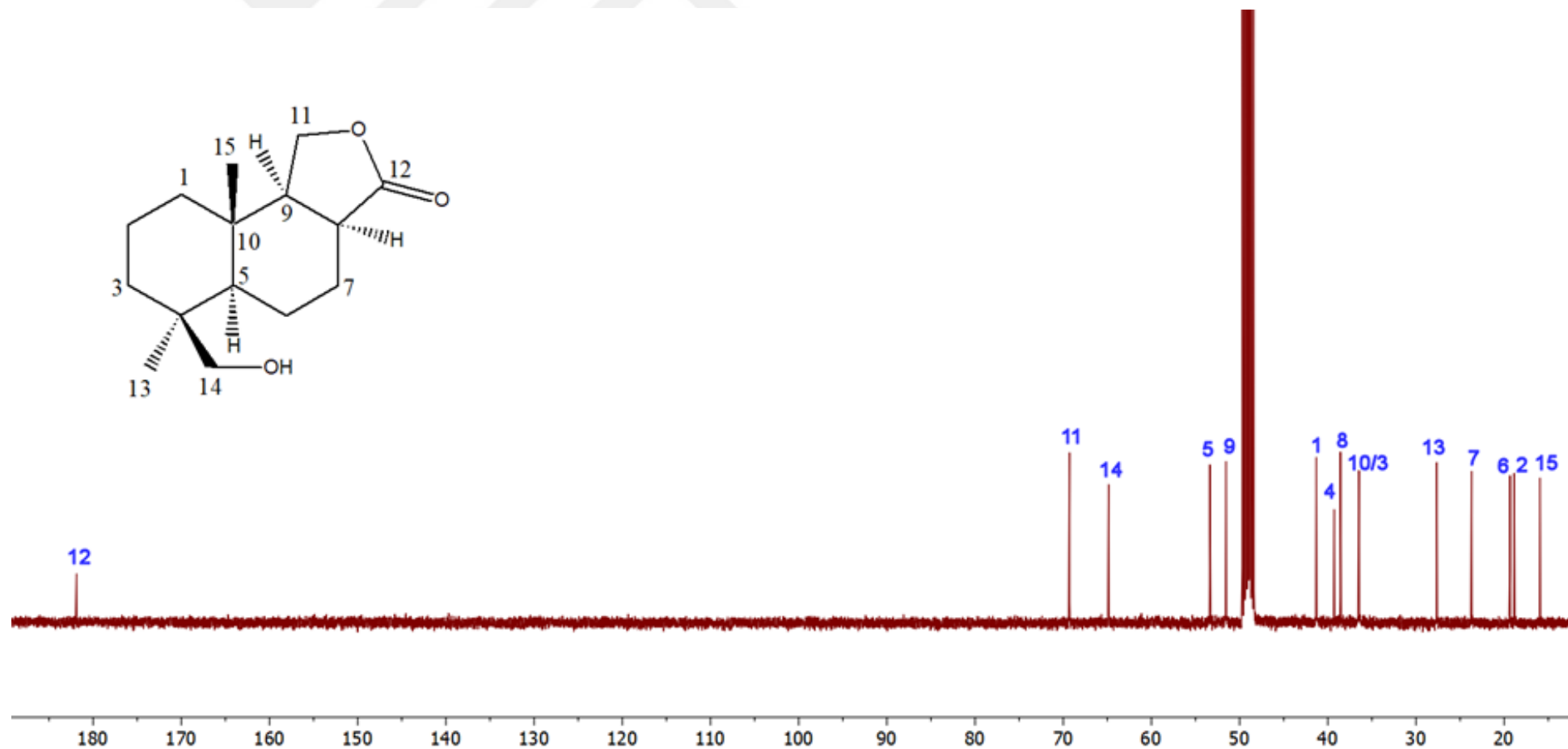
Table 97. ^{13}C and ^1H NMR data of sisymbriifolins A (**22**) and B (**23**) (CD_3OD ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

22				23	
C/H Atom	Multiplicity	$\delta_{\text{C}}(\text{ppm})$	$\delta_{\text{H}}(\text{ppm}), J (\text{Hz})$	$\delta_{\text{C}}(\text{ppm})$	$\delta_{\text{H}}(\text{ppm}), J (\text{Hz})$
1	CH_2	41.3	1.78 m 1.00 m	41.2	1.80 m 1.10 m
2	CH_2	18.8	1.54 m 1.40 m	19.0	1.61 m 1.44 m
3	CH_2	36.4	1.90 m 0.96 m	37.5	1.83 m 1.07 m
4	C	39.4	-	38.7	-
5	CH	53.3	1.04 dd (12.8, 4.3)	53.4	1.13 dd (12.8, 4.3)
6	CH_2	19.3	1.63 m 1.24 m	19.7	1.67 m 1.32 m
7	CH_2	23.7	2.21 m 1.68 m	23.8	2.25 m 1.74 m
8	CH	38.5	2.69 t (7.7)	38.2	2.72 t (7.7)
9	CH	51.5	2.18 m	51.6	2.21 m
10	C	36.5	-	36.7	-
11	CH_2	69.3	4.26 d (9.7) 4.16 dd (9.7, 5.4)	69.4	4.29 d (9.7) 4.18 dd (9.7, 5.4)
12	C	181.7	-	181.9	-
13	CH_3	27.6	0.95 s	28.1	1.06 s
14	CH_2	64.8	3.70 d (11.1) 3.27 d (11.1)	67.8	4.43 d (11.1) 3.95 d (11.1)
15	CH_3	15.9	0.80 s	15.9	0.88 s
<i>Caffeoyl (C-14)</i>					
1'	C	-	-	127.8	-
2'	CH	-	-	115.2	7.03 d (2.0)
3'	C	-	-	147.0	-
4'	C	-	-	149.9	-
5'	CH	-	-	116.7	6.77 d (8.2)
6'	CH	-	-	123.2	6.94 dd (8.2, 2.0)
α	CH	-	-	115.3	6.24 d (15.9)
β	CH	-	-	147.1	7.52 d (15.9)
CO	C	-	-	169.6	-

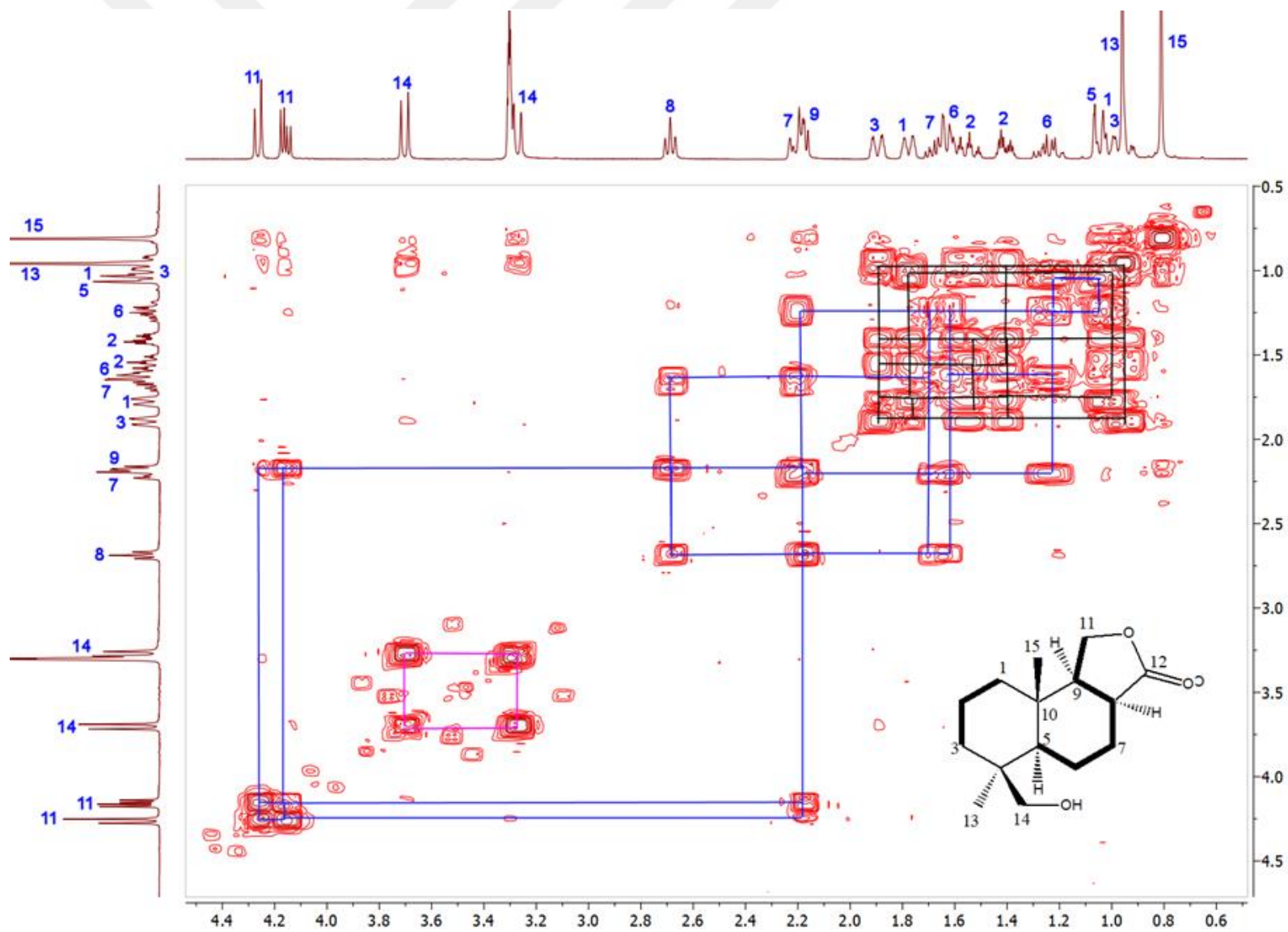
^aAssignments are based on COSY, HSQC, HMBC and NOESY experiments.



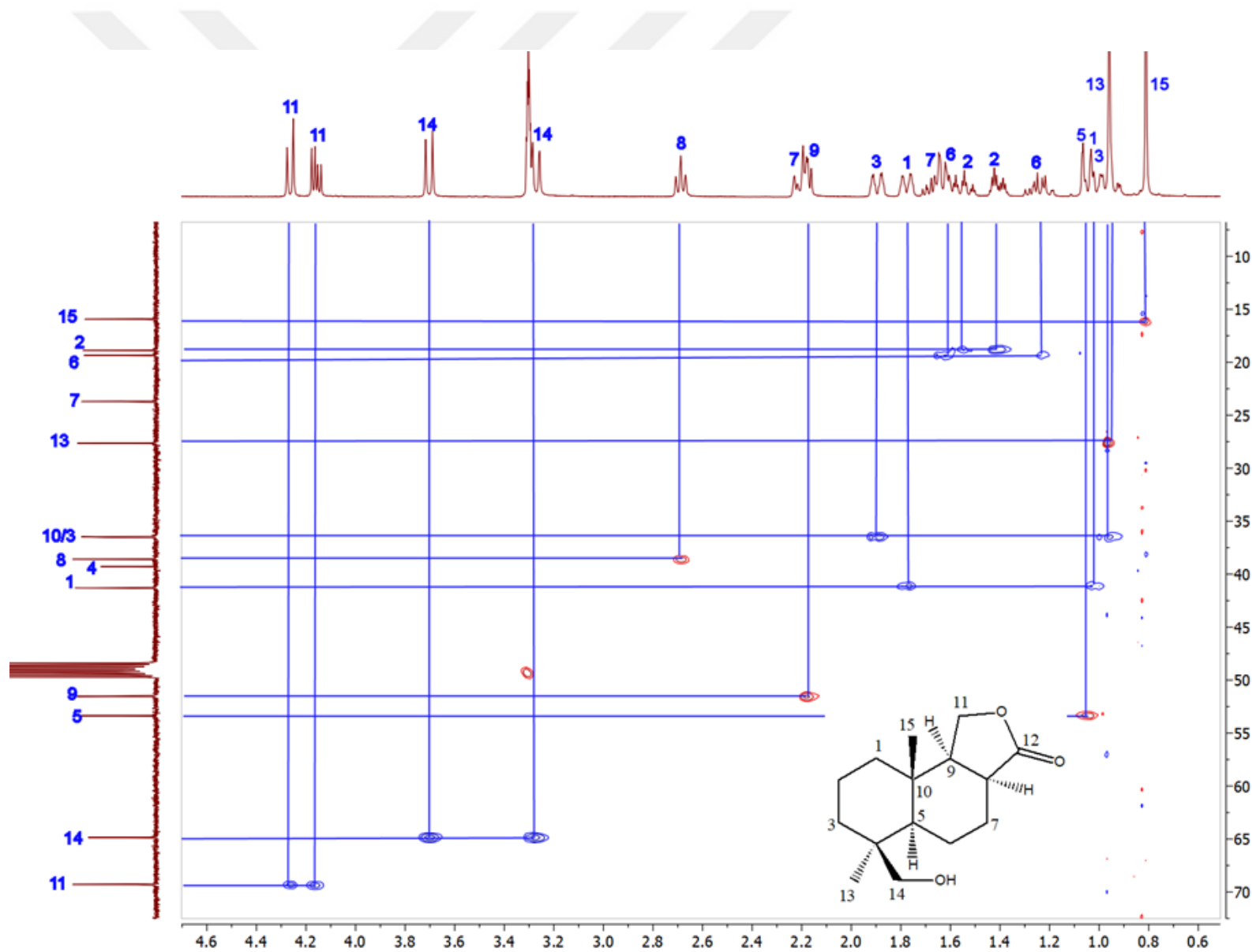
Spectrum 91. ¹H NMR Spectrum of Sisymbriifolin A (22) (CD₃OD, 400 MHz)



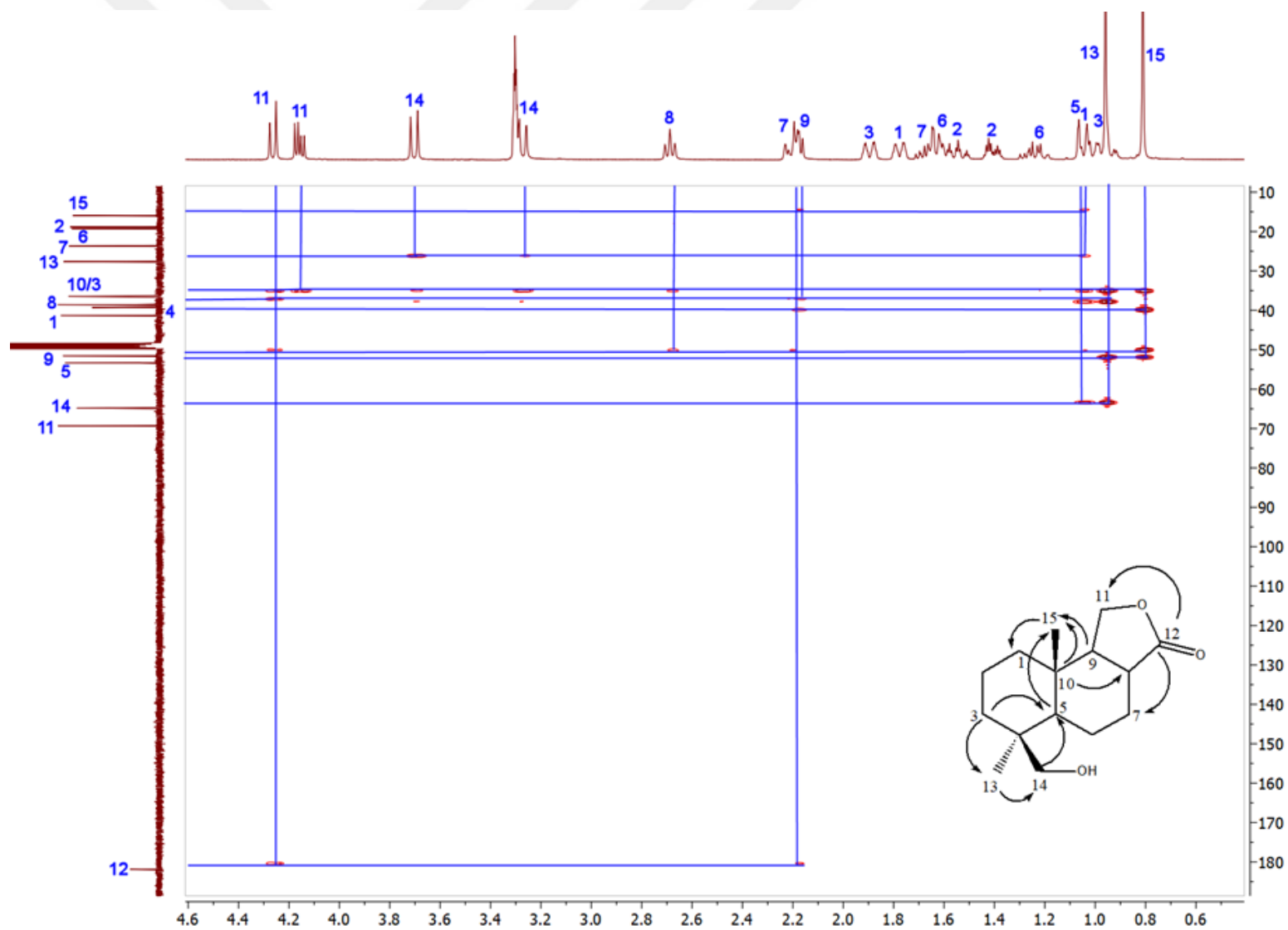
Spectrum 92. ^{13}C NMR Spectrum of Sisymbriifolin A (22) (CD_3OD , 100 MHz)



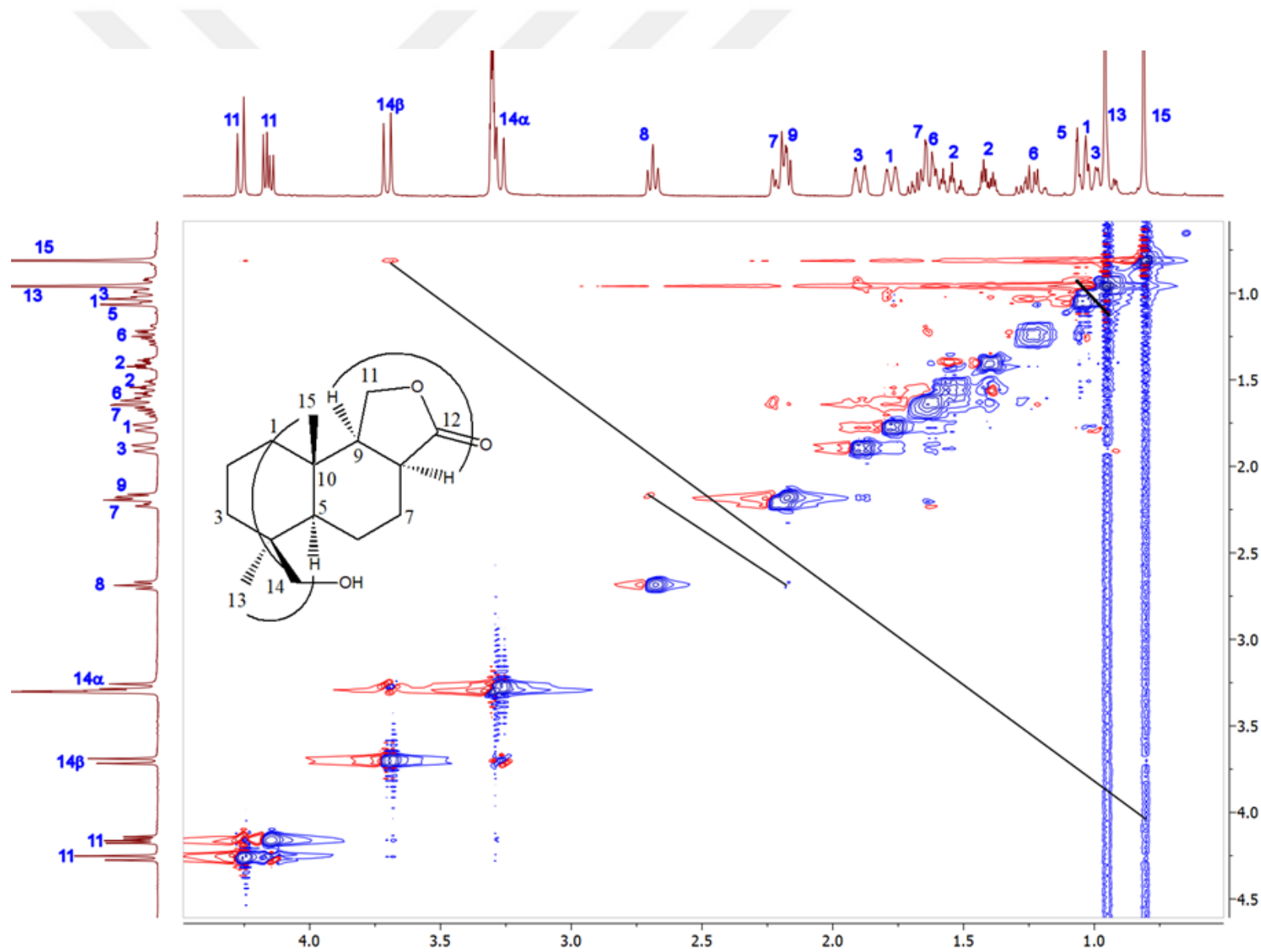
Spectrum 93. COSY Spectrum of Sisymbriifolin A (22)



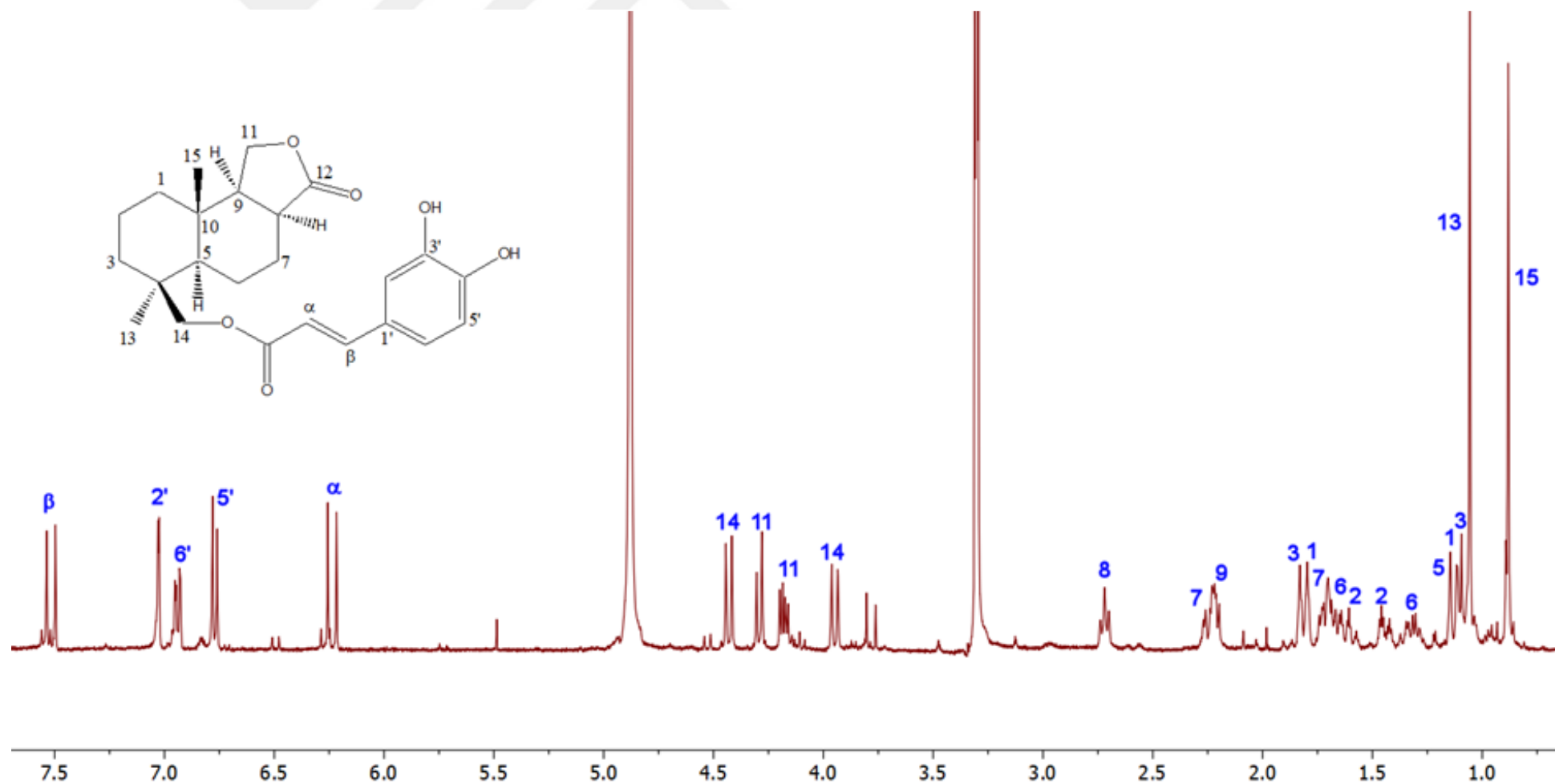
Spectrum 94. HSQC Spectrum of Sisymbriifolin A (22)



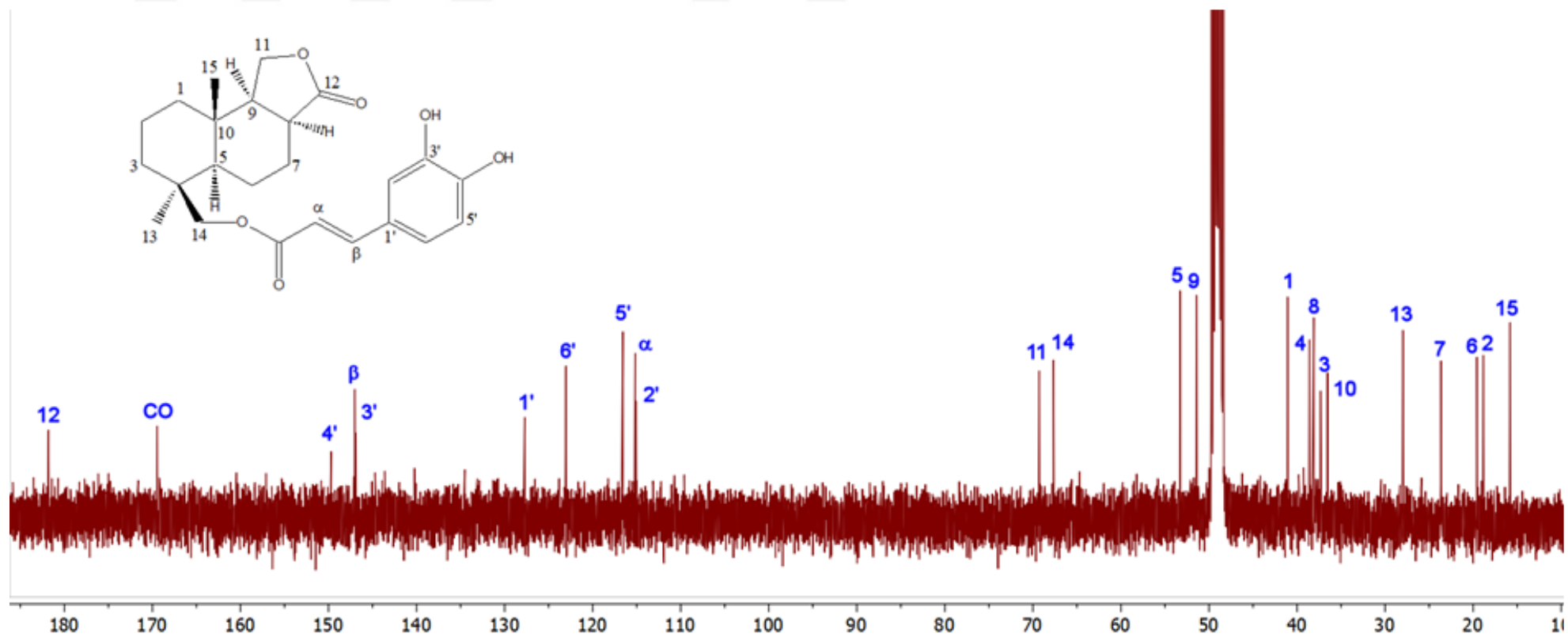
Spectrum 95. HMBC Spectrum of Sisymbriifolin A (22)



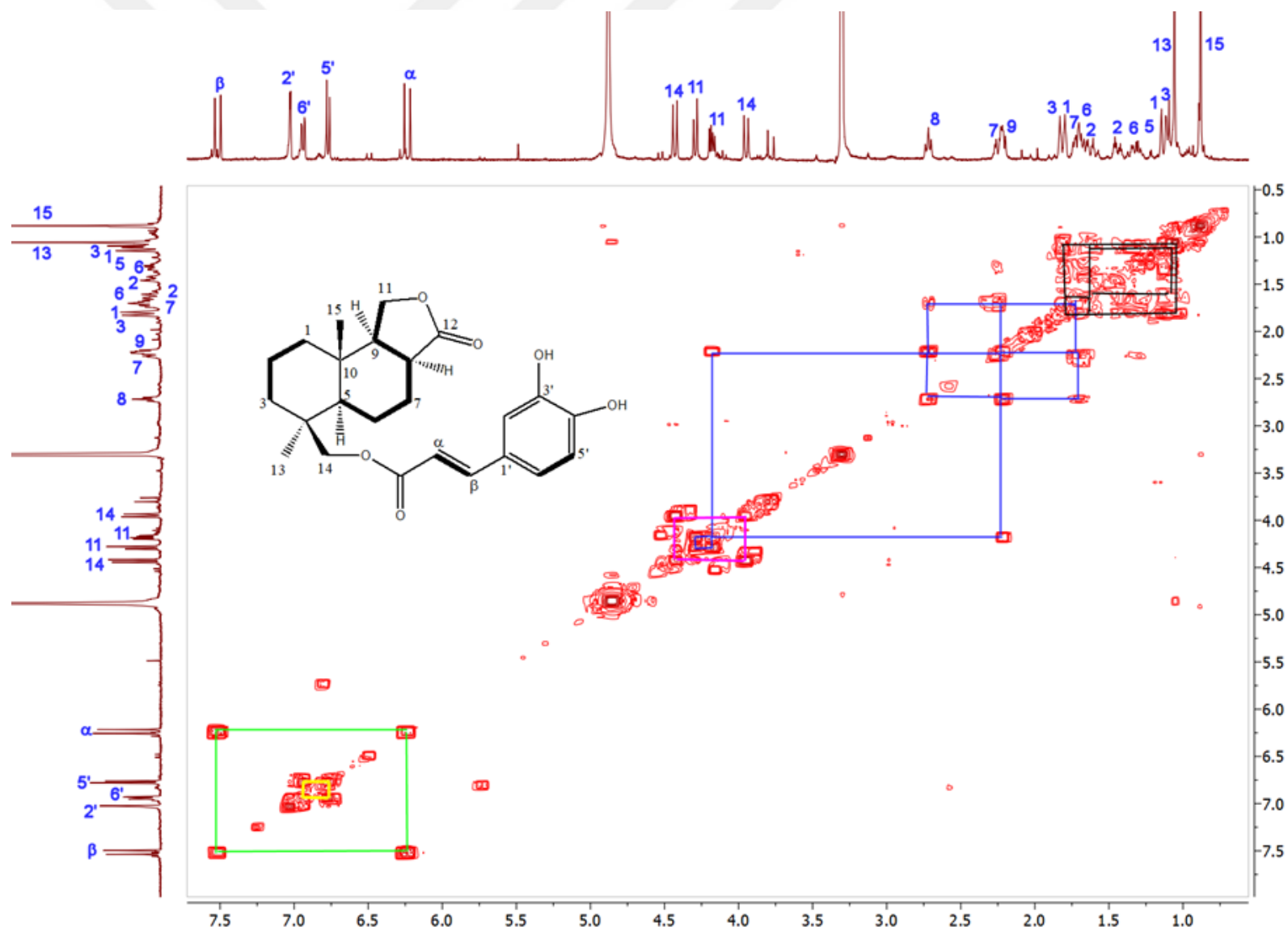
Spectrum 96. NOESY Spectrum of Sisymbriifolin A (22)



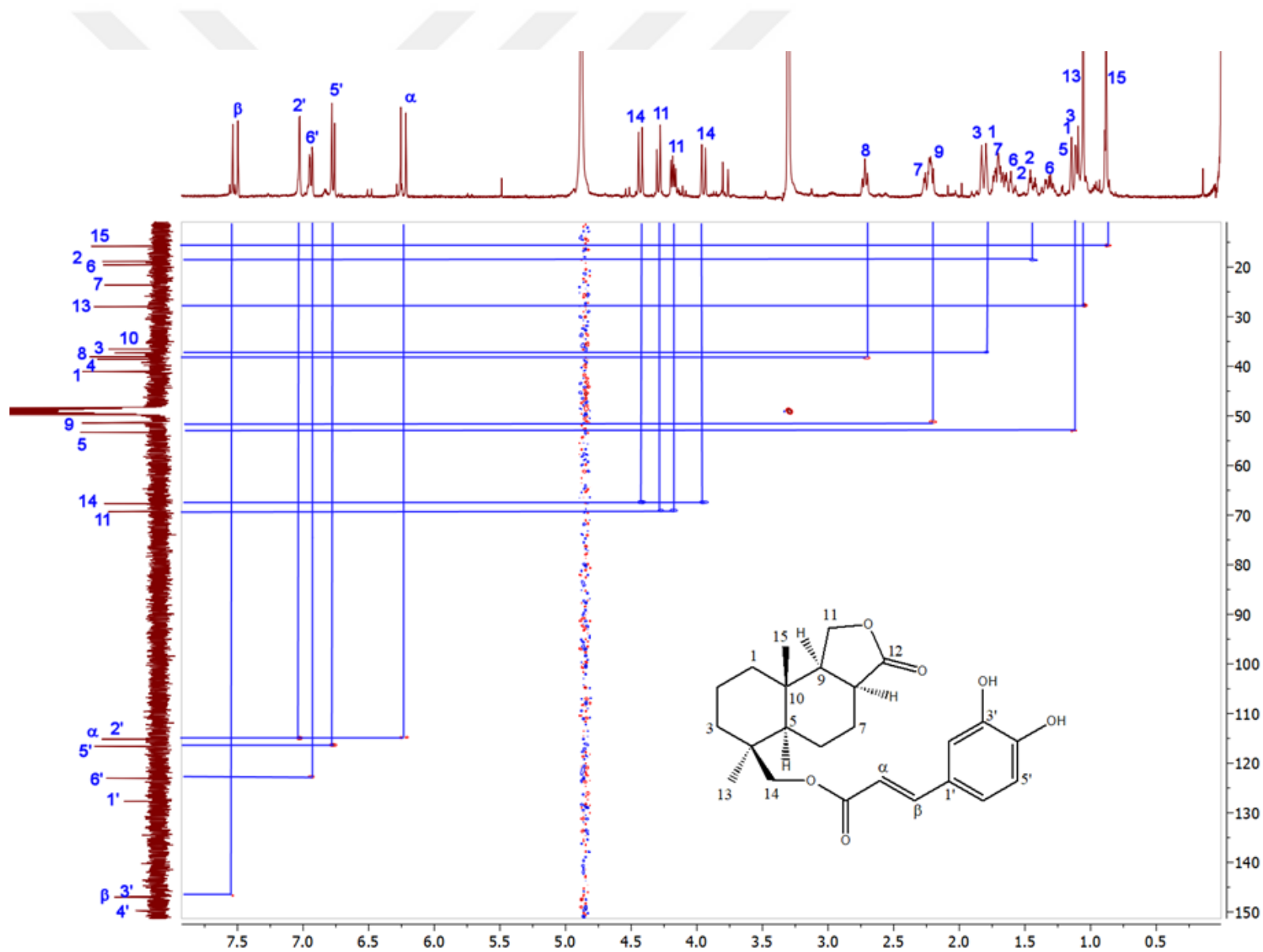
Spectrum 97. ^1H NMR Spectrum of Sisymbriifolin B (23) (CD_3OD , 400 MHz)



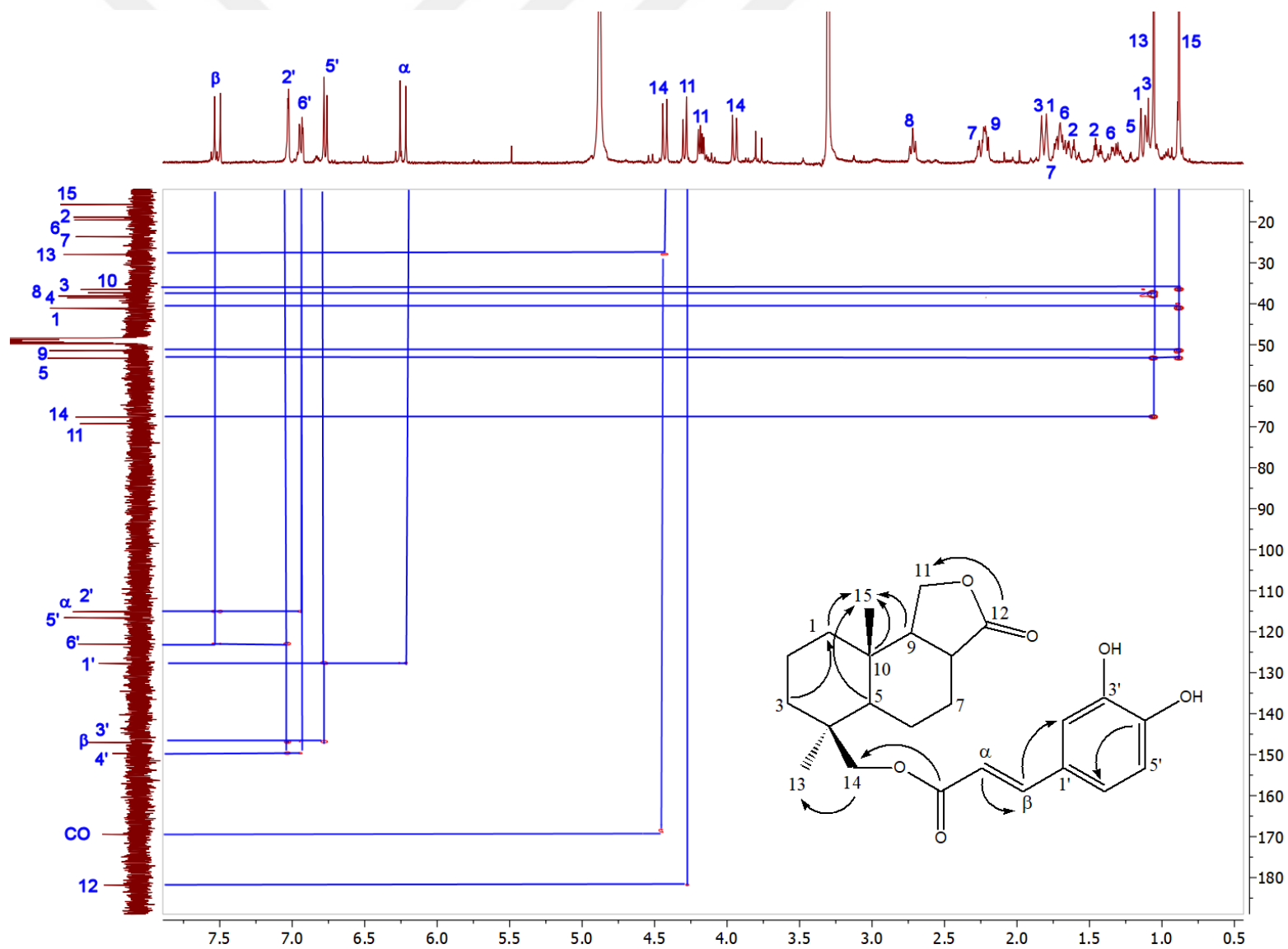
Spectrum 98. ^{13}C NMR Spectrum of Sisymbriifolin B (**23**) (CD_3OD , 100 MHz)



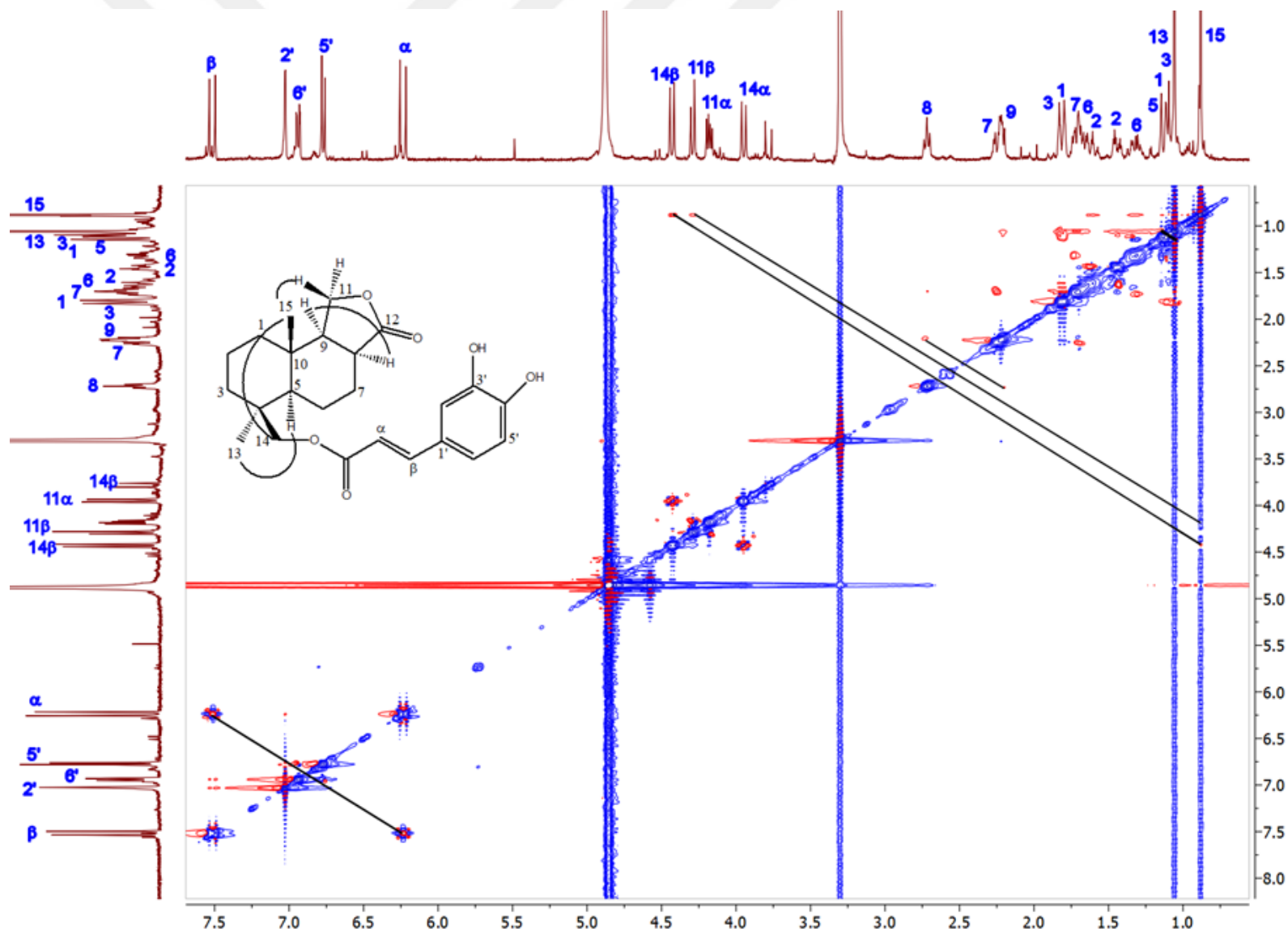
Spectrum 99. COSY Spectrum of Sisymbriifolin B (23)



Spectrum 100. HSQC Spectrum of Sisymbriifolin B (23)

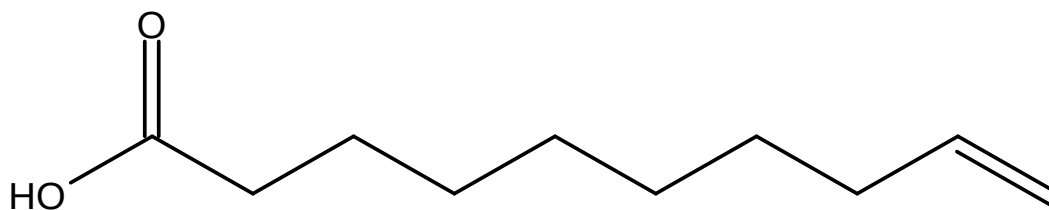


Spectrum 101. HMBC Spectrum of Sisymbriifolin B (23)



Spectrum 102. NOESY Spectrum of Sisymbriifolin B (23)

4.5.1.3.2. Fatty Acid



9-DECENOIC ACID= CAPROLEIC ACID (24): C₁₀H₁₈O₂ (MW:316.39)

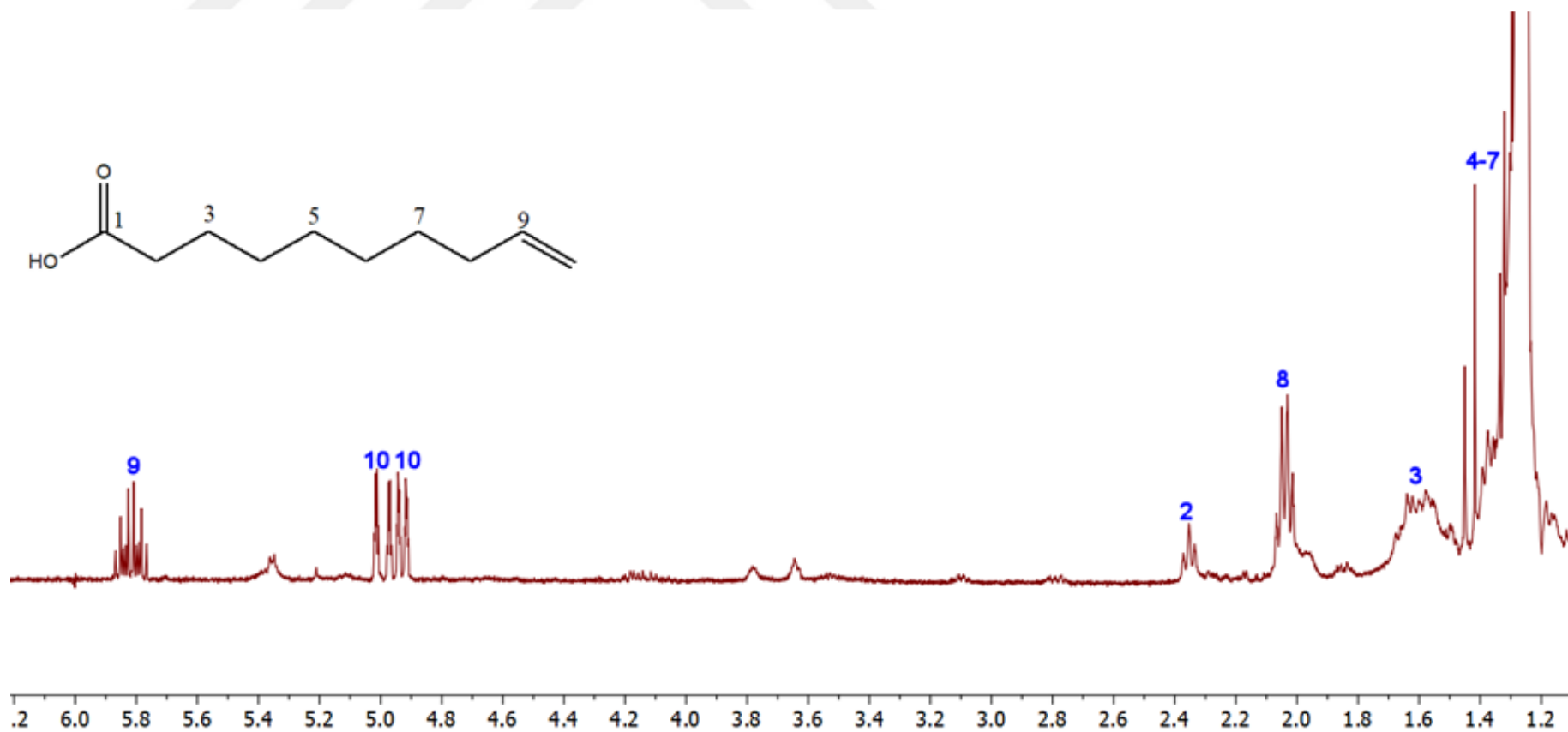
UV $\lambda_{\max}$ (MeOH) nm:	207
IR $\nu_{\max}$ (KBr) cm ⁻¹ :	3342 (OH), 1743 (C=O), 1609 (olefinic, C=C)
¹ H NMR:	Table 98, Spectrum 103
¹³ C NMR:	Table 98, Spectrum 104

Compound **24** was isolated as colorless oil. The UV spectrum showed absorption maximum at 207 nm, and its IR spectrum contained absorption bands corresponding to hydroxyl (3342 cm⁻¹), carboxylic acid carbonyl (1743 cm⁻¹) and olefinic (1609 cm⁻¹) functionalities. The ¹H and ¹³C NMR findings (Table 98) of **24** superimposed with those reported for 9-decenoic acid (caproleic acid) (260).

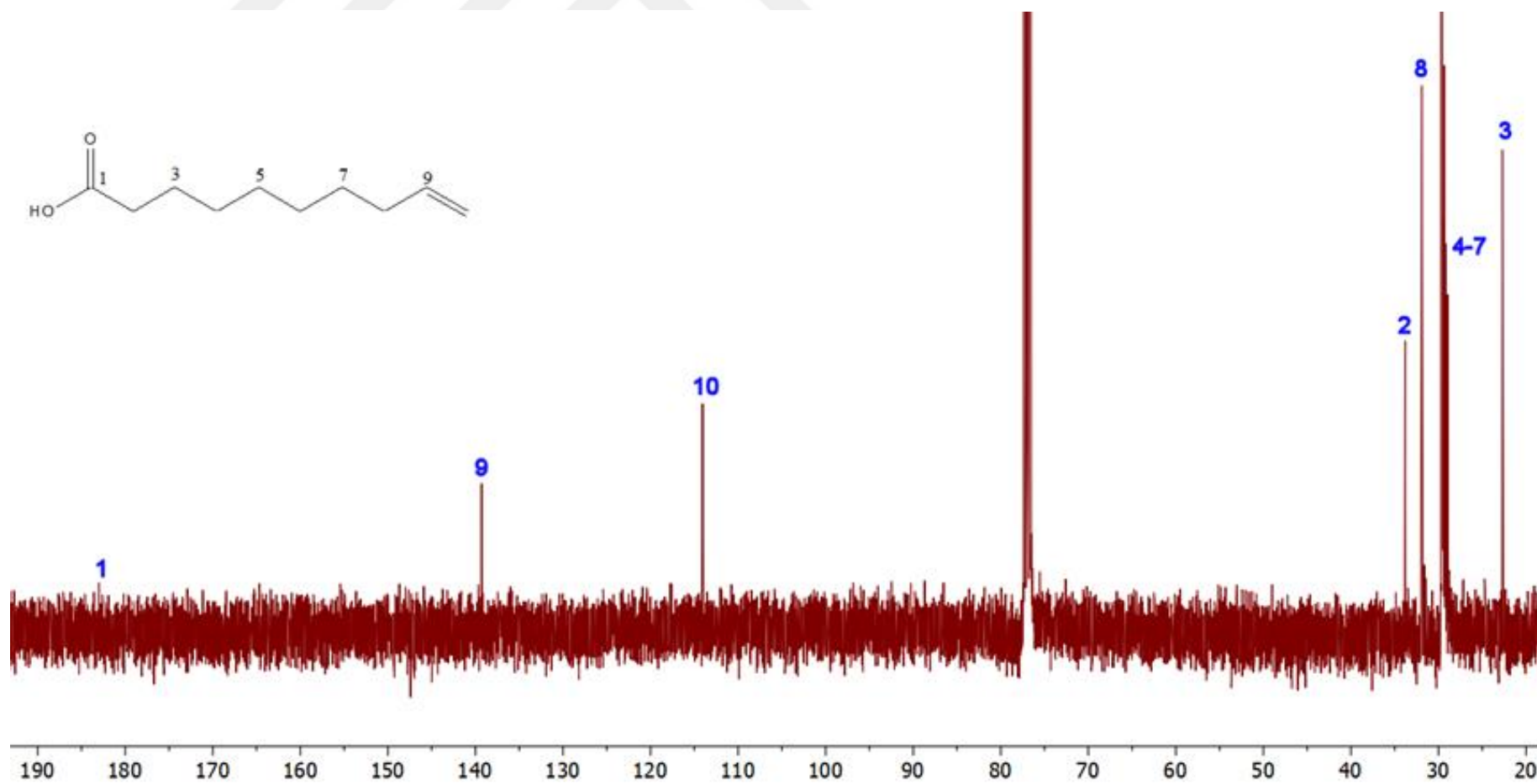
Table 98. ^{13}C and ^1H NMR data of 9-decenoic acid (**24**) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)

C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
1	C	183.0	-
2	CH_2	33.8	2.35 t (7.4)
3	CH_2	22.7	1.59 †
4	CH_2	29.7-29.0	1.39-1.25 †
5	CH_2	29.7-29.0	1.39-1.25 †
6	CH_2	29.7-29.0	1.39-1.25 †
7	CH_2	29.7-29.0	1.39-1.25 †
8	CH_2	31.9	2.04 m
9	CH	139.3	5.82 ddt (16.9, 10.1, 6.7)
10	CH_2	114.1	4.99 m 4.93 m

†: Overlapped signals.



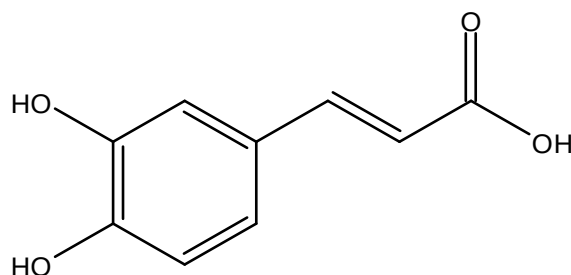
Spectrum 103. ^1H NMR Spectrum of 9-Decenoic acid (**24**) (CDCl_3 , 400 MHz)



Spectrum 104. ¹³C NMR Spectrum of 9-Decenoic acid (24) (CDCl₃, 100 MHz)

4.5.1.4. Compounds Isolated from Cytotoxic Main Fractions of *V. speluncaria*

4.5.1.4.1. Phenylpropane Derivative



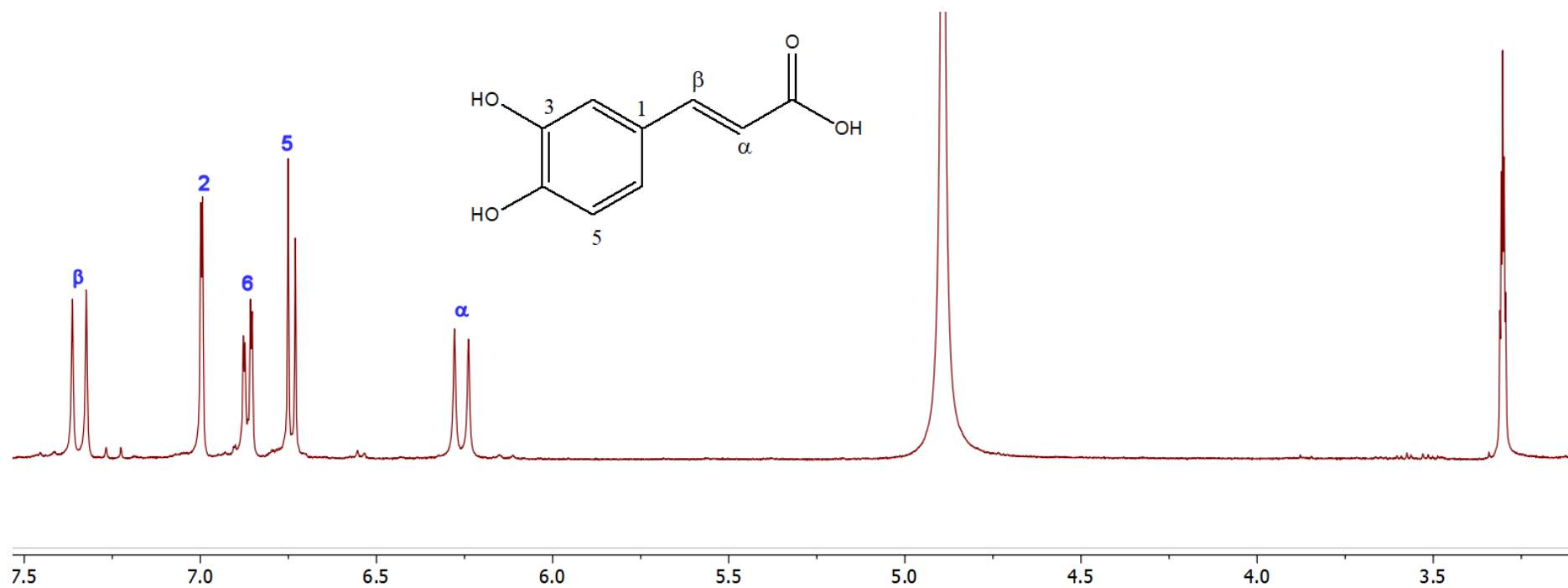
CAFFEIC ACID (26): C₉H₈O₄ (MW:180.16)

UV λ_{max} (MeOH) nm:	217, 298, 325
IR ν_{max} (KBr) cm ⁻¹ :	3435 (OH), 1645 (C=O), 1620, 1600 (olefinic, C=C), 1531 (aromatic, C=C)
ESI-MS m/z :	179.26 [M-H] ⁻
¹ H NMR:	Table 99, Spectrum 105

Compound **26** was purified as yellow solid. The molecular formula of **26** was found to be C₉H₈O₄ via the ESI-MS ion at m/z 179.26 [M-H]⁻. The UV spectrum indicated absorption maxima at 217, 298 and 325 nm, typical for a phenylpropane derivative. The IR spectrum revealed the presence of hydroxyl (3435 cm⁻¹), carbonyl (1645 cm⁻¹), olefinic (1620 and 1600 cm⁻¹) and aromatic (1531 cm⁻¹) functionalities. The ¹H NMR data (Table 99 and Spectrum 105) was found to be similar with those reported for caffeic acid (261).

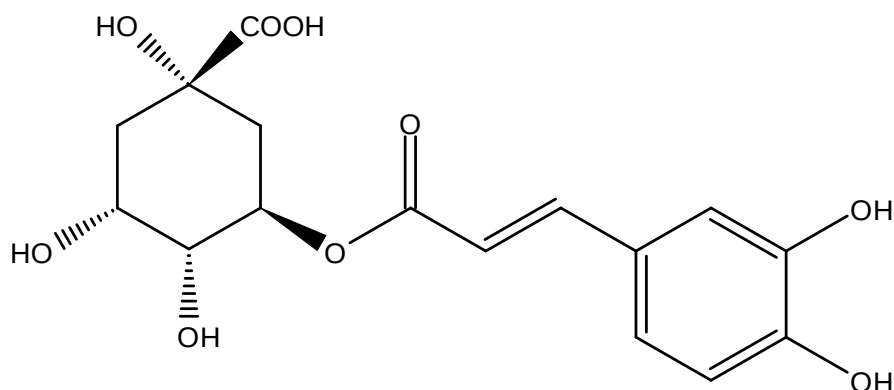
Table 99. ^1H NMR data of caffeic acid (**26**) (CD_3OD ; ^1H : 400 MHz)

C/H atom	Multiplicity	δ_{H} (ppm), J (Hz)
1	C	-
2	CH	7.0 d (2.0)
3	C	-
4	C	-
5	CH	6.74 d (8.2)
6	CH	6.87 dd (8.2, 2.0)
α	CH	6.26 d (15.9)
β	CH	7.35 d (15.9)
CO	C	-



Spectrum 105. ^1H NMR Spectrum of Caffeic acid (**26**) (CD_3OD , 400 MHz)

4.5.1.4.2. Caffeoyl quinic acid derivatives



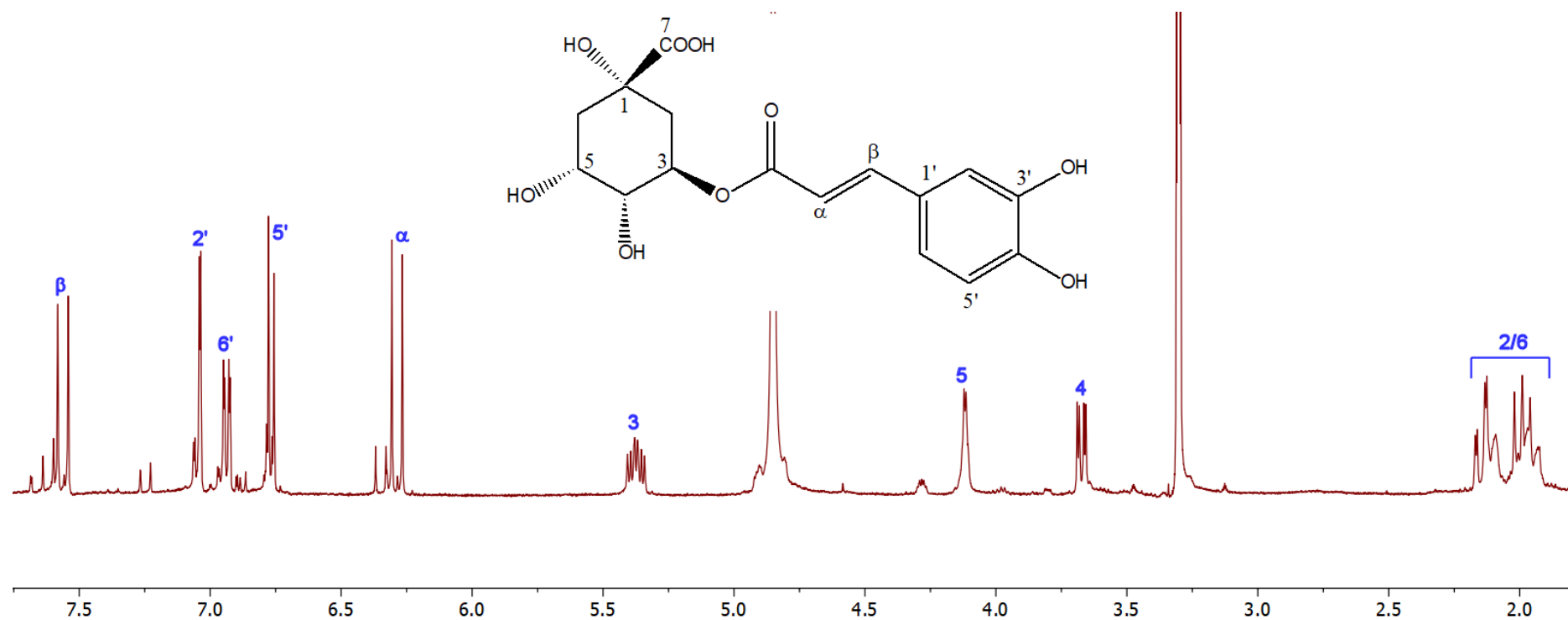
CHLOROGENIC ACID (27): C₁₆H₁₈O₉ (MW:354.31)

UV λ_{max} (MeOH) nm:	217, 298, 325
ESI-MS m/z :	377.82 [M+Na] ⁺ , 355.92 [M+H] ⁺
IR ν_{max} (KBr) cm ⁻¹ :	3470, 3324 (OH), 1686 (C=O), 1639, 1600 (olefinic, C=C), 1520 (aromatic, C=C)
¹ H NMR:	Table 100, Spectrum 106

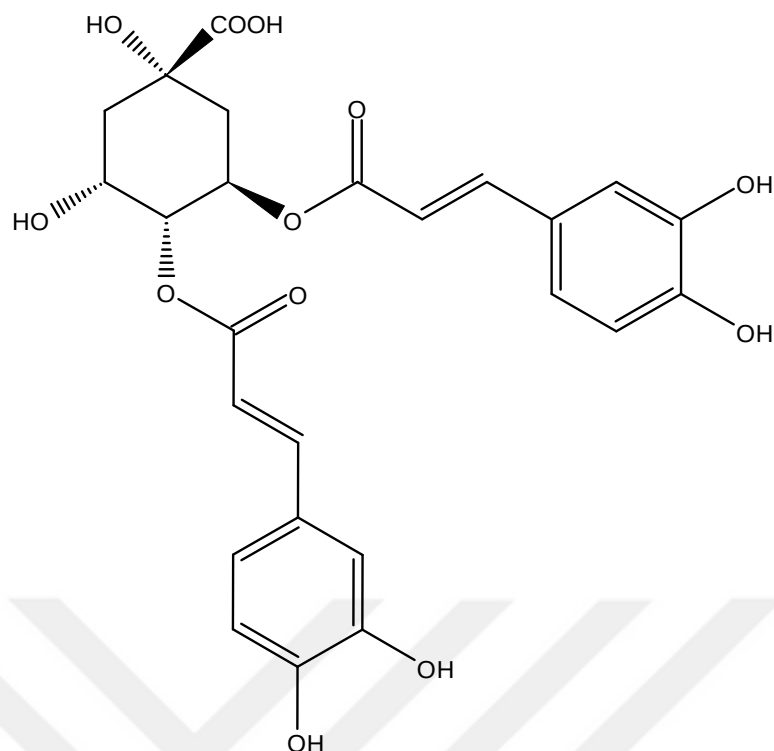
Compound **27** was isolated as white solid. Its molecular formula was deduced to be C₁₆H₁₈O₉, on the basis of pseudomolecular ion peaks at m/z 377.82 [M + Na]⁺ and m/z 355.92 [M + H]⁺ in the ESI-MS spectrum, as well as NMR data. The UV spectrum indicated absorption maxima at 217, 298 and 325 nm. The IR spectrum showed absorption bands due to hydroxyl (3470 and 3324 cm⁻¹), carbonyl (1686 cm⁻¹) and olefinic (1639 and 1600 cm⁻¹) as well as aromatic ring (1520 cm⁻¹). The ¹H NMR spectrum (Spectrum 106) contained signals due to a (*E*)-caffeoyl and quinic acid. Compound **27** was elucidated as chlorogenic acid by comparing its ¹H NMR and MS data with those reported in the literature (261).

Table 100. ^1H NMR data of chlorogenic acid (**27**) (CD_3OD ; ^1H : 400 MHz)

C/H Atom	Multiplicity	δ_{H} (ppm), J (Hz)
1	C	-
2	CH_2	2.18-1.88 m
3	CH	5.37 m
4	CH	3.67 dd (9.9, 3.2)
5	CH	4.10 m
6	CH_2	2.18-1.88 m
7	C	-
<i>Caffeoyl (C-3)</i>		
1'	C	-
2'	CH	7.03 d (2.0)
3'	C	-
4'	C	-
5'	CH	6.75 d (8.2)
6'	CH	6.92 dd (8.2, 2.0)
α	CH	6.28 d (15.9)
β	CH	7.56 d (15.9)
CO	C	-



Spectrum 106. ^1H NMR Spectrum of Chlorogenic acid (27) (CD_3OD , 400 MHz)



3,4-DICAFFEOYLQUINIC ACID (28): C₂₅H₂₄O₁₂(MW:516.45)

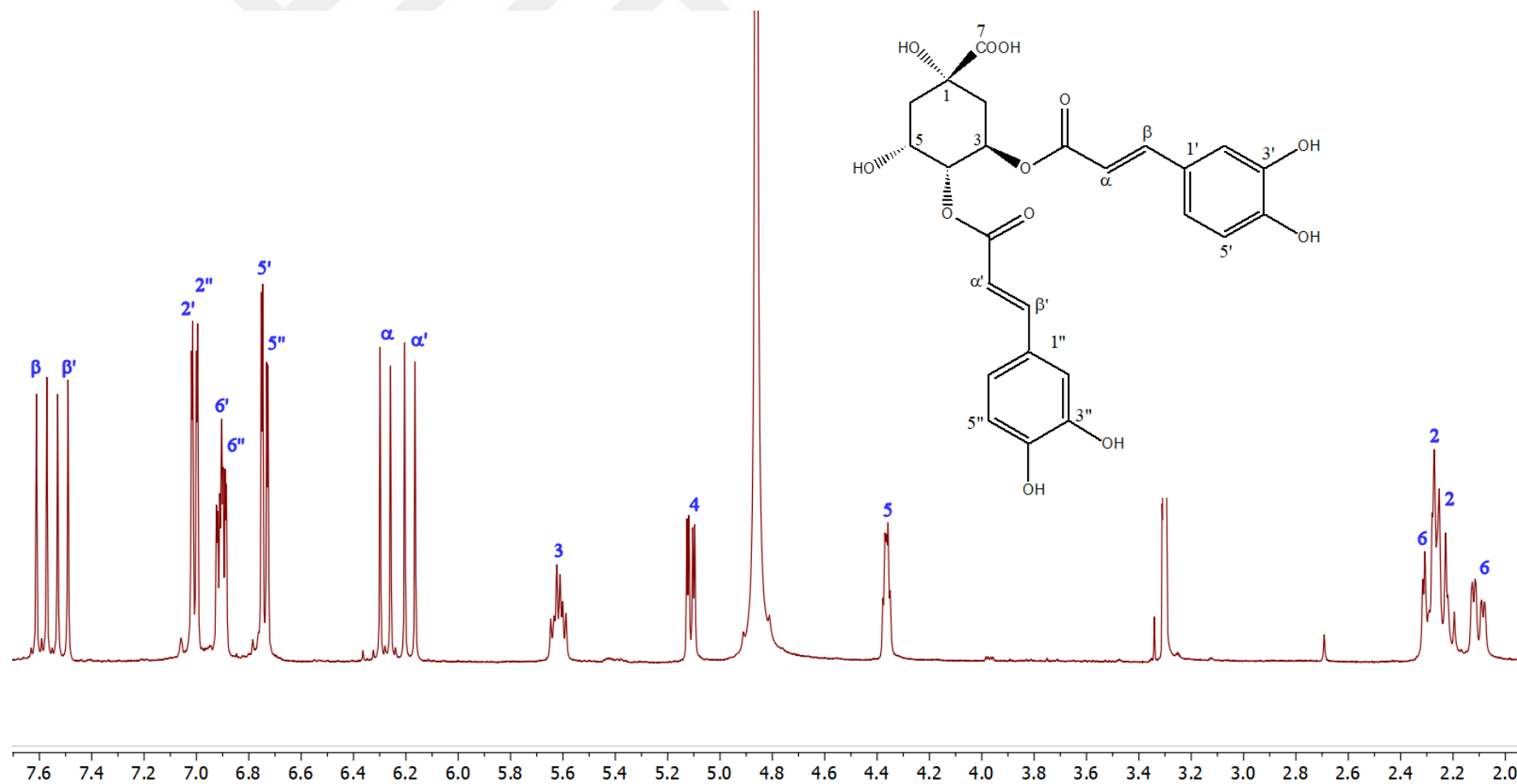
UV λ_{max} (MeOH) nm:	218, 243, 298, 330
ESI-MS m/z :	515.75 [M-H] ⁻
IR ν_{max} (KBr) cm ⁻¹ :	3573, 3204 (OH), 1695 (C=O), 1630 (olefinic, C=C), 1599, 1521 (aromatic, C=C)
¹ H NMR:	Table 101, Spectrum 107
¹³ C NMR:	Table 101, Spectrum 108

Compound **28** was obtained as white solid, with the molecular formula C₂₅H₂₄O₁₂ as established via negative ESI-MS at m/z 515.75 [M-H]⁻. Its MS data was 162 molecular mass unit bigger than that of **27**, indicating the presence of an additional (*E*)-caffeoyl unit in **28**. The UV spectrum showed absorption maxima at 218, 243, 298, 330 nm. The IR spectrum suggested the presence of hydroxy (3573 and 3204 cm⁻¹), carbonyl (1695 cm⁻¹), olefinic (1630 cm⁻¹) and aromatic ring (1599 and 1521 cm⁻¹) functionalities. The ¹H NMR data (Table 101) of **28**, was very similar to those of compound **27** (chlorogenic acid), except for a presence of additional caffeoyl moiety at 4th position in **28**. Taken together, ¹H and ¹³C NMR findings of **28** superimposed with those reported for 3,4-dicafeoylquinic acid in the literature (261).

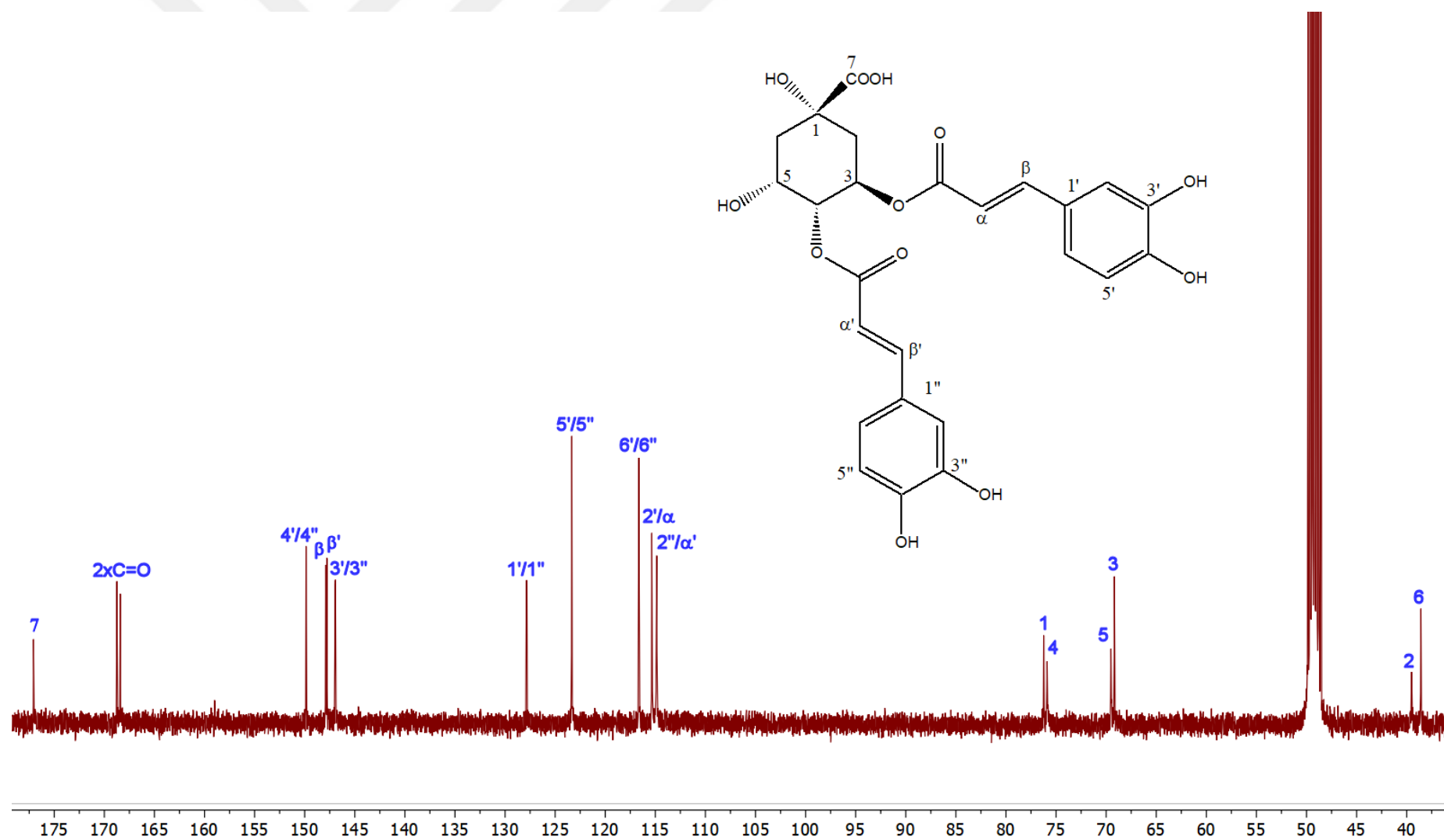
Table 101. ^{13}C and ^1H NMR data of 3,4-dicaffeoylquinic acid (**28**) (CD_3OD ; ^{13}C : 100 MHz, ^1H : 400 MHz)

C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
1	C	76.2	-
2	CH_2	39.5	2.27 † 2.23 †
3	CH	69.2	5.62 m
4	CH	75.9	5.11 dd (9.0, 3.0)
5	CH	69.5	4.36 m
6	CH_2	38.6	2.30 m 2.10 dd (14.3, 4.7)
7	C	177.1	-
<i>Caffeoyl (C-3)</i>			
1'	C	127.9	-
2'	CH	115.3	7.02 d (2.0)
3'	C	146.9	-
4'	C	149.9	-
5'	CH	123.3	6.75 d (8.2)
6'	CH	116.6	6.91 dd (8.2, 2.0)
α	CH	115.3	6.28 d (15.9)
β	CH	147.9	7.59 d (15.9)
CO	C	168.7	-
<i>Caffeoyl (C-4)</i>			
1''	C	127.8	-
2''	CH	114.9	7.00 d (2.0)
3''	C	146.9	-
4''	C	149.9	-
5''	CH	123.3	6.73 d (8.2)
6''	CH	116.6	6.89 d (8.2, 2.0)
α'	CH	114.9	6.18 d (15.9)
β'	CH	147.8	7.51 d (15.9)
CO	C	168.4	-

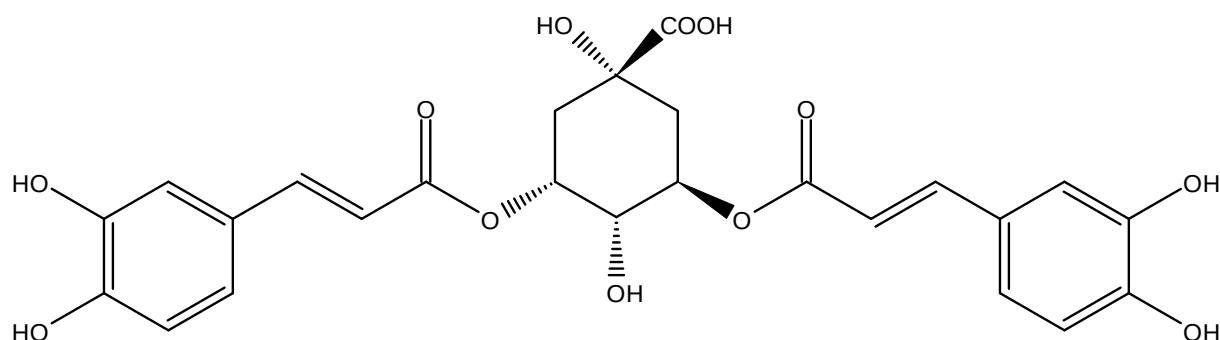
† Overlapped signals.



Spectrum 107. ^1H NMR Spectrum of 3,4-Dicaffeoylquinic acid (**28**) (CD_3OD , 400 MHz)



Spectrum 108. ^{13}C NMR Spectrum of 3,4-Dicaffeoylquinic acid (**28**) (CD_3OD , 100 MHz)



3,5-DICAFFEOYLQUINIC ACID (29): C₂₅H₂₄O₁₂(MW:516.45)

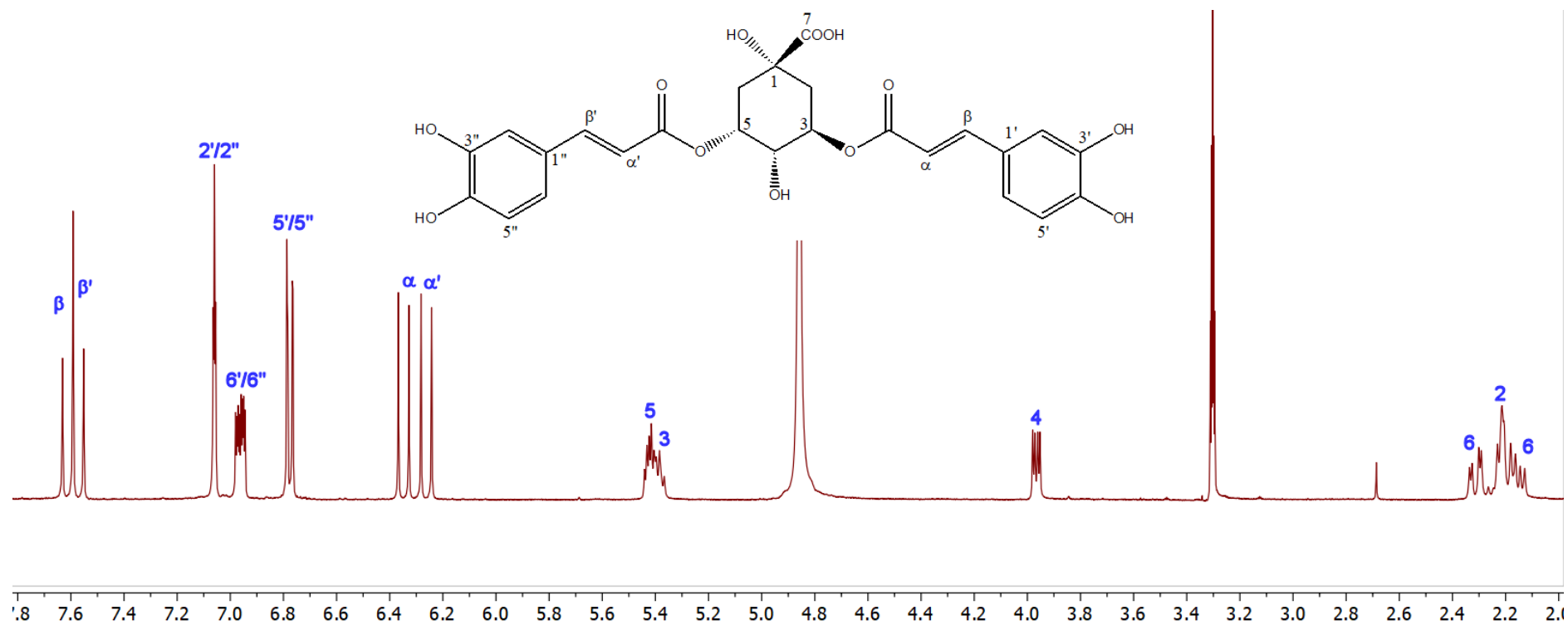
UV λ_{max} (MeOH) nm:	218, 244, 298, 330
ESI-MS m/z :	515.89 [M-H] ⁻
IR ν_{max} (KBr) cm ⁻¹ :	3290 (OH), 1696 (C=O), 1630 (olefinic, C=C), 1601, 1522 (aromatic, C=C)
¹ H NMR:	Table 102, Spectrum 109

Compound **29**, as a white solid, was found to possess the same molecular formula with those of **28** (3,5-dicaffeoylquinic acid). The UV spectrum indicated absorption maxima at 218, 244, 298 and 330 nm, whereas the IR spectrum possessed absorption bands belonging to hydroxyl (3290 cm⁻¹), carbonyl (1696 cm⁻¹), olefinic (1630 cm⁻¹) and aromatic ring (1601 and 1522 cm⁻¹). The similarities in ¹H NMR data of **29** and **28** (Tables 102 and 101) also demonstrated that both compounds are structurally analogous, differing only by the location of the second caffeoyl moiety in their structure. The second caffeoyl moiety was substituted at C-5 in **29**, instead of C-4 substitution in **28**. Also, the spectroscopic findings of **29** was found to be compatible with the those previously reported for 3,5-dicaffeoylquinic acid (261).

Table 102. ¹H NMR data of 3,5-dicaffeoylquinic acid (**29**) (CD₃OD; ¹H: 400 MHz)

C/H Atom	Multiplicity	δ _H (ppm), J (Hz)
1	C	-
2	CH ₂	2.21 †
3	CH	5.38 m †
4	CH	3.97 dd (7.6, 3.3)
5	CH	5.42 m †
6	CH ₂	2.32 dd (13.9, 3.8) 2.15 m
7	C	-
<i>Caffeoyl (C-3)</i>		
1'	C	-
2'	CH	7.06 d (2.0)
3'	C	-
4'	C	-
5'	CH	6.78 d (8.2)
6'	CH	6.97 dd (8.2, 2.0)
α	CH	6.35 d (15.9)
β	CH	7.61 d (15.9)
CO	C	-
<i>Caffeoyl (C-5)</i>		
1''	C	-
2''	CH	7.06 d (2.0)
3''	C	-
4''	C	-
5''	CH	6.77 d (8.2)
6''	CH	6.95 dd (8.2, 2.0)
α'	CH	6.26 d (15.9)
β'	CH	7.57 d (15.9)
CO	C	-

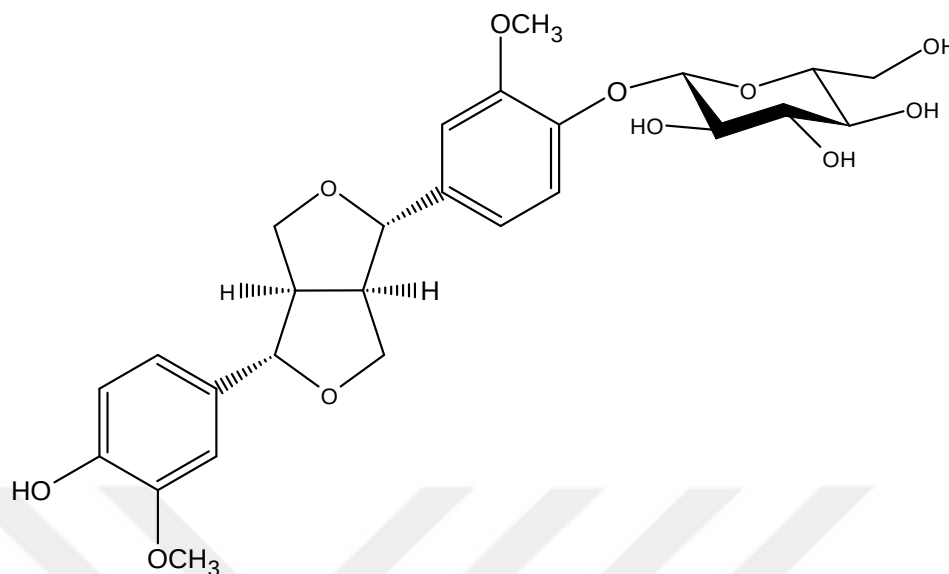
†: Overlapped signals.



Spectrum 109. ^1H NMR Spectrum of 3,5-Dicaffeoylquinic acid (**29**) (CD_3OD , 400 MHz)

4.5.1.4.3. Lignan Derivatives

4.5.1.4.3.A. Lignan glycoside



PINORESINOL 4-O- β -D-GLUCOPYRANOSIDE (25): C₂₆H₃₂O₁₁ (MW:520.53)

UV $\lambda_{\max}$ (MeOH) nm:	228, 280
ESI-MS m/z :	519.92 [M-H] ⁻
IR $\nu_{\max}$ (KBr) cm ⁻¹ :	3378 (OH), 1605, 1515 (aromatic, C=C)
¹ H NMR:	Table 103, Spectrum 110
¹³ C NMR:	Table 103, Spectrum 111
COSY:	Spectrum 112
HSQC:	Spectrum 113
HMBC:	Spectrum 114

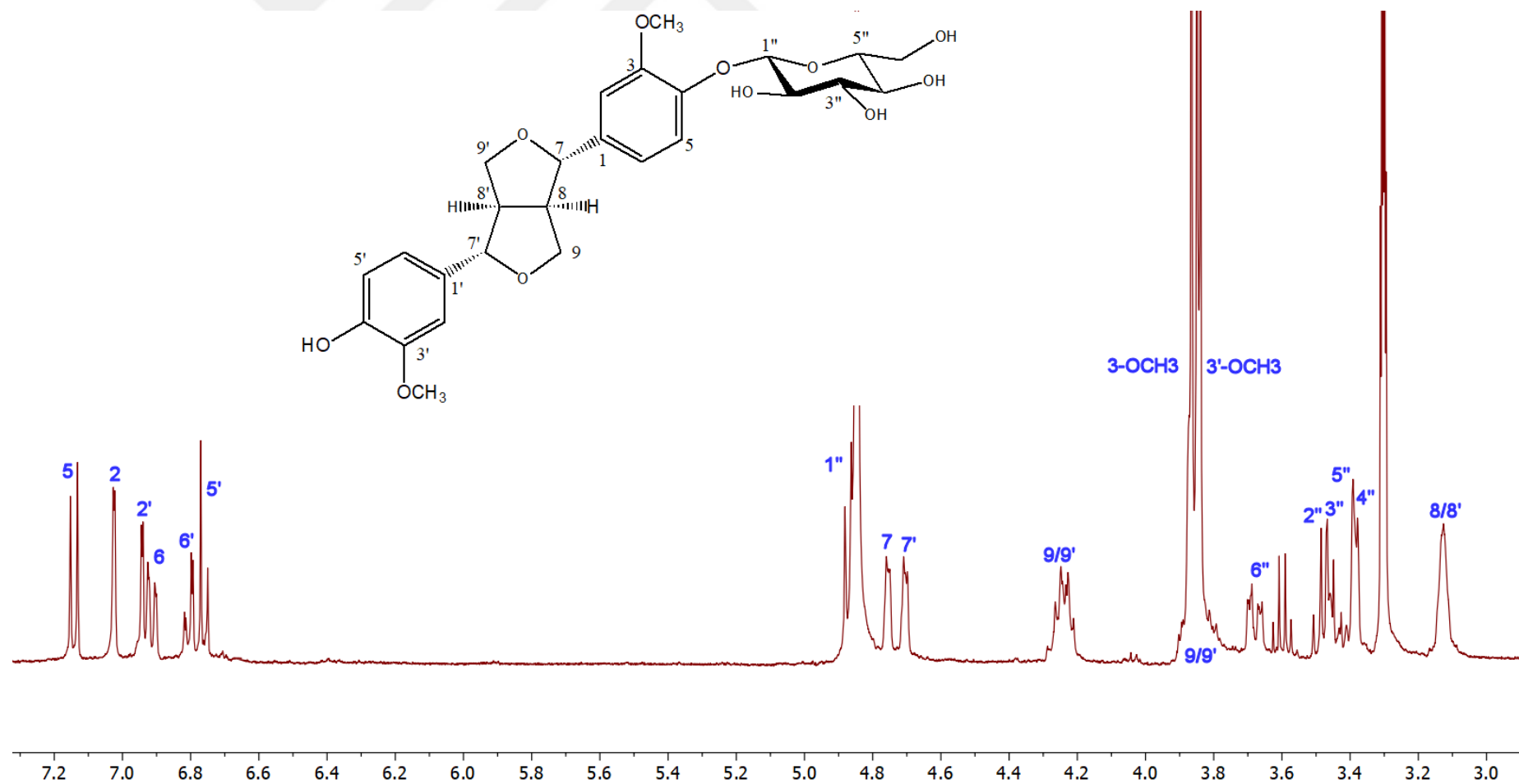
Compound **25** was isolated as amorphous white solid. The molecular formula, C₂₆H₃₂O₁₁, was deduced from the pseudomolecular ion peak at 519.92 [M-H]⁻ in the negative ion ESI-MS. The UV spectrum gave maxima at 228 and 280 nm. The IR spectrum exhibited absorption bands corresponding to hydroxyl (3378 cm⁻¹) and aromatic ring (1605 and 1515 cm⁻¹) functionalities. The 1D and 2D (COSY, HSQC and HMBC) NMR findings (Table 103, Spectra 110-114) of **25** were found to be similar to those of **14** (8-hydroxypinoresinol) except for the presence of a sugar unit at 4th position as well as absence of hydroxyl group at 8th position in **25**. Overall, the spectroscopic features of **25** were superimposed with those of pinoresinol 4-O- β -D glucopyranoside (262).

Table 103. ^{13}C and ^1H NMR data of pinoresinol 4-*O*- β -D glucopyranoside (**25**) (CD_3OD ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

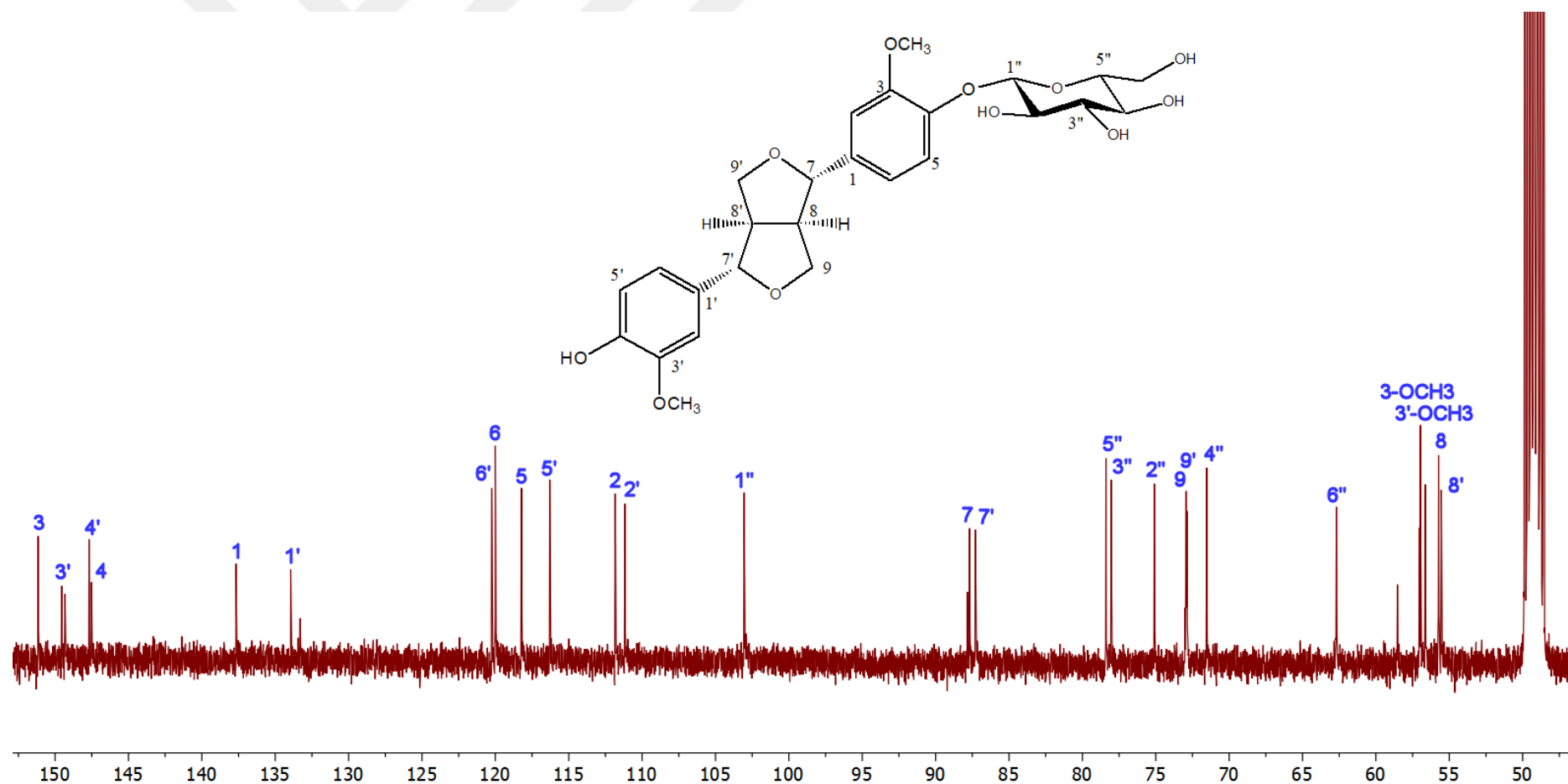
C/H Atom	Multiplicity	δ_{C} ppm	δ_{H} ppm, <i>J</i> (Hz)
<i>Aglycone</i>			
1	C	137.7	-
2	CH	111.8	7.02 d (1.7)
3	C	151.2	-
4	C	147.5	-
5	CH	118.2	7.14 d (8.3)
6	CH	120.0	6.91 dd (8.3, 1.7)
7	CH	87.3	4.75 d (4.1)
8	CH	55.7	3.13 m
9	CH ₂	72.9	4.24 † 3.86 †
3-OCH ₃	CH ₃	57.0	3.86 s
1'	C	133.9	-
2'	CH	111.2	6.94 d (1.8)
3'	C	149.6	-
4'	C	147.7	-
5'	CH	116.3	6.76 d (8.1)
6'	CH	120.3	6.81 dd (1.8, 8.1)
7'	CH	87.7	4.70 d (4.3)
8'	CH	55.5	3.13 m
9'	CH ₂	72.9	4.24 † 3.86 †
3'-OCH ₃	CH ₃	56.6	3.84 s
<i>Glucose</i>			
1''	CH	103.0	4.87 d (7.5)
2''	CH	75.1	3.48 †
3''	CH	78.0	3.47 †
4''	CH	71.5	3.38 †
5''	CH	78.4	3.39 †
6''	CH ₂	62.7	3.86 † 3.68 dd (12.0, 3.6)

^aAssignments are based on COSY, HSQC, HMBC and NOESY experiments.

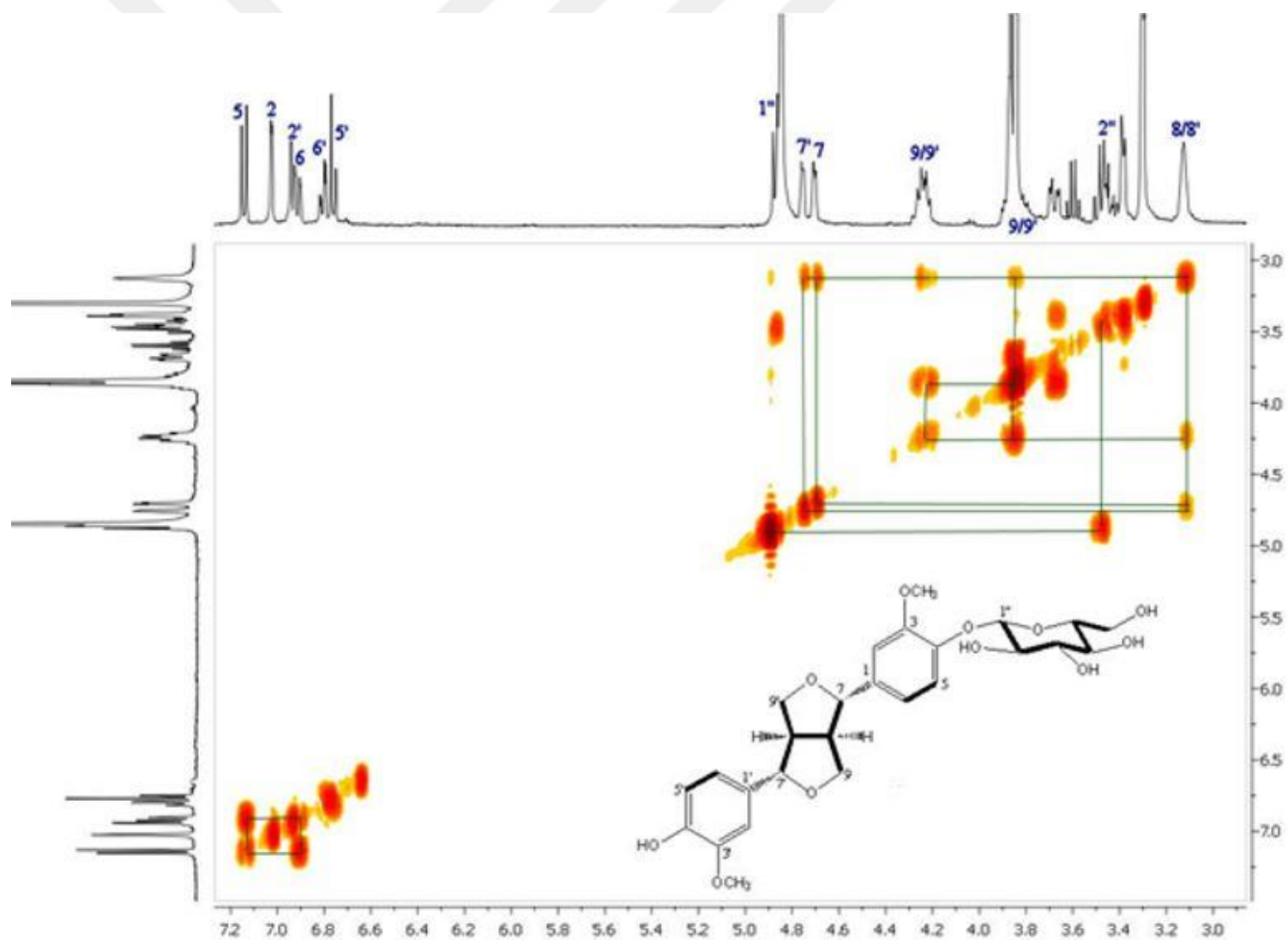
†: Overlapped signals.



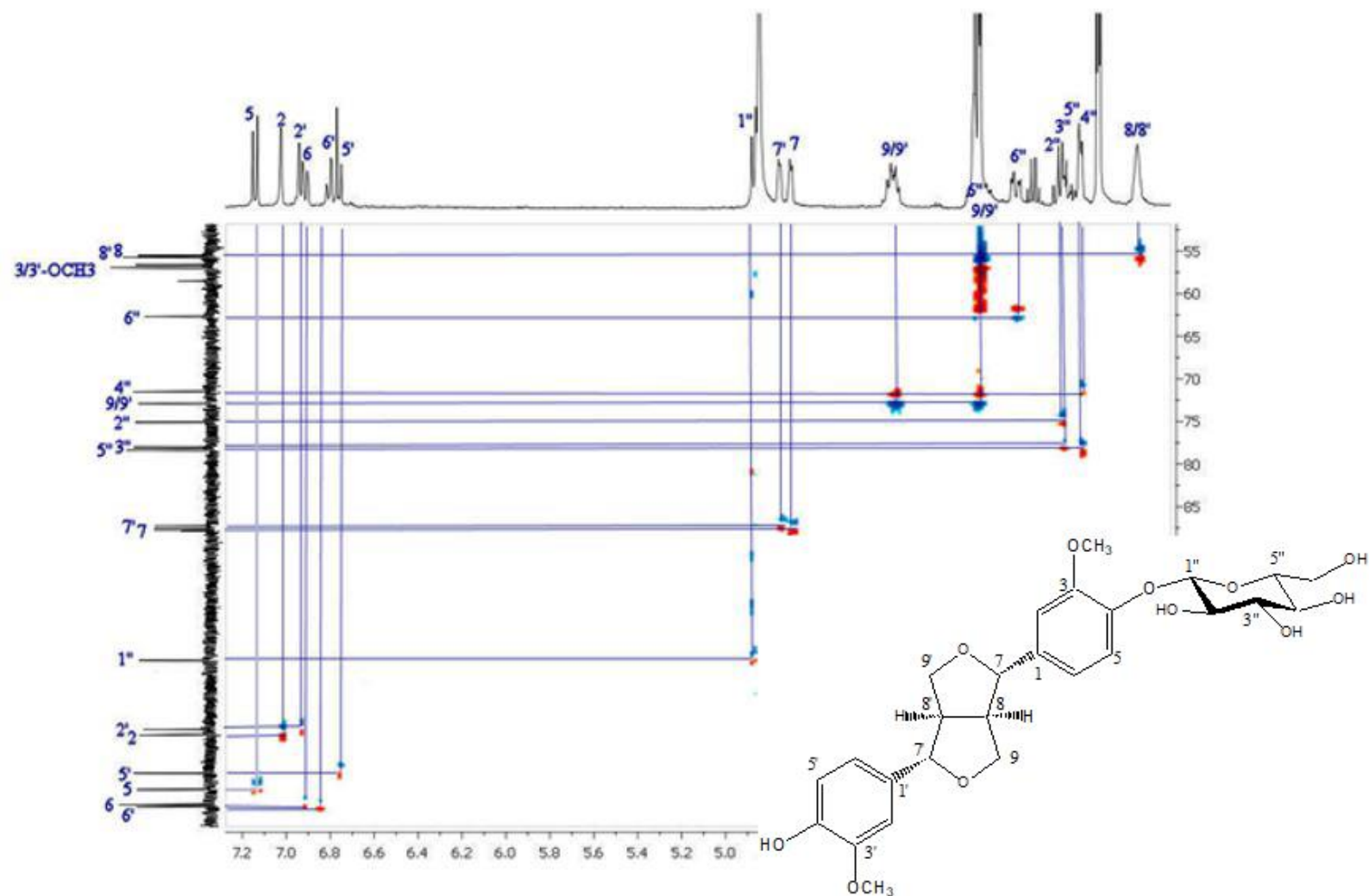
Spectrum 110. ^1H NMR Spectrum of Pinoresinol 4-O- β -D glucopyranoside (25) (CD₃OD, 400 MHz)



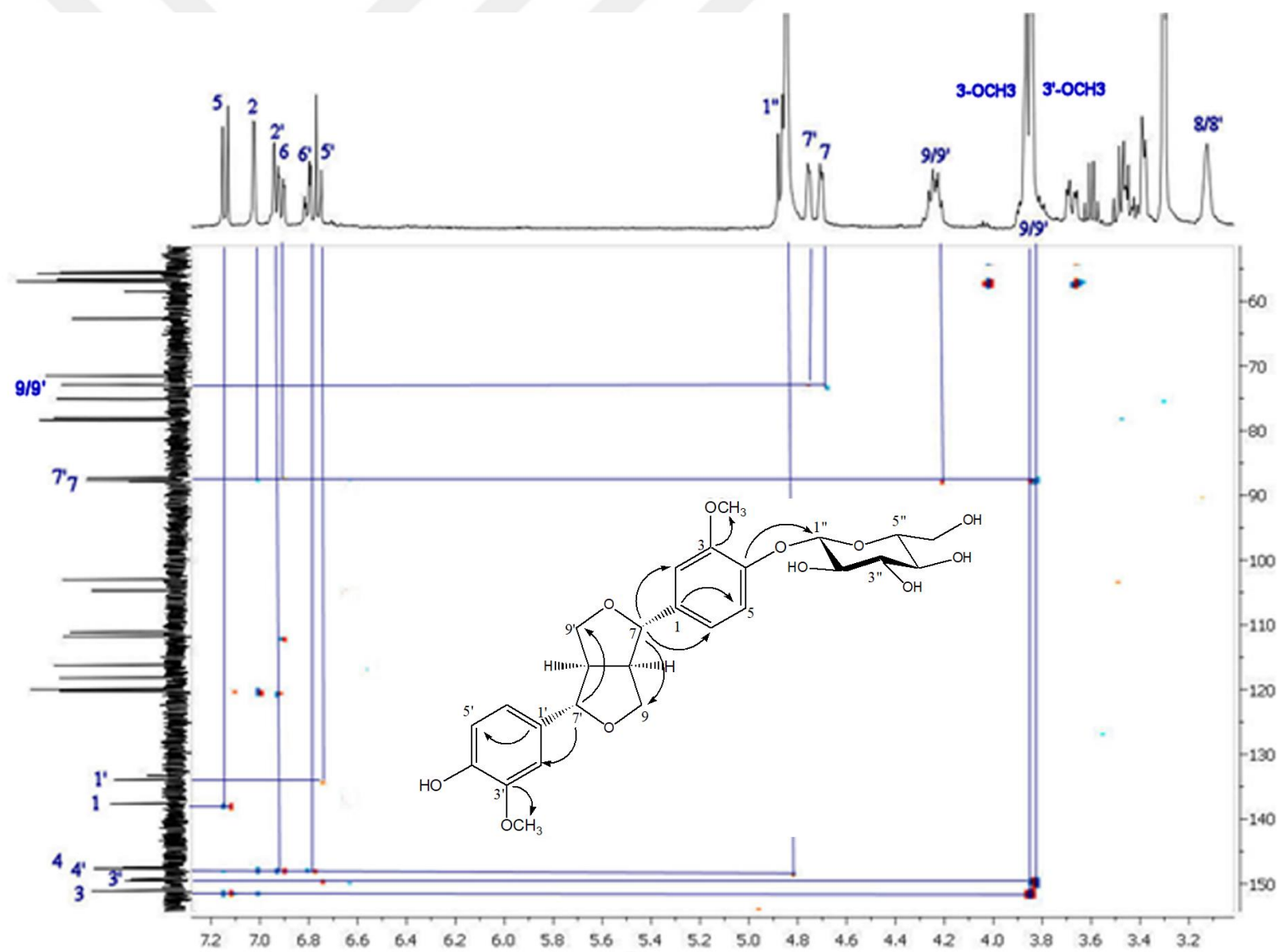
Spectrum 111. ^{13}C NMR Spectrum of Pinoresinol 4-O-β-D-glucopyranoside (25) (CD_3OD , 100 MHz)



Spectrum 112. COSY Spectrum of Pinoresinol 4-O-β-D glucopyranoside (25)

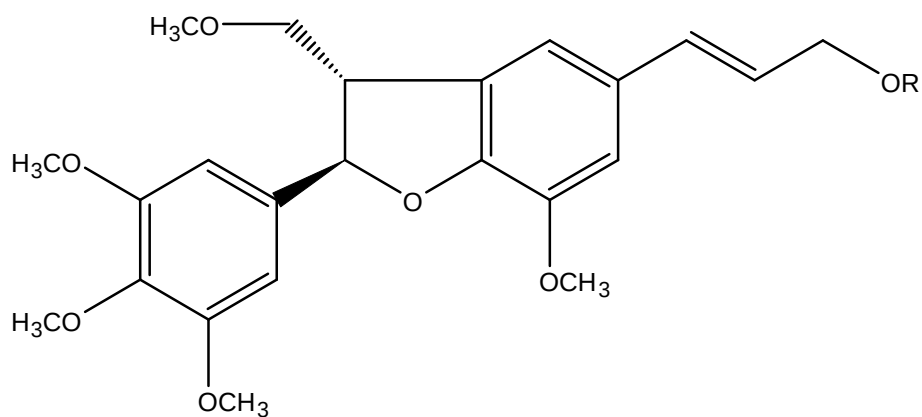


Spectrum 113. HSQC Spectrum of Pinoresinol 4-O-β-D glucopyranoside (25)



Spectrum 114. HMBC Spectrum of Pinoresinol 4-O-β-D glucopyranoside (25)

4.5.1.4.3.B. Neolignans



R

30: H

31: CH₃

SPELUNCARIN A (30): C₂₃H₂₈O₇ (MW:416.4642)

SPELUNCARIN B (31): C₂₄H₃₀O₇ (MW: 430.4908)

	Speluncarin A (30)	Speluncarin B (31)
UV $\lambda_{\max}$ (MeOH) nm:	207, 277	207, 225, 278
IR $\nu_{\max}$ (KBr) cm ⁻¹ :	3573 (OH), 1676 (olefinic, C=C), 1593 (aromatic, C=C)	1676 (olefinic, C=C), 1592 (aromatic, C=C)
HR-ESI-MS m/z :	417.1893 [M+H] ⁺ , (calcd for C ₂₃ H ₂₉ O ₇ ⁺ , 417.1908)	431.2045 [M+H] ⁺ , (calcd for C ₂₄ H ₃₁ O ₇ ⁺ , 431.2064)
¹ H NMR:	Table 104, Spectrum 115	Table 104, Spectrum 121
¹³ C NMR:	Table 104, Spectrum 116	Table 104, Spectrum 122
COSY:	Spectrum 117	Spectrum 123
HSQC:	Spectrum 118	Spectrum 124
HMBC:	Spectrum 119	Spectrum 125
NOESY:	Spectrum 120	Spectrum 126

SPELUNCARIN A (30)

Compound **30** was obtained as a colorless oil. The molecular formula, $C_{23}H_{28}O_7$, was established by HRESIMS (m/z 417.1893 $[M+H]^+$, calcd for $C_{23}H_{29}O_7^+$, 417.1908) and NMR analyses. The UV spectrum showed absorbances at λ_{max} 207 and 277 nm, and the IR spectrum displayed absorption bands characteristic for hydroxyl (3573 cm^{-1}), olefinic bond (1676 cm^{-1}) and aromatic ring (1593 cm^{-1}) functionalities. The ^1H NMR spectrum of **30** (Table 104) showed signals due to two 1,3,4,5-tetrasubstituted benzene rings [δ_{H} 6.89 (br s) and 6.87 (br s); δ_{H} 6.63 (s, 2H)], a disubstituted *trans*-olefin [δ_{H} 6.55 (d, $J = 15.6\text{ Hz}$) and 6.24 (dt, $J = 15.6, 6.1\text{ Hz}$), two oxymethylene [δ_{H} 3.69 and 3.63, (each br d, $J = 10.2\text{ Hz}$); δ_{H} 4.30 (2H, dd, $J = 5.9, 1.4\text{ Hz}$), two methine [δ_{H} 5.51 (d, $J = 5.8\text{ Hz}$) and 3.67 (m)], and five methoxy groups [δ_{H} 3.91 (3H, s), 3.83 (6H, s), 3.82 (3H, s) and 3.41 (3H, s)]. ^{13}C NMR spectrum of **30** contained resonances arising from five methoxy groups [δ_{C} 60.8, 59.1, 56.1 (2C), and 55.9], and 18 further signals, including 12 aromatic [δ_{C} 153.2 (2xC), 147.9, 144.4, 137.6, 136.8, 130.8, 128.5, 115.2, 110.3, 103.0 (2xC)], two olefinic (δ_{C} 131.4 and 126.3), two oxymethylene (δ_{C} 74.7 and 63.8) and two methine signals (δ_{C} 88.6 and 51.3), which are characteristic for a dehydrodiconiferyl alcohol type neolignan skeleton (263). The COSY spectrum of **30** (Spectrum 117) indicated the presence of two spin systems for the side chains of both phenylpropane units [(H-7/H-8/H₂-9) and (H-7'/H-8'/H₂-9')]. Detailed analysis of HSQC (Spectrum 118) and HMBC (Spectrum 119) spectra led the structure to be a dihydrobenzofuran type neolignan. The cross-peak between δ_{C} 131.4 (C-7') and δ_{H} 6.87 (H-2')/ δ_{H} 6.89 (H-6') determined the attachment point of phenylpropene side chain is C-1'. Similarly, the long-range coupling of the methine carbon at δ_{C} 88.6 (C-7) with the aromatic equivalent proton signals at δ_{H} 6.63 (H-2/6) confirmed the position of the aromatic ring to be C-7. These NMR findings were found to be similar with those of woorenogenin, reported from *Coptis japonica* (264) except for the presence of an additional methoxy signal at δ_{H} 3.41 in **30**. The locations of the methoxy groups were clarified unambiguously by the following cross-peaks in the HMBC spectrum; CH_3O -3/5 and C-3/5; CH_3O -4 and C-4; CH_3O -3' and C-3'; CH_3O -9 and C-9 (Spectrum 119).

The relative stereochemistry at C-7 and C-8 were determined as *trans* by the strong cross-peaks of H-7/H₂-9 and H-8/H-2/6 and a weak NOE between H-7 and H-8 in the NOESY spectrum (Spectrum 120) as well as due to the coupling constant of H-7 and H-8 ($J_{7,8} = 5.8\text{ Hz}$)

(265). Therefore, the chemical structure of **30** was proposed as depicted and given the trivial name **speluncarin A** based on the species name of *V. speluncaria*.



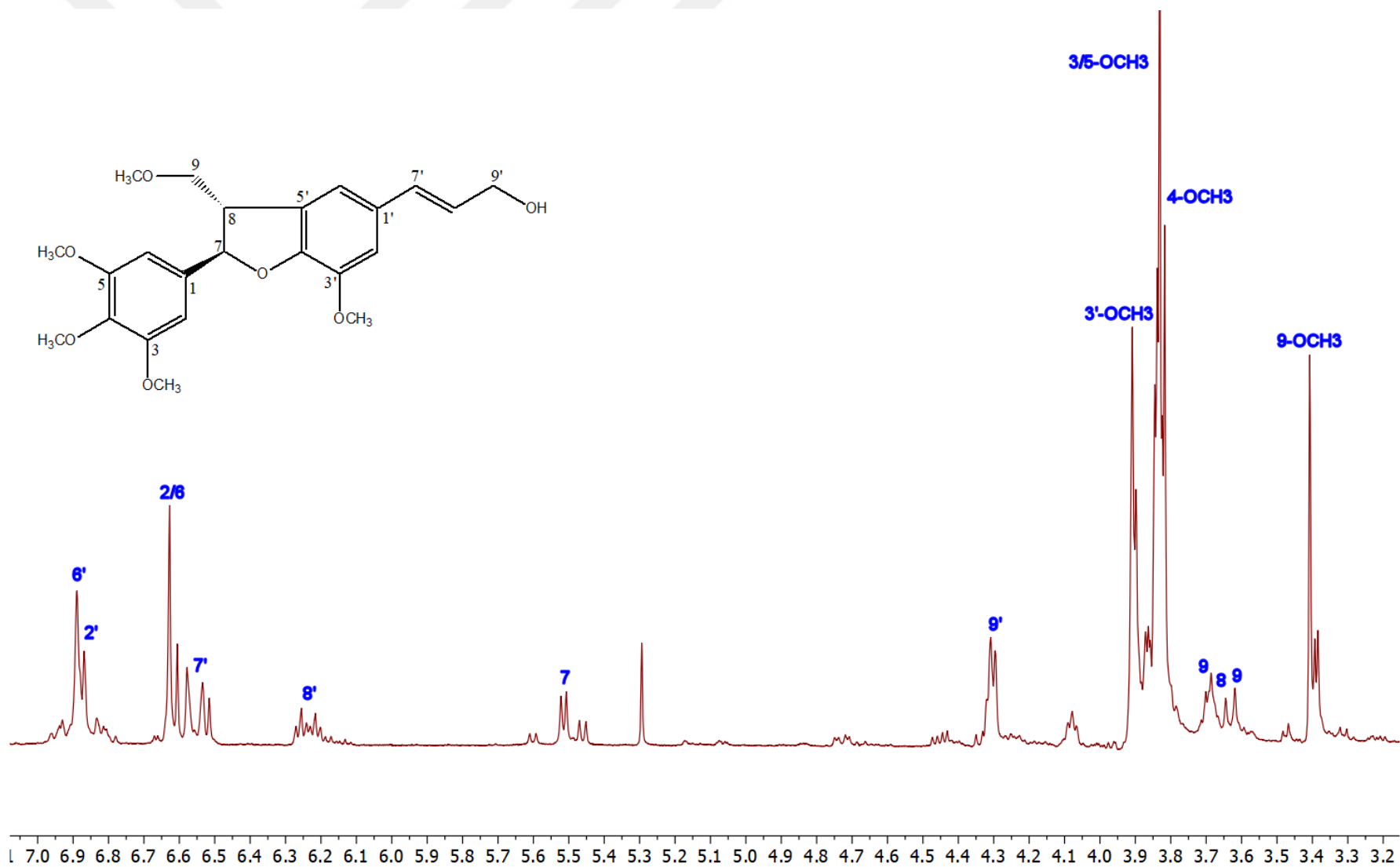
SPELUNCARIN B (31)

Compound **31** was obtained as a colorless oil. It gave a molecular formula of $C_{24}H_{30}O_7$, as determined from the HRESIMS data (m/z 431.2045 ($[M+H]^+$ calcd for $C_{24}H_{31}O_7^+$, 431.2064) and ^{13}C NMR spectrum. Its UV and IR spectra have resembled to those of **30**. The 1H and ^{13}C NMR data (Table 104) of **31**, identified by the aid of detailed analysis of COSY (Spectrum 123), HSQC (Spectrum 124) and HMBC (Spectrum 125) spectra, were found to be similar to those of **30**, except for an additional methoxy signal at δ_H 3.38 and δ_C 57.8 in the NMR spectra of **31**. Moreover, the oxymethylene carbon signal at δ_C 73.2 (C-9') was found to be shifted downfield around 10 ppm, indicating the location of the additional methoxy group to be at C-9'. The key HMBC correlation (Spectrum 125) between C-9' (δ_C 73.2) and OCH_3 -9' (δ_H 3.38) confirmed the location of the methoxy group. Accordingly, compound **31** was identified as a new neolignan and named as **speluncarin B**.

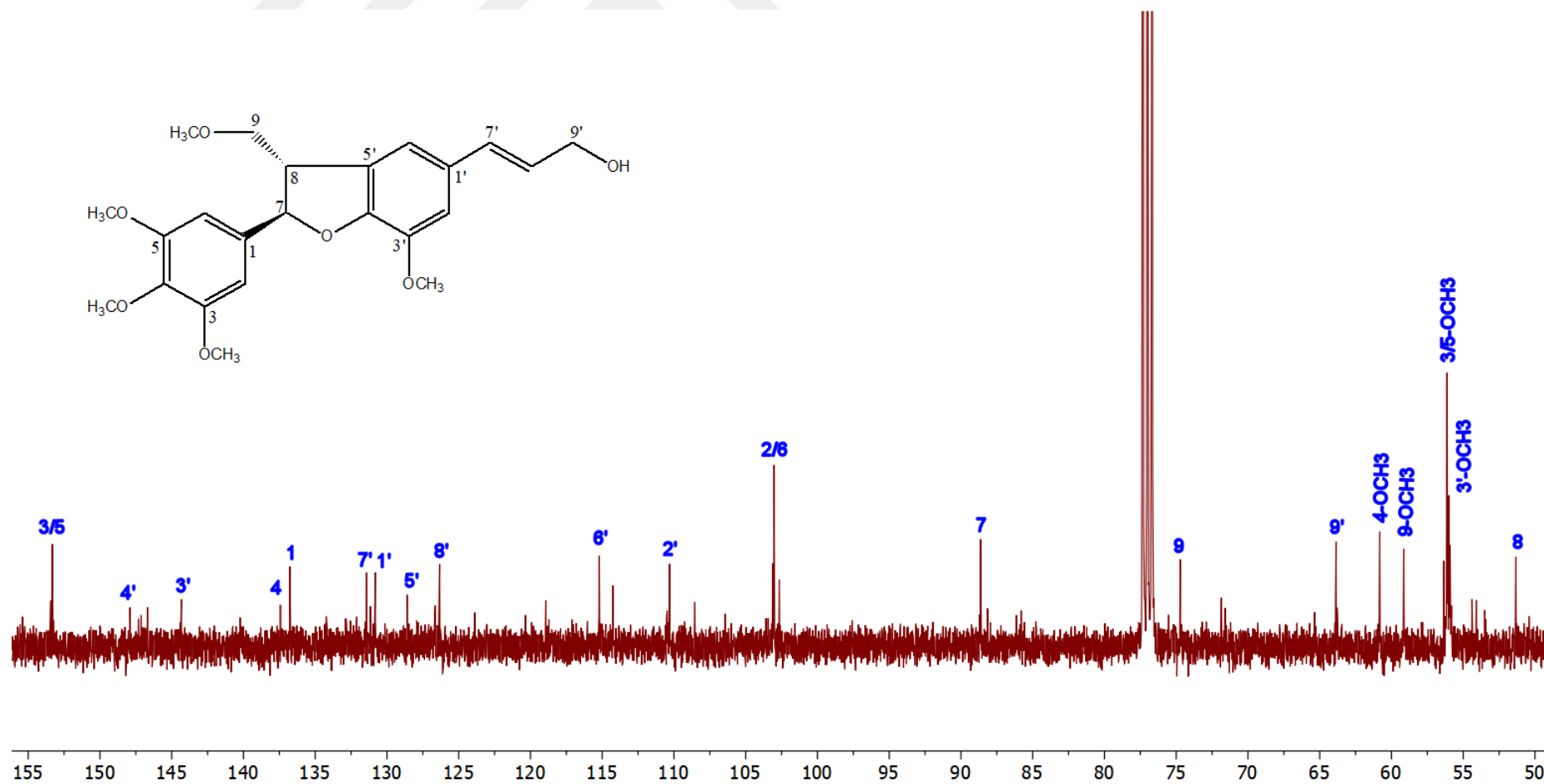
Table 104. ^{13}C and ^1H NMR data of speluncarins A (**30**) and B (**31**) (CDCl_3 ; ^{13}C : 100 MHz, ^1H : 400 MHz)^a

C/H Atom	Multiplicity	30		31	
		δ_{C} (ppm)	δ_{H} (ppm), J (Hz)	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
1	C	136.8	-	136.8	-
2	CH	103.0	6.63 s	103.1	6.63 s
3	C	153.2	-	153.3	-
4	C	137.6	-	137.7	-
5	C	153.2	-	153.3	-
6	CH	103.0	6.63 s	103.1	6.63 s
7	CH	88.6	5.51 d (5.8)	88.6	5.51 d (5.9)
8	CH	51.3	3.67 m	51.4	3.67 m
9	CH ₂	74.7	3.69 br.d (10.2)	74.7	3.69 br.d (10.5)
			3.63 br.d (10.2)		3.63 br.d (10.5)
3/5-OCH ₃	CH ₃	56.1	3.83 s	56.1	3.83 s
4-OCH ₃	CH ₃	60.8	3.82 s	60.8	3.82 s
9-OCH ₃	CH ₃	59.1	3.41 s	59.0	3.40 s
1'	C	130.8	-	130.8	-
2'	CH	110.3	6.87 s	110.3	6.87 s
3'	C	144.4	-	144.3	-
4'	C	147.9	-	147.9	-
5'	C	128.5	-	128.5	-
6'	CH	115.2	6.89 s	115.2	6.89 s
7'	CH	131.4	6.55 d (15.6)	132.6	6.55 d (15.8)
8'	CH	126.3	6.24 dt (15.6, 6.1)	123.8	6.14 dt (15.8, 6.2)
9'	CH ₂	63.8	4.30 dd (5.9, 1.4)	73.2	4.07 dd (6.2, 1.1)
3'-OCH ₃	CH ₃	55.9	3.91 s	56.0	3.90 s
9'-OCH ₃	-	-		57.8	3.38 s

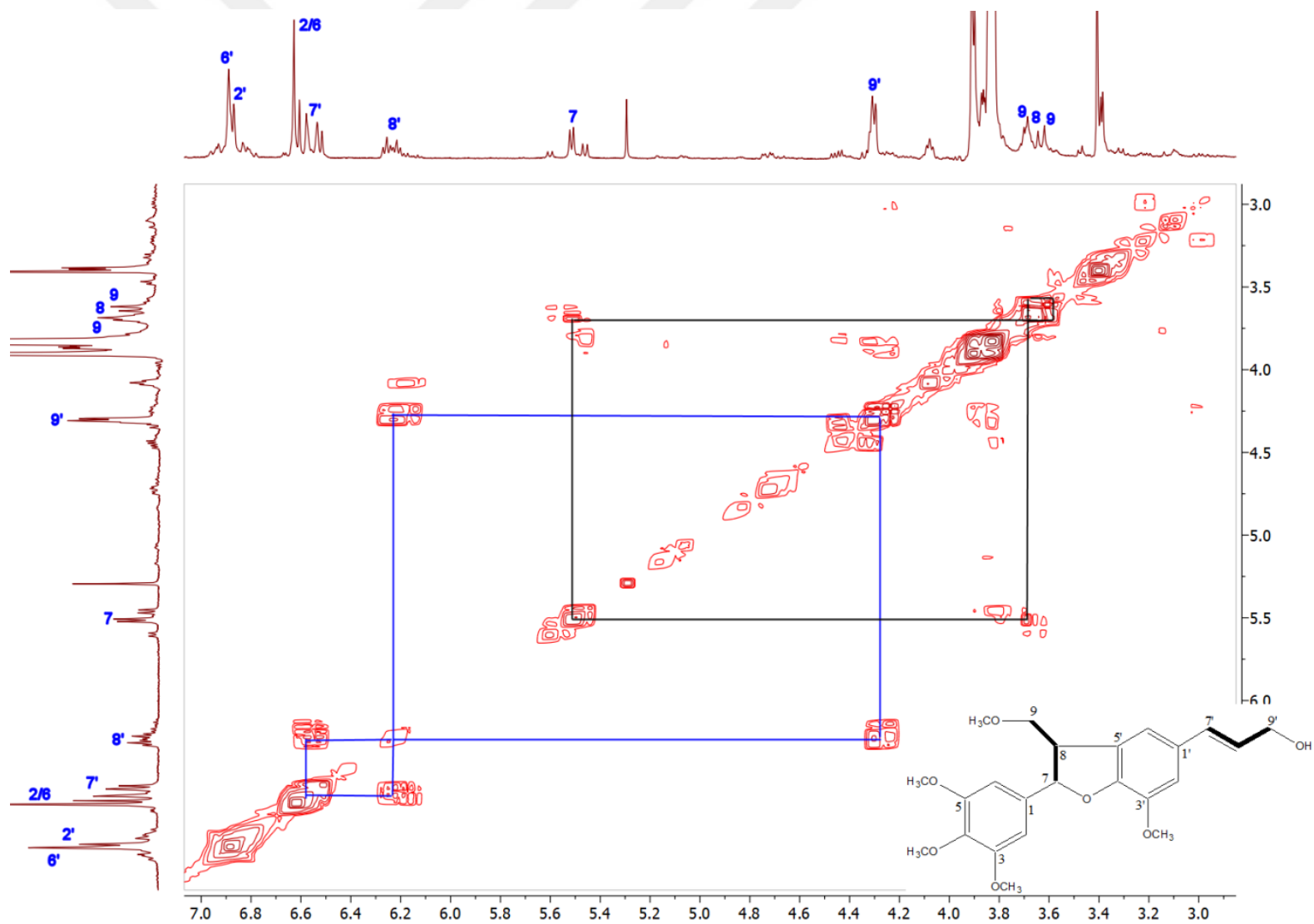
^aAssignments are based on COSY, HSQC, HMBC and NOESY experiments.



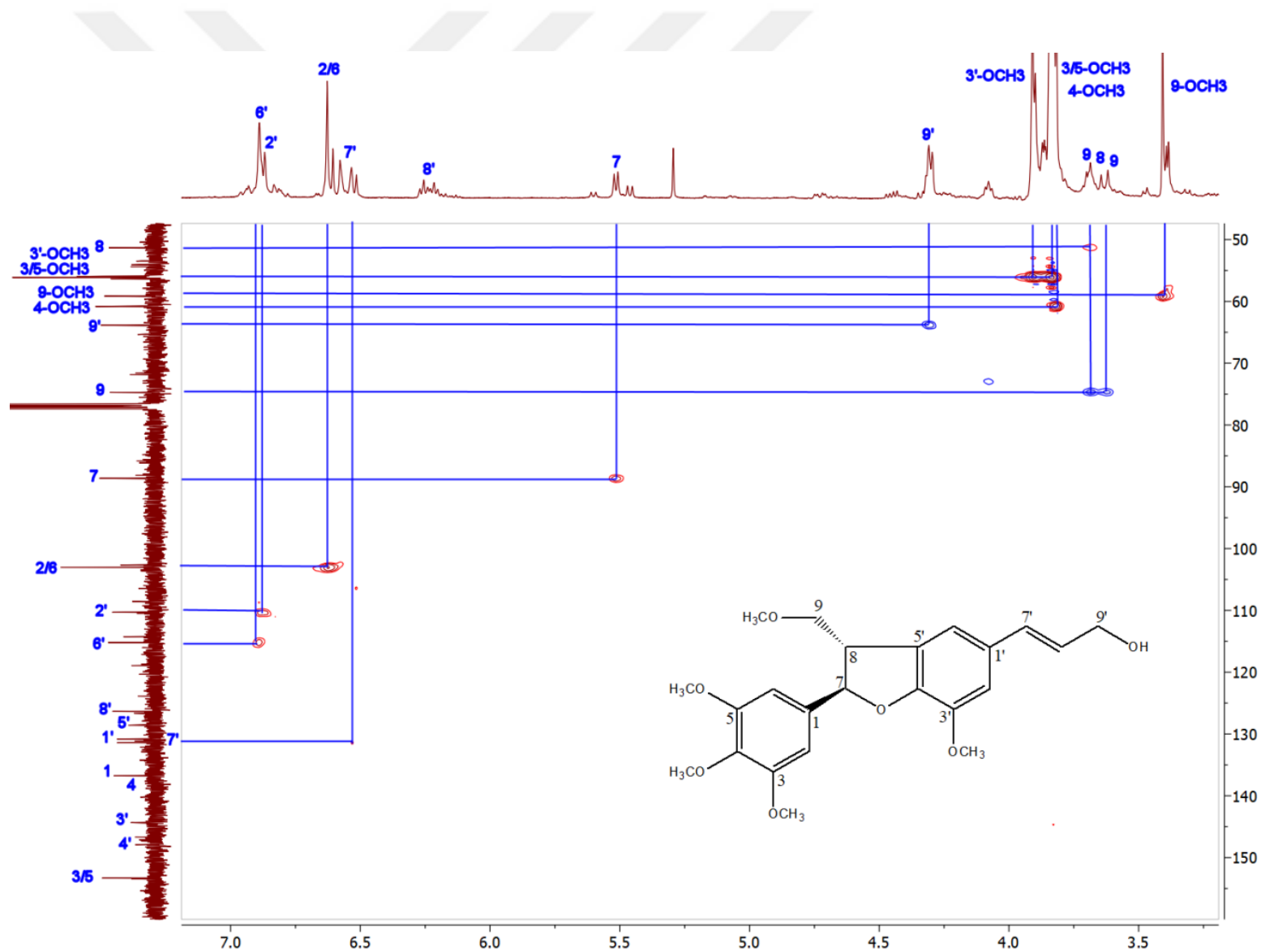
Spectrum 115. ¹H NMR Spectrum of Speluncarin A (30) (CDCl₃, 400 MHz)



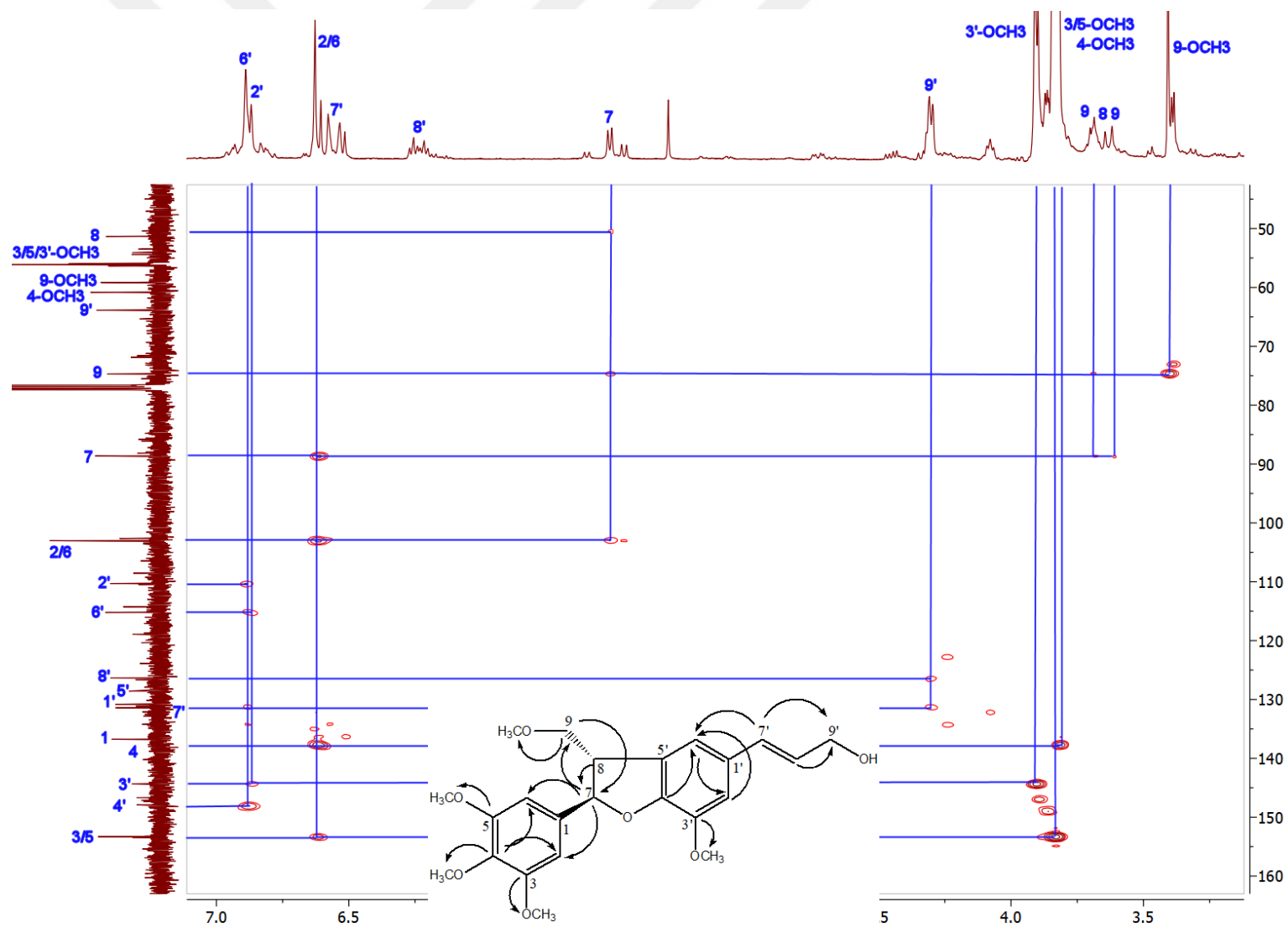
Spectrum 116. ^{13}C NMR Spectrum of Speluncarin A (30) (CDCl_3 , 100 MHz)



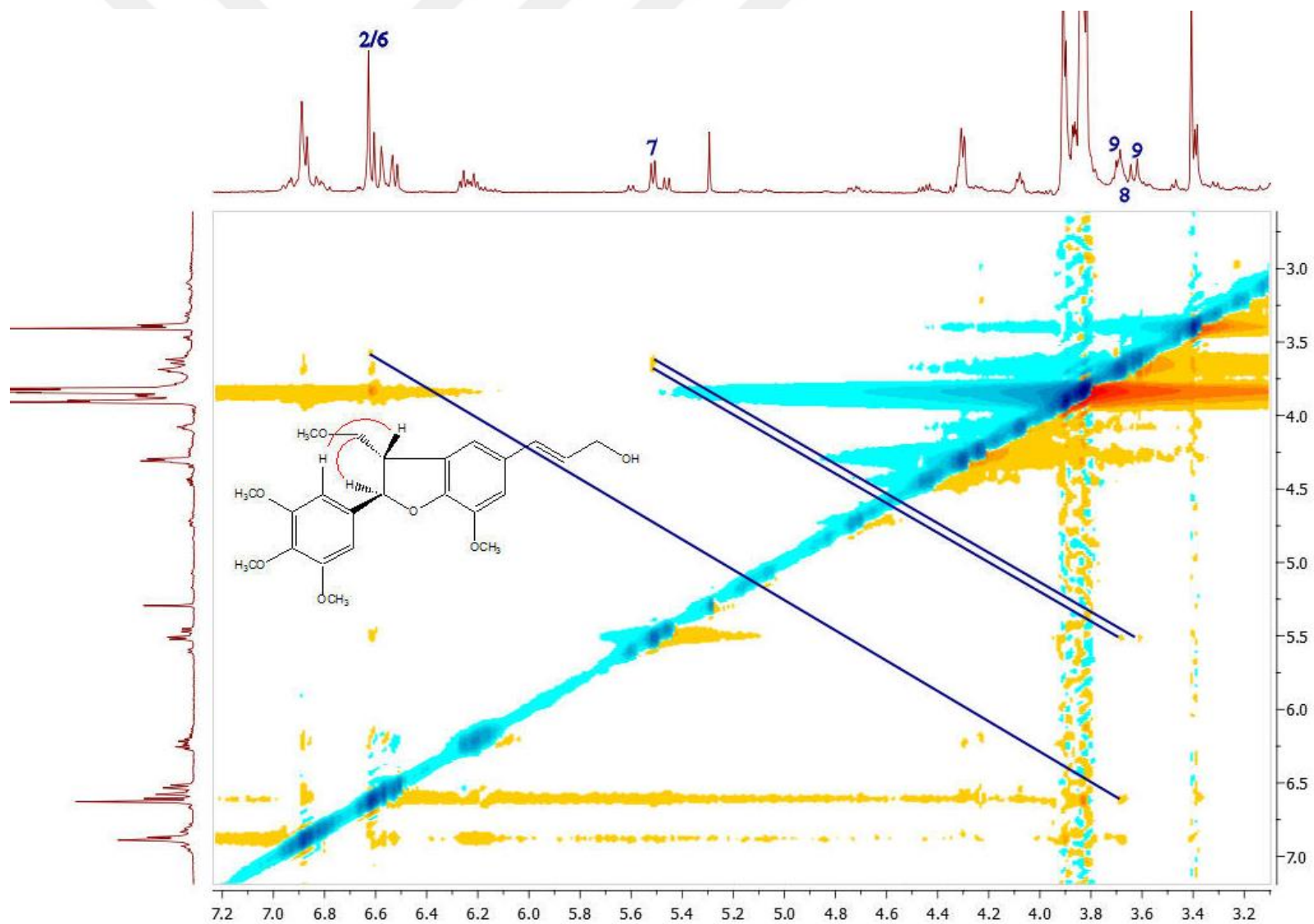
Spectrum 117. COSY Spectrum of Speluncarin A (30)



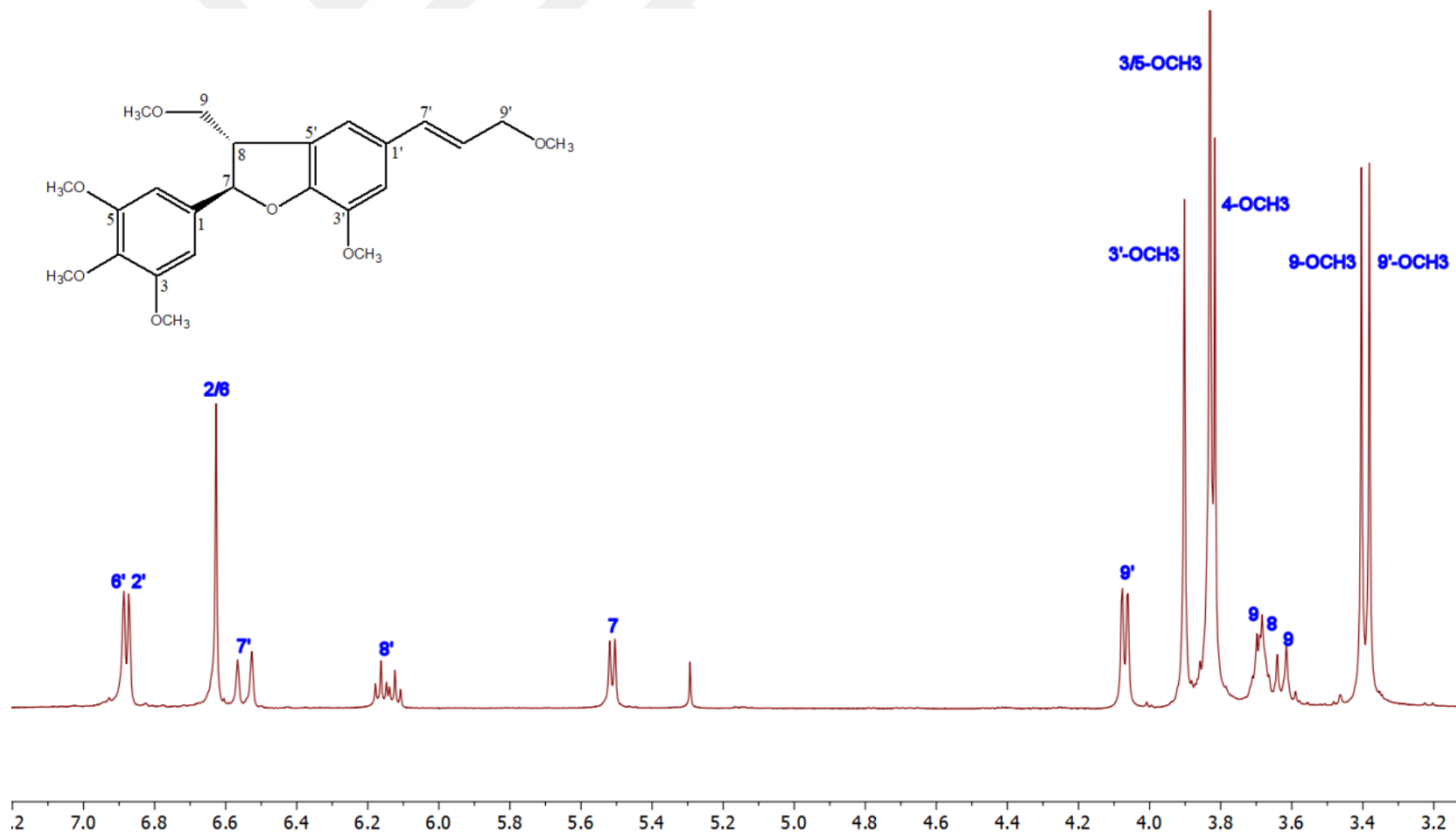
Spectrum 118. HSQC Spectrum of Speluncarin A (30)



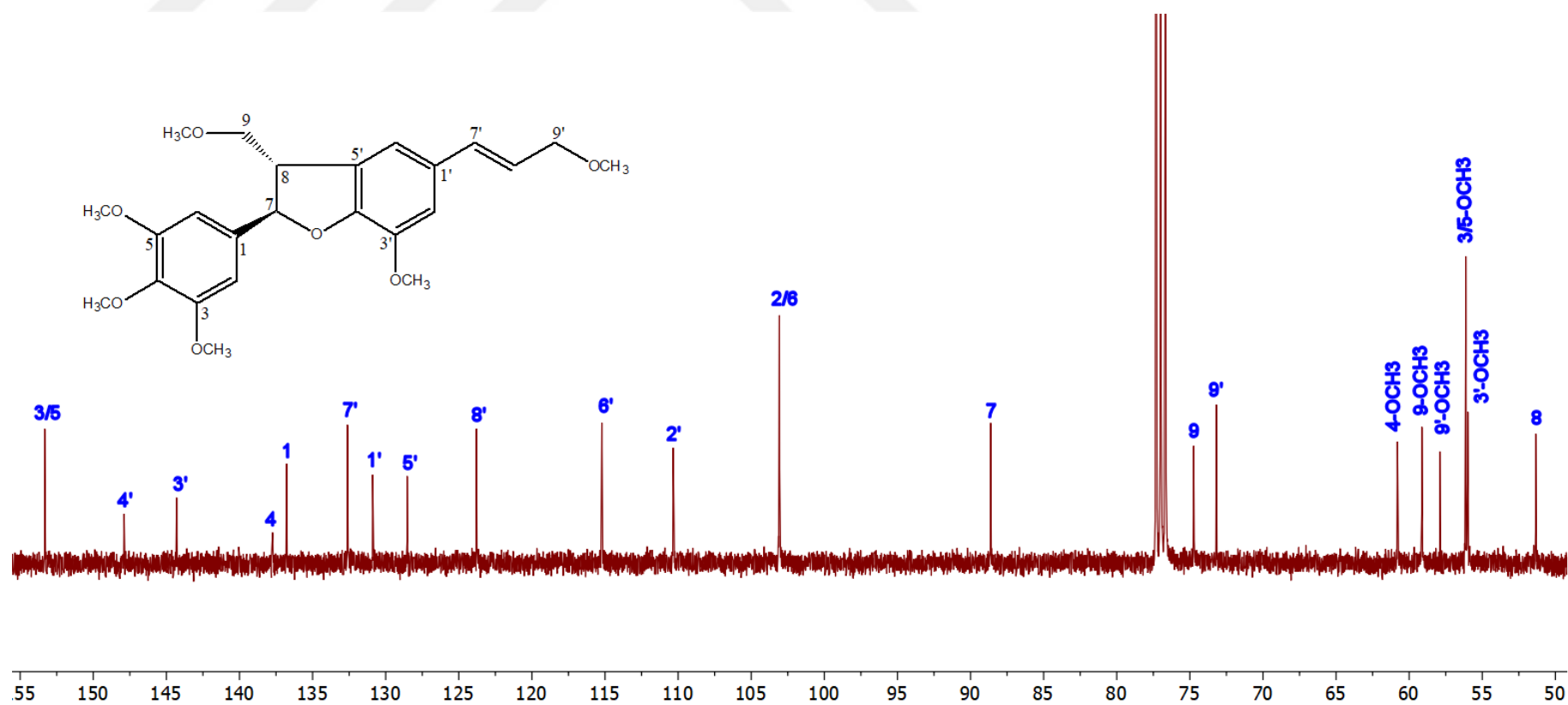
Spectrum 119. HMBC Spectrum of Speluncarin A (30)



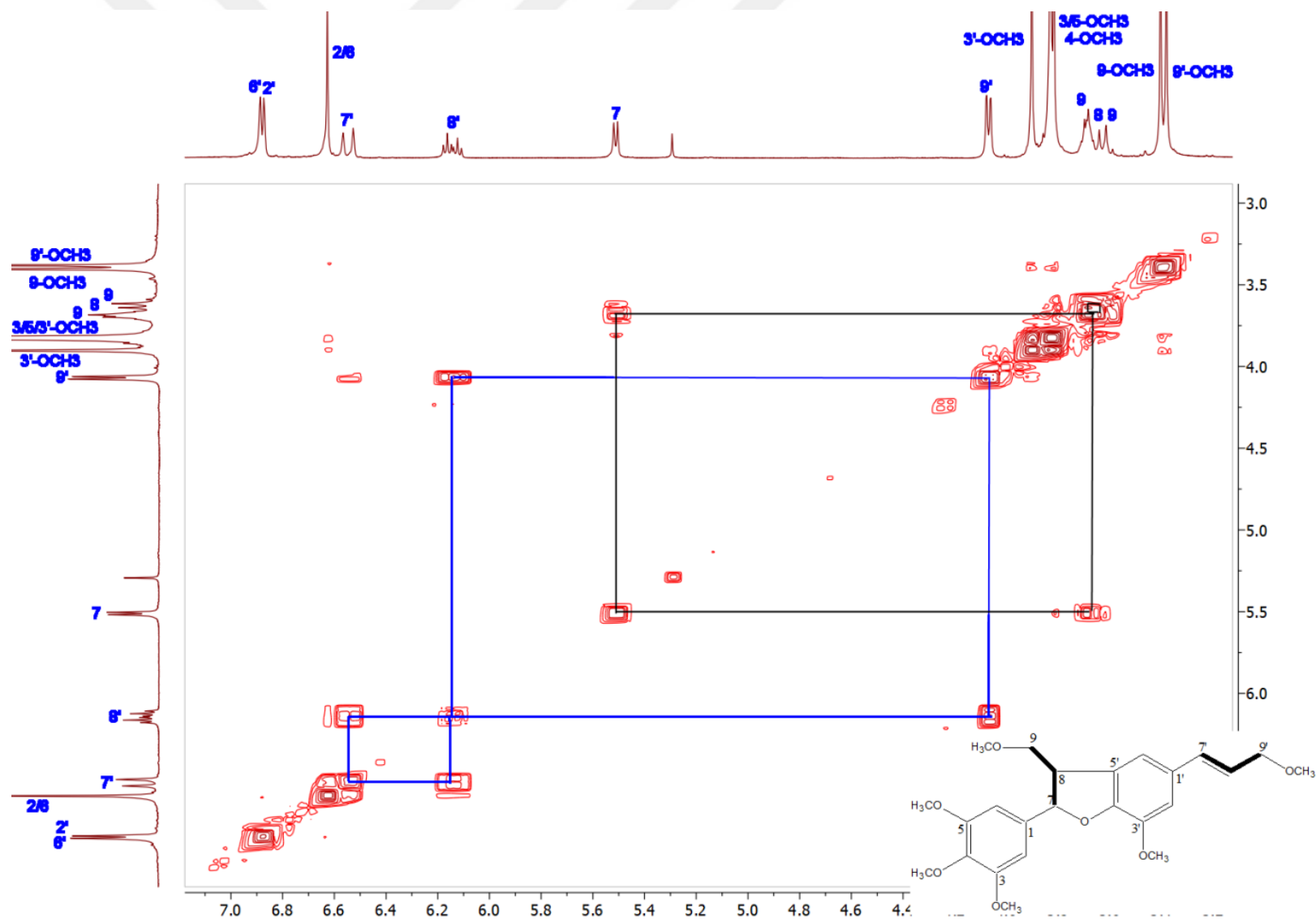
Spectrum 120. NOESY Spectrum of Speluncarin A (30)



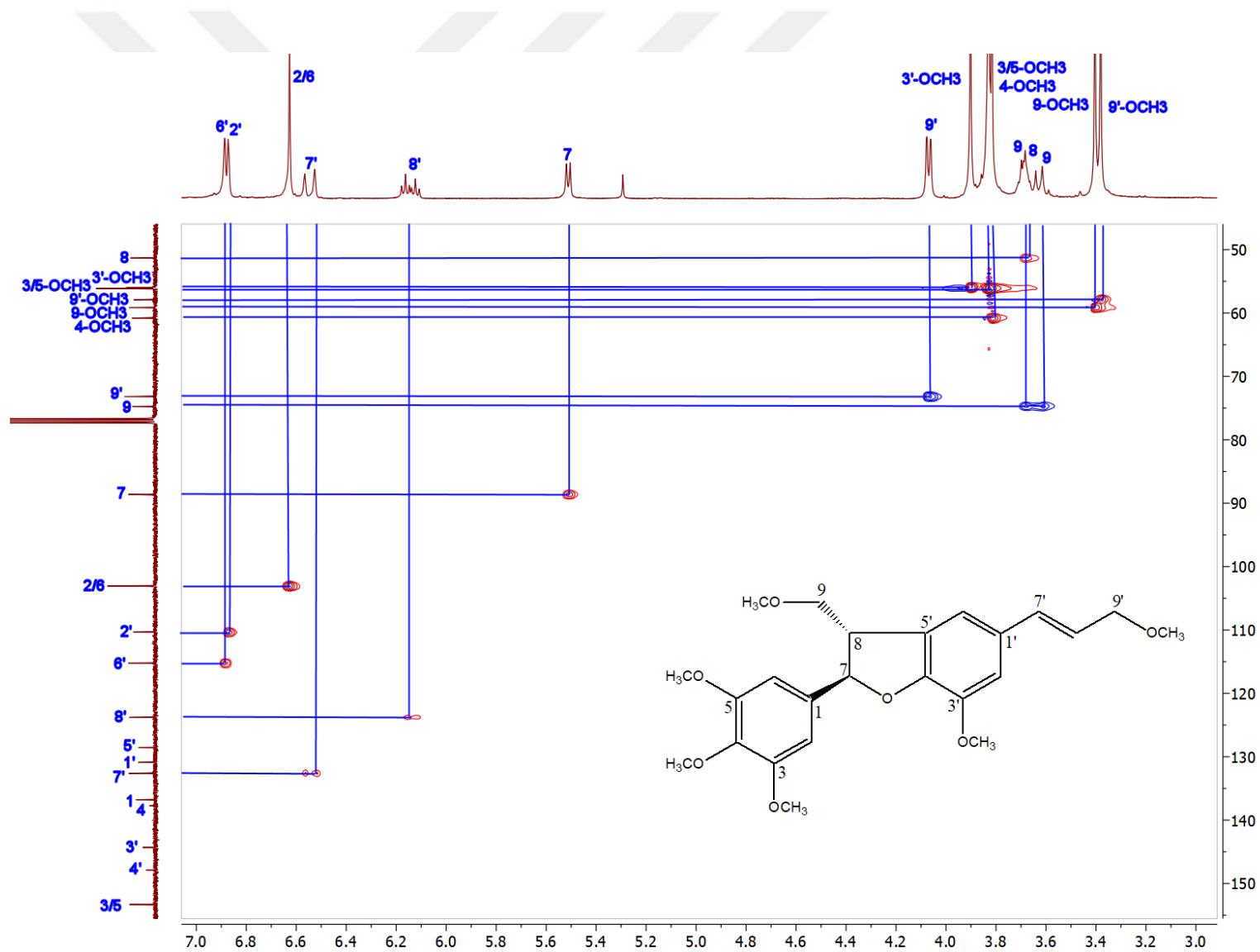
Spectrum 121. ¹H NMR Spectrum of Speluncarin B (31) (CDCl₃, 400 MHz)



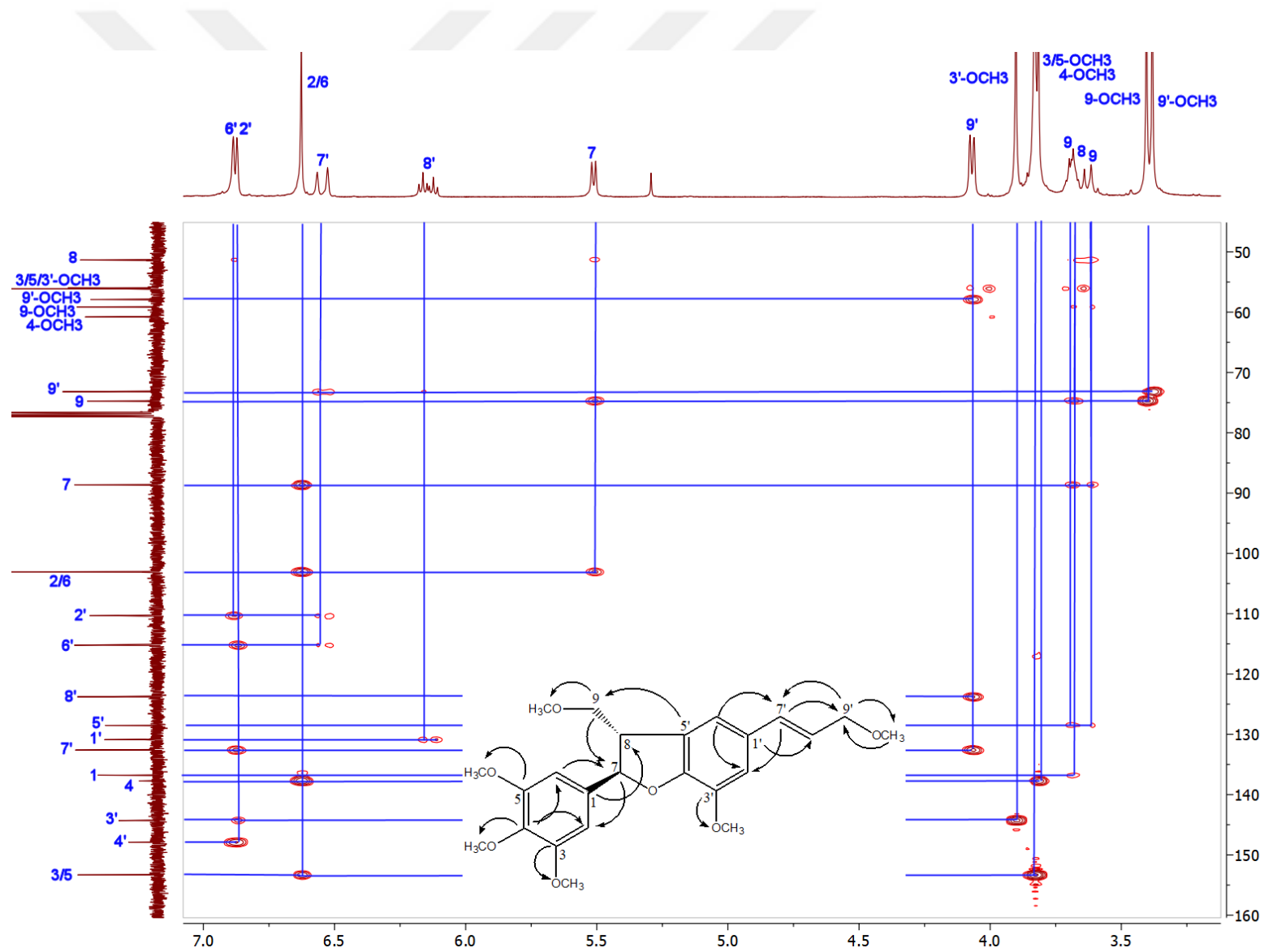
Spectrum 122. ¹³C NMR Spectrum of Speluncarin B (31) (CDCl₃, 100 MHz)



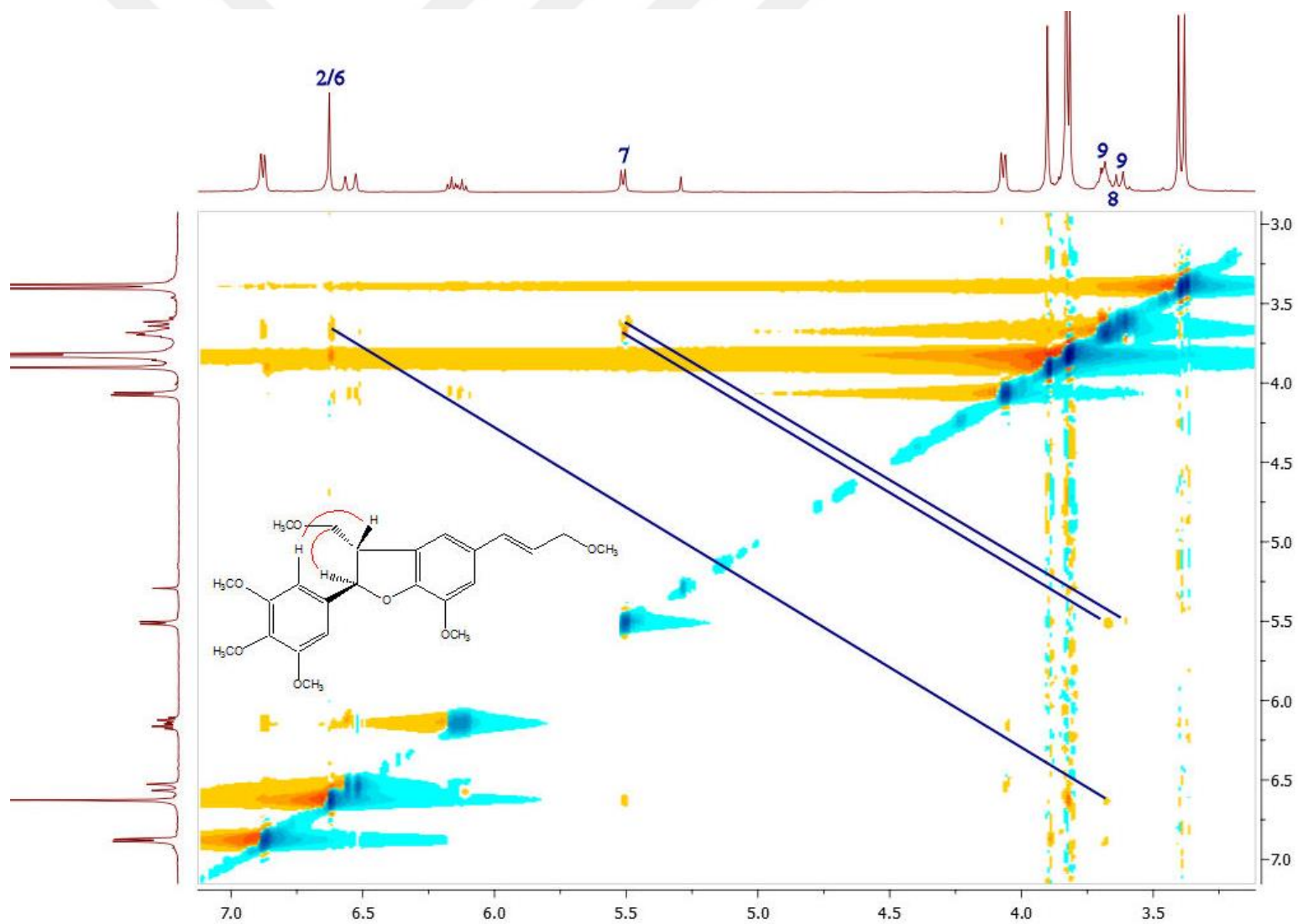
Spectrum 123. COSY Spectrum of Speluncarin B (31)



Spectrum 124. HSQC Spectrum of Speluncarin B (31)

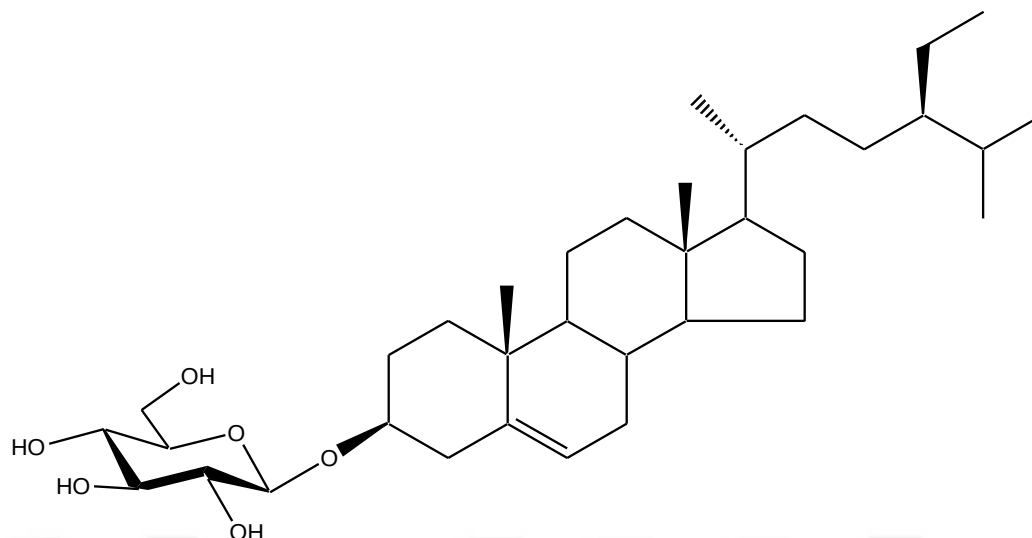


Spectrum 125. HMBC Spectrum of Speluncarin B (31)



Spectrum 126. NOESY Spectrum of Speluncarin B (31)

4.5.1.4.3. Phytosterol glycoside



β -SITOSTEROL 3-O- β -D-GLUCOPYRANOSIDE (DAUCOSTEROL) (32):

C₃₅H₆₀O₆ (MW:576.85)

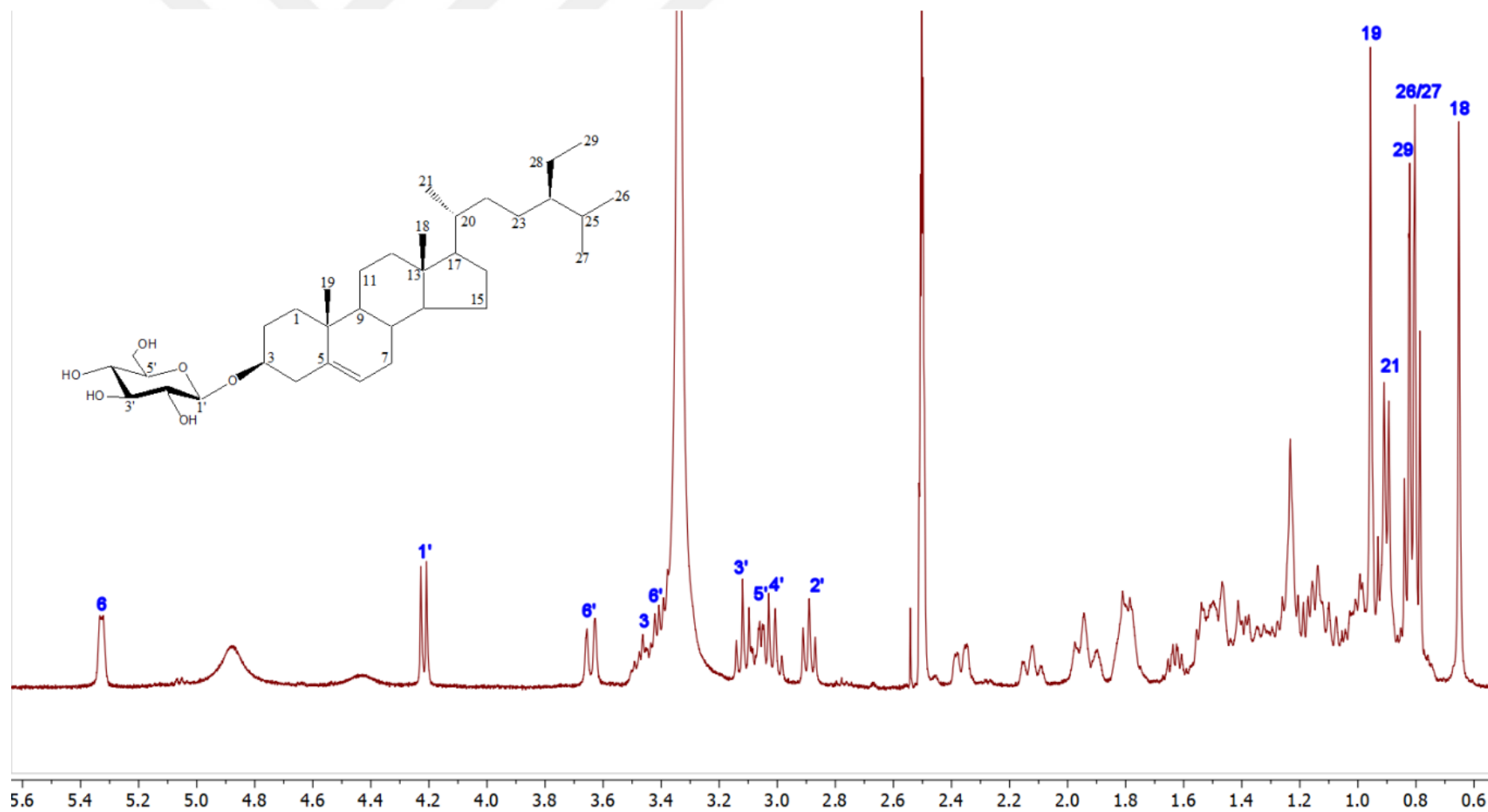
IR $\nu_{\max}$ (KBr) cm ⁻¹ :	3402 (OH), 1527 (olefinic, C=C)
¹ H NMR:	Table 105, Spectrum 127
¹³ C NMR:	Table 105, Spectrum 128

Compound **32** was obtained as white solid. Its IR spectrum demonstrated the presence of absorption bands for hydroxyl (3402 cm⁻¹) and olefinic (1527 cm⁻¹) functional groups. Its ¹H and ¹³C NMR spectra exhibited characteristic signals for a phytosterol core and a β -glucopyranose moieties. It was identified as by comparison of the 1D NMR data of **32** (Table 105, Spectra 127 and 128) with those reported for β -sitosterol 3-O- β -D-glucopyranoside in the literature (206).

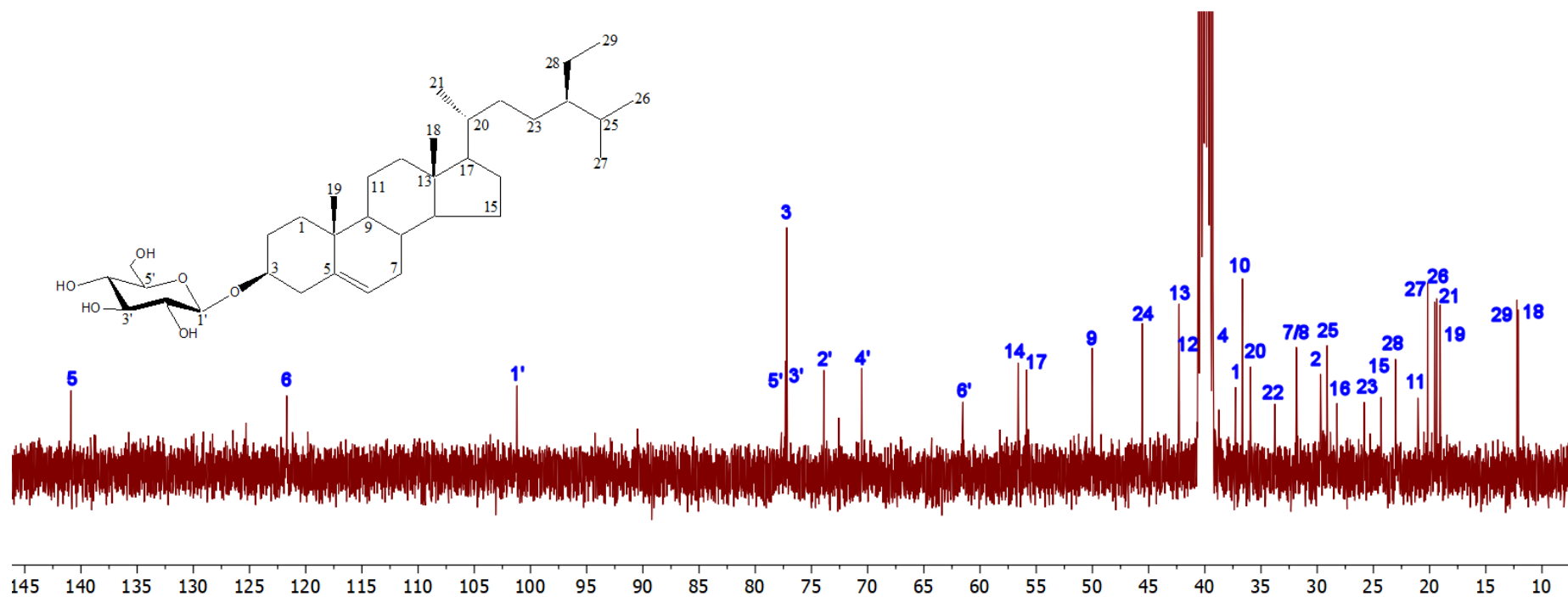
Table 105. ^{13}C and ^1H NMR data of β -sitosterol 3-O- β -D-glucopyranoside (Daucosterol) (**32**) (DMSO- d_6 ; ^{13}C : 100 MHz, ^1H : 400 MHz)

C/H Atom	Multiplicity	δ_{C} (ppm)	δ_{H} (ppm), J (Hz)
1	CH ₂	37.2	1.79 †, 0.96 †
2	CH ₂	29.7	1.79 †, 1.47 †
3	CH	77.2	3.46 †
4	CH ₂	39.4	2.37 dd (13.2, 3.1), 2.12 †
5	C	140.9	-
6	CH	121.7	5.33 d (2.6)
7	CH ₂	31.8	1.94 †, 1.51 †
8	CH	31.8	1.41 †
9	CH	50.0	0.87 †
10	C	36.7	-
11	CH ₂	21.0	1.47 †
12	CH ₂	40.1	1.94 †, 1.10 †
13	C	42.3	-
14	CH	56.6	0.96 †
15	CH ₂	24.3	1.55 †, 1.01 †
16	CH ₂	28.2	1.79 †, 1.23 †
17	CH	55.8	1.08 †
18	CH ₃	12.1	0.65 s
19	CH ₃	19.4	0.96 s
20	CH	35.9	1.34 †
21	CH ₃	19.1	0.90 d (6.4)
22	CH ₂	33.8	1.27 †, 0.96 †
23	CH ₂	25.8	1.16 †
24	CH	45.6	0.92 †
25	CH	29.1	1.63 m
26	CH ₃	19.5	0.79 d (6.7)
27	CH ₃	20.2	0.79 d (6.7)
28	CH ₂	23.0	1.25 †
29	CH ₃	12.2	0.82 t (7.2)
<i>Glucose (C-3)</i>			
1'	CH	101.2	4.22 d (7.7)
2'	CH	73.9	2.89 †
3'	CH	77.2	3.12 t (8.6)
4'	CH	70.5	3.02 †
5'	CH	77.3	3.06 †
6'	CH ₂	61.5	3.64 d (10.9), 3.39 †

†: Overlapped signals.



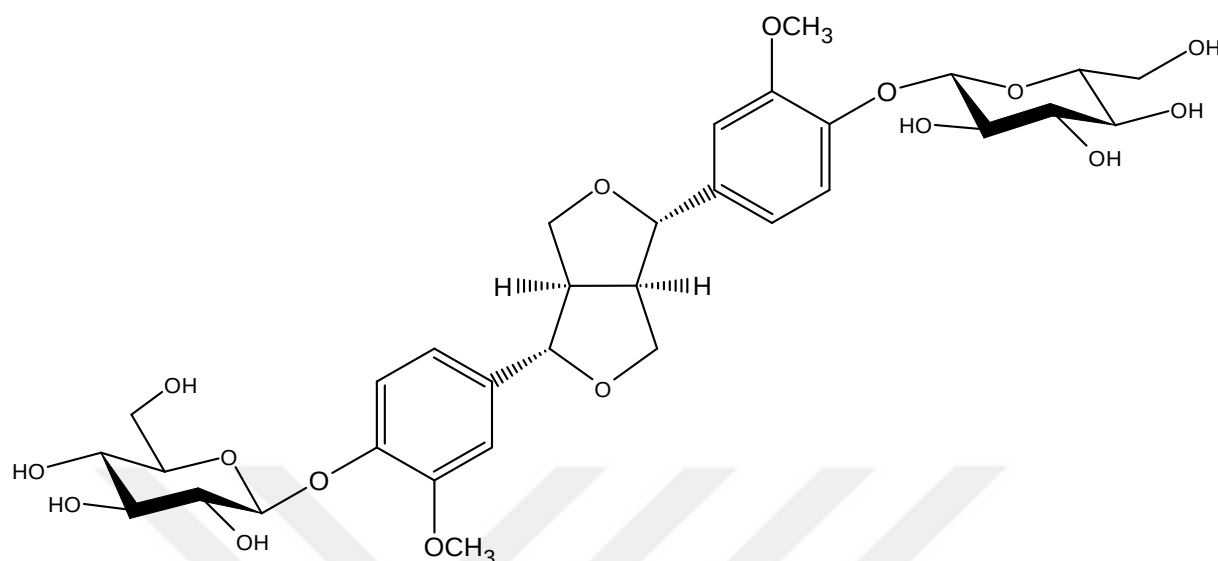
Spectrum 127. ^1H NMR Spectrum of β -Sitosterol 3-O- β -D-glucopyranoside (32) ($\text{DMSO}-d_6$, 400 MHz)



Spectrum 128. ^{13}C NMR Spectrum of β -Sitosterol 3-O- β -D-glucopyranoside (32) ($\text{DMSO}-d_6$, 100 MHz)

4.5.1.5. Compounds Isolated from *n*-BuOH Subextract of *V. speluncaria*

4.5.1.5.1. Lignan glycosides



PINORESINOL 4,4'-DI-O- β -D-GLUCOPYRANOSIDE (**36**): C₃₂H₄₂O₁₆ (MW:682.6)

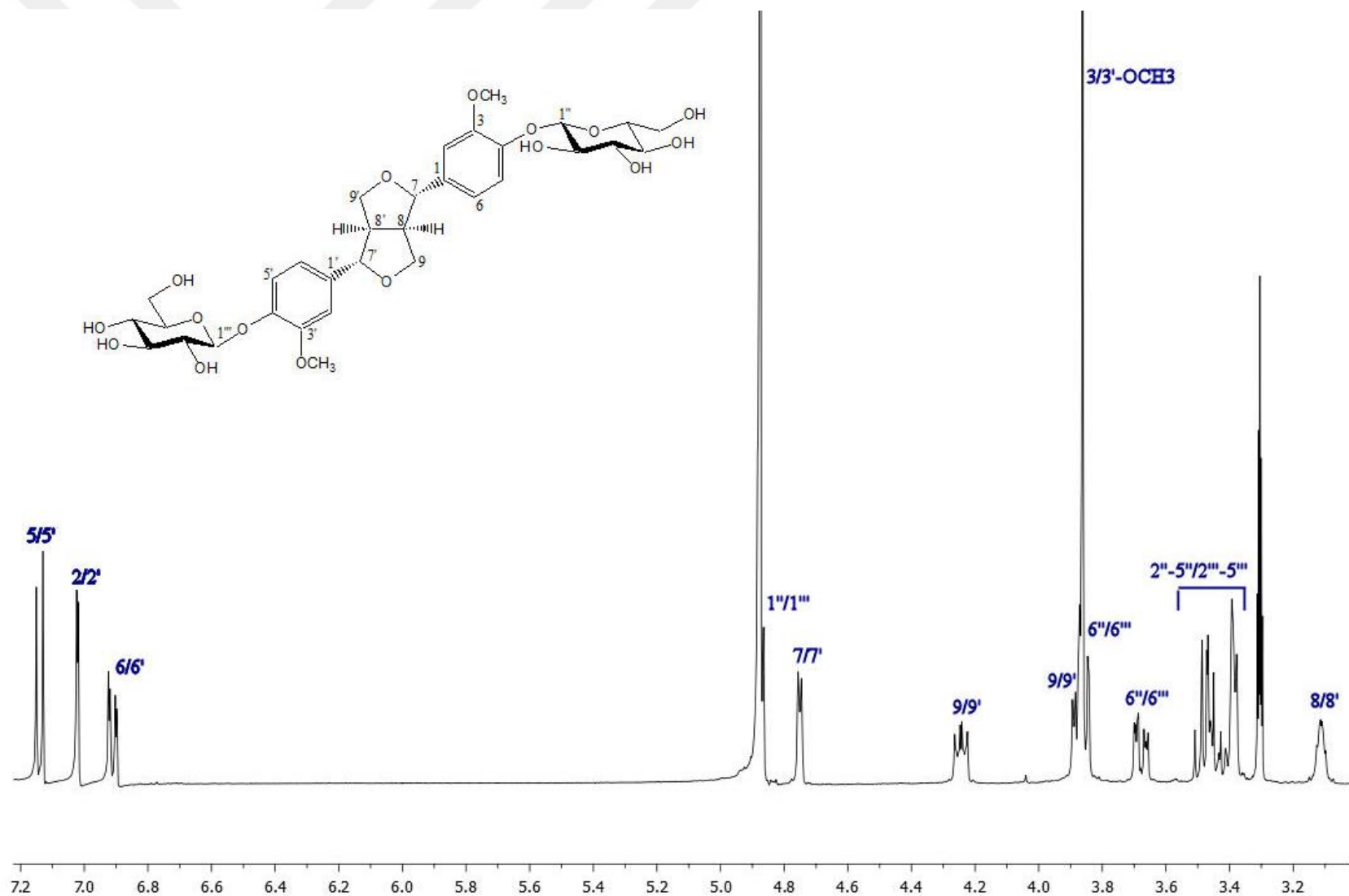
UV $\lambda_{\max}$ (MeOH) nm:	228, 278
ESI-MS m/z :	706.34 [M+Na] ⁺
IR $\nu_{\max}$ (KBr) cm ⁻¹ :	3374 (OH), 1596, 1516 (aromatic, C=C)
¹ H NMR:	Table 106, Spectrum 129
¹³ C NMR:	Table 106, Spectrum 130

Compound **36** was isolated as amorphous white solid with a molecular formula C₃₂H₄₂O₁₆, as established by ESI-MS at m/z 706.34 [M+Na]⁺. The UV spectrum showed absorption maxima at 228 and 278 nm. The absorption bands belonging to hydroxyl (3374 cm⁻¹) and aromatic ring (1596 and 1516 cm⁻¹) were observed in IR spectrum. Its 1D NMR data (Table 106, Spectra 129 and 130) of **36** demonstrated that **36** had a symmetrical lignan glycoside structure due to the overlapped signals. A careful inspection of its NMR and MS data led to the identification of **36** to be pinoresinol 4,4'-di-O- β -D-glucopyranoside (266).

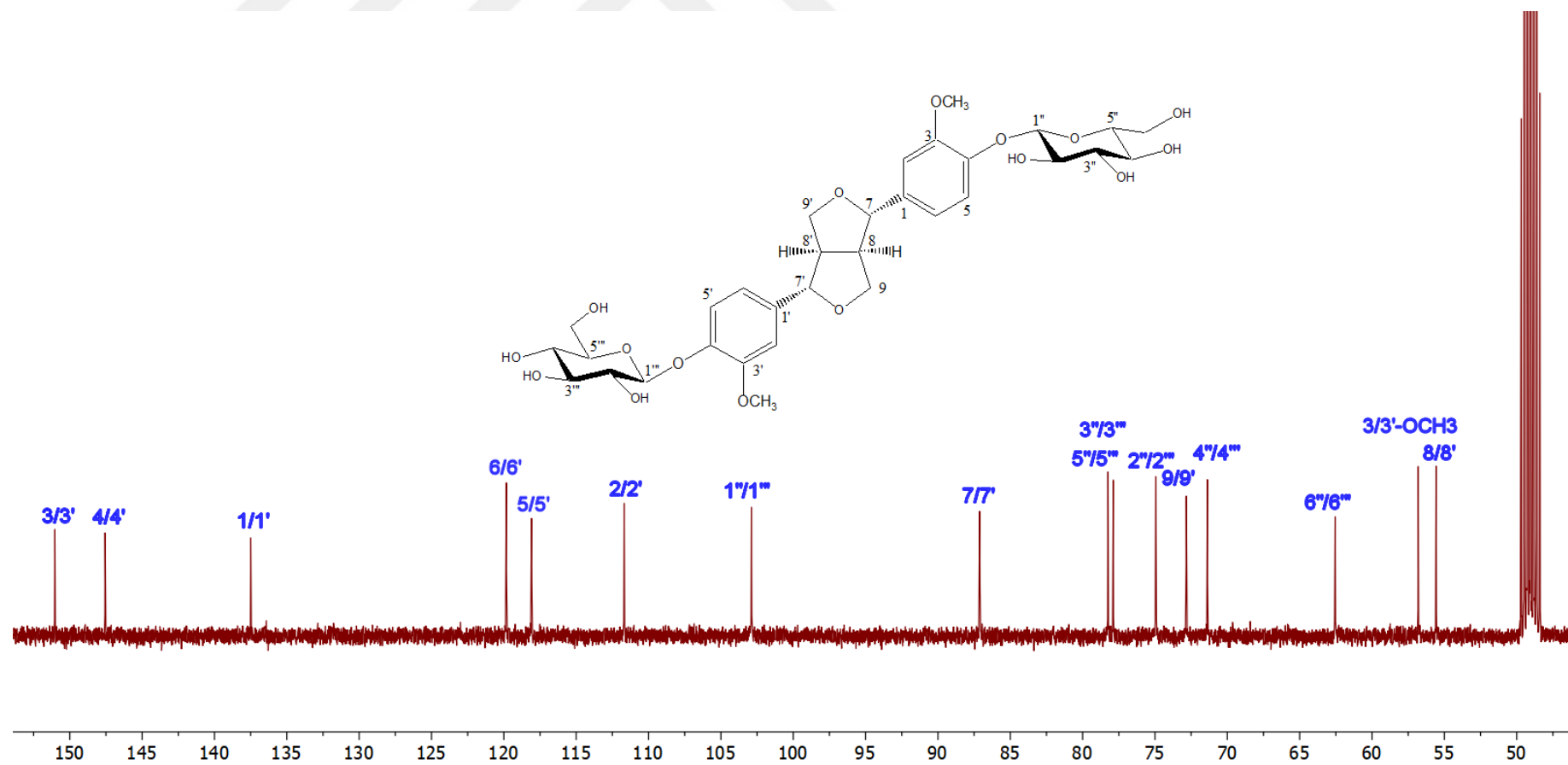
Table 106. ^{13}C and ^1H NMR data of pinoresinol 4,4'-di-*O*- β -D-glucopyranoside (**36**) (CD_3OD ; ^{13}C : 100 MHz, ^1H : 400 MHz)

C/H Atom	Multiplicity	δ_{C} ppm	δ_{H} ppm, <i>J</i> (Hz)
<i>Aglycone</i>			
1/1'	C	137.5	-
2/2'	CH	111.7	7.02 d (1.9)
3/3'	C	151.0	-
4/4'	C	147.6	-
5/5'	CH	118.1	7.14 d (8.4)
6/6'	CH	119.8	6.91 dd (8.4, 1.9)
7/7'	CH	87.1	4.75 d (4.2)
8/8'	CH	55.6	3.11 m
9/9'	CH ₂	72.8	4.24 dd (9.0, 6.9) 3.88 dd (9.0, 3.7)
3-OCH ₃ /3'-OCH ₃	CH ₃	56.8	3.86 s
<i>Glucose (C-4/4')</i>			
1''/1'''	CH	102.9	4.87 d (7.3)
2''/2'''	CH	75.0	3.47-3.38 †
3''/3'''	CH	77.9	3.51 t (9.0)
4''/4'''	CH	71.4	3.47-3.38 †
5''/5'''	CH	78.2	3.47-3.38 †
6''/6'''	CH ₂	62.5	3.85 † 3.68 dd (12.0, 5.3)

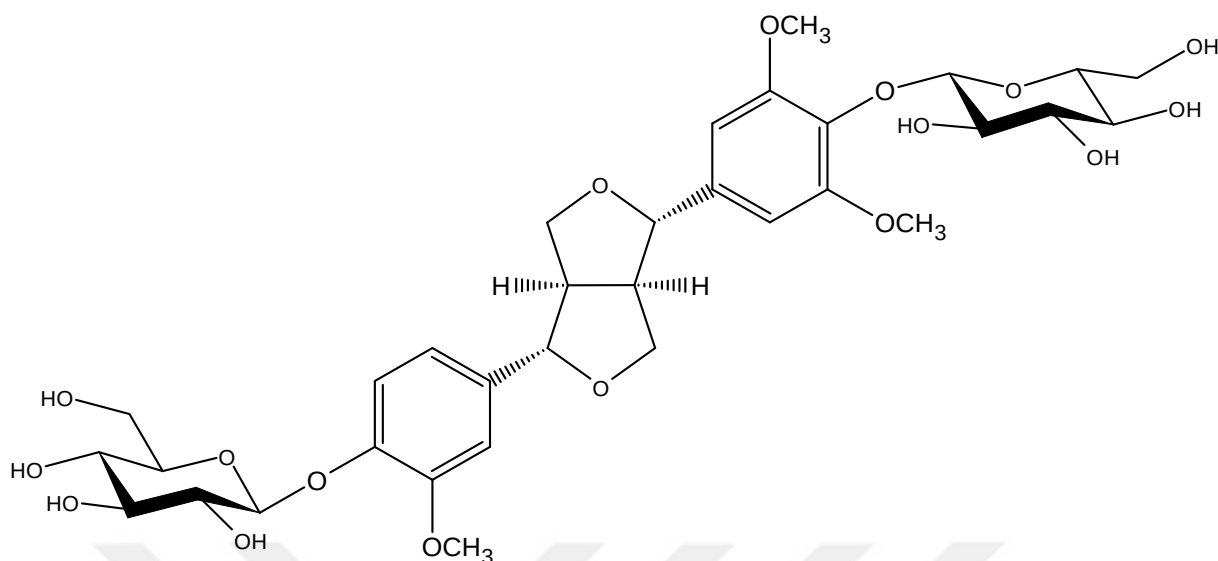
†: Overlapped signals.



Spectrum 129. ^1H NMR Spectrum of Pinoresinol 4,4'-di-O- β -D-glucopyranoside (36) (CD_3OD , 400 MHz)



Spectrum 130. ¹³C NMR Spectrum of Pinoresinol 4,4'-di-O-β-D-glucopyranoside (36) (CD₃OD, 100 MHz)



MEDIORESINOL DI-O- β -D-GLUCOPYRANOSIDE (37): C₃₃H₄₄O₁₇ (MW:712.69)

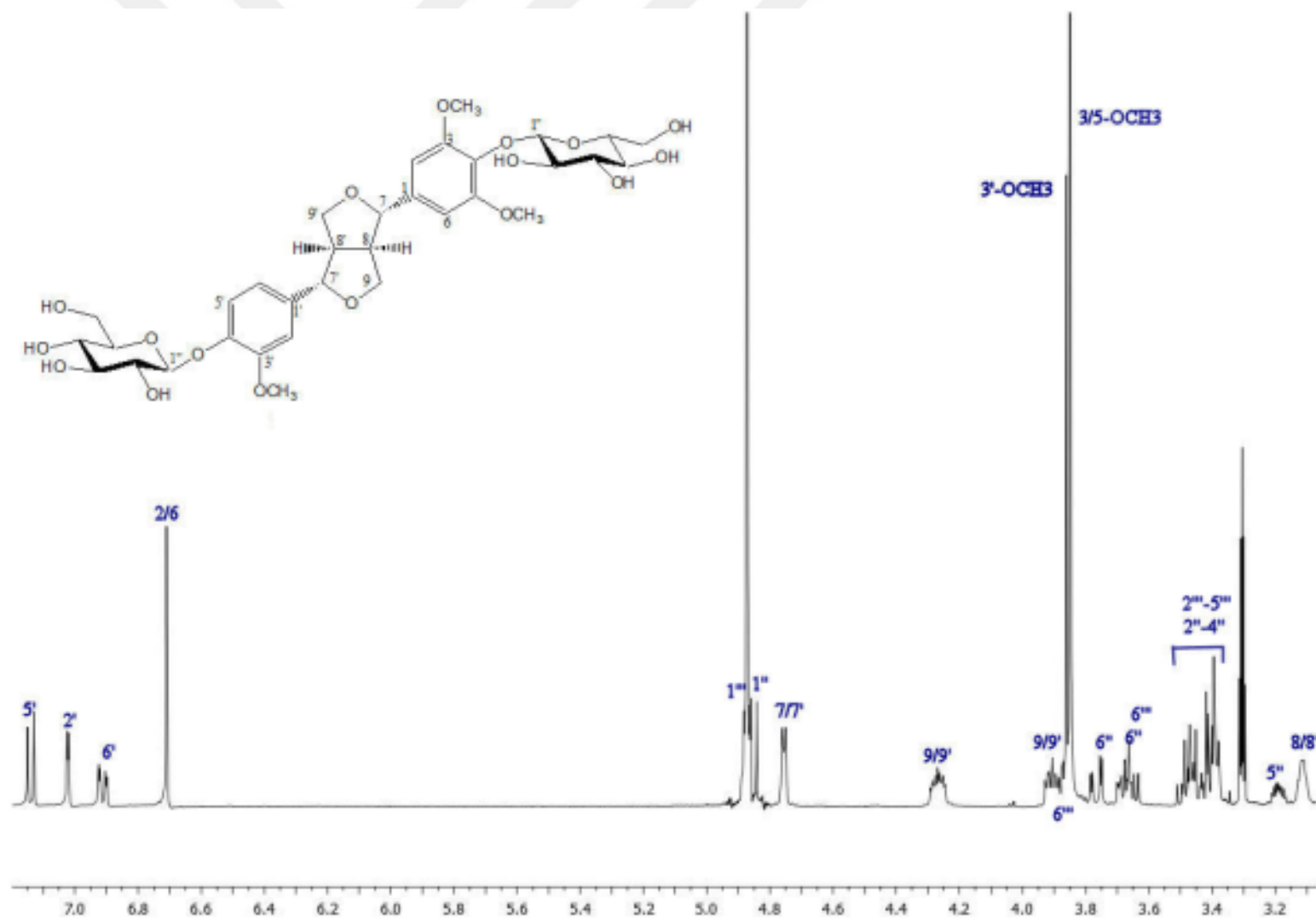
UV λ_{max} (MeOH) nm:	227, 277
ESI-MS m/z :	736.43 [M+Na] ⁺
IR ν_{max} (KBr) cm ⁻¹ :	3408 (OH), 1597, 1513 (aromatic, C=C)
¹ H NMR:	Table 107, Spectrum 131
¹³ C NMR:	Table 107, Spectrum 132

Compound **37** was obtained as white solid and its molecular formula C₃₃H₄₄O₁₇ was deduced from a pseudomolecular ion at m/z 736.43 [M+Na]⁺ in the ESI-MS, with IHD of 12. Its UV and IR spectra were characteristic for a lignan structure. The ¹H and ¹³C NMR spectra (Table 107), contained signals due to an furafuranoid lignan skeleton, and sugar units. The ¹H and ¹³C NMR data (Table 107) of **37** were found to be consistent with the those of medioresinol di-O- β -D-glucopyranoside presented on the literature (206).

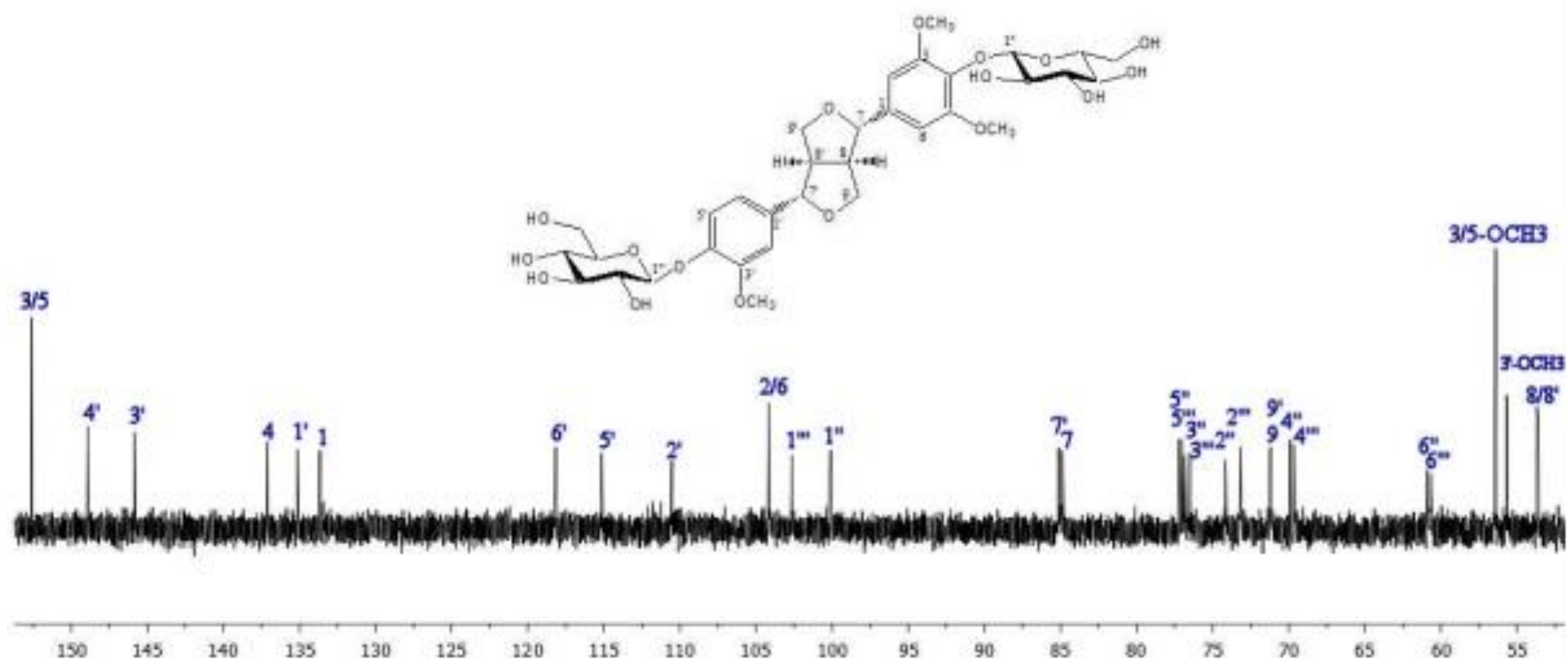
Table 107. ^{13}C and ^1H NMR data of medioresinol di-*O*- β -D-glucopyranoside (**37**) (CD_3OD ; ^{13}C : 100 MHz, ^1H : 400 MHz)

C/H Atom	Multiplicity	δ_c ppm	δ_H ppm, J (Hz)
<i>Aglycone</i>			
1	C	133.7	-
2	CH	104.2	6.71 s
3	C	152.6	-
4	C	137.2	-
5	C	152.6	-
6	CH	104.2	6.71 s
7	CH	84.9	4.76 d (4.1)
8	CH	53.6	3.11 m
9	CH ₂	71.2	4.27 dd (9.2, 6.8)
3/5-OCH ₃	CH ₃	56.4	3.91 m
1'	C	135.1	3.85 s
2'	CH	110.2	-
3'	C	145.9	7.02 d (1.9)
4'	C	148.9	-
5'	CH	115.2	-
6'	CH	118.2	7.14 d (8.4)
7'	CH	85.1	6.91 dd (8.4, 1.8)
8'	CH	53.7	4.76 d (4.1)
9'	CH ₂	71.3	3.11 m
3'-OCH ₃	CH ₃	55.7	4.27 dd (9.2, 6.8)
<i>Glucose (C-4)</i>			3.91 m
1''	CH	100.1	3.86 s
2''	CH	74.2	4.85 d (7.5)
3''	CH	76.9	3.50-3.38 †
4''	CH	69.9	3.50-3.38 †
5''	CH	77.2	3.50-3.38 †
6''	CH ₂	60.7	3.19, m
<i>Glucose (C-4')</i>			3.77 dd (12.0, 2.4)
1'''	CH	102.7	4.87 d (7.4)
2'''	CH	73.2	3.50-3.38 †
3'''	CH	76.5	3.50-3.38 †
4'''	CH	69.7	3.50-3.38 †
5'''	CH	77.0	3.50-3.38 †
6'''	CH ₂	60.9	3.87 †
			3.66 dd (12.0, 5.1)

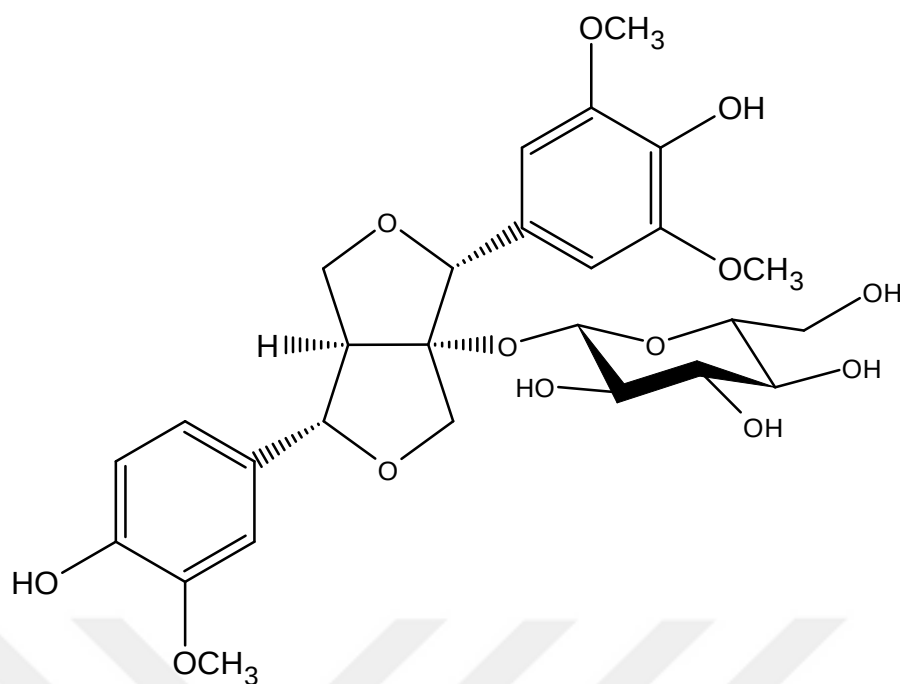
†: Overlapped signals.



Spectrum 131. ¹H NMR Spectrum of Medioresinol di-O-β-D-glucopyranoside (37) (CD₃OD, 400 MHz)



Spectrum 132. ¹³C NMR Spectrum of Medioresinol di-O-β-D-glucopyranoside (37) (CD₃OD, 100 MHz)



FRAXIRESINOL 1-O- β -D-GLUCOPYRANOSIDE (38): C₂₇H₃₄O₁₃ (MW:566.55)

UV λ_{max} (MeOH) nm:	233, 280
ESI-MS m/z :	590.20 [M+Na] ⁺
IR ν_{max} (KBr) cm ⁻¹ :	3303 (OH), 1641, 1583 (aromatic, C=C)
¹ H NMR:	Table 108, Spectrum 133
¹³ C NMR:	Table 108, Spectrum 134
HMBC:	Spectrum 135

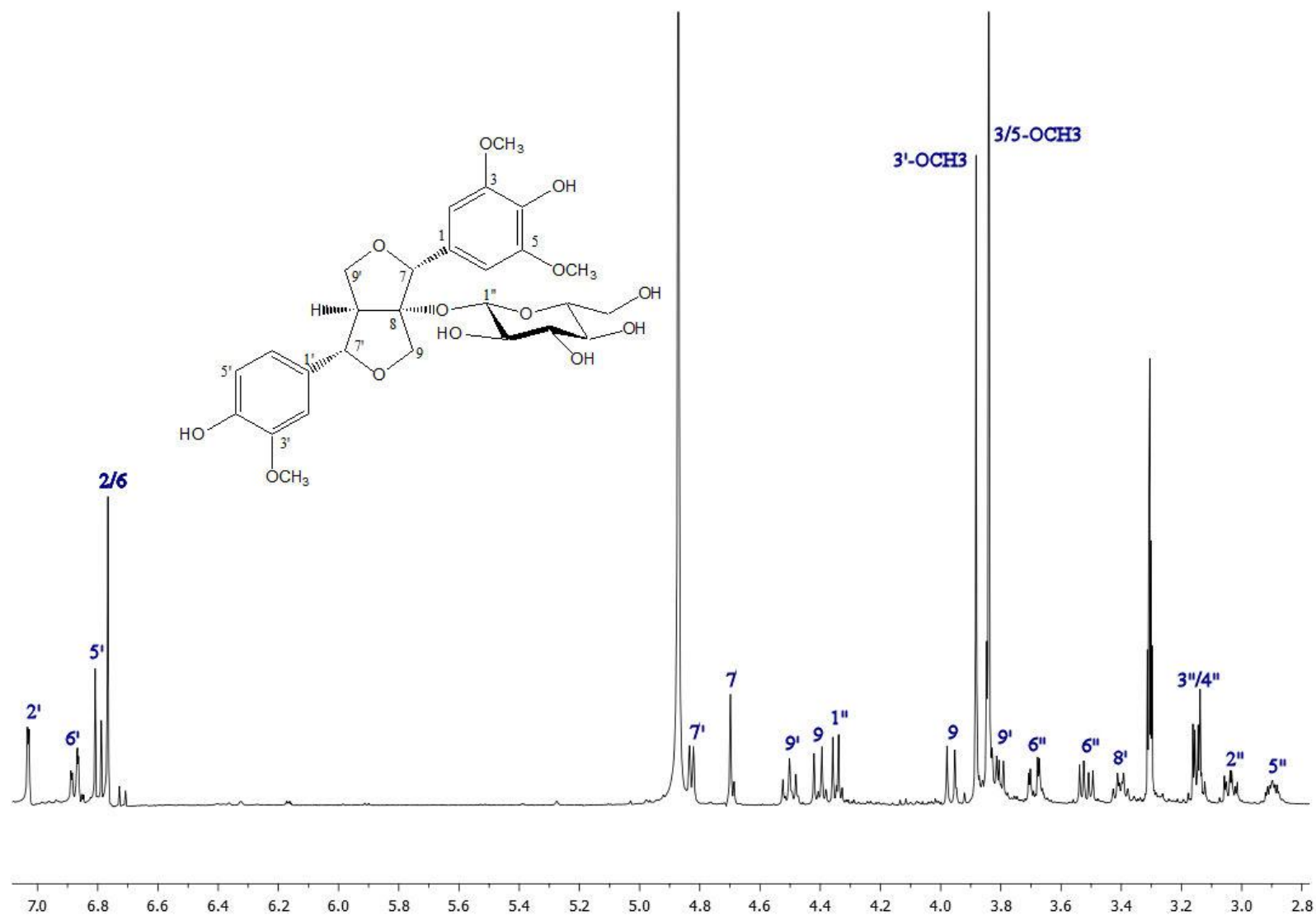
Compound **38** was obtained as white amorphous solid, and it possessed the molecular formula C₂₇H₃₄O₁₃ based on its pseudomolecular ion peak at m/z 590.20 [M+Na]⁺ in positive ESI-MS, displaying 11 degrees of unsaturation. The UV spectrum gave maxima at 233 and 280 nm, whereas the IR spectrum indicated absorption bands due to the hydroxyl (3303 cm⁻¹) and aromatic ring (1641 and 1583 cm⁻¹) groups. The 1D NMR findings (Table 108, Spectra 133 and 134) secured by HMBC spectrum led to the identification of **38** as fraxiresinol 1-O- β -D-glucopyranoside in the literature (267).

Table 108. ^{13}C and ^1H NMR data of fraxiresinol 1-*O*- β -D-glucopyranoside (**38**) (CD_3OD ; ^{13}C : 100 MHz, ^1H : 400 MHz)

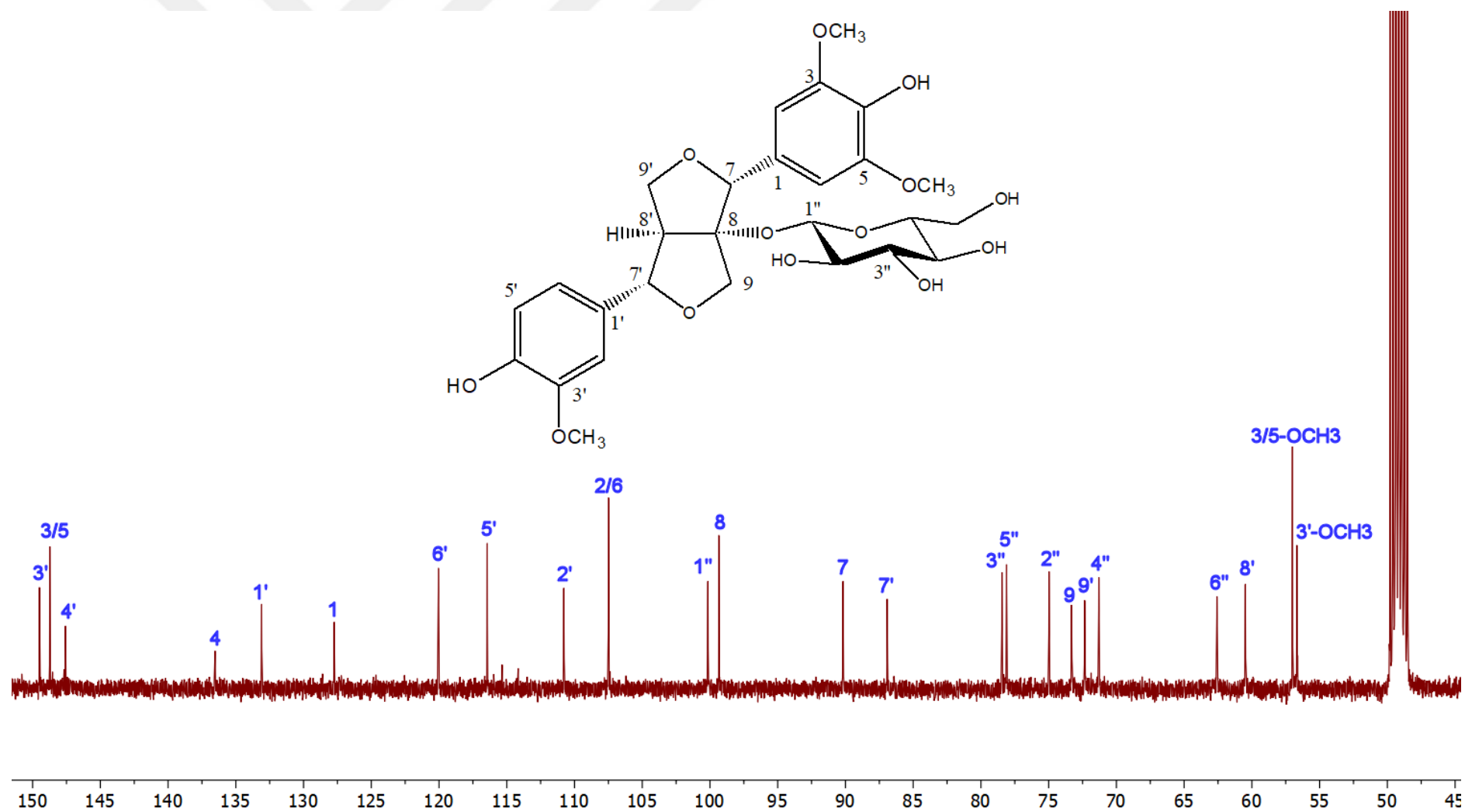
C/H Atom	Multiplicity	δ_{C} ppm	δ_{H} ppm, <i>J</i> (Hz)
<i>Aglycone</i>			
1	C	127.7	-
2	CH	107.5	6.77 s
3	C	148.7	-
4	C	136.5	-
5	C	148.7	-
6	CH	107.5	6.77 s
7	CH	90.2	4.70 s
8	C	99.3	-
9	CH ₂	73.3	4.41 d (10.4) 3.97 d (10.4)
3/5-OCH ₃	CH ₃	57.0	3.84 s
1'	C	133.1	-
2'	CH	110.8	7.03 d (1.8)
3'	C	149.5	-
4'	C	147.6	-
5'	CH	116.4	6.80 d (8.1)
6'	CH	120.0	6.88 dd (8.1, 1.7)
7'	CH	86.7	4.83 d (5.4)
8'	CH	60.5	3.40 m
9'	CH ₂	72.3	4.50 t (8.7) 3.81 dd (9.1, 6.6)
3'-OCH ₃	CH ₃	56.7	3.88 s
<i>Glucose (C-4')</i>			
1''	CH	100.2	4.35 d (7.7)
2''	CH	75.0	3.04 dd (9.3, 7.7)
3''	CH	78.4	3.15 †
4''	CH	71.3	3.15 †
5''	CH	78.1	2.90 m
6''	CH ₂	62.6	3.69 dd (12.0, 2.3) 3.52 dd (12.0, 5.7)

^aAssignments are based on HSQC and HMBC experiments.

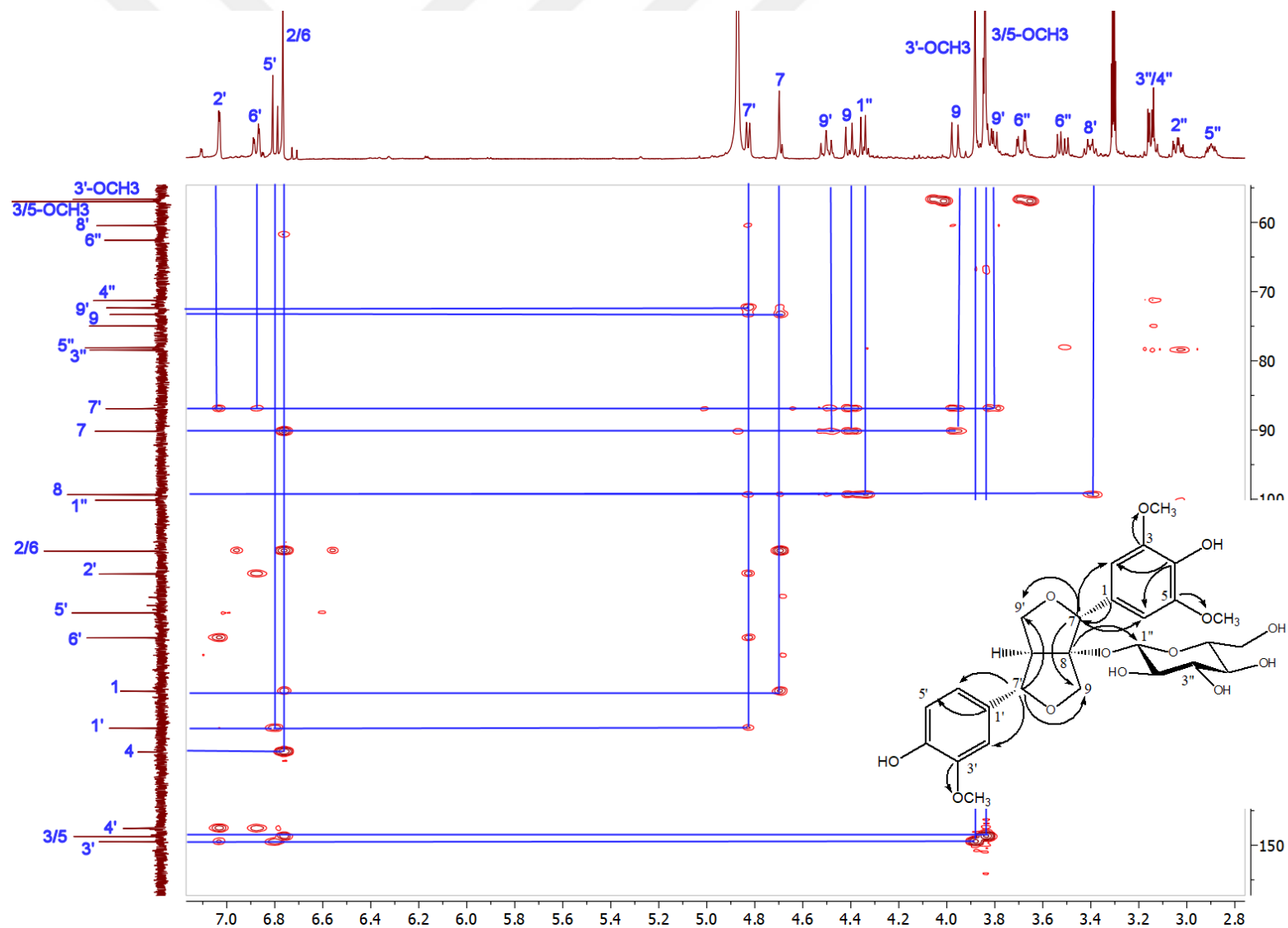
†: Overlapped signals.



Spectrum 133. ¹H NMR Spectrum of Fraxiresinol 1-O-β-D-glucopyranoside (**38**) (CD₃OD, 400 MHz)



Spectrum 134. ¹³C NMR Spectrum of Fraxiresinol 1-O-β-D-glucopyranoside (**38**) (CD₃OD, 100 MHz)



Spectrum 135. HMBC Spectrum of Fraxiresinol 1-O- β -D-glucopyranoside (**38**)

4.6. *In vitro* Cytotoxic Activity Results of Isolated Compounds

The *in vitro* cytotoxic effects of 32 different compounds isolated from the cytotoxic main fractions of *V. alliariifolia* (**1-14**), *V. sisymbriifolia* (**1, 3, 9** and **15-24**) and *V. speluncaria* (**11** and **25-32**) were investigated by MTS assay towards the same cancer cell lines (A549, MCF7, HGC27 and PC3 for *V. alliariifolia* and *V. sisymbriifolia* compounds; U87 and MCF7 for *V. speluncaria* compounds). The results are expressed as IC₅₀ values (μM) in the Tables 109-111 and graphs showing cell viability in Figures 10-19. The compounds having IC₅₀ values less than 40 μM were considered as active, whereas compounds with IC₅₀ values below 10 μM were regarded as the potent ones. Compounds (**33-38**) isolated from inactive *n*-BuOH subextracts of *V. alliariifolia* and *V. speluncaria* were not included in any cytotoxic studies.

4.6.1. *In vitro* Cytotoxic Activity Results of Isolated Compounds from *V. alliariiifolia*

Table 109. Cytotoxic activities of compounds isolated from *V. alliariiifolia* against A549, MCF7, HGC27 and PC3 cancer cell lines by MTS assay.

Compound	Cell Lines, IC ₅₀ (μM)				
	A549	MCF7	HGC27	PC3	HUVEC
Didrovaltrate (1)	26.7±4.6	3.9±0.5	12.6±2.0	69.3±5.7	14.8±2.7
IVHD-Valtrate (2)	12.5±1.5	3.8±0.4	10.2±2.3	22.2±2.9	8.7±1.8
Valtrate (3)	7.5±1.3	2.5±0.6	2.3±0.1	9.7±2.0	3.4±0.4
1-β-Acevaltrate (4)	20.7±4.3	2.6±0.5	11.9±3.3	9.9±2.0	13.8±3.1
Seneciovaltrate (5)	35.0±5.3	12.2±2.5	9.8±1.9	28.7±4.6	14.9±3.8
Valtrate hydrine B2 (6)	20.6±4.8	3.7±0.5	6.4±1.0	3.7±0.8	11.2±3.0
Isovaleroxyvaltrate hydrine (7)	11.8±2.1	8.4±2	7.6±1.6	57.5±5.5	10.5±3.2
Valerosidate (8)	41.9±5.3	28.7±2.6	>100	>100	94.3±8.9
Baldrinal (9)	>100	22.9±3.7	>100	50.5±6.1	57.9±9.4
Coniferyl aldehyde (10)	>100	74.3±7.3	71.6±2.4	>100	97.2±5.4
Coniferin (11)	>100	>100	>100	>100	>100
7-Deisovaleroylvaltrate (12)	66.4±8.3	13.4±1.4	69.9±9.1	33.7±2.1	10.9±2.0
Valeriotriate B (13)	>100	61.1±6.3	>100	>100	>100
8-Hydroxypinoresinol (14)	>100	>100	>100	>100	>100
Paclitaxel*	40.5±2.7	2.3±0.6	35.1±2.6	20.5±2.7	75.2±3.2

* Positive Control (nM)

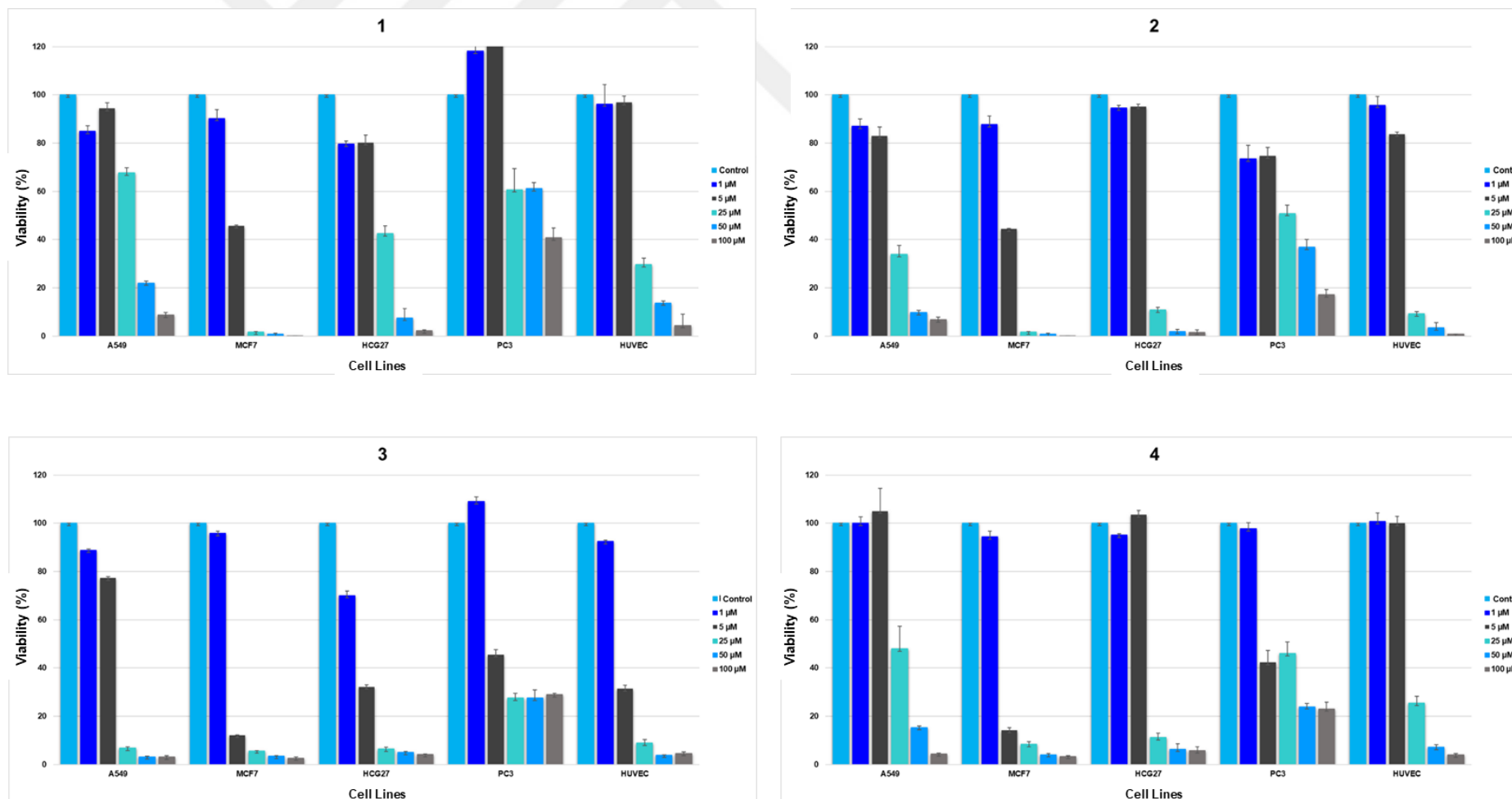


Figure 10. The cell growth inhibition (%) of compounds **1-4** at different concentrations on A549, MCF7, HGC27, PC3 and HUVEC cell lines.

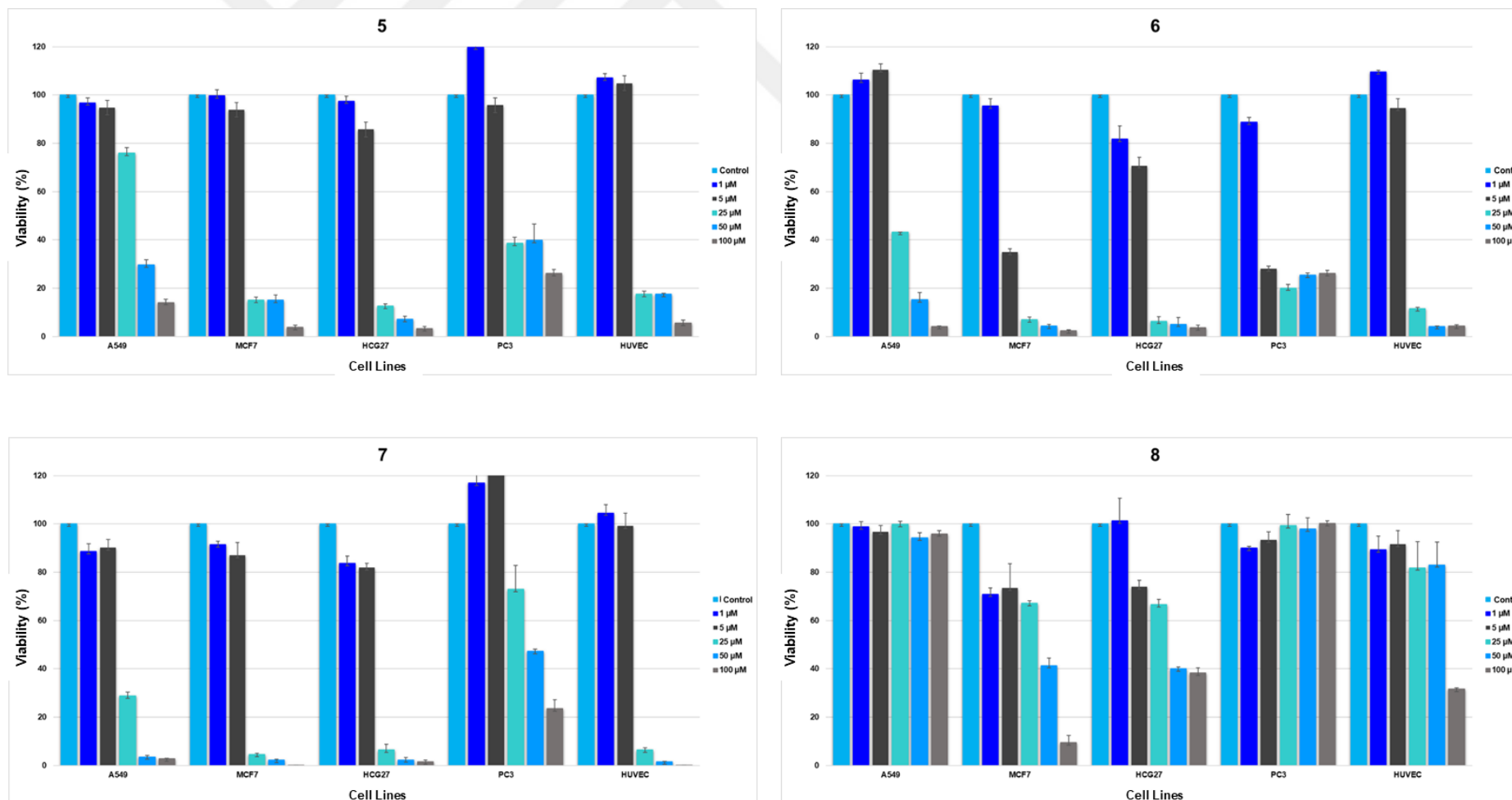


Figure 11. The cell growth inhibition (%) of compounds 5-8 at different concentrations on A549, MCF7, HGC27, PC3 and HUVEC cell lines.

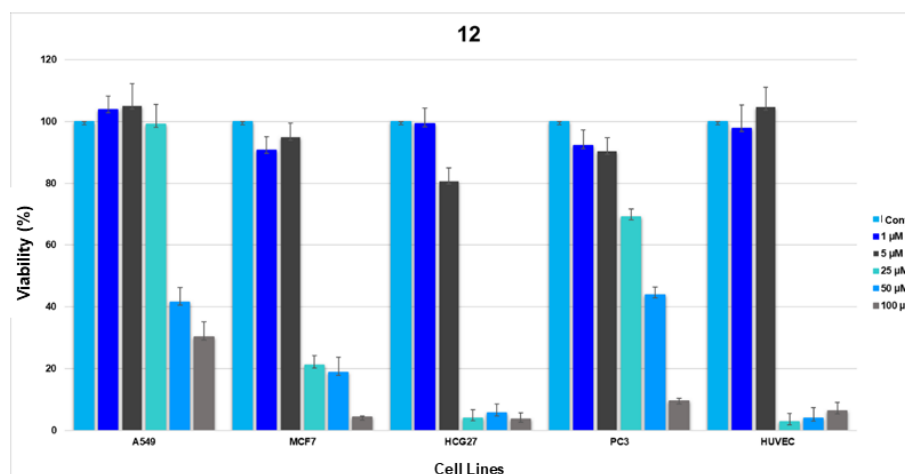
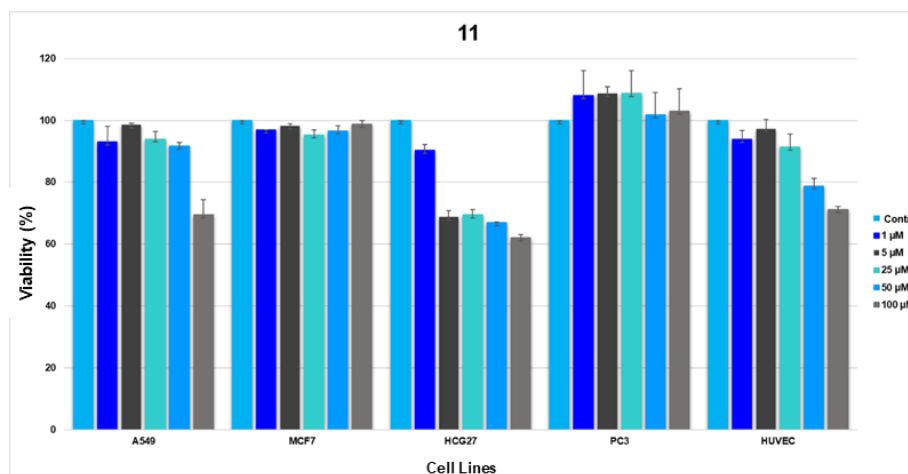
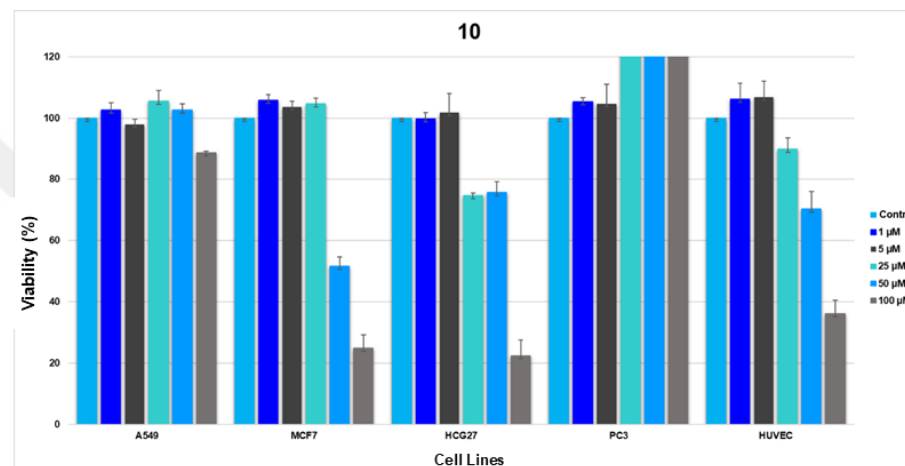
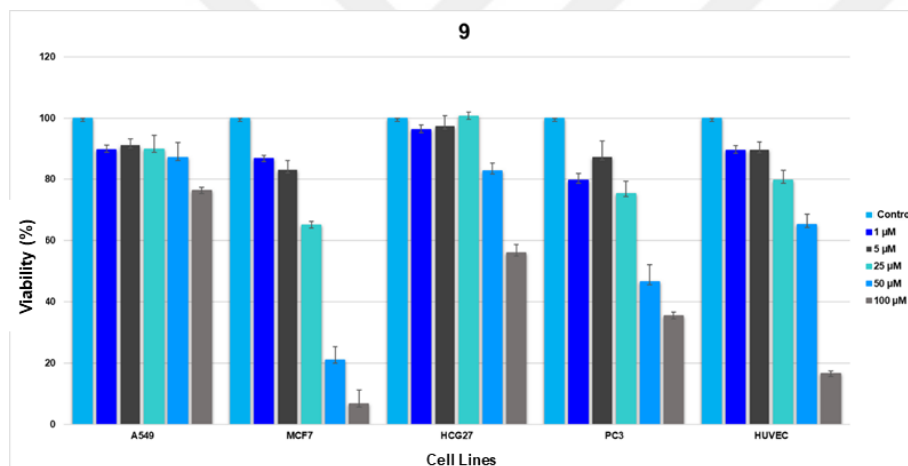


Figure 12. The cell growth inhibition (%) of compounds **9-12** at different concentrations on A549, MCF7, HGC27, PC3 and HUVEC cell lines.

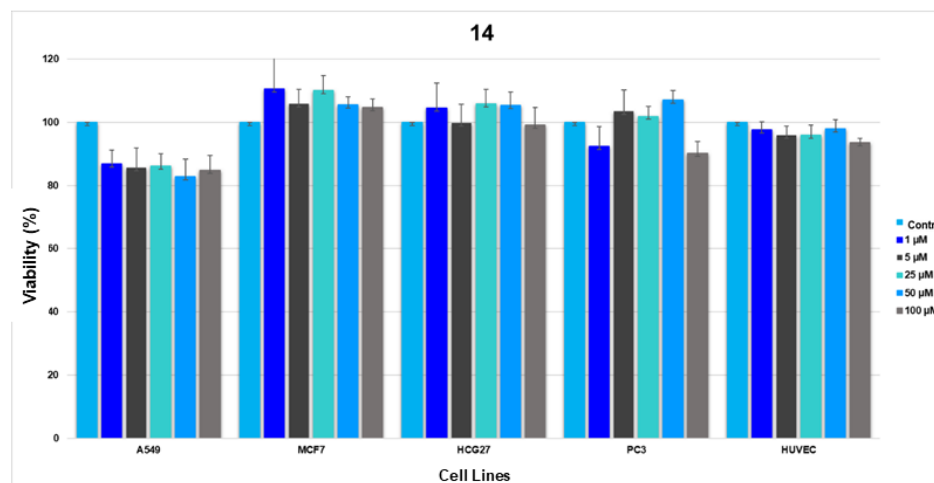
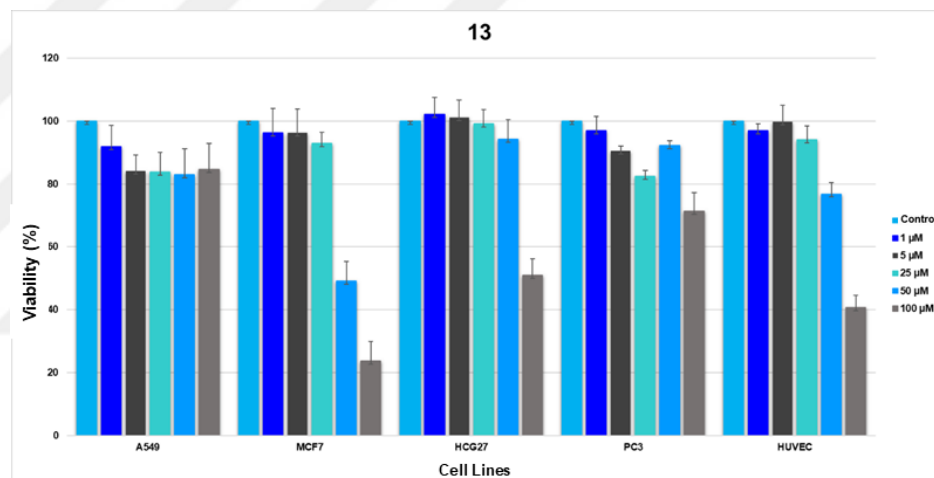


Figure 13. The cell growth inhibition (%) of compounds **13-14** at different concentrations on A549, MCF7, HGC27, PC3 and HUVEC cell lines.

Among the tested isolates, **1-7**, possessing non-glycosidic ester iridoid structure, were found to be particularly responsible for the cytotoxic effect of *V. alliariifolia* against the tested cancer cell lines (Table 109). More specifically, compounds **1-7** showed cytotoxic activity against A549 cancer cell line in the range of 7.5-35.0 μM , being the most potent one was valtrate (**3**) (IC_{50} : 7.5 μM). In MCF7 cancer cell line, compounds **1-9** and **12** demonstrated cytotoxic effects (IC_{50} : 2.5-28.7 μM), being didrovaltrate (**1**) (IC_{50} : 3.9 μM), IVHD-valtrate (**2**) (IC_{50} : 3.8 μM), valtrate (**3**) (IC_{50} : 2.5 μM), 1- β -acevaltrate (**4**) (IC_{50} : 2.6 μM), valtrate hydrine B2 (**6**) (IC_{50} : 3.7 μM) and isovaleroxyvaltrate hydrine (**7**) (IC_{50} : 8.4 μM) as the most active ones. Also, compounds **1-7** were found to possess cytotoxic activity against HGC27 cancer cell line with IC_{50} values ranging from 2.3 to 12.6 μM . Amongst, valtrate (**3**), seneciovaltrate (**5**), valtrate hydrine B2 (**6**) and isovaleroxyvaltrate hydrine (**7**) were found to be most potent ones (IC_{50} : 2.3-9.8 μM). Against PC3 cells, compounds **2-6** and **12** exerted cytotoxic activity with the IC_{50} values varied from 3.7 to 33.7 μM and the most potent cytotoxic compounds were valtrate (**3**) (IC_{50} : 9.7 μM), 1- β -acevaltrate (**4**) (IC_{50} : 9.9 μM) and valtrate hydrine B2 (**6**) (IC_{50} : 3.7 μM). In addition, compounds **1-7** and **12** exhibited cytotoxic effect on a healthy cell line, namely HUVEC in the range of 3.4 to 14.9 μM . Among the tested compounds, some of them displayed relatively selective cytotoxicity on certain cancer cell lines such as **1-9** for MCF7, **1** and **3-7** for HGC27, **4** and **6** for PC3. However, a more accurate interpretation of the selectivity of the compounds can be accomplished by testing the cytotoxicity of compounds against healthy lung, breast, gastric and prostate cell lines. Furthermore, MCF7 cancer cells were found to be the most sensitive cell lines against the tested compounds, except for **10**, **11**, **13** and **14** based on the MTS assay findings (Table 109).

4.6.2. *In vitro* Cytotoxic Activity Results of Isolated Compounds from *V. sisymbriifolia*

Table 110. Cytotoxic activities of compounds isolated from *V. sisymbriifolia* against A549, MCF7, HGC27 and PC3 cancer cell lines by MTS assay.

Compound	Cell Lines, IC ₅₀ (μM)				
	A549	MCF7	HGC27	PC3	HUVEC
Didrovaltrate (1)	26.7±4.6	3.9±0.5	12.6±2.0	69.3±5.7	14.8±2.7
Valtrate (3)	7.5±1.3	2.5±0.6	2.3±0.1	9.7±2.0	3.4±0.4
Baldrinal (9)	>100	22.9±3.7	>100	50.5±6.1	57.9±9.4
1-α-Acevaltrate (15)	19.6±2.0	5.9±0.6	12.6±1.7	6.4±0.6	11.4±1.3
7-Homovaltrate (16)	17.5±2.4	3.1±0.2	3.7±0.4	2.3±0.2	3.9±0.5
Sisymbriifolivaltrate (17)	99.2±1.4	12.3±0.6	51.4±1.1	23.1±0.9	32.6±0.6
Sisymbriifolioside (18)	>100	>100	>100	>100	>100
Patriscabrol (19)	>100	>100	>100	>100	>100
11-Ethoxyviburtinal (20)	>100	84.4±2.0	>100	>100	>100
Bornyl caffeate (21)	>100	64.8±1.8	>100	66.7±5.7	>100
Sisymbriifolin A (22)	>100	>100	>100	>100	>100
Sisymbriifolin B (23)	98.4±0.5	68.1±1.5	44.7±1.1	65.5±0.9	74.9±1.2
9-Decenoic acid (24)	>100	>100	>100	>100	>100
Paclitaxel*	40.5±2.7	2.3±0.6	35.1±2.6	20.5±2.7	75.2±3.2

* Positive Control (nM)

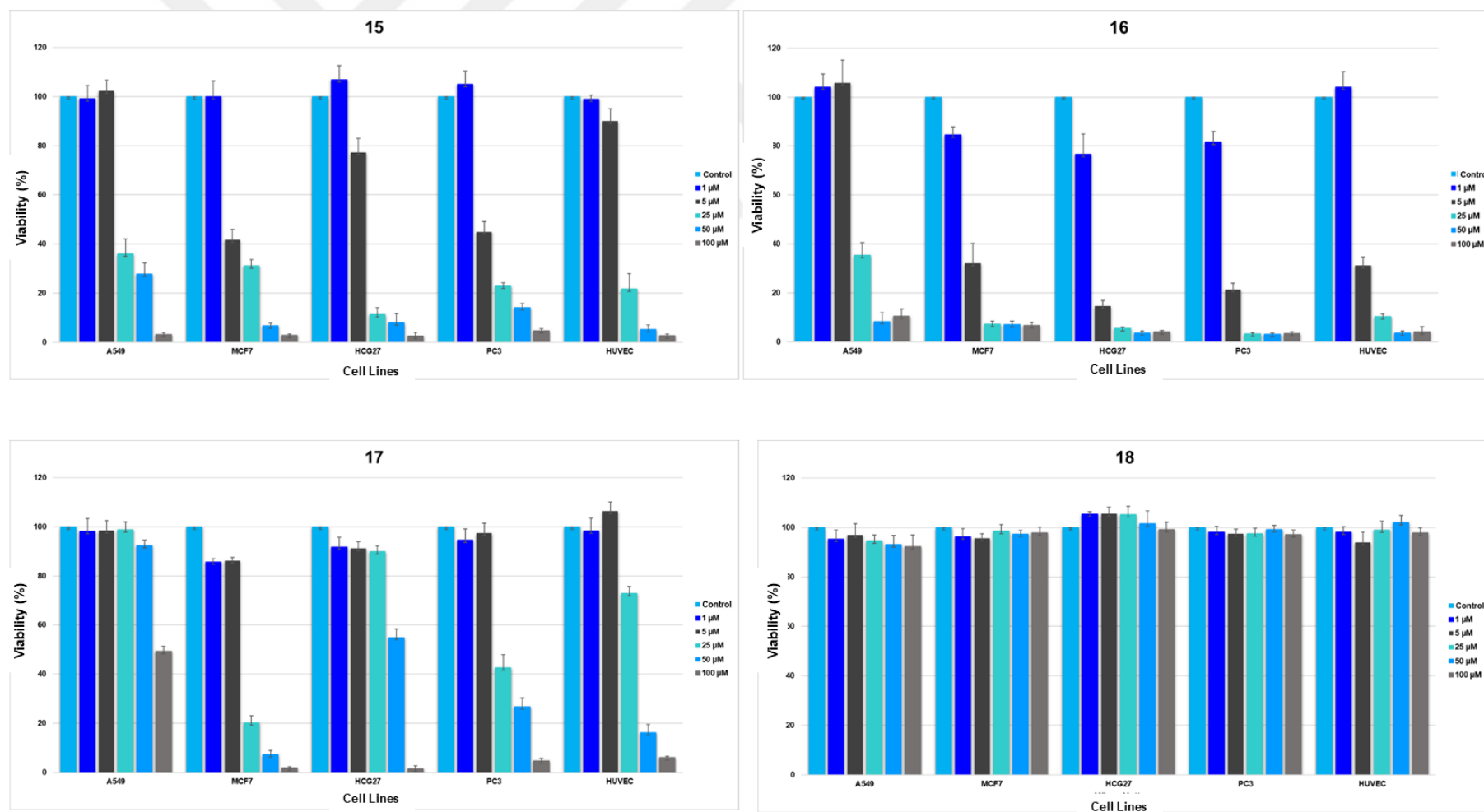


Figure 14. The cell growth inhibition (%) of compounds **15-18** at different concentrations on A549, MCF7, HGC27, PC3 and HUVEC cell lines.

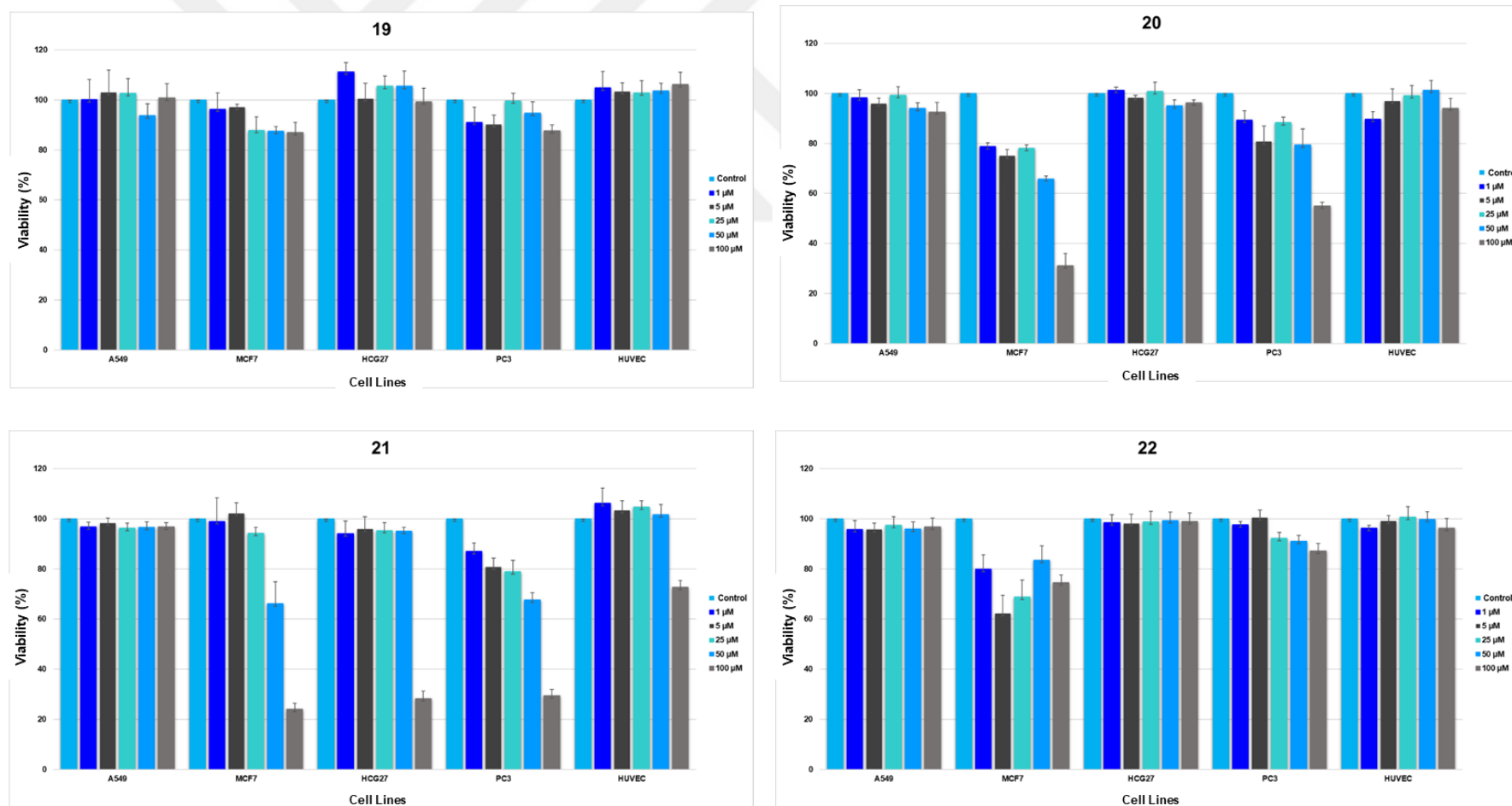


Figure 15. The cell growth inhibition (%) of compounds **19-22** at different concentrations on A549, MCF7, HGC27, PC3 and HUVEC cell lines.

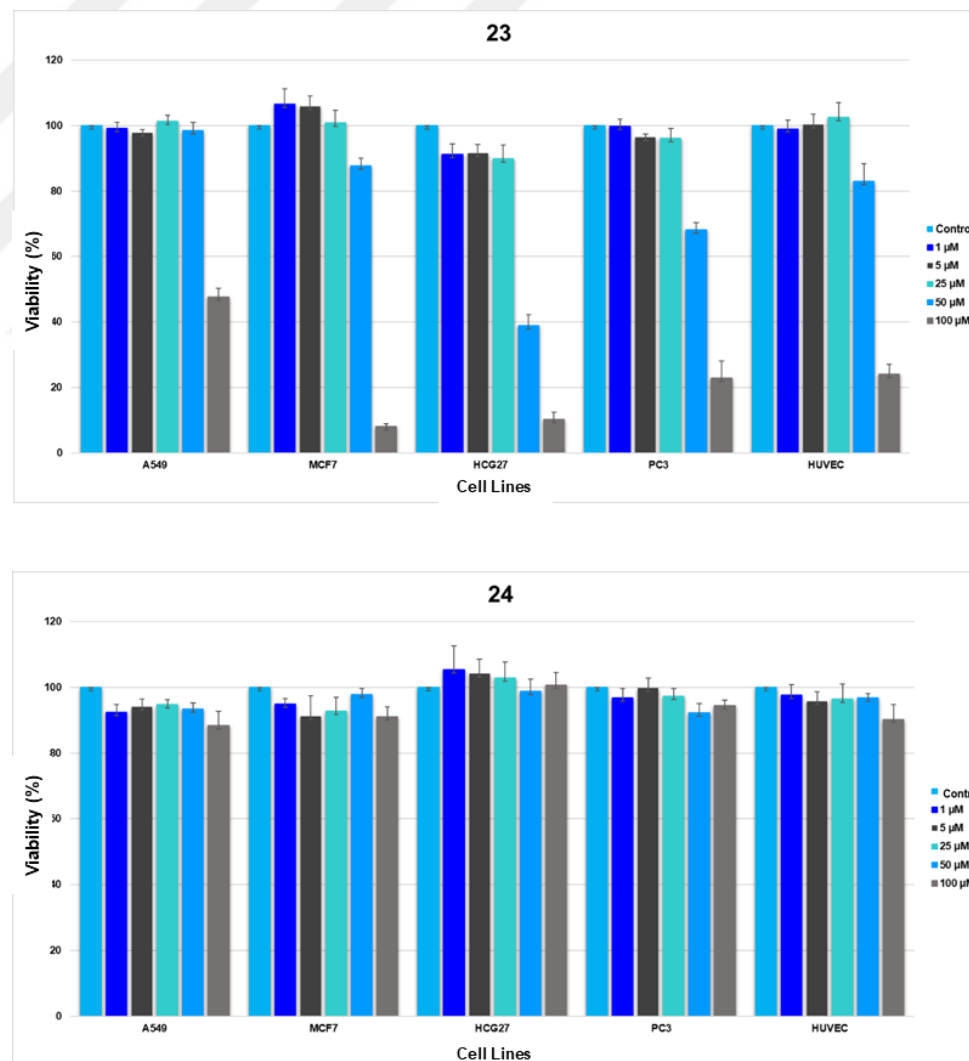


Figure 16. The cell growth inhibition (%) of compounds **23** and **24** at different concentrations on A549, MCF7, HGC27, PC3 and HUVEC cell lines.

As given in Table 110, compounds with non-glycosidic ester structure (**1**, **3** and **15-17**) were found to exhibit cytotoxic activity against the tested cell lines. When the cytotoxicity results of the compounds were evaluated according to tested cell lines, compounds **1**, **3**, **9** and **15**, **16** exerted cytotoxic bioactivities against A549 cancer cells with IC₅₀ values in the range of 7.5-26.7 μ M and the strongest of which was valtrate (**3**) (IC₅₀: 7.5 μ M). Further, compounds **1**, **3**, **9** and **15-17** showed considerable cytotoxic effects on MCF7 cancer cell line (IC₅₀: 2.5-22.9 μ M), being didrovaltrate (**1**), valtrate (**3**), 1- α -acevaltrate (**15**) and 7-homovaltrate (**16**) as the potent ones with the IC₅₀ values ranging from 2.5 to 5.9 μ M. Against HGC27 cells, compounds **1**, **3**, **15** and **16** were found to be cytotoxically active with the IC₅₀ values in the range of 2.3 to 12.6 μ M, and the most potent of them were valtrate (**3**) and homovaltrate (**16**) with the IC₅₀ values of 2.3 and 3.7 μ M, respectively. In PC3 cancer cell lines, compounds **3**, **15**, **16** and **17** exhibited cytotoxic activities in varying degrees (IC₅₀: 2.3-23.1 μ M), being valtrate (**3**) (IC₅₀: 9.7 μ M), 1- α -acevaltrate (**15**) (IC₅₀: 6.4 μ M) and 7-homovaltrate (**16**) (IC₅₀: 2.3 μ M) were the most active ones. All these active compounds (**1**, **3**, **9** and **15-17**) except for baldrinal (**9**) displayed cytotoxicity against HUVEC cells (IC₅₀: 3.4-32.6 μ M). Considering the selectivity of these compounds, compounds **1**, **3**, **9** and **15-17** for MCF7 cells, compounds **1** and **3** for HGC27 cells and compounds **15-17** for PC3 cells were found to be slightly selective molecules. Inclusion of healthy cells of respective cancer cell lines instead of HUVECs might provide precise information about selectivity of the tested compounds.

4.6.3. *In vitro* Cytotoxic Activity Results of Isolated Compounds from *V. speluncaria*

Table 111. Cytotoxic activities of compounds isolated from *V. speluncaria* against U87 and MCF7 cancer cell lines by MTS assay.

Compound	Cell Lines, IC ₅₀ (μM)		
	U87	MCF7	HUVEC
Coniferin (11)	>100	>100	>100
Pinoresinol 4- <i>O</i> -β-D-glucopyranoside (25)	>100	>100	>100
Caffeic acid (26)	>100	>100	>100
Chlorogenic acid (27)	>100	>100	>100
3,4-Dicaffeoylquinic acid (28)	>100	>100	>100
3,5-Dicaffeoylquinic acid (29)	>100	>100	>100
Speluncarin A (30)	>100	>100	>100
Speluncarin B (31)	>100	>100	>100
β-sitosterol 3- <i>O</i> -β-D-glucopyranoside (32)	>100	>100	>100
Paclitaxel*	>100	2.3±0.6	75.2±3.2

* Positive Control (nM)

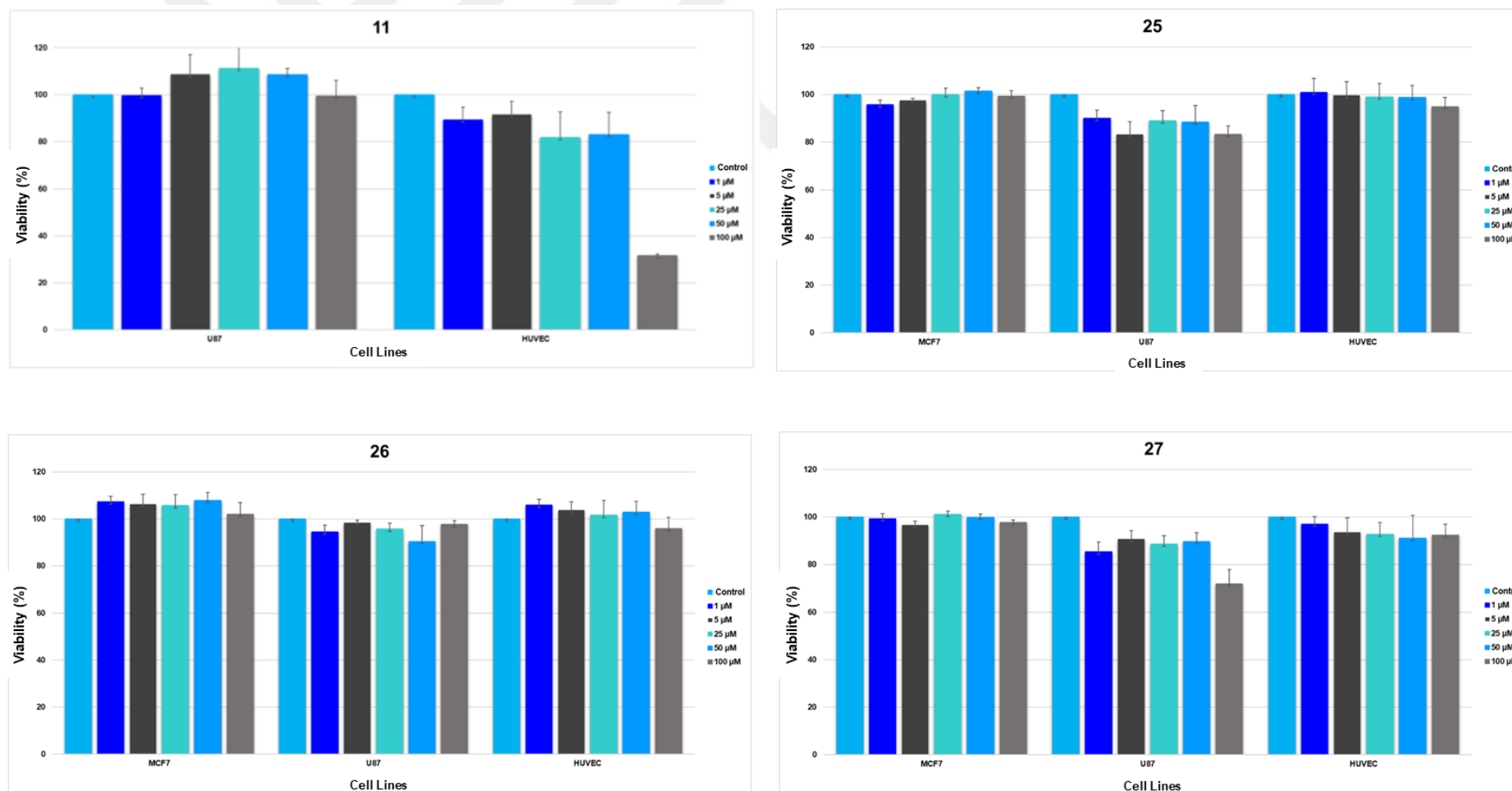


Figure 17. The cell growth inhibition (%) of compounds **11**, **25-27** at different concentrations on U87, MCF7 and HUVEC cell lines.

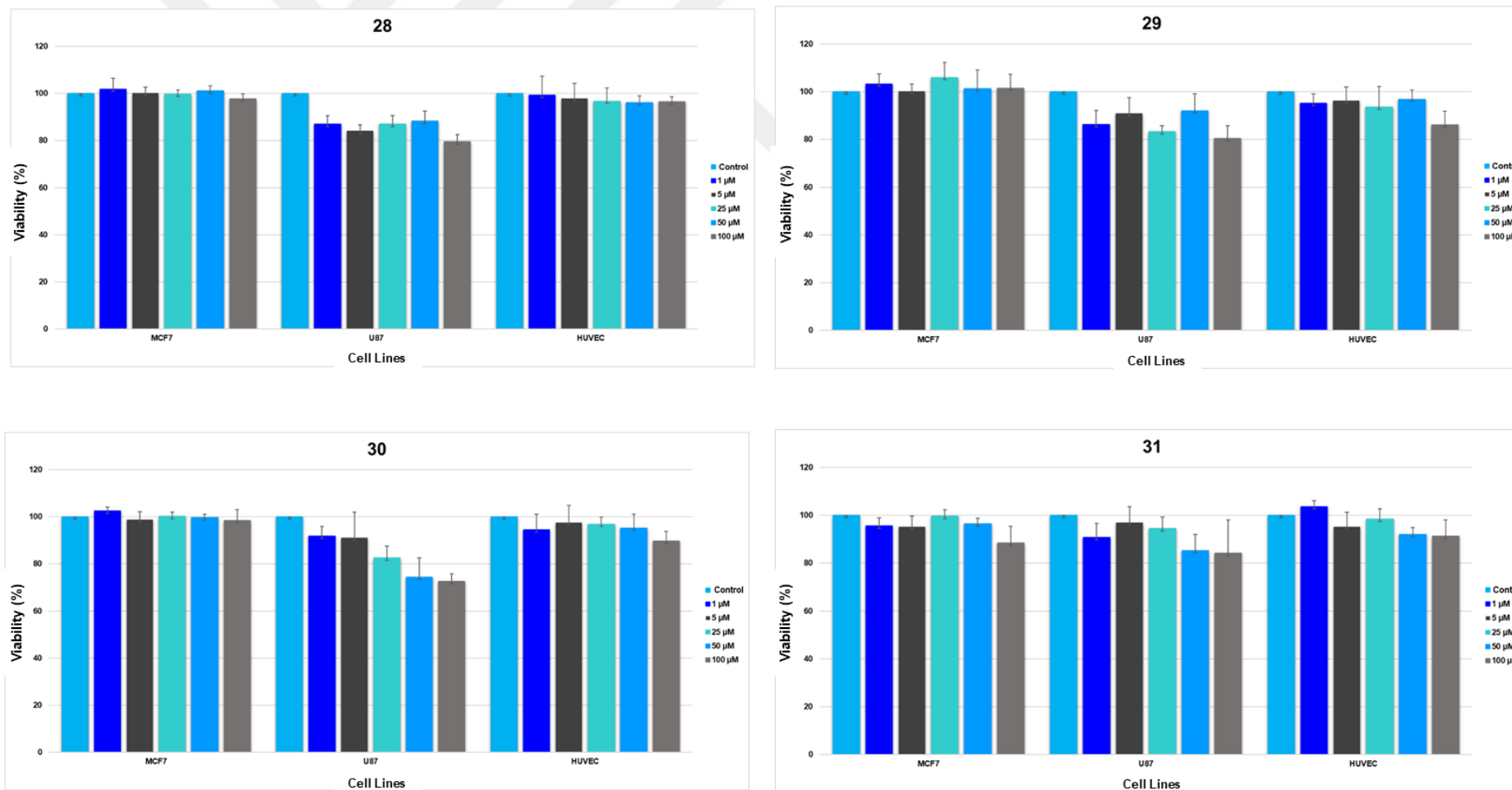


Figure 18. The cell growth inhibition (%) of compounds **28-31** at different concentrations on U87, MCF7 and HUVEC cell lines.

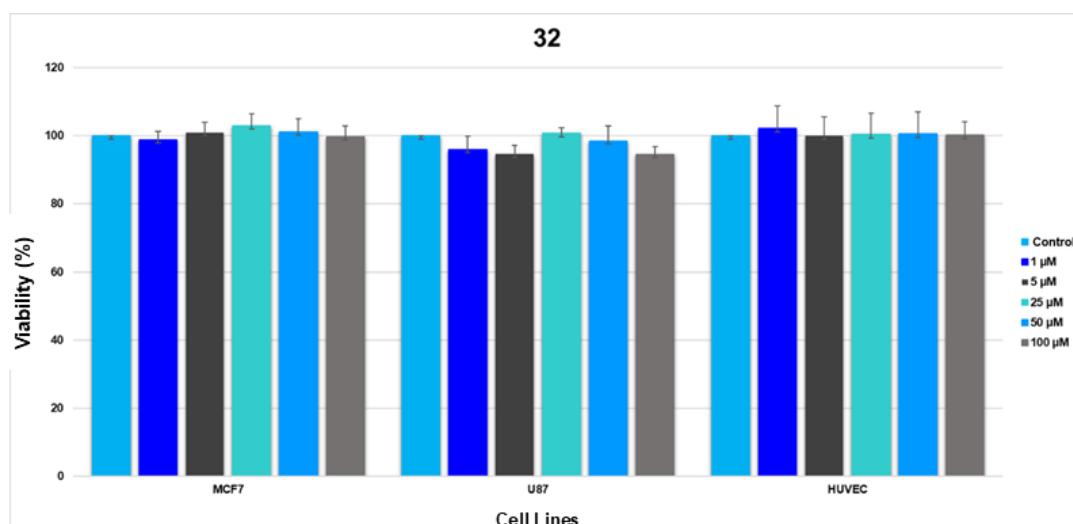


Figure 19. The cell growth inhibition (%) of compound **32** at different concentrations on U87, MCF7 and HUVEC cell lines.

The cytotoxic effects of compounds (**11**, **25-32**) isolated from cytotoxic subextracts of *V. speluncaria* were investigated only against MCF7 and U87 cancer cell lines. Although, all compounds (**11**, **25-32**) failed to show any cytotoxic activity against MCF7 cancer cell line within the tested concentration, compounds **27-30** demonstrated approximately 20-25% inhibition on U87 cancer cells at 100 μ M (Figure 18). However, the IC_{50} values of tested compounds were not calculated within the tested concentration range (Table 111). Iridoids, particularly non-glycosidic ester derivatives, were found to be responsible for the cytotoxicity of other tested *Valeriana* species. However, these types of compounds were not isolated from *V. speluncaria*. On the other hand, the fact that the cytotoxic effect observed in the main extract and main fractions was not detected for the isolated compounds can be explained by the demonstration of a synergistic effect of compounds in the extracts and fractions as were in some bioactivity-guided isolation studies (268,269).

4.7. Result of Cell Death Mechanisms of Active Compounds

In the light of MTS assay findings, a total of eight compounds (**2**, **4-6**, **15-17**) with IC_{50} values close to or lower than 10 μ M were chosen for further analysis to unveil their cell death mechanisms, including apoptosis, necrosis, autophagy and DNA content analysis (Table 112). In these mechanistic studies, the cell line in which each compound showed potent cytotoxicity was selected, and the IC_{50} values of these compounds in the relevant cell line was used for the working concentration. Accordingly, only **3** for A549 cells; **2-4**, **6** and **15-17** for MCF7 cells; **2-5**, **15** and **16** for HGC27 cells; and **3**, **4**, **6**, **15** and **16** for PC3 cells were chosen for further

mechanistic studies. Although didrovaltrate (**1**) showed significant cytotoxic activity against MCF7 cells (IC_{50} : 3.7 μ M), it was not included in mechanistic studies since it was not stable due to its crystalline form. Despite the fact that valtrate hydrine B2 (**6**) had potent cytotoxicity against HGC27 cells, it was only incorporated in mechanistic studies conducted on MCF7 and PC3 cancer cells because it was obtained in small amounts. Apoptosis, necrosis and viability of the cancer cell lines treated with tested compounds were evaluated with Annexin V/PI staining and then analyzed with flow cytometry. In addition, the effect of the compounds on cell cycle progression and cellular division was evaluated with DNA content analysis by using flow cytometry. In addition, the expression levels of proteins involved in necrosis, autophagy and cell cycle progression were also measured by flow cytometry.

Table 112. Compounds used in the mechanistic studies and their IC_{50} values in the relevant cancer cell lines

Compound	Cell Lines, IC_{50} (μ M)			
	A549	MCF7	HGC27	PC3
IVHD-Valtrate (2)		3.8 $\pm$ 0.4	10.2 $\pm$ 2.3	
Valtrate (3)	7.5 $\pm$ 1.3	2.5 $\pm$ 0.6	2.3 $\pm$ 0.1	9.7 $\pm$ 2.0
1- β -acevaltrate (4)		2.6 $\pm$ 0.5	11.9 $\pm$ 3.3	9.9 $\pm$ 2.0
Seneciovaltrate (5)			9.8 $\pm$ 1.9	
Valtrate hydrine B2 (6)		3.7 $\pm$ 0.5		3.7 $\pm$ 0.8
1- α -acevaltrate (15)		5.9 $\pm$ 0.6	12.6 $\pm$ 1.7	6.4 $\pm$ 0.6
7-Homovaltrate (16)		3.1 $\pm$ 0.2	3.7 $\pm$ 0.4	2.3 $\pm$ 0.2
Sisymbriifolivaltrate (17)		12.3 $\pm$ 0.6		

4.7.1. Evaluation of Apoptosis, Necrosis and Viability

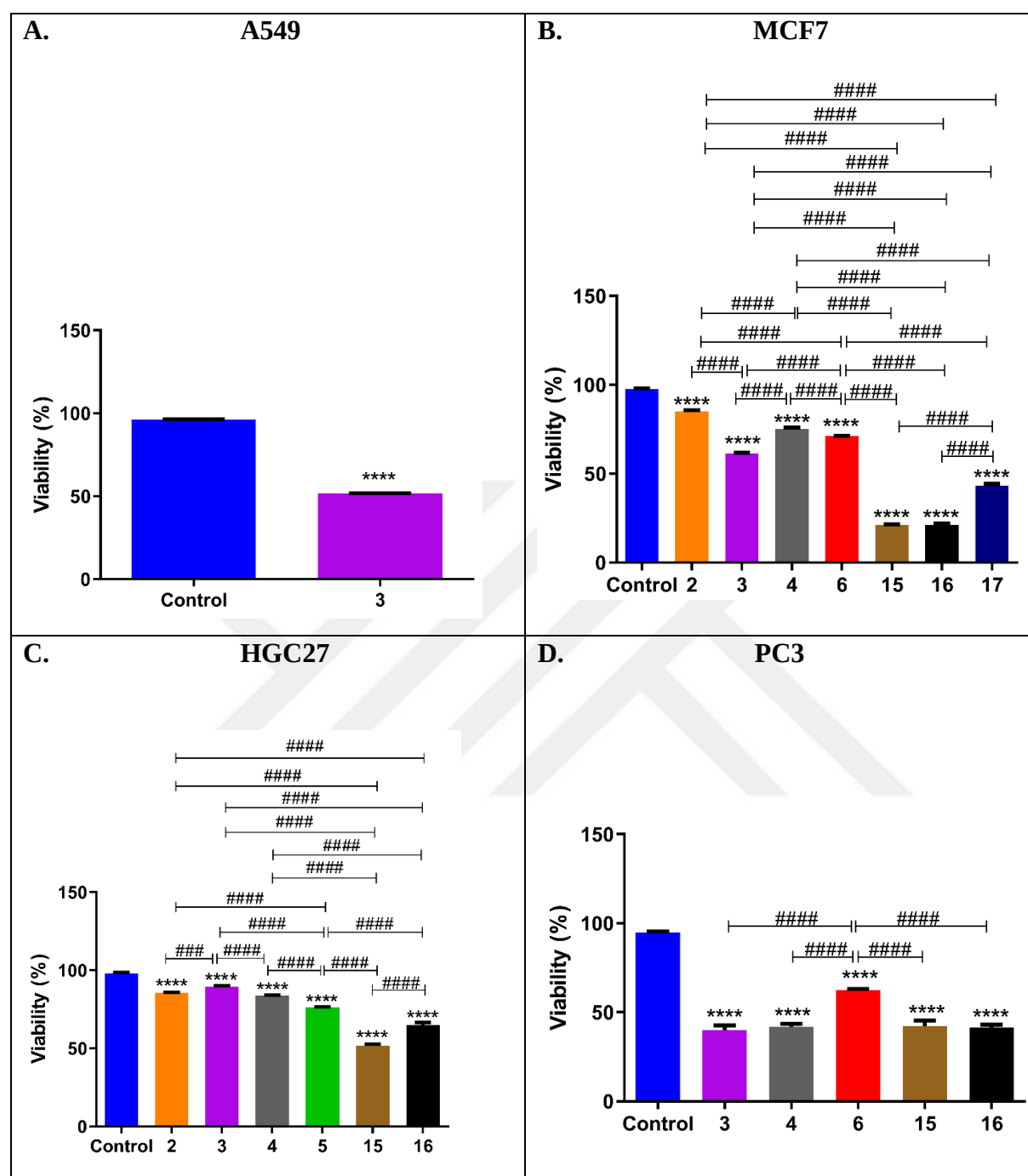


Figure 20. Effects of compounds on cell viability in (A) A549, (B) MCF7, (C) HGC27 and (D) PC3 cell lines.

(Comparison with control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$; Comparison with other compounds: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ and #### $p < 0.0001$).

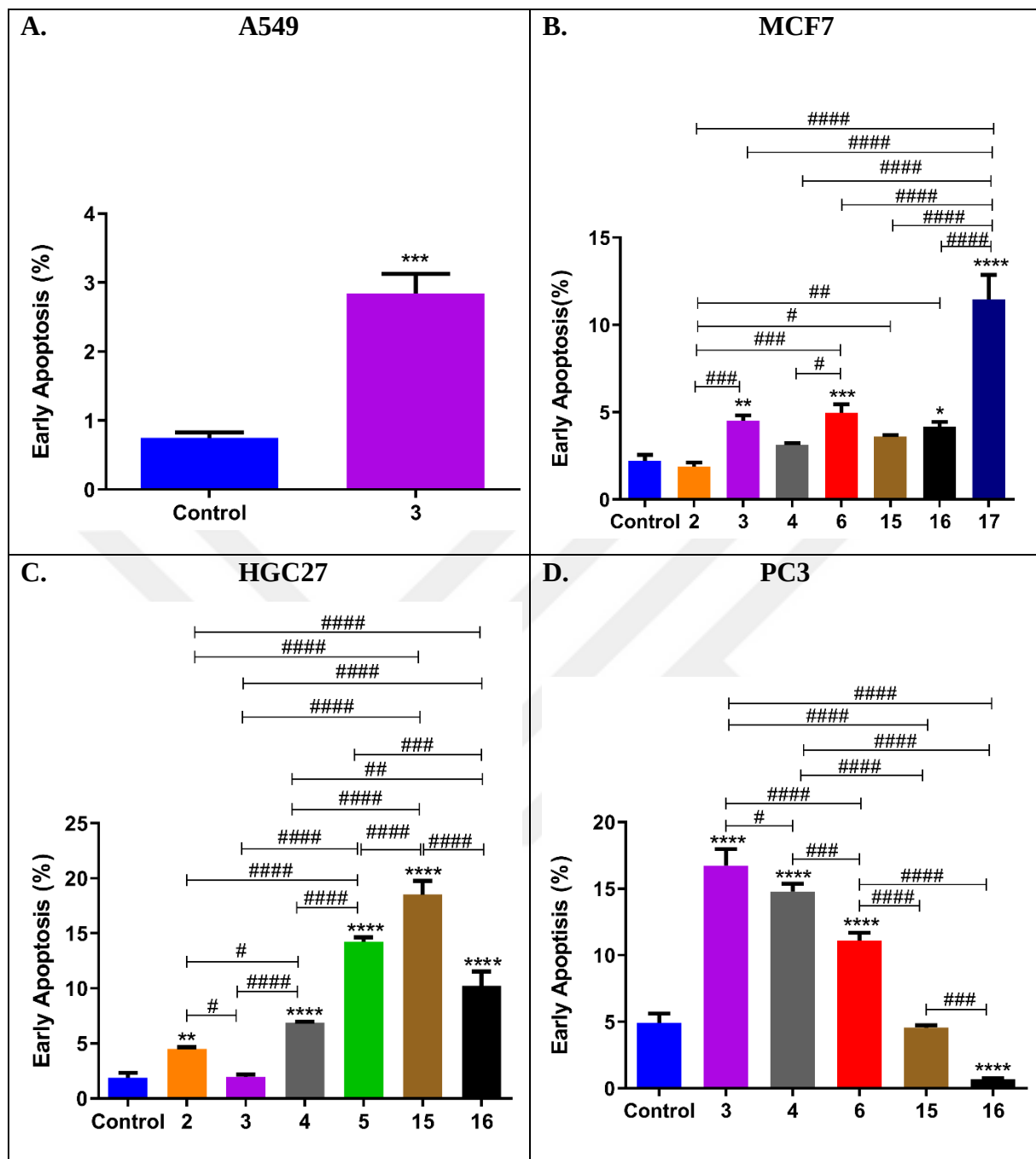


Figure 21. Effects of compounds on early apoptosis in (A) A549, (B) MCF7, (C) HGC27 and (D) PC3 cell lines.

(Comparison with control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$; Comparison with other compounds: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ and #### $p < 0.0001$).

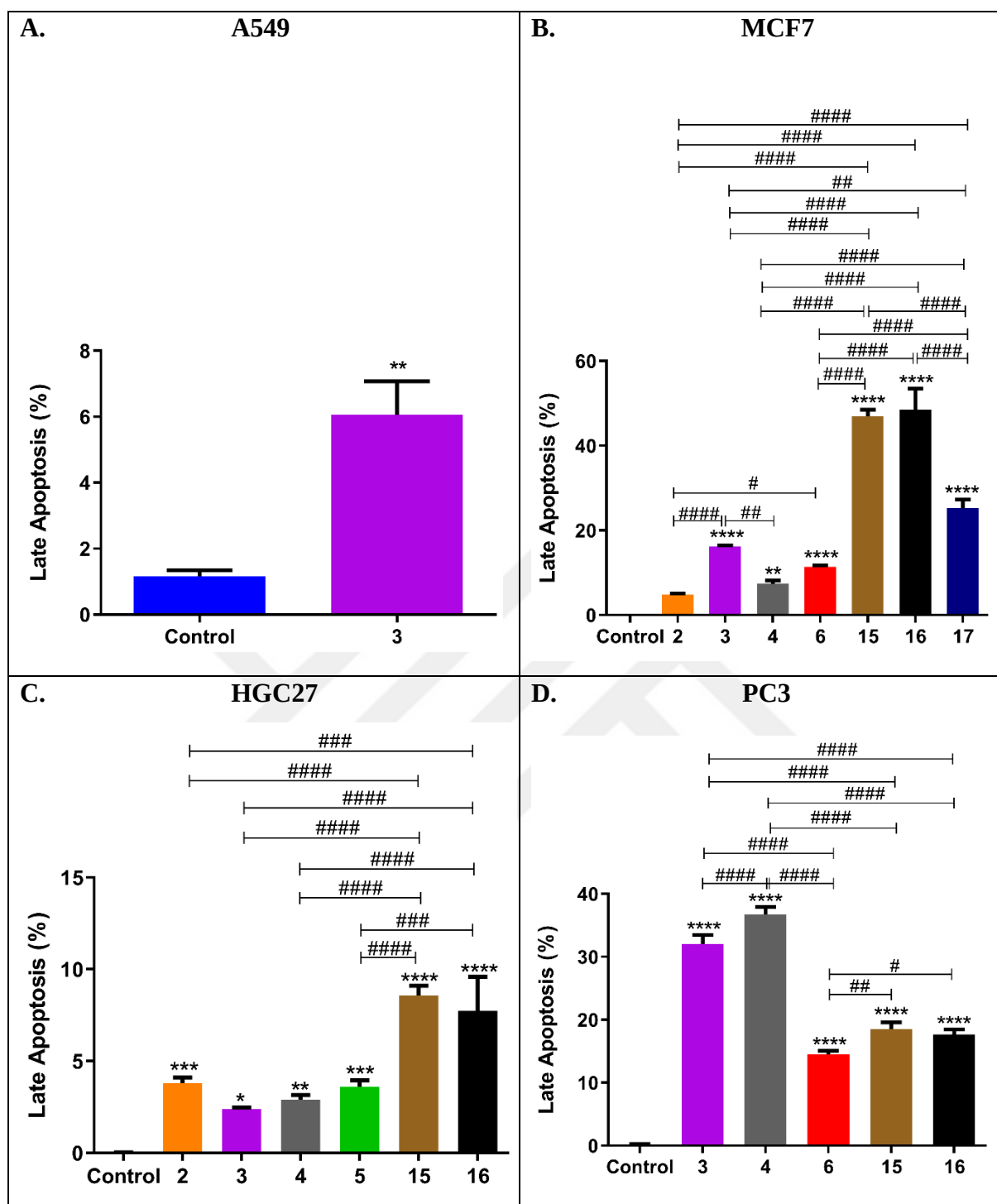


Figure 22. Effects of compounds on late apoptosis in (A) A549, (B) MCF7, (C) HGC27 and (D) PC3 cell lines.

(Comparison with control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$; Comparison with other compounds: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ and #### $p < 0.0001$).

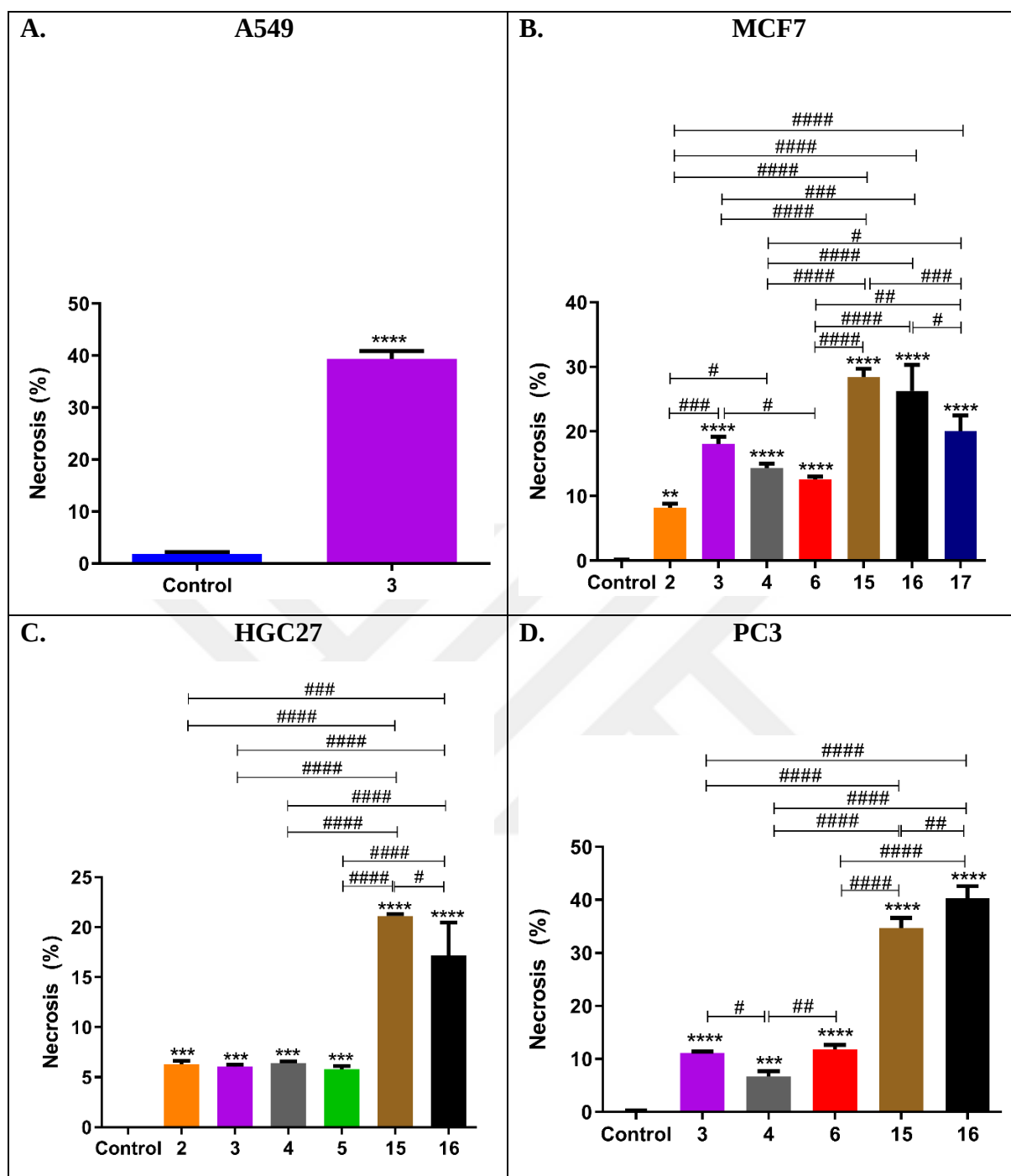


Figure 23. Effects of compounds on necrosis in (A) A549, (B) MCF7, (C) HGC27 and (D) PC3 cell lines.

(Comparison with control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$; Comparison with other compounds: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ and #### $p < 0.0001$).

All tested compounds reduced viability of cancer cell lines significantly compared to control ($p < 0.05$) (Figure 20). Valtrate (**3**), the only compound tested in the A549 cell line, inhibited cell viability by approximately 50%. On the other hand, more than one compounds were tested in other cell lines, and the effects of these compounds were compared among themselves. The compounds showed the highest reduction in cell viability were determined as **15** and **16** for both MCF7 and HGC cells, and **3**, **4**, **15** and **16** for PC3 cells (figs. 20B-D). When comparing the effects of each compound based on their tested cell lines, compounds **15** and **16** led to significant increase in both apoptosis and necrosis levels and decrease cell viability in both MCF7 and HGC27 cancer cell lines ($p < 0.05$). Thus, 1- α -acevaltrate (**15**) and 7-homovaltrate (**16**), which exhibited the highest activity against both MCF7 and HGC27 cells, simultaneously promoted both these two pathways. Similarly, valtrate (**3**) concurrently caused the activation of both apoptotic and necrotic pathways, together with reducing cell viability ($p < 0.05$). In PC3 cells, it was determined that compounds **3** and **4** induced apoptosis, whereas compounds **15** and **16** activated necrotic pathway.

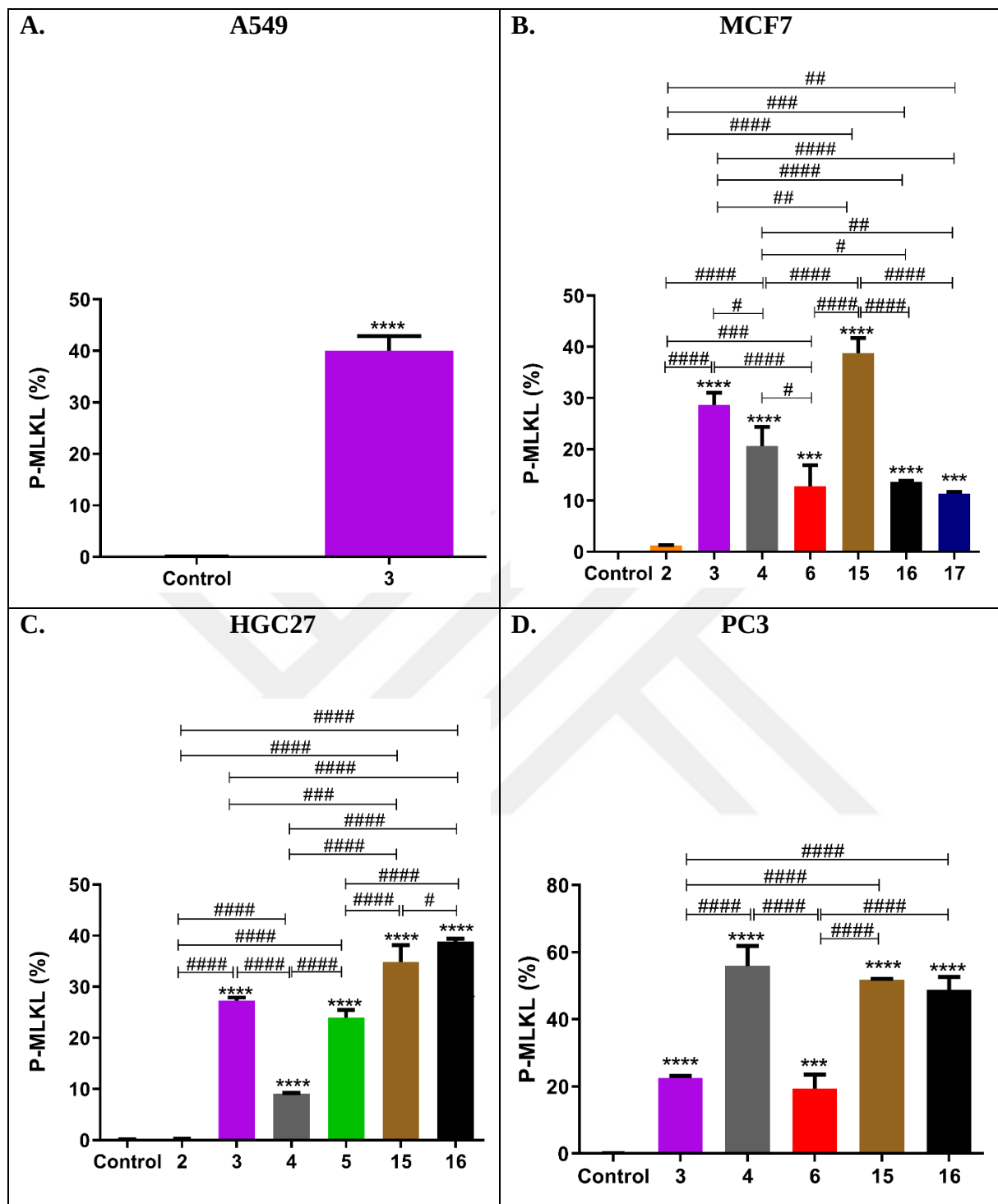


Figure 24. Effects of compounds on p-MLKL protein expression in (A) A549, (B) MCF7, (C) HGC27 and (D) PC3 cell lines.

(Comparison with control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$; Comparison with other compounds: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ and #### $p < 0.0001$).

In addition to evaluation of necrosis with Annexin V/PI staining, p-MLKL/MLKL ratio of tested compounds were also used for assessment of necrosis, which is calculated by dividing the p-MLKL levels to the MLKL levels. Results revealed that all tested compounds, except for compound **2** ($p>0.05$), significantly increased the p-MLKL protein ratio in all tested cell lines ($p<0.05$) (Figure 24). 1- α -acevaltrate (**15**) significantly increased the p-MLKL protein ratios compared to other tested compounds in the MCF7 cells ($p<0.05$) (Figure 24B). In the HGC27 cancer cell line, the compounds that increased the p-MLKL protein ratio at the highest levels were **15** and **16** (Figure 24C). Among them, the effects of **16** on the p-MLKL ratio were significantly higher in comparison with those of **15** ($p<0.05$). On the other hand, compounds **4**, **15** and **16** significantly increased p-MLKL protein levels comparing to others in PC3 cancer cell line ($p<0.05$) (Figure 24D). However, there was no significant difference between these three compounds ($p>0.05$).

4.7.2. DNA Content Analysis

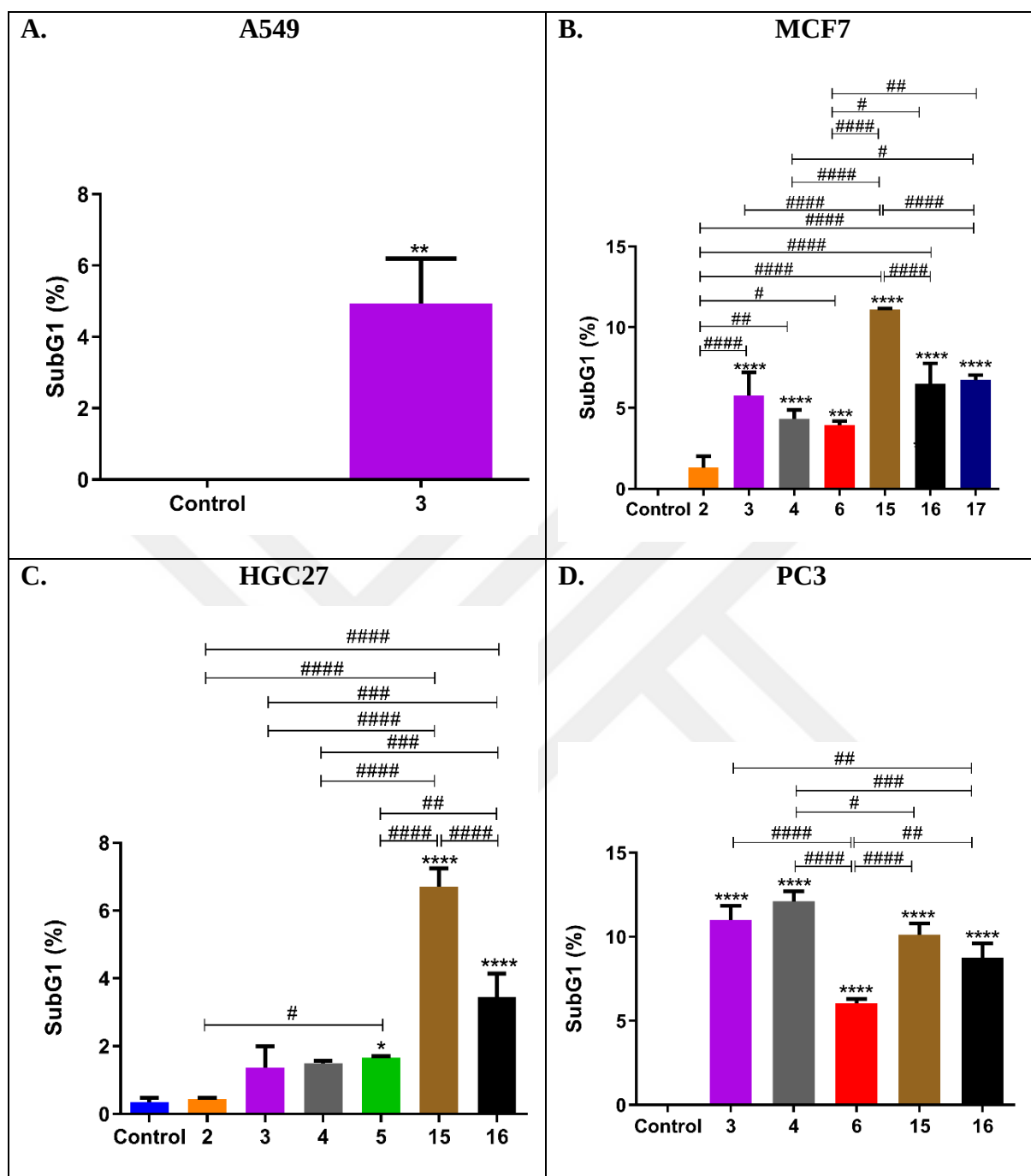


Figure 25. Effects of compounds on the cell population ratio at the SubG₁ phase of the cell cycle in (A) A549, (B) MCF7, (C) HGC27, and (D) PC3 cell lines.

(Comparison with control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$; Comparison with other compounds: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ and #### $p < 0.0001$).

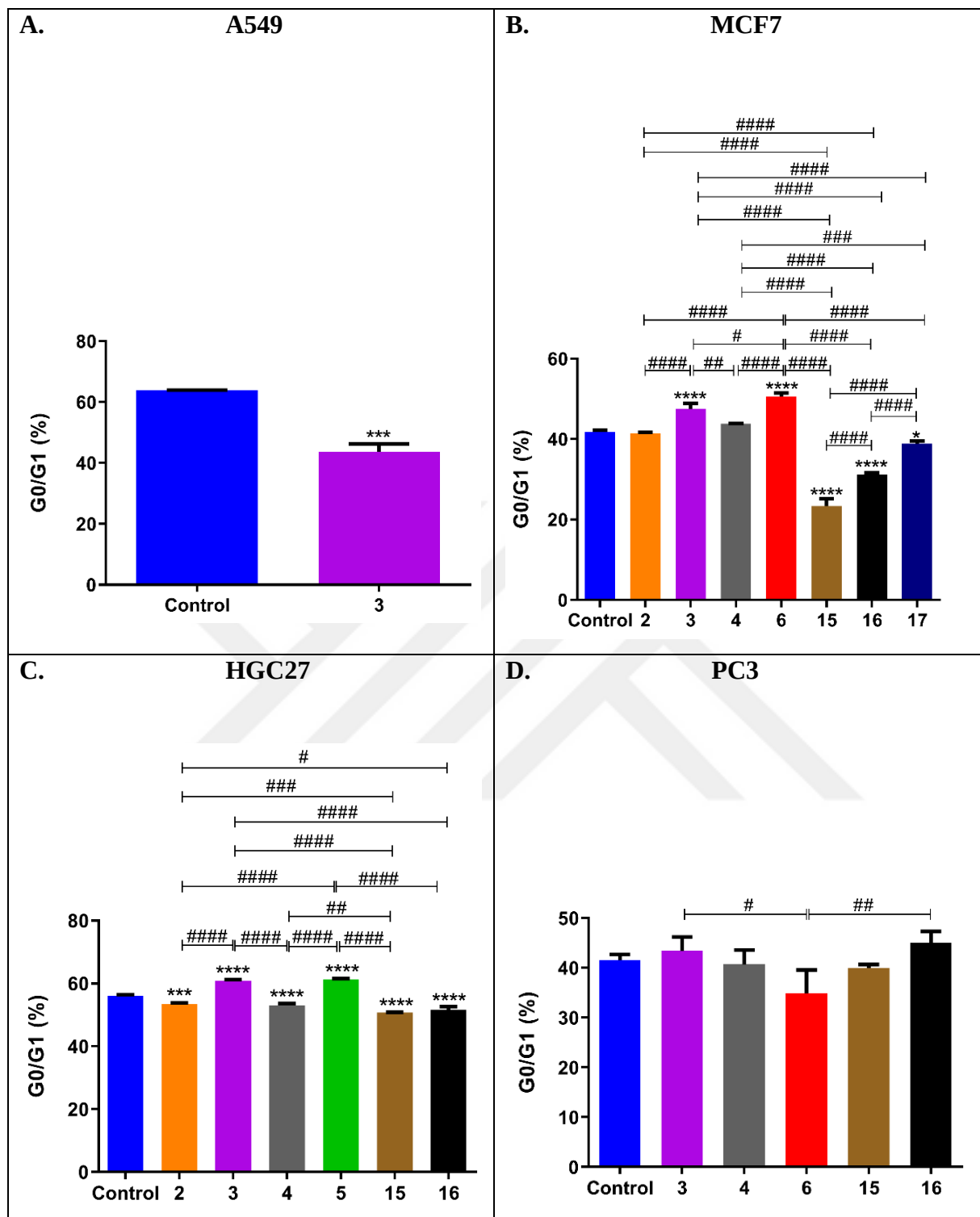


Figure 26. Effects of compounds on the cell population ratio at the G₀/G₁ phase of the cell cycle in (A) A549, (B) MCF7, (C) HGC27, and (D) PC3 cell lines.

(Comparison with control group: *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001; Comparison with other compounds: #p<0.05, ##p<0.01, ###p<0.001 and ####p<0.0001).

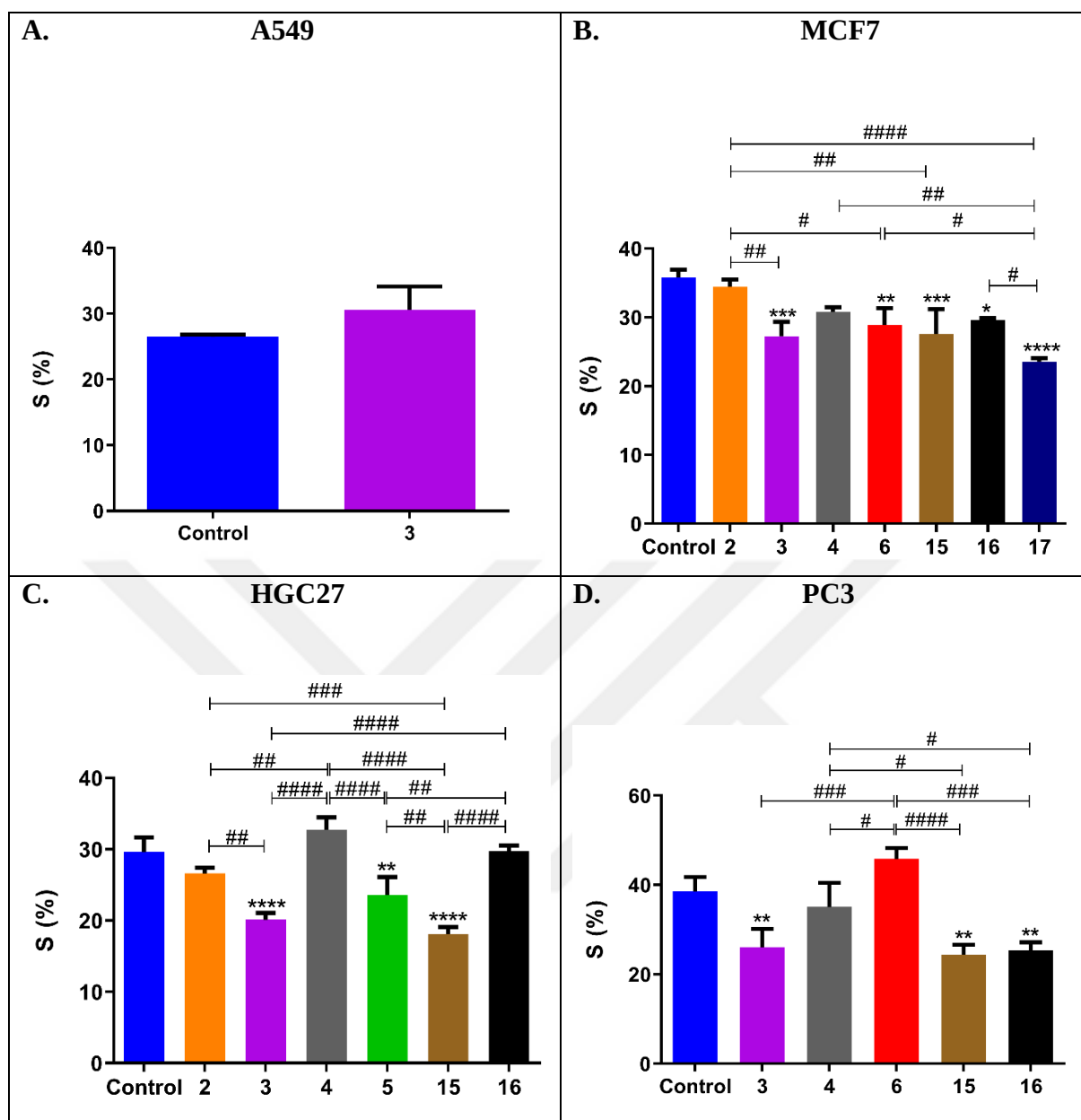


Figure 27. Effects of compounds on the cell population ratio at the S phase of the cell cycle in (A) A549, (B) MCF7, (C) HGC27, and (D) PC3 cell lines.

(Comparison with control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$; Comparison with other compounds: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ and #### $p < 0.0001$).

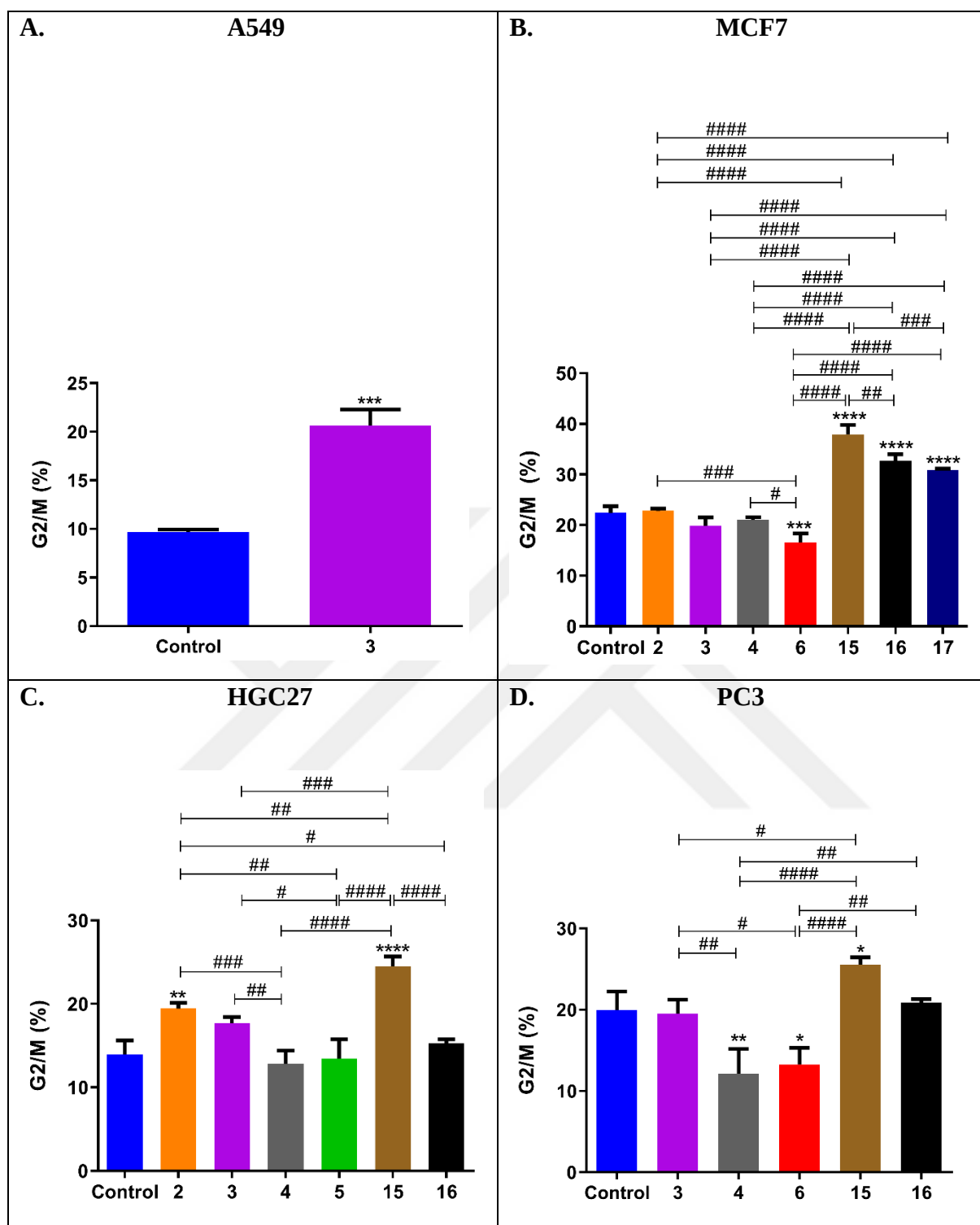


Figure 28. Effects of compounds on the cell population ratio at the G₂/M phase of the cell cycle in (A) A549, (B) MCF7, (C) HGC27, and (D) PC3 cell lines.

(Comparison with control group: *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001; Comparison with other compounds: #p<0.05, ##p<0.01, ###p<0.001 and ####p<0.0001).

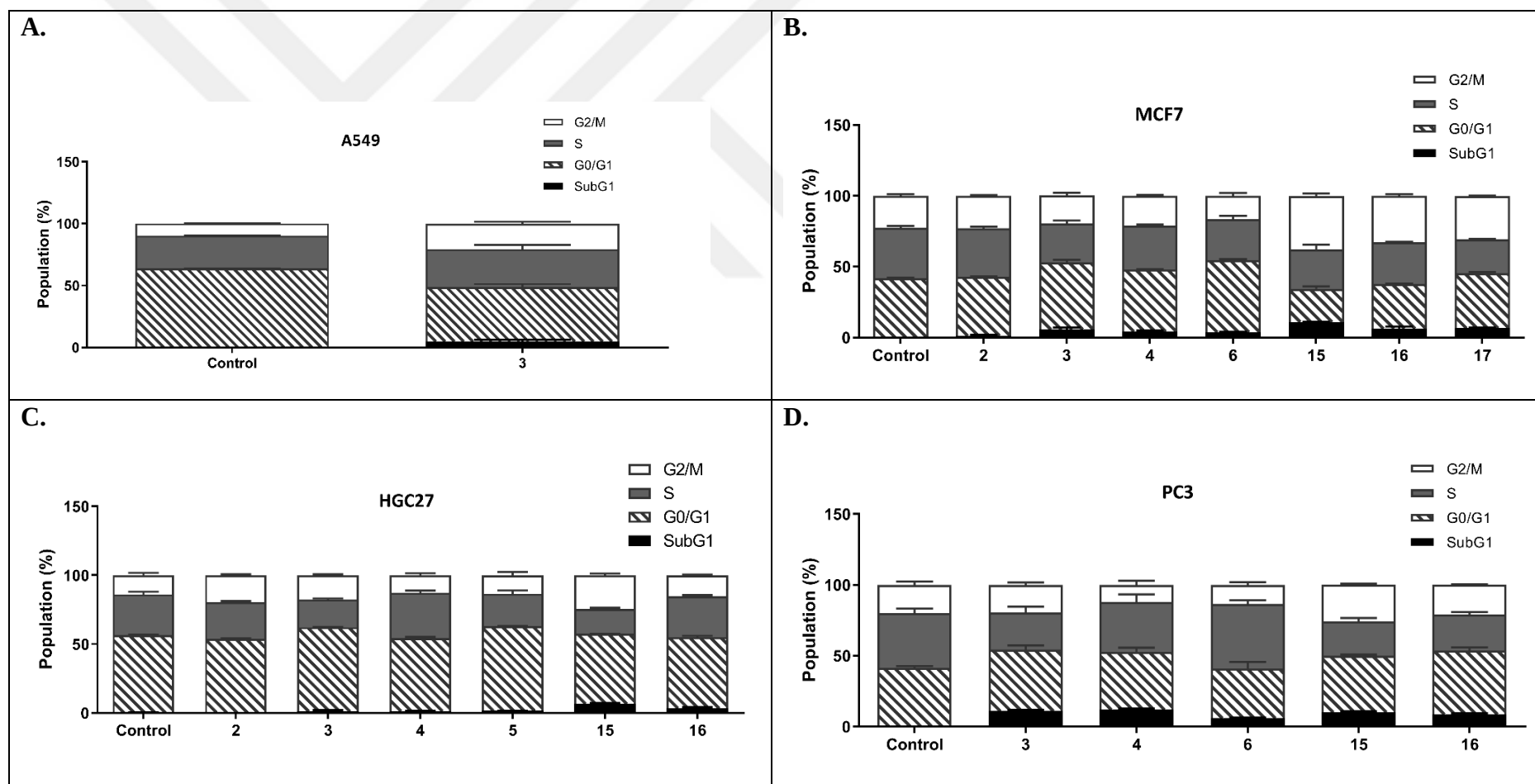


Figure 29. A. DNA content analysis of A549 cells upon treatment with **3** or without treatment, B. DNA content analysis of MCF7 cells upon treatment with **2-4, 6** and **15-17** or without treatment, C. DNA content analysis of HGC27 cells upon treatment with **2-5, 15** and **16** or without treatment, D. DNA content analysis of PC3 cells upon treatment with **3, 4, 6, 15** and **16** or without treatment.

Based on the DNA content analysis which are presented in Figures 25-29, all tested compounds except from IVHD-valtrate (**2**) led to accumulation of cells in SubG₁ phase significantly ($p < 0.05$) in A549, MCF7 and PC3 cancer cells, whereas significant accumulation was also observed in SubG₁ phase upon treatment of HGC27 cells (Figure 25) with compound **5**, **15** and **16** ($p < 0.05$). **3** and **6** caused the accumulation of MCF7 cells at G₀/G₁, while compounds **15-17** led to accumulation of cells in G₂/M phase statistically significant ($p < 0.05$). Valtrate (**3**) not only increased cell accumulation in G₂/M phase ($p < 0.05$) but also decreased the both S and G₀/G₁ phases in A549 cells ($p < 0.05$) (Figures 26A, 27A and 28A). In PC3 cells, treatment with **15** arrested the cells in the G₂/M phase, whereas there were no compounds that promoted accumulation in the G₀/G₁ phase. Compounds **2** and **15** significantly increased accumulation in G₂/M, while similarly **3** and **5** led to cell accumulation in G₀/G₁ phases statistically significant, based on HGC27 cells DNA content findings ($p < 0.05$). Although none of the tested compounds resulted in significant accumulation in the S phase, only compounds **4** and **6** showed a non-significant increase in HGC27 and PC3 cell lines, respectively ($p > 0.05$). Conversely, significant reductions in cell population at the S phase was observed in MCF7 cells separately treated with **3**, **6**, **15-17** (Figure 27B); in HGC27 cells separately treated with **3**, **5**, **15** and **16** (Figure 27C); in PC3 cells separately treated with **3**, **15** and **16** ($p < 0.05$) (Figure 27D).

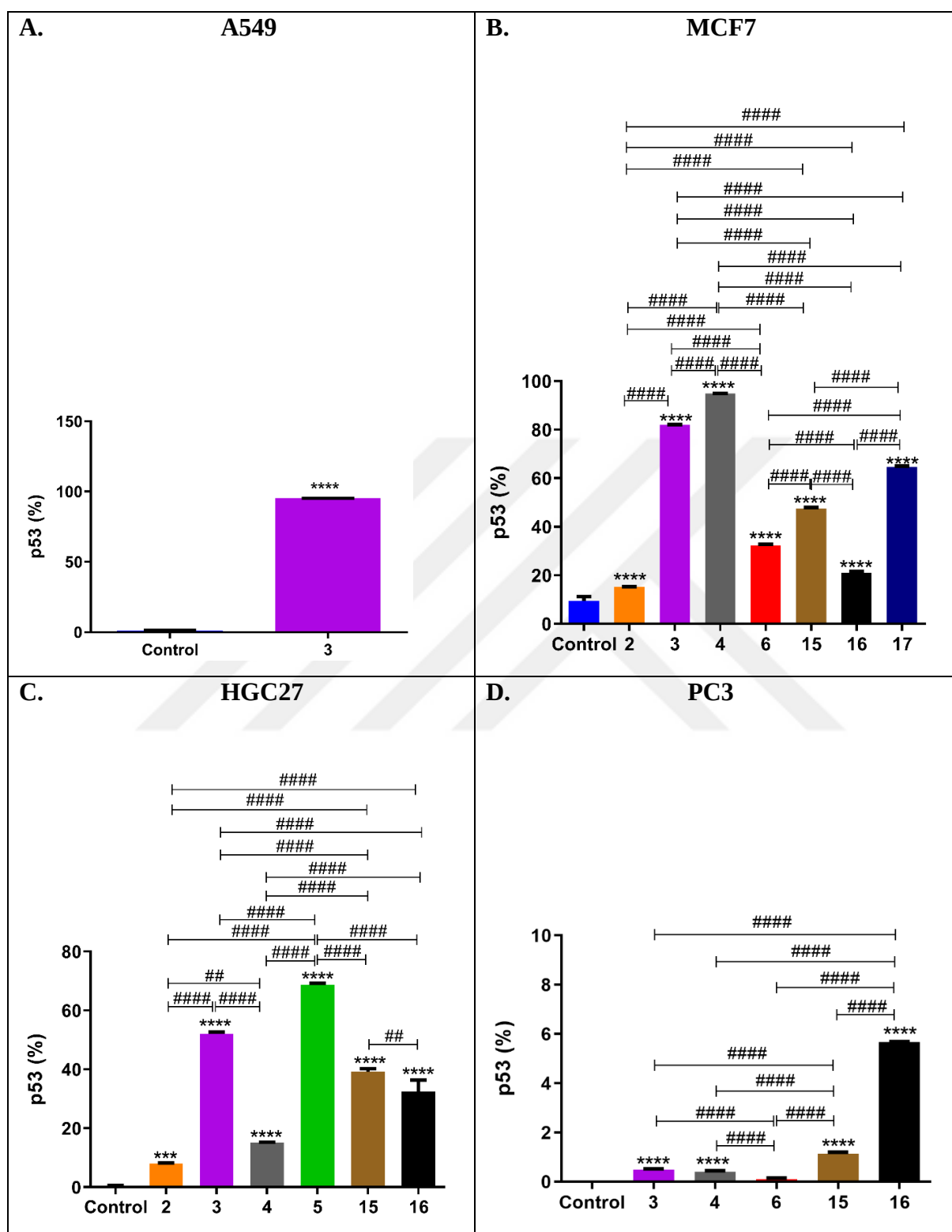


Figure 30. Effects of compounds on p53 protein expression in (A) A549, (B) MCF7, (C) HGC27 and (D) PC3 cell lines.

(Comparison with control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$; Comparison with other compounds: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ and #### $p < 0.0001$).

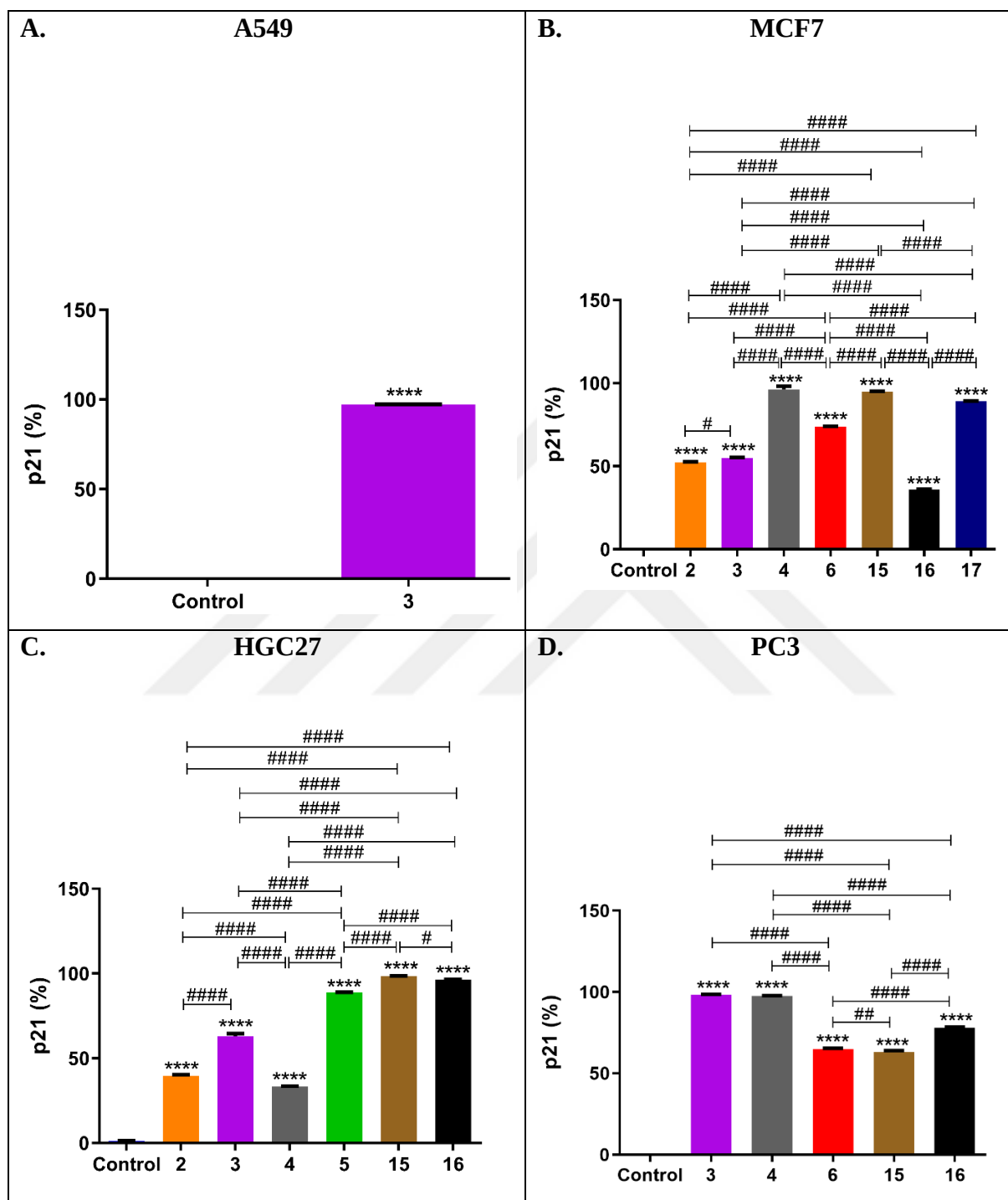


Figure 31. Effects of compounds on p21 protein expression in (A) A549, (B) MCF7, (C) HGC27 and (D) PC3 cell lines.

(Comparison with control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$; Comparison with other compounds: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ and #### $p < 0.0001$).

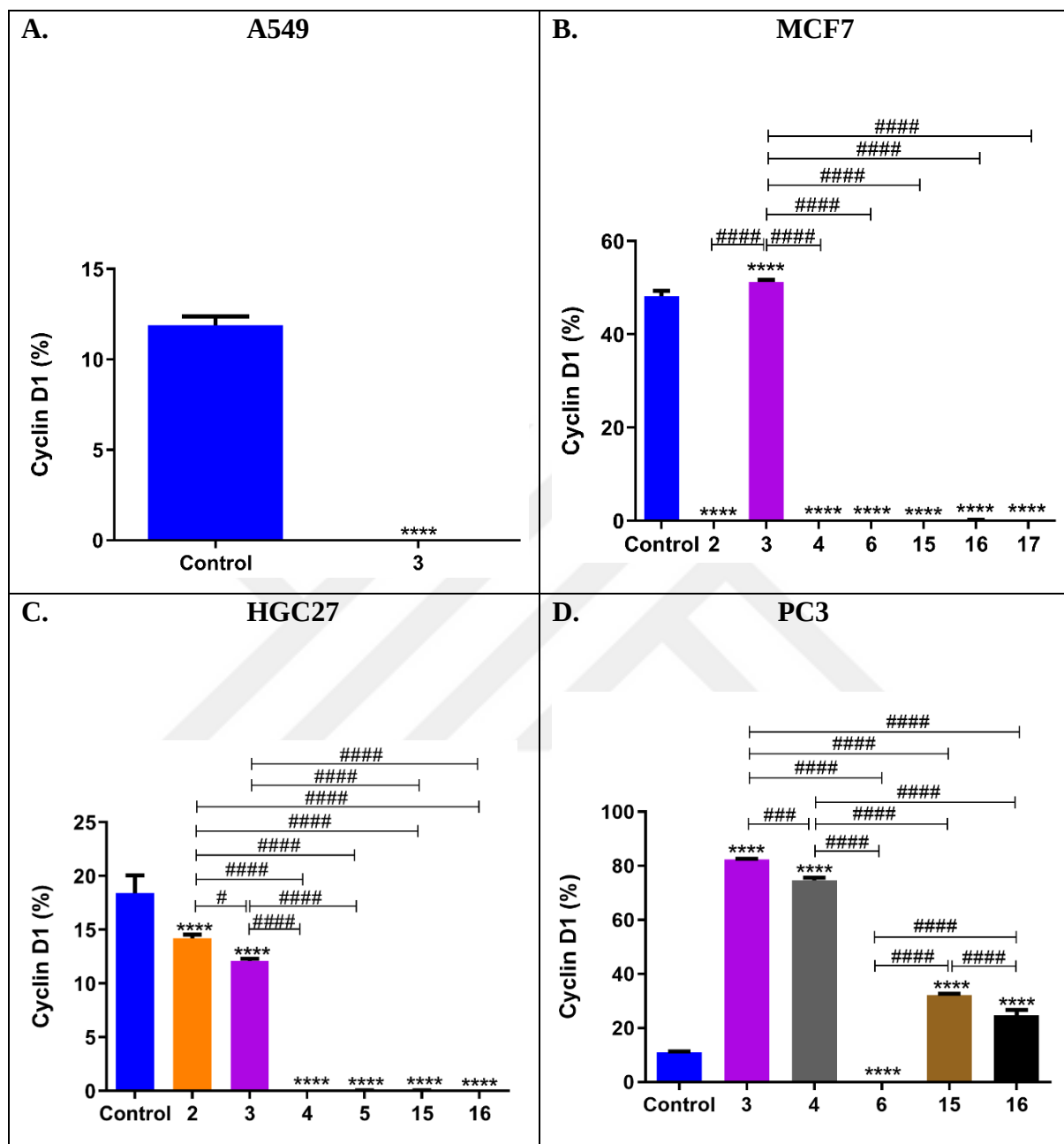


Figure 32. Effects of compounds on cyclin D1 protein expression in (A) A549, (B) MCF7, (C) HGC27 and (D) PC3 cell lines.

(Comparison with control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$; Comparison with other compounds: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ and #### $p < 0.0001$).

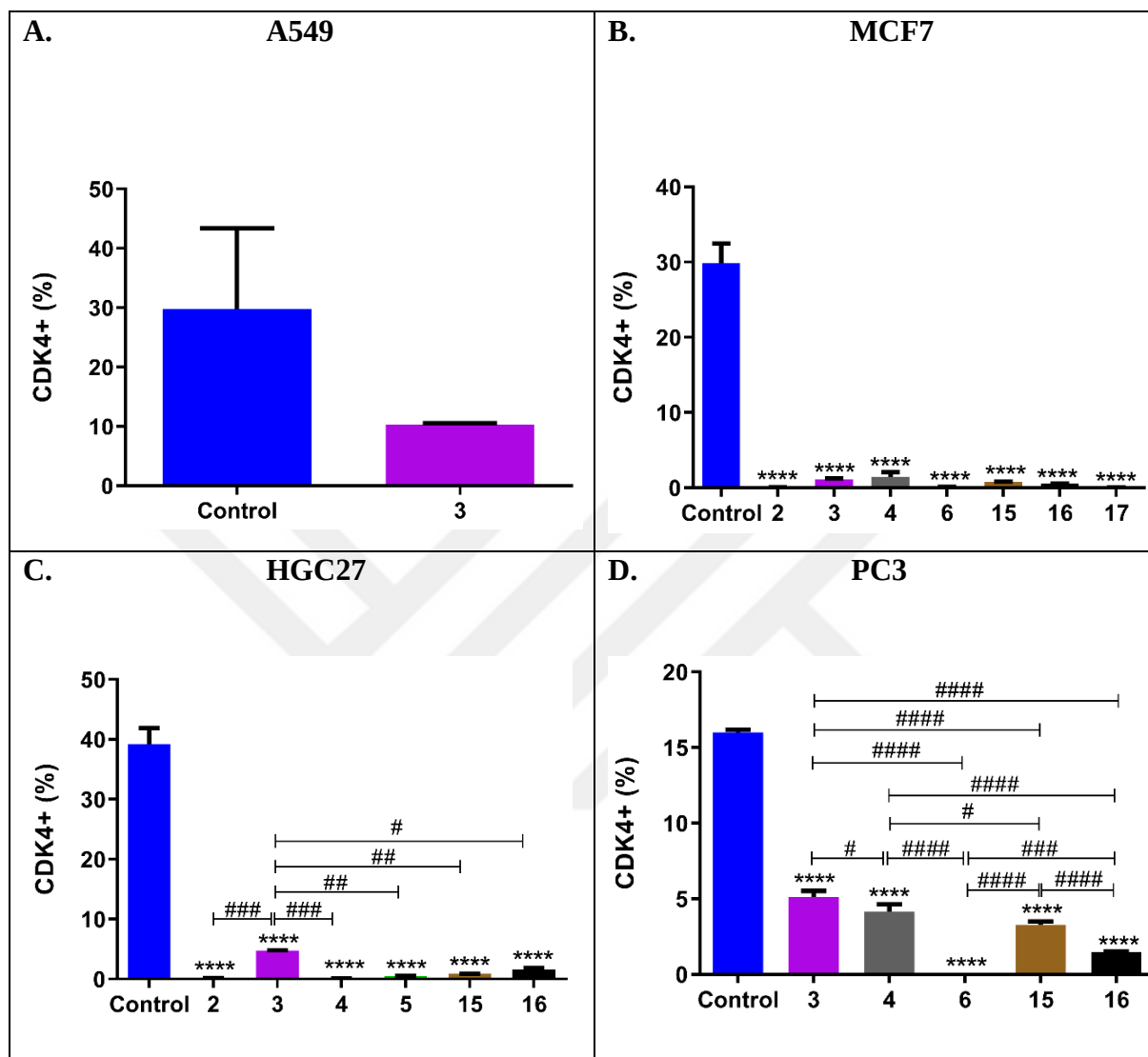


Figure 33. Effects of compounds on CDK4 protein expression in (A) A549, (B) MCF7, (C) HGC27 and (D) PC3 cell lines.

(Comparison with control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$; Comparison with other compounds: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ and #### $p < 0.0001$).

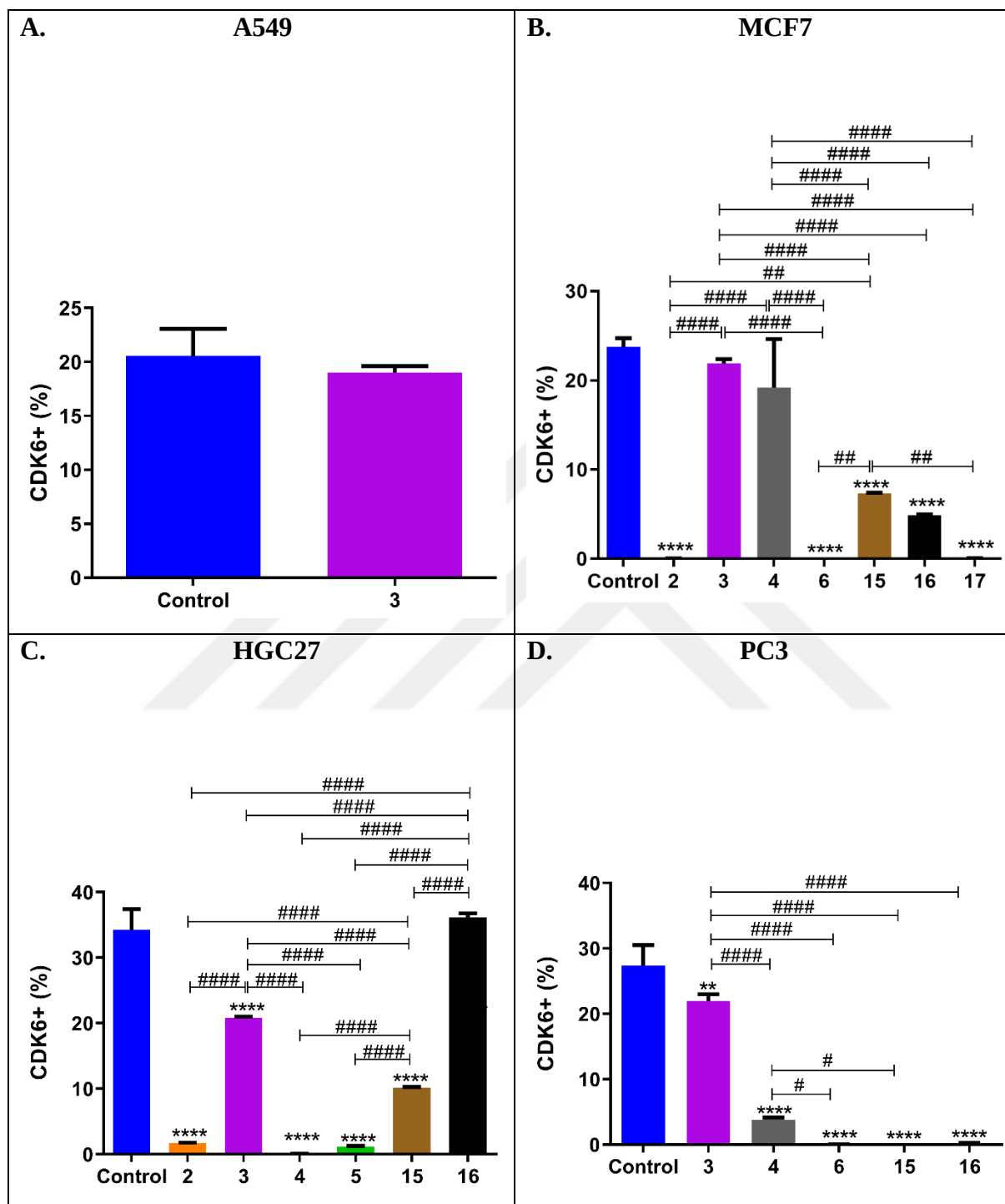


Figure 34. Effects of compounds on CDK6 protein expression in (A) A549, (B) MCF7, (C) HGC27 and (D) PC3 cell lines.

(Comparison with control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$; Comparison with other compounds: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ and #### $p < 0.0001$).

Effects of the compounds on cell cycle progression were also determined through estimating the levels of cell cycle proteins (p21, p53, cyclin D1, CDK4 and CDK6) by flow cytometry (Figures 30-34). According to results, all tested compounds induced expression of p21 genes statistically significant in all the tested cancer cells ($p < 0.05$) (Figure 31). Compounds causing the highest increase in cell lines were found to be valtrate (**3**) in A549 cells, 1- β -acevaltrate (**4**) and 1- α -acevaltrate (**15**) in MCF7 cells, 1- α -acevaltrate (**15**) and 7-homovaltrate (**16**) in HGC27 cells, and valtrate (**3**) and 1- β -acevaltrate (**4**) in PC3 cells ($p < 0.05$). Except for the valtrate hydrine B2 (**6**) against PC3 cells, the p53 levels were increased with individual treatment of all compounds in tested all cell lines (Figure 30). All tested compounds significantly decreased the CDK4 protein levels in MCF7, HGC27 and PC3 cell lines compared to control ($p < 0.05$) (Figure 33B-D). It was observed that the suppressive effects of valtrate (**3**) on CDK4 protein levels were lower than all other compounds in HGC27 and PC3 cancer cells ($p < 0.05$). Also, a slight reduction in CDK4 protein levels was observed in A549 upon **3** treatment comparing with control, though it was not significant ($p > 0.05$). Similarly, the reduction in CDK6 protein levels were found to be nonsignificant when MCF7 cells and A549 cells are treated with **3** ($p > 0.05$). Although, valtrate (**3**) caused decrease in CDK6 protein levels in both HGC27 and PC3 cells as statistically significant, **3** showed less reduction comparing with compounds having a significant reduction in those of cell lines ($p < 0.05$). Moreover, compounds **2**, **5**, **6** and **15** decreased the protein levels of both CDK4 and CDK6 significantly in all tested cell lines. Considering the effects of compounds on cyclin D1 protein levels, compound **3** in MCF7 cells as well as compounds **3**, **4**, **15** and **16** in PC3 cells increased the levels of Cyclin D1 significantly ($p < 0.05$). On the contrary, all the other tested compounds significantly suppressed the cyclin D1 protein levels against tested cell lines, except for the aforementioned compounds with their cell lines ($p < 0.05$).

4.7.3. Evaluation of Autophagy

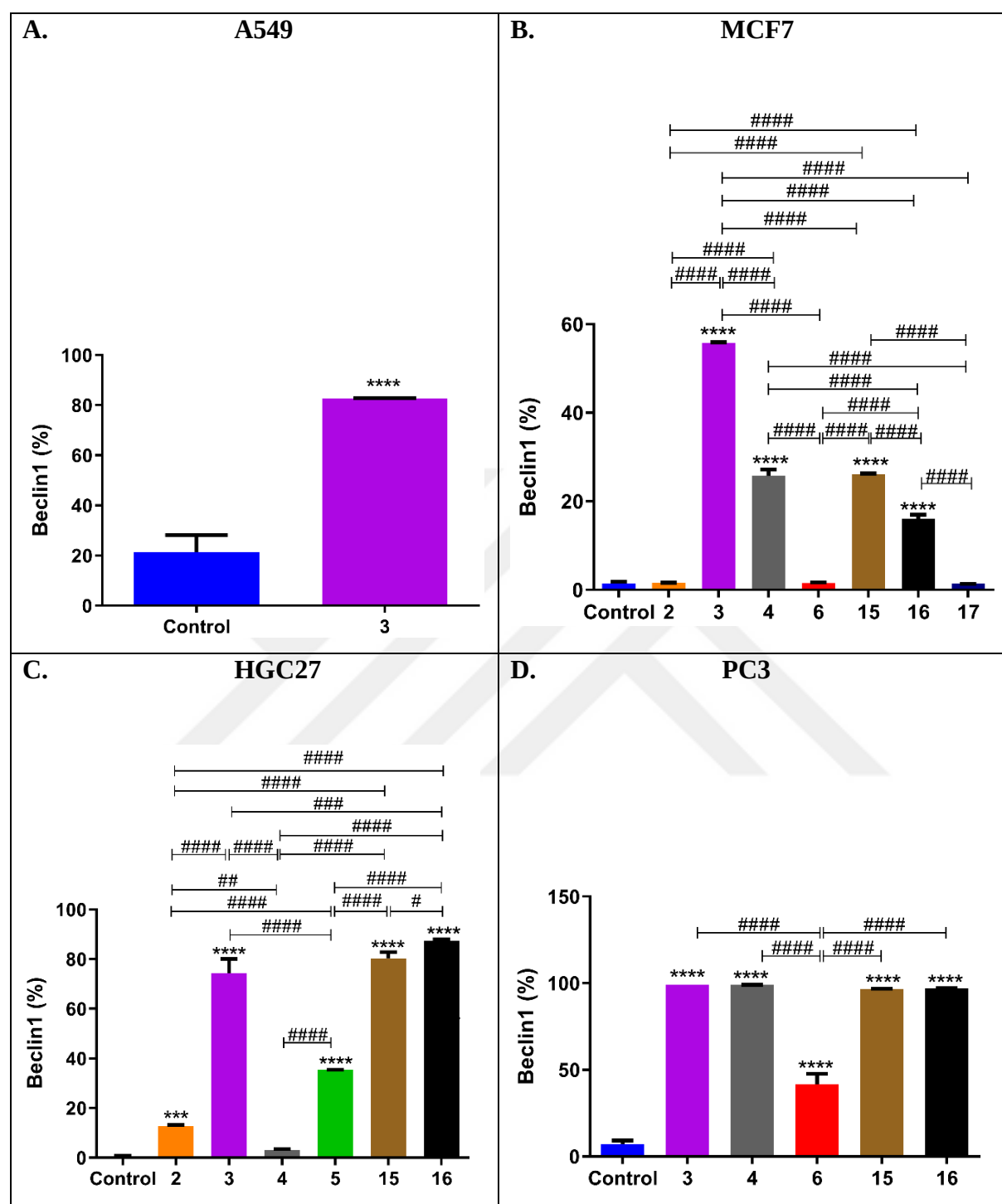


Figure 35. Effects of compounds on Beclin1 protein expression in (A) A549, (B) MCF7, (C) HGC27 and (D) PC3 cell lines.

(Comparison with control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$; Comparison with other compounds: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ and #### $p < 0.0001$).

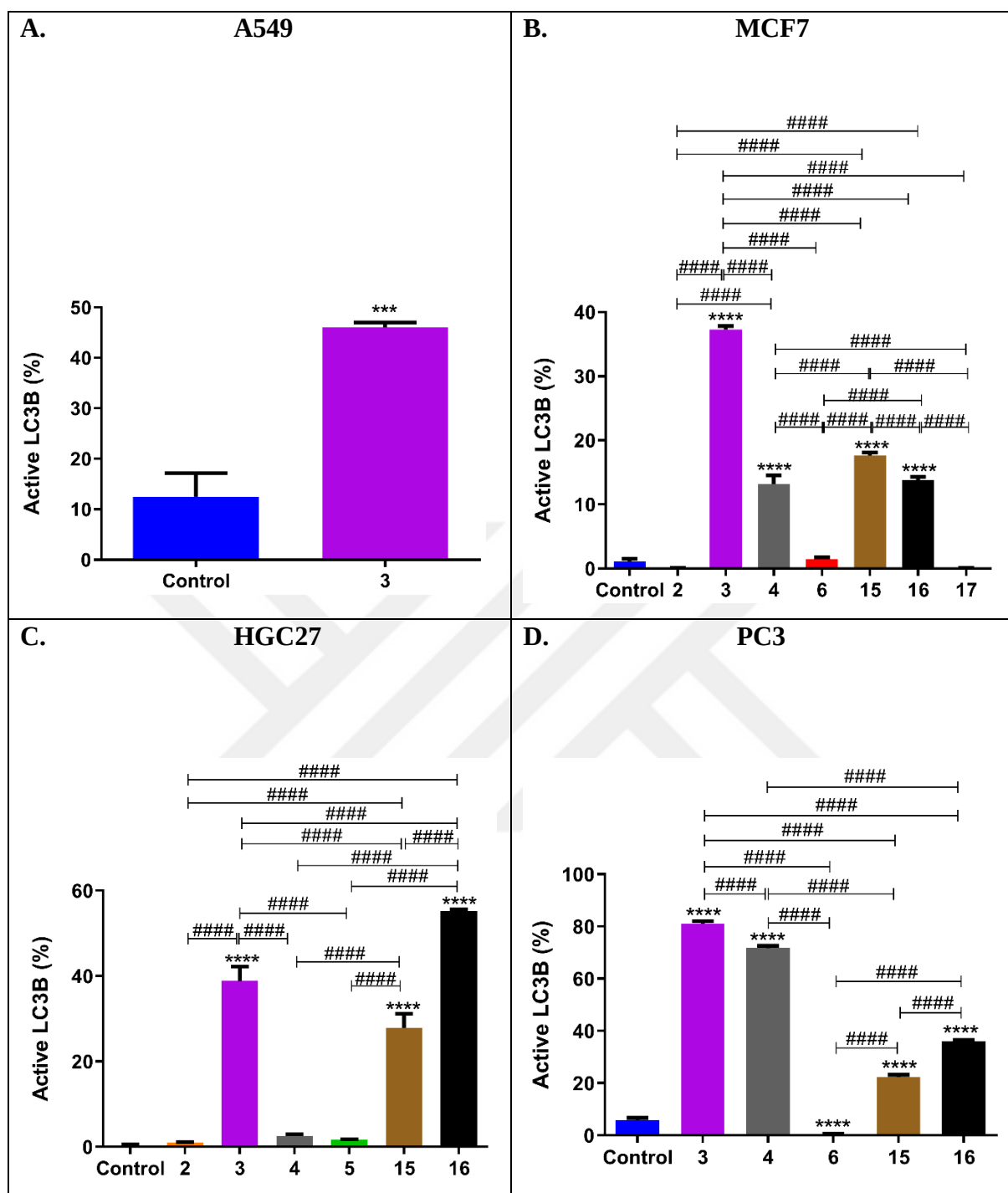


Figure 36. Effects of compounds on active LC3B protein expression in (A) A549, (B) MCF7, (C) HGC27 and (D) PC3 cell lines.

(Comparison with control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$; Comparison with other compounds: # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ and #### $p < 0.0001$).

Beclin-1 and LC3B protein levels, which are recognized autophagic pathway biomarkers, were measured using flow cytometry to evaluate the impact of substances on autophagic pathways. When the protein levels of Beclin-1 and autophagosome marker active LC3B in the autophagic pathway are evaluated together, valtrate (**3**), 1- β -acevaltrate (**4**), 1- α -acevaltrate (**15**) and 7-homovaltrate (**16**) led to an increase in expression of both two autophagy related proteins significantly in cancer cell lines ($p < 0.05$) except for **4** in HGC27 (Figures 35 and 36). In MCF7 cells, **3** was determined to be most active compound compared to other ones ($p < 0.05$). Similarly, **3** increased active LC3B level in PC3 cells in comparison with other compounds ($p < 0.05$) (Figure 36). However, no statistically significant differences between Beclin-1 protein levels after treatment with **3**, **4**, **15** and **16** in PC3 cells was observed. Comparisons between tested and control groups in HGC27 cells revealed that **2**, **3**, **5**, **15** and **16** caused a significant elevation of Beclin1 levels (Figure 35C) whereas a significant increase in active LC3B levels (Figure 36C) were observed for compounds **3**, **15** and **16**. Amongst, 7-homovaltrate (**16**) caused the most potent induction of both Beclin-1 and active LC3B levels significantly, when compared with tested compounds in HGC27 cells ($p < 0.05$).

5. DISCUSSION and CONCLUSION

Cancer, an important public health concern, is one of the leading causes of death worldwide. According to the statistics of GLOBOCAN 2020, there have been reported 19.3 million new cases of cancer and roughly 10 million deaths occurred due to different cancer types including female breast (11.7% of cases), lung (11.4% of cases), prostate (7.3% of cases) and gastric (5.6% of cases) cancers (1). Surgery, radiotherapy, and chemotherapy as well as their combinations have been widely used for the traditional treatment of cancer, nowadays. Considering the possible side effects and multi-drug resistance of current chemotherapy methods, new alternative treatment strategies are required to fight against cancer. (7). In recent years, there has been a considerable demand for more selective and effective new anticancer agents with fewer side effects. Thus, many researchers have focused on natural products (10). Cumulative evidence has shown that many plant-based compounds have played a crucial role in the drug development process due to their diverse mechanisms of action (270). Between 1940 and 2014, approximately 49% of approved small anticancer drugs were obtained either from natural sources or from their semi-synthesis (8). Currently, more than half of the drugs used clinically for cancer chemotherapy are phytochemicals or their derivatives such as camptothecin, paclitaxel, irinotecan, homoharringtonine, vinorelbine, omacetaxine, teniposide, vinblastine, etoposide, vincristine, mepesuccinate, vindesine, and topotecan (9). Compared to the high expense and adverse effects of most synthetic drugs, medicinal plants as a source of bioactive compounds still retain their huge potential in the discovery of new anticancer molecules. Thus, this study was designed to search for new potential natural-based anticancer drug leads.

The genus *Valeriana* L. (Caprifoliaceae, formerly Valerianaceae family) consist of more than 200 species distributed particularly in North America, Asia and Europe. Some members of this genus, especially *V. jatamansi* and *V. officinalis* are traditionally utilized for their anxiolytic and sedative effects (11,12). Previous studies on various extracts of *Valeriana* species as well as the isolated compounds revealed a broad range of pharmacological activities such as anticonvulsant, neuroprotective, anti-inflammatory, anxiolytic, antidepressant, antibacterial, cytotoxic and antitumor (14–20,26).

In the flora of Türkiye, there are 13 species of the genus *Valeriana* growing wild, three of are endemic including *V. speluncaria* Boiss. (33,34). Recent phytochemical and pharmacological studies on *Valeriana* species revealed that the isolated compounds, especially

non-glycosidic ester iridoids and sesquiterpenoids, exhibit significant cytotoxic and antitumor activities (22–24,27,28,32,109,111). Considering the potential cytotoxic effects of compounds and extracts obtained from *Valeriana* species, this study was designed to obtain the cytotoxic compounds from the underground parts of three naturally grown *Valeriana* species, namely *V. alliariiifolia*, *V. sisymbriiifolia* and *V. speluncaria* via *in vitro* activity-guided fractionation. Besides, the mechanisms behind the cytotoxic activity of most potent isolates were also unraveled. In addition to the main subjects of this study, chromatographic studies were also performed on the *n*-BuOH subextracts to obtain major secondary metabolites in order to make a contribution to the chemotaxonomy of genus.

Firstly, the crude EtOH extracts prepared from the underground parts of *V. alliariiifolia*, *V. sisymbriiifolia* and *V. speluncaria* were tested for their cytotoxic effects against A549, MCF7, HGC27, PC3 and U87 cancer cell lines as well as a healthy cell line (HUVEC) using MTS colorimetric assay. The cytotoxic activity results (Tables 63 and 64) showed that crude EtOH extracts of *V. alliariiifolia* (IC₅₀: 12.04-30.75 µg/mL) and *V. sisymbriiifolia* (IC₅₀: 7.46 to 12.56 µg/mL) exhibited remarkable cytotoxic activity against A549, MCF7, HGC27 and PC3 cancerous cells. *V. sisymbriiifolia* was found to be more cytotoxic than *V. alliariiifolia*. However, both crude extracts were inactive against U87 cell line (IC₅₀: >100 µg/mL). EtOH extract of *V. alliariiifolia* (against HUVEC; IC₅₀: 39.09 µg/mL) was found to be more selective, than that of *V. sisymbriiifolia* (against HUVEC; IC₅₀: 17.6 µg/mL) due to exhibition of less cytotoxicity on HUVEC cell lines. The crude EtOH extract of *V. speluncaria* was found to be potent cytotoxic against MCF7 and U87 (IC₅₀: 5.09 and 2.01 µg/mL, respectively) (Table 65) showing differences from the other species. More importantly, this extract can be considered as selective cytotoxic due to having weak cytotoxicity towards HUVEC with the IC₅₀ value of 83.17 µg/mL. Among the tested cancer cell lines, MCF7 was determined to be the most susceptible cell line (Tables 63-65) and the EtOH extract of *V. speluncaria* displayed most cytotoxic activity with the IC₅₀ values of 5.09 µg/mL. Accordingly, A549, MCF7, HGC27 and PC3 cancer cell lines were selected for further cytotoxicity studies for *V. alliariiifolia* and *V. sisymbriiifolia*, whereas U87 and MCF7 cells were chosen for the relevant studies on *V. speluncaria* extracts.

This study constitutes the first report on the investigation of the cytotoxic effects of the extracts obtained from *V. alliariiifolia*, *V. sisymbriiifolia* and *V. speluncaria*. On the other hand, despite the fact that the cytotoxicity of the isolates from *Valeriana* species is well known, there are few studies on the cytotoxic activity of the extract obtained from *Valeriana* species. For

instance, in the previous studies, Lin and colleagues carried out isolation studies on the whole parts of *V. jatamansi* through cytotoxic-guided fractionation. It was found that hydroalcoholic extract (95% EtOH) of *V. jatamansi* exerted cytotoxic effect on four cancer cell lines including A549 (IC₅₀: 22.3 µg/mL), PC-3M (IC₅₀: 18.8 µg/mL), Bel 7402 (IC₅₀: 25.2 µg/mL) and HCT-8 (IC₅₀: 29.4 µg/mL) by MTT assay (187). In addition, Bos et al. demonstrated the cytotoxic activity of *V. wallichii*, *V. officinalis* and *V. edulis* ssp. *procera* tinctures (underground parts, 70% EtOH) against GLC4 and COLO320 cancerous cells (28). Taken together with our results and those previously reported, the crude extracts of *Valeriana* species were shown to possess cytotoxicity against different cancer cell lines.

In the present study, after the successful partition of each crude EtOH extract of plants, CHCl₃, EtOAc, *n*-BuOH and rH₂O subextracts were yielded. These subextracts were also evaluated for their cytotoxic activity against the same cancer cell lines as mentioned above. Among the tested subextracts of *V. alliarifolia*, CHCl₃ subextract exhibited noteworthy cytotoxic activity against A549, MCF7, HGC27 and PC3 cancer cell lines with IC₅₀ values varying from 2.5 to 21.5 µg/mL, while EtOAc subextract indicated cytotoxic effect only against MCF7 with IC₅₀ value of 12.2 µg/mL (Table 63). However, remarkable activity was not observed in *n*-BuOH and rH₂O subextracts (IC₅₀: >100 µg/mL). Apart from the A549 cells, CHCl₃ subextract (IC₅₀: 2.5-12.0 µg/mL) was found to be more cytotoxic than its crude EtOH extract (IC₅₀: 12.04-30.75 µg/mL) and EtOAc extract (IC₅₀: 12.2-66.6 µg/mL) against all tested cell lines. Conversely, CHCl₃ subextract (IC₅₀: 21.5 µg/mL) was found to have weaker cytotoxicity than crude EtOH extract (IC₅₀: 15.1 µg/mL) on A549 cancerous cells. Further, the effects of crude EtOH extract and EtOAc subextract demonstrated equipotent bioactivity against the MCF7 cancer cell line with IC₅₀ values of 12.04 and 12.2 µg/mL, respectively. On the other hand, CHCl₃ and EtOAc subextracts of *V. sisymbriifolia* showed pronounced cytotoxic activity against A549, MCF7, HGC27 and PC3 cancer cell lines with IC₅₀ values in the range of 2.4 to 44.1 µg/mL. Similar to *V. alliarifolia* subextracts, *n*-BuOH and rH₂O subextracts of *V. sisymbriifolia* were inactive against all tested cancer cell lines (IC₅₀: >100 µg/mL). Likewise, CHCl₃ subextract (IC₅₀: 2.4-5.3 µg/mL) of *V. sisymbriifolia* exerted stronger cytotoxic activity on MCF7, HGC27 and PC3 cells in comparison with the crude EtOH extract (IC₅₀: 10.16-12.56 µg/mL) and EtOAc subextract (IC₅₀: 7.5-16.3 µg/mL). However, against A549 cancerous cells, crude EtOH extract (IC₅₀: 7.46 µg/mL) was found to possess more cytotoxicity than CHCl₃ and EtOAc subextracts (IC₅₀: 11.6 and 44.1 µg/mL, respectively). Besides, the subextracts of *V. speluncaria* were tested against U87 and MCF7 cancer cell lines.

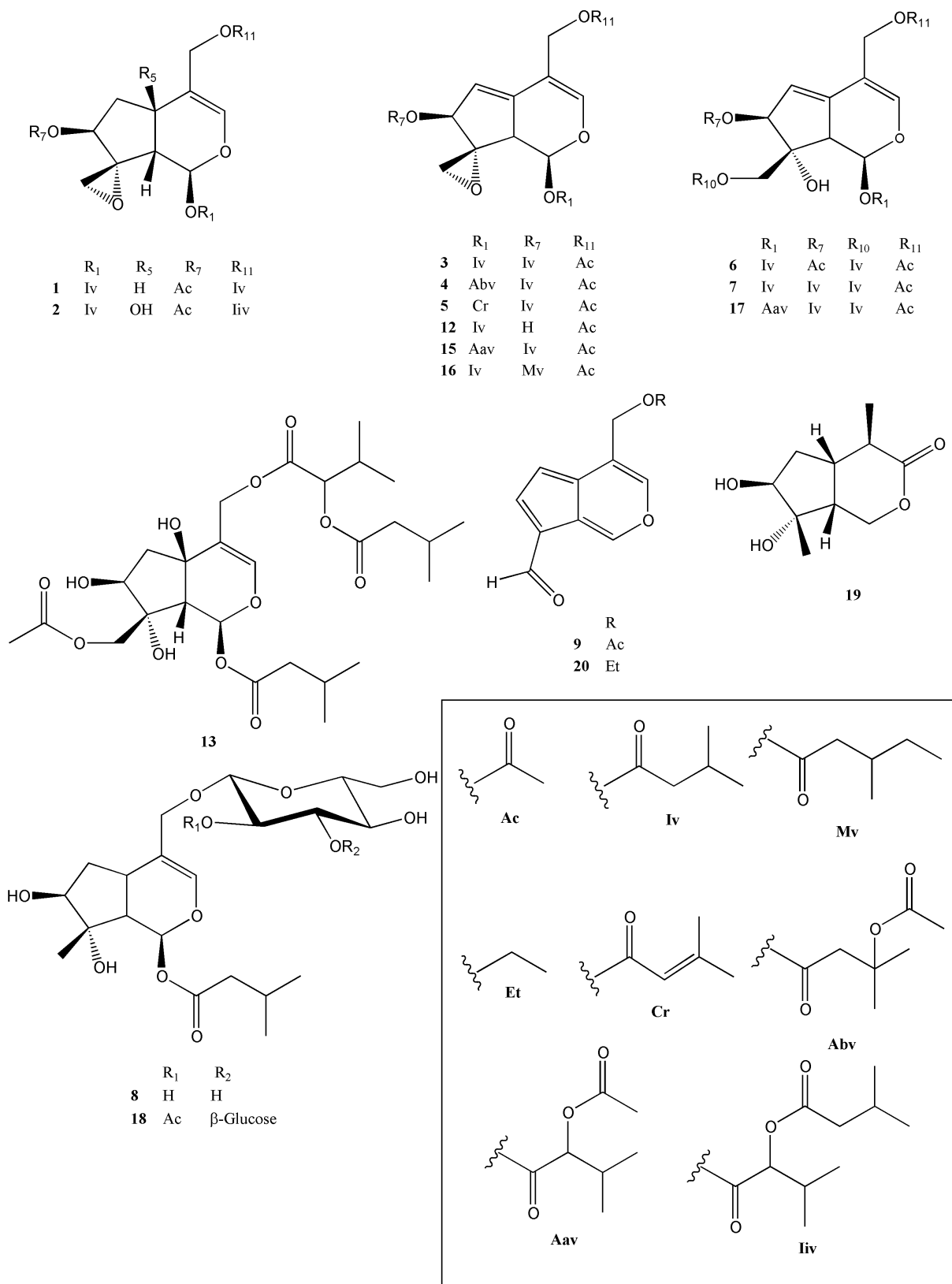
Despite the potent cytotoxic activity of *V. speluncaria* EtOH extract on U87 and MCF7 cancer cells (IC₅₀: 2.01 and 5.09 µg/mL, respectively), weak cytotoxic effects were observed in its subextracts. The reason why the cytotoxic effects of the subextracts were weaker than that of the crude EtOH extract can be explained by the loss of the synergistic effect between the compounds in the crude extract upon partition. Comparing the activities of the subextracts of *V. speluncaria*, CHCl₃ and EtOAc subextracts (IC₅₀: 48.8-81.0 µg/mL) were found to be cytotoxic against two tested cancer cell lines, whereas *n*-BuOH subextract displayed weak cytotoxic effects on only MCF7 cells with IC₅₀ value of 65.5 µg/mL. Although the EtOAc subextract (IC₅₀: 48.8 µg/mL) was more active than the *n*-BuOH and CHCl₃ (IC₅₀: 73.7 µg/mL) subextracts against MCF7 cells, CHCl₃ subextract (IC₅₀: 70.7 µg/mL) exhibited relatively higher cytotoxicity than EtOAc subextract (IC₅₀: 81.0 µg/mL) on glioblastoma (U87) cancerous cell line. When the cytotoxic activity of all subextracts from the three species were compared, the CHCl₃ and EtOAc subextracts of plants displayed higher cytotoxic activity than the others. However, the cytotoxic activity of CHCl₃ and EtOAc subextracts from *V. speluncaria* were extremely weaker than those of *V. alliariifolia* and *V. sisymbriifolia*. This situation could be associated with the differences in the chemical composition of *V. speluncaria*. Considering the almost similar cytotoxicity results observed in subextracts from *V. alliariifolia* and *V. sisymbriifolia*, it can be interpreted that these active subextracts could have close proximity to each other in terms of their chemical contents.

The cytotoxically active CHCl₃ and EtOAc subextracts of *V. alliariifolia*, were fractionated over SiO₂ and Sephadex LH-20 column chromatographies, respectively to afford the main fractions that were also tested by MTS assay. According to the activity results, frs. A-D from CHCl₃ and fr. 1 from EtOAc were selected for further chromatographic separations in order to isolate potential cytotoxic compounds. The activity-guided isolation studies on *V. alliariifolia* led to the isolation of didrovaltrate (**1**), IVHD-valtrate (**2**), valtrate (**3**), 1-β-acevaltrate (**4**), seneciovaltrate (**5**), valtrate hydrine B2 (**6**), isovaleroxyvaltrate hydrine (**7**), baldrinal (**9**), coniferyl aldehyde (**10**), 7-deisovaleroylvaltrate (**12**), valeriotriate B (**13**) and 8-hydroxypinoresinol (**14**) from the cytotoxic CHCl₃ subextract (frs. A-D), while valerosidate (**8**) and coniferin (**11**) were purified from the cytotoxic fraction of EtOAc (fr. 1). In order to make a contribution to the phytochemical composition of this plant, *n*-BuOH subextract was separated to afford coniferin (**11**), eriocitrin (**33**), xiecaoside E (**34**) and 8-hydroxypinoresinol 4'-*O*-β-glucopyranoside (**35**) (271).

Similar chromatographic methods were applied to the cytotoxic subextracts of *V. sisymbriifolia* to obtain main fractions. Based on the MTS assay results, frs. A-E from CHCl₃ and, frs. 1 and 2 from EtOAc subextract were selected for further isolation steps to obtain cytotoxic metabolites. Accordingly, *V. sisymbriifolia* CHCl₃ subextract afforded didrovaltrate (**1**), valtrate (**3**), baldrinal (**9**), 1- α -acevaltrate (**15**), sisymbriifolivaltrate (**17**), 11-ethoxyviburtinal (**20**), bornyl caffeate (**21**), sisymbriifolins A (**22**) and B (**23**), 9-decenoic acid (**24**), while valtrate (**3**), baldrinal (**9**), 1- α -acevaltrate (**15**), 7-homovaltrate (**16**), sisymbriifolioside (**18**) and patriscabrol (**19**) were isolated from EtOAc subextract.

CHCl₃ and EtOAc subextracts of *V. speluncaria* were subjected to SiO₂ and Sephadex LH-20 CCs, respectively to yield main fractions. According to MTS assay results, frs. C-E of CHCl₃ subextract, and frs. 2 and 3 of EtOAc subextract were subjected for further purification studies. Chromatographic separations on active fractions of CHCl₃ subextract (frs. C-E) resulted in the purification of pinoresinol 4-*O*- β -D-glucopyranoside (**25**), speluncarins A (**30**) and B (**31**), daucosterol (**32**), whereas coniferin (**11**), pinoresinol 4-*O*- β -D-glucopyranoside (**25**), caffeic acid (**26**), chlorogenic acid (**27**), 3,4-dicaffeoylquinic acid (**28**), and 3,5-dicaffeoylquinic acid (**29**) were successively purified from cytotoxic EtOAc subextract (frs. 2 and 3). Besides the activity-guided fractionation studies, the isolation on *n*-BuOH subextract yielded pinoresinol 4-*O*- β -D-glucopyranoside (**25**), 8-hydroxypinoresinol 4'-*O*- β -glucopyranoside (**35**), pinoresinol 4,4'-di-*O*- β -D-glucopyranoside (**36**), medioresinol di-*O*- β -D-glucopyranoside (**37**), and fraxiresinol 1-*O*- β -D-glucopyranoside (**38**) (272).

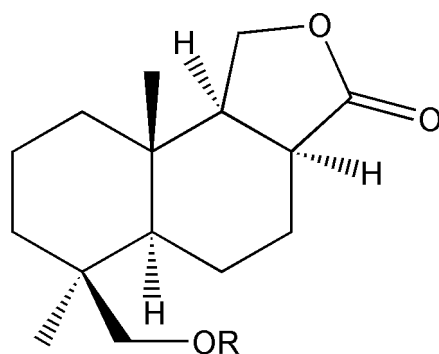
Totally 38 compounds (**1-38**) including six previously unreported ones (**17**, **18**, **22**, **23**, **30** and **31**) were purified from *V. alliariifolia*, *V. sisymbriifolia* and *V. speluncaria* by using numerous successive chromatographic techniques. The structure elucidation of the isolates was accomplished by extensive spectroscopic experiments including IR, UV, 1D (¹H and ¹³C) and 2D (HSQC, COSY, HMBC and NOESY) NMR as well as MS/HRESIMS analysis. Overall, the chromatographic separations led to the isolation of compounds **1-14**, **33-35** from *V. alliariifolia*, **1**, **3**, **9**, **15-24** from *V. sisymbriifolia*, **11**, **25-32**, **35-38** from *V. speluncaria*. Some compounds were isolated from more than one species, namely compounds **1**, **3**, and **9** were obtained from *V. alliariifolia* and *V. sisymbriifolia*, whereas compounds **11** and **35** were purified from *V. alliariifolia* and *V. speluncaria* (Table 72).



It is well-documented that iridoids are the main components of *Valeriana* species and totally 259 different iridoids have been characterized from the genus *Valeriana* till now (273). In this study, 17 iridoid derivatives, namely didrovaltrate (**1**), IVHD-valtrate (**2**), valtrate (**3**),

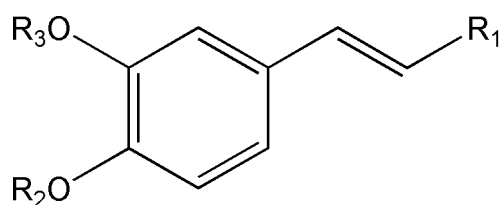
1- β -acevaltrate (**4**), seneciovaltrate (**5**), valtrate hydrine B2 (**6**), isovaleroxyvaltrate hydrine (**7**), valerosidate (**8**), baldrinal (**9**), 7-deisovaleroylvaltrate (**12**), valeriotriate B (**13**), 1- α -acevaltrate (**15**), 7-homovaltrate (**16**), sisymbriifolivaltrate (**17**), sisymbriifolioside (**18**) and patriscabrol (**19**) and 11-ethoxyviburtinal (**20**) were obtained as a result of successive chromatographic separations. These compounds can be classified into subclasses of iridoids namely, non-glycosidic ester iridoids (**1-7**, **12**, **13**, **15-17**), iridoid glycosides (**8** and **18**), iridolactone (**19**) and baldrinals (**9** and **20**). Among the non-glycosidic esters, two subgroups commonly found in the genus *Valeriana*, including valepotriates (**1-5**, **12**, **15** and **16**) and valtrate hydrines (**6**, **7**, **13** and **17**) were determined in this study. Compounds **17** and **18** were isolated from *V. sisymbriifolia* as new compounds and named as sisymbriifolivaltrate (**17**) and sisymbriifolioside (**18**), respectively. Compound **17** possessed diene-type valtrate hydrine structure, and its derivatives esterified with different acyl groups were previously reported in the genus *Valeriana* (111,162,166,248). Also, the structure of **17** was very similar to that of isovaleroxyvaltrate hydrine (**7**), except for the presence of α -acetoxisovaleroxy group at 1th position in **17**, instead of isovaleroxy moiety in **7**. On the other hand, compound **18**, which is a diglycosidic ester iridoid, had the same skeleton as valerosidate (**8**) that was isolated from *V. alliariifolia*, having a second glucose and acetyl moiety. Although compound **12** was previously prepared by semi-synthesis, it is being reported from a natural source for the first time in this study and named 7-deisovaleroylvaltrate (246). Meanwhile, the lack of acyl group at 7th position is rarely observed in diene-type valepotriates, only 7-epi-deacetylisovaltrate has been reported upto date (167). Thus, 7-deisovaleroylvaltrate (**12**) constitutes the second deacetyl derivative of diene-valepotriates. Among the isolated iridoids, didrovaltrate (**1**), valtrate (**3**) and baldrinal (**9**) were yielded from both *V. alliariifolia* and *V. sisymbriifolia*. Among the isolated iridoids, valerosidate (**8**), 7-deisovaleroylvaltrate (**12**) and valeriotriate B (**13**) are being reported for the first time from *V. alliariifolia*, whereas it is the first report on the occurrence of 1- α -acevaltrate (**15**), 7-homovaltrate (**16**), sisymbriifolivaltrate (**17**), sisymbriifolioside (**18**), patriscabrol (**19**) and 11-ethoxyviburtinal (**20**) in *V. sisymbriifolia*.

Bornyl caffeate (**21**), the only monoterpene ester, is being reported from a *Valeriana* species for the second time. Also, it was previously isolated from *V. wallichii* (59).



R
22: H
23: (*E*)-Caffeoyl

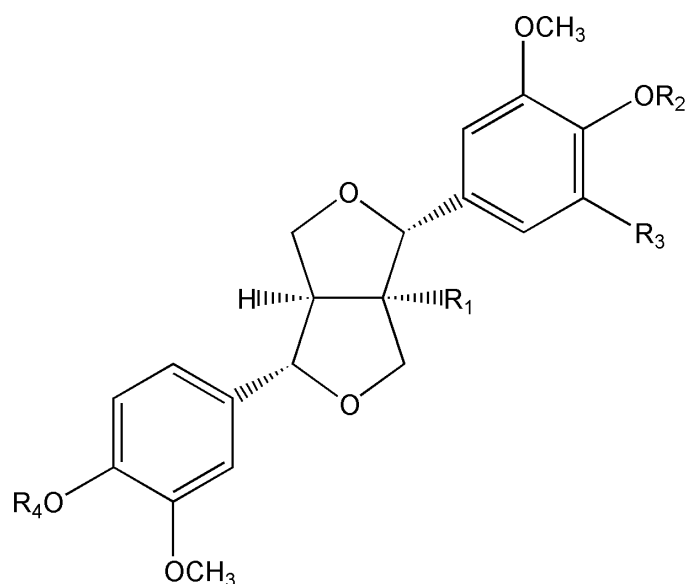
Two unreported sesquiterpene lactones, sisymbriifolins A (**22**) and B (**23**), were obtained from *V. sisymbriifolia*. Structurally, they can be classified under the drimane-type sesquiterpene lactone group (258). Despite the identification of approximately 80 sesquiterpenes from *Valeriana* species up to date, compounds with sesquiterpene lactone skeletons are reported for the second time from the genus *Valeriana*. The first drimane-type sesquiterpene lactone from the genus *Valeriana*, cinnamolide, has recently been reported from *V. jatamansi* (274).



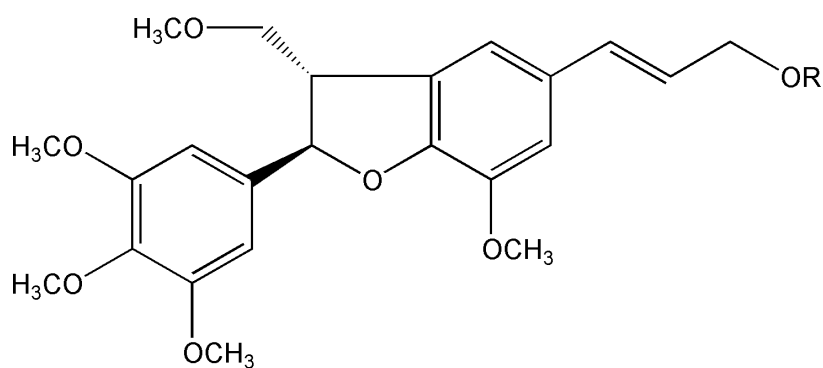
	R ₁	R ₂	R ₃
10	CHO	H	CH ₃
11	CH ₂ OH	β-Glc	CH ₃
26	COOH	H	H
34	CH ₂ OH	Rut	CH ₃

In this study four phenylpropane derivatives were obtained including coniferyl aldehyde (**10**), coniferin (**11**), caffeic acid (**26**) and xiecaoside E (**34**). It is well-established that coniferyl alcohol, one of the monolignol derivatives, participates in not only lignin but also lignan biosynthesis and moreover, it is generated from coniferyl aldehyde via the dehydrogenase enzymes (275). Considering that lignin is found in hard tissues like roots and rhizomes, it is not

surprising that coniferyl alcohol derivatives including coniferyl aldehyde (**10**), coniferin (**11**) and xiecaoside E (**34**) were encountered in the underground parts of three *Valeriana* species. Coniferin (**11**) was isolated from both *V. alliariifolia* and *V. speluncaria*. While coniferyl aldehyde (**10**) and xiecaoside E (**34**) were yielded from *V. alliariifolia*, caffeic acid (**26**) was purified from *V. speluncaria* in this study. All these isolated phenylpropanoids are being reported for the first time from these species.

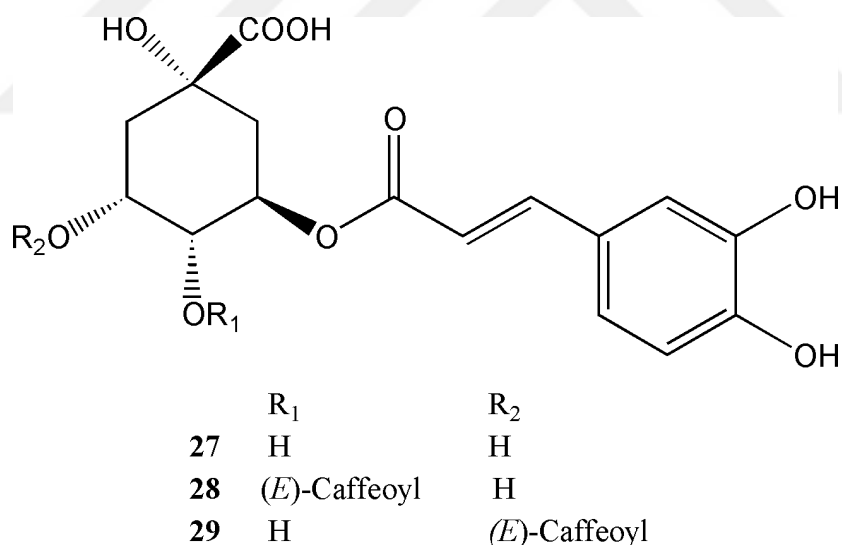


	R ₁	R ₂	R ₃	R ₄
14	OH	H	H	H
25	H	β-Glc	H	H
35	OH	H	H	β-Glc
36	H	β-Glc	OCH ₃	β-Glc
37	<i>O</i> -β-Glc	H	OCH ₃	H
38	H	β-Glc	H	β-Glc

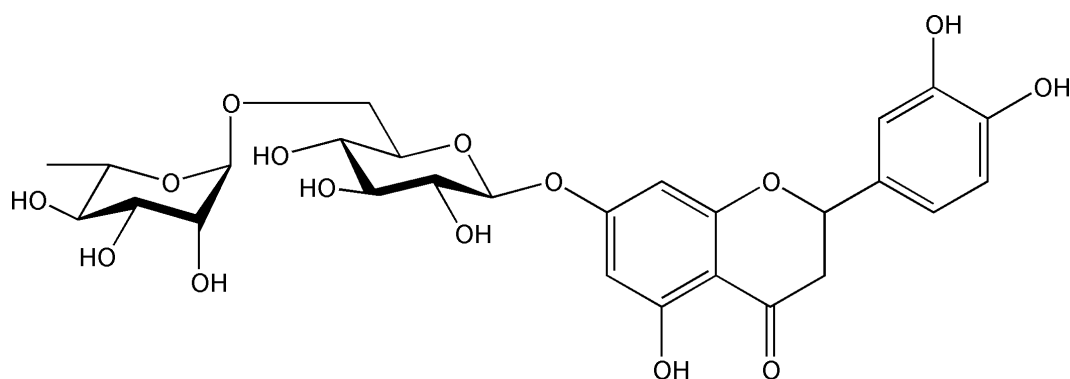


	R
30:	H
31:	CH ₃

According to the previous phytochemical studies on *Valeriana* species, more than 50 lignan derivatives have been identified till now (273). Six lignan derivatives were obtained in this study namely 8-hydroxypinoresinol (**14**), pinoresinol 4-O- β -D-glucopyranoside (**25**), 8-hydroxypinoresinol 4'-O- β -D-glucopyranoside (**35**), pinoresinol 4,4'-di-O- β -D-glucopyranoside (**36**), medioresinol di-O- β -D-glucopyranoside (**37**), and fraxiresinol 1-O- β -D-glucopyranoside (**38**) and two neolignan derivatives, speluncarins A (**30**) and B (**31**), were obtained. Among them, speluncarins A (**30**) and B (**31**) two previously undescribed neolignans, were isolated from *V. speluncaria*. All isolated lignans are being reported for the first time from *V. alliariifolia* and *V. speluncaria*, similar to those of phenylpropanes isolated in this study. Besides, 8-hydroxypinoresinol 4'-O- β -D-glucopyranoside (**35**), previously isolated from *V. wallichii*, *V. officinalis* and *V. prionophylla* was afforded from both *V. alliariifolia* and *V. speluncaria*, and also it was reported for the first time from both species (118,125,204). Furthermore, this is the first study on the isolation of fraxiresinol 1-O- β -D-glucopyranoside (**38**) from the genus *Valeriana*.



Caffeoylquinic acid esters namely, chlorogenic acid (**27**), 3,4-dicaffeoylquinic acid (**28**) and 3,5 dicaffeoylquinic acid (**29**) were obtained from only *V. speluncaria* among the studied species. Furthermore, these are reported for the first time from *V. speluncaria*. Meanwhile, it is the first report on the isolation of 3,4-dicaffeoylquinic acid (**28**) in the genus *Valeriana*, while chlorogenic acid (**27**) and 3,5 dicaffeoylquinic acid (**29**) were previously reported from *V. dioscorides* (12).



33

Eriocitrin (**33**), a flavanone derivative, was obtained from *n*-BuOH subextract of *V. alliariifolia*. According to previous phytochemical studies on *Valeriana* species, 40 flavonoids were identified, five of which belong to flavanone subclass (273). However, eriocitrin (**33**) is being reported for the first time from the genus *Valeriana* in our study.

The only phytosterol isolated within this study was daucosterol (**32**), also known as β -sitosterol 3-*O*- β -D-glucopyranoside. This compound was also obtained from *V. wallichii* in previous studies (191,206).

Considering the chemotaxonomic importance of the isolated compounds, eriocitrin (**33**) may serve as chemotaxonomic marker for *V. alliariifolia* within the genus due to being a new compound for the genus *Valeriana*. Similarly, sisymbriifolins A (**22**) and (**23**), which are new drimane-type sesquiterpene lactones, may be chemotaxonomic markers that could be used to chemically distinguish *V. sisymbriifolia* from other *Valeriana* species because of being reported for the first time in this species. On the other hand, according to the phylogenetic studies on members of some *Valeriana* and *Patrinia* species, both genera moved from their former family Valerianaceae to new family, Caprifoliaceae (276). In addition, “*Valeriana*-type” ester iridoids or derivatives such as homobaldrinal, isovaltrate, jatamanin A and 11-ethoxyviburtinal, as well as iridolactones, especially patriscabrol and patriscabroside I that were yielded from genus *Valeriana* were also isolated from *Patrinia* species (277). Thus, the occurrence of such iridoids within these genera alludes to the chemotaxonomic relationship between *Valeriana* and *Patrinia*. Therefore, our phytochemical results on *V. sisymbriifolia* supported this reclassification based on phylogenetic studies owing to the isolation of patriscabrol and 11-ethoxyviburtinal from the genus *Valeriana*.

According to the *in vitro* cytotoxic activity test results conducted on isolates using MTS assay (Tables 109 and 110), didrovaltrate (**1**), IVHD-valtrate (**2**), valtrate (**3**), 1- β -acevaltrate

(4), seneciovaltrate (5), valtrate hydrine B2 (6), isovaleroxyvaltrate hydrine (7), valerosidate (8), baldrinal (9) and 7-deisovaleroylvaltrate (12) from *V. alliariifolia*; didrovaltrate (1), valtrate (3), baldrinal (9), 1- α -acevaltrate (15), 7-homovaltrate (16), sisymbriifolivaltrate (17) from *V. sisymbriifolia* were found to be active compounds responsible for the cytotoxicity of relevant species having IC₅₀ values less than 40 μ M against at least one of the tested cancer cell line. None of the isolates from *V. speluncaria* exhibited significant cytotoxicity against the tested cancer cell lines, despite possessing cytotoxicity of the crude extract and main fractions from which the compounds purified (Tables 65 and 111). This issue can be speculated by the fact that the compound in the crude extract or main fractions interact synergistically with each other, producing a cytotoxic bioactivity. Similar results were also reported in some previous cytotoxic-guided isolation studies (268,269).

When the cytotoxic activities of the compounds are compared according to cancer cell lines, only valtrate (3) (IC₅₀: 7.5 μ M) demonstrated potent cytotoxic effect towards A549. Against MCF7 cells, valtrate (3) (IC₅₀: 2.5 μ M) was the strongest cytotoxic molecule followed by 1- β -acevaltrate (4) (IC₅₀: 2.6 μ M), valtrate hydrine B2 (6) (IC₅₀: 3.7 μ M), IVHD-valtrate (2) (IC₅₀: 3.8 μ M), didrovaltrate (1) (IC₅₀: 3.9 μ M), isovaleroxyvaltrate hydrine (7) (IC₅₀: 8.4 μ M). Similarly, valtrate (3) (IC₅₀: 2.3 μ M) displayed the best cytotoxicity on HGC27 cell line, compared to other potent cytotoxic compounds including IVHD-valtrate (2) (IC₅₀: 10.2 μ M), seneciovaltrate (5) (IC₅₀: 9.2 μ M), valtrate hydrine B2 (6) (IC₅₀: 11.9 μ M), isovaleroxyvaltrate hydrine (7) (IC₅₀: 8.4 μ M), 1- α -acevaltrate (15) (IC₅₀: 12.6 μ M) and 7-homovaltrate (16) (IC₅₀: 3.7 μ M). Among the tested compounds, valtrate (3) (IC₅₀: 9.7 μ M), 1- β -acevaltrate (4) (IC₅₀: 9.9 μ M), valtrate hydrine B2 (6) (IC₅₀: 3.7 μ M), 1- α -acevaltrate (15) (IC₅₀: 6.4 μ M) and 7-homovaltrate (16) (IC₅₀: 2.3 μ M) were found to be cytotoxically most potent isolates against PC3 cancer cell line. It is clear that valtrate (3) showed the best cytotoxicity against all tested cancer cell lines with IC₅₀ values in the range of 2.3 to 9.7 μ M, being MCF7 the most sensitive cell line.

In the light of MTS assay findings, iridoids (1-9, 12 and 15-17) exhibited pronounced cytotoxic activity against the tested cancer cell lines with IC₅₀ values lower than 40 μ M. These active compounds except for valerosidate (8), were non-glycosidic iridoids which bear isovaleroyl derivatives or acetyl group. The detailed structure-activity relationships (SAR) of the isolated iridoids are discussed below.

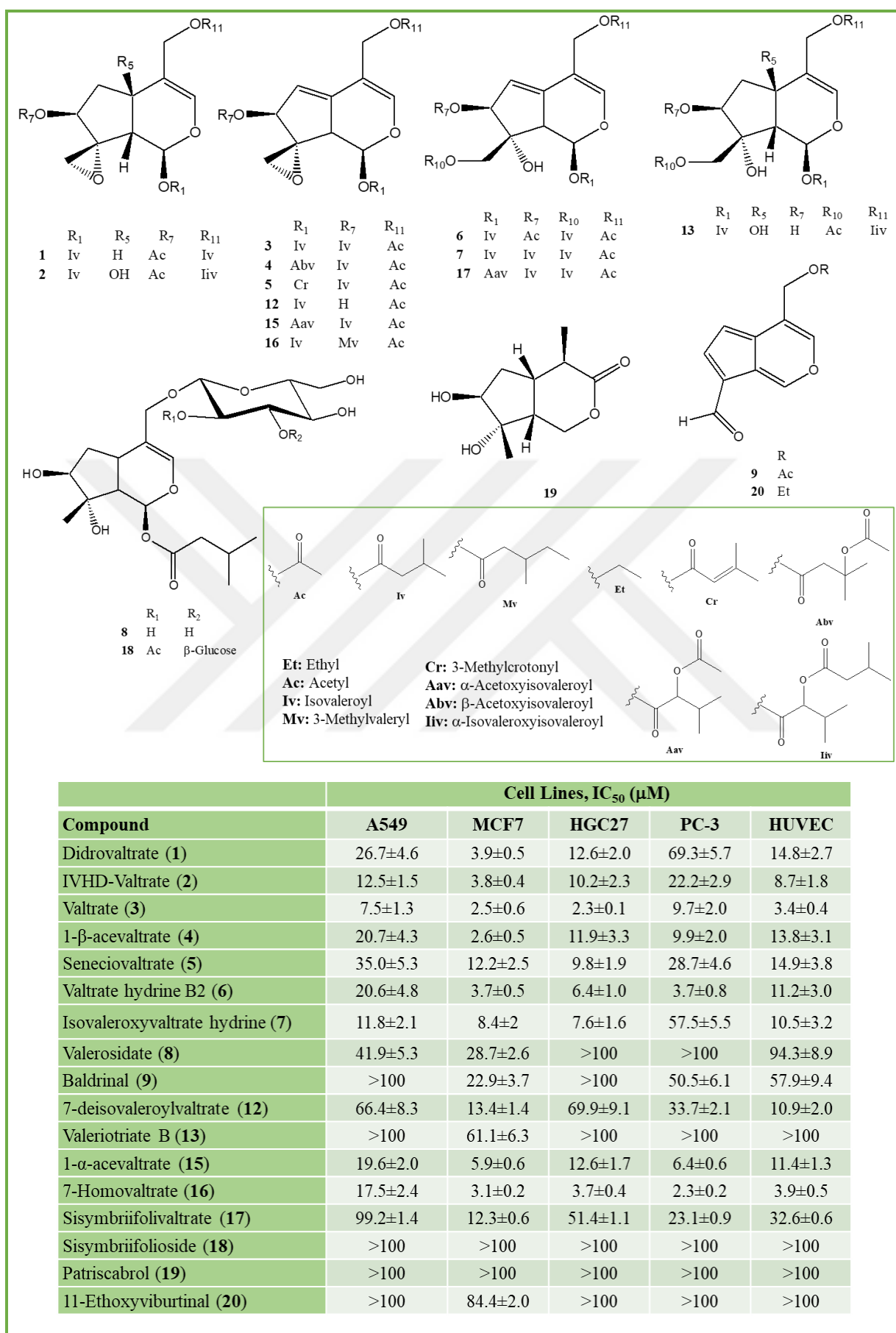


Figure 37. Chemical structures of the iridoids and their IC₅₀ values in MTS assay.

It was also reported that among the compounds obtained from *Valeriana* species up to now, particularly iridoids were found to be the most active metabolites in *in vitro* studies (17,22–24). In terms of SAR, specifically non-glycosidic ester iridoids exhibited the best activity, while iridoid glycosides from *Valeriana* species demonstrated weak to moderate cytotoxic activities in different cancer cells (12,200). In our study, it was demonstrated that compounds showing cytotoxic effects towards at least one of the tested cancerous cells possessed iridoid skeleton (**1-9**, **12** and **15-17**). Among these compounds, only valerosidate (**8**) contained sugar moiety and it exhibited cytotoxic activity against only MCF7 cell line moderately (IC₅₀: 28.7 μ M). However, sisymbriifolioside (**18**), a diglycosidic analogue of valerosidate (**8**), was found to be inactive on all tested cancer cells (IC₅₀: >100 μ M). This reduction in cytotoxicity on MCF7 cells demonstrated that number of sugar molecules is also important factor for cytotoxicity of valerosidate nucleus bearing compounds in MCF7 cancer cell line. Conversely, compounds that were determined to have potent cytotoxic effects belong to monoene (**1** and **2**) and diene (**3-5**, **12**, **15**, **16**) type valepotriates as well as valtrate hydrines (**6**, **7** and **17**). Such compounds were also reported to be the ones that were especially responsible for the *in vitro* cytotoxic bioactivities of *Valeriana* species (17,22,107,111). It is worth to mention that, the occurrence of one (C₃-C₄) or more double bonds (C₃-C₄ and (C₅-C₆) in their cyclopentanopyran rings significantly enhanced their cytotoxic effect which was also consistent with results of this study (22,111). Moreover, valepotriates and valtrate hydrines are similar in terms of esterification with different acyl groups, whereas they differ from each other containing oxirane group or not (40). Generally, these acyl groups are substituted at C-1, -7 and -11 positions in valepotriates, whereas they are attached at C-1, -7, -10 and -11 in valtrate hydrines. In addition, studies have shown that the cytotoxicity may vary depending on the binding position and structural diversity of the acyl moieties in the molecules (111,166). This interpretation was supported by the results of this thesis. It was demonstrated that valtrate (**3**) (IC₅₀: 2.3-9.7 μ M) containing isovaleroyl group at C-1, was found to be most cytotoxic compound against the tested cancer cell lines. However, 1- β -acevaltrate (**4**) (IC₅₀: 2.6-20.7 μ M), seneciovaltrate (**5**) (IC₅₀: 9.8-35.0 μ M), 1- α -acevaltrate (**15**) (IC₅₀: 5.9-19.6 μ M) which bears different acyl groups at C-1, showed broadly less cytotoxic effects than valtrate (**3**). Accordingly, isovaleroxyvaltrate hydrine (**7**) (IC₅₀: 7.6-57.5 μ M) displayed stronger cytotoxic activities than its analogue namely sisymbriifolivaltrate (**17**) (IC₅₀: 12.3-99.2 μ M) which contains α -acetoxysovaleroyl moiety at C-1 instead of isovaleroyl group in compound **7**. According to these findings, it can be deduced that the presence of isovaleroyl group in the 1st position in valepotriate and valtrate hydrines is the key factor, the best cytotoxicity of such

iridoids. Conversely, when the esterification was not observed at any of the stated positions in valepotriates and valtrate hydrines, the activity was found to be severely reduced in this study. Based on the MTS assay results (Table 109, Figure 37), valtrate (**3**) (IC_{50} : 2.3-9.7 μ M) bearing isovaleroyl group at C-7 position, were found to be more cytotoxic than 7-deisovaleroylvaltrate (**12**) (IC_{50} : 13.4-69.9 μ M) that lacks acyl group at the same position. Similarly, valeriotriate B (**13**) that does not contain any acyl group at C-7 position exhibited weak cytotoxic effects (IC_{50} : 61.1- >100 μ M) when compared with the other valtrate hydrines derivatives (**6**, **7** and **17**) (IC_{50} : 3.7- 99.2 μ M) that bear acyl groups at same positions. Bounthanh et al. stated that the presence of epoxide function between 8th and 10th position is not crucial for the cytotoxic effects (278). Therefore, our results are correlated with these findings due to strong cytotoxicity of valepotriate (**1-6**) and valtrate hydrine (**6**, **7** and **17**) structures. However, valeriotriate B (**13**) (IC_{50} : 61.1- >100 μ M), a monoene type valtrate hydrine, exhibited weaker cytotoxic activity against tested cell lines, compared to IVHD-valtrate (**2**) (IC_{50} : 3.8-22.2 μ M). Moreover, it was structurally similar to compound **2**, differing by the absence of acetyl group at 7th position as well as absence of an epoxide group. Considering that the presence of epoxide function is not necessary for cytotoxicity, the reason for the higher activity of IVHD-valtrate (**2**) than valeriotriate B (**7**) can be explained by the presence of the acetyl group at C-7. According to aforementioned findings, the presence of at least one double bond in the iridoid skeleton, the absence of sugar moiety as well as the lacking any free OH group on the cyclopentanopyran ring are key factors that increase the cytotoxic activity of iridoids. It can be concluded that iridoids lacking sugar moiety, carrying at least one double bond in their basic skeleton, and esterified with at least three aliphatic acyl groups (three for valepotriates, four for valtrate hydrines) showed the potent *in vitro* cytotoxic effects. On the other hand, it has been reported in many studies that cytotoxically active valepotriates are degraded to baldrinal derivatives under certain conditions, resulting in a significant reduction in their cytotoxic effects (22,28). Likewise, baldrinal (**9**) (IC_{50} : 22.9- >100 μ M) and 11-ethoxyviburtinal (**20**) (IC_{50} : 84.4- >100 μ M), obtained in our study, demonstrated a weak cytotoxicity comparing to their parent molecules valepotriates.

Within the scope of this study, the cytotoxicity of compounds **1-24** were evaluated against A549, MCF7, HGC27 and PC3 cancerous cells whereas compounds **11**, **25-32** were tested for their cytotoxic effects on U87 and MCF7 cancer cell lines. Comparing with the previous cytotoxicity studies, the *in vitro* cytotoxic activities of seneciovaltrate (**7**), isovaleroxyvaltrate hydrine (**7**), valerosidate (**8**), baldrinal (**9**), 7-deisovaleroylvaltrate (**12**), 1-

α -acevaltrate (**15**), 7-homovaltrate (**16**), patriscabrol (**19**), 11-ethoxyviburtinal (**20**), caproleic acid (**24**) were investigated for the first time against A549, MCF7, HGC27 and PC3 cell lines, apart from new compounds (compounds **17**, **18**, **22**, **23**, **30** and **31**). Amongst, compounds **7**, **8**, **15**, **16**, **20** and **24** were investigated for the first time in terms of their cytotoxic potential. On the other hand, the cytotoxic effect of pinoresinol 4-O- β -D-glucopyranoside (**25**) on U87 and MCF7 as well as of 3,4-dicaffeoylquinic acid (**28**) and 3,5-dicaffeoylquinic acid (**29**) against U87 were studied for the very first time. On the other hand, HGC27 cell line was used for the first time to investigate the cytotoxic bioactivities of all tested compounds (compounds **1-24**). Taken together, this study was the first report showing cytotoxic activities of didrovaltrate (**1**), IVHD-valtrate (**2**), 1- β -acevaltrate (**4**), seneciovaltrate (**5**), valtrate hydrin B2 (**6**) and valeriotriate B (**13**) against PC3 and MCF7 cells as well as only 8-hydroxypinoresinol (**14**) and bornyl caffeate (**21**) against PC3 cells. Further, the cytotoxic potential of valtrate (**3**) against PC3 cell line was tested for the first time.

Lin et al. investigated the cytotoxic activities of 22 different acylated iridoids including valeriotriate B, IVHD-valtrate, didrovaltrate, valtrate and 1- β -acevaltrate isolated from *V. jatamansi* against lung (A549) and metastatic prostate (PC-3M) cancer cell lines using MTT assay. The results of this study demonstrated that 1- β -acevaltrate (IC₅₀: 2.9 μ M), valtrate (IC₅₀: 3.7 μ M) and IVHD-valtrate (IC₅₀: 7.4 μ M) exhibited potent cytotoxicity against lung (A549) cancer cells, whereas valeriotriate B and didrovaltrate were inactive. In addition, 1- β -acevaltrate, valtrate, IVHD-valtrate, didrovaltrate and valeriotriate B displayed pronounced cytotoxic effects against PC-3M cells with IC₅₀ values of 1.4, 1.6, 1.9, 3.8 and 3.9 μ M, respectively (166). According to the MTS assay results in this thesis, only valtrate (**3**) was found to be the potent cytotoxic compound against A549 cells with IC₅₀ value of 7.5 μ M. Although, didrovaltrate (**1**) (IC₅₀: 26.7 μ M), IVHD-valtrate (**2**) (IC₅₀: 12.5 μ M) and 1- β -acevaltrate (**4**) (IC₅₀: 20.7 μ M) showed relatively weaker cytotoxicity against A549 cells than valtrate (**3**), while valeriotriate B (**13**) was inactive (IC₅₀: >100 μ M). On the other hand, valtrate (**3**) and 1- β -acevaltrate (**4**) also exhibited remarkable bioactivities on PC3 cell lines with IC₅₀ values of 9.7 and 9.9 μ M, respectively. However, didrovaltrate (**1**) (IC₅₀: 69.3 μ M), and IVHD-valtrate (**2**) (IC₅₀: 22.2 μ M) indicated less cytotoxicity against the same cancer cell line. Valeriotriate B (**13**) (IC₅₀: >100 μ M) did not yield any IC₅₀ values within the tested concentration range against PC3 cells, similar to its result on A549. When our results and the findings of Lin et al. (2009) were compared, not only the *in vitro* cytotoxicity methods but also the incubation period of the treated cells were different. This situation may be the reason why the cytotoxicity of

didrovaltrate (**1**), IVHD-valtrate (**2**), valtrate (**3**) and 1- β -acevaltrate (**4**) against A549 cells were found to be less than the results of the Lin and colleagues. Valeriotriate B (**13**) was found inactive against A549 cells based on the findings of both the present study and that of Lin's. On the contrary, it is well documented that PC-3M (metastatic prostate cancer) cells are more aggressive and metastatic than PC3 cells (279). In addition to the aforementioned differences in the study conducted by Lin et al., the variations in cytotoxic activities of mentioned iridoids against both prostate cancer cells (PC3 and PC-3M) could also explain by this circumstance.

In a study conducted by Tian and colleagues, valtrate was investigated in terms of its cytotoxic effects against a panel of cancer cell lines, namely SK-OV-3, PANC1, LNCaP, A2780, MCF7, MDA-MB-231, MDA-MB-453, MDA-MB-468 cells as well as a healthy breast epithelial cells (MCF10-A) by MTT method. Among these cancer cell lines, MCF7 was the most sensitive to valtrate with IC₅₀ value of 9.27 μ M (107). Within this study, valtrate (**3**) (IC₅₀: 2.5 μ M) showed potent cytotoxic activity against the same cell line based on the MTS assay findings that is compatible with the stated study. More specifically, valtrate (**3**) was found to be approximately four times more cytotoxic against MCF7 cells than the results of a study conducted by Tian and co-workers (2018). This circumstance might be due to the different incubation periods. The selectivity of the tested compounds was evaluated by comparing the results on cancer cells with those on HUVEC. Some of the potent cytotoxic compounds (**1-7** and **15-17**) were also found to be cytotoxic against HUVEC (IC₅₀: 3.4-32.6 μ M). Among all compounds tested, **1** (IC₅₀: 3.9 μ M) and **17** (IC₅₀: 12.3 μ M) have already shown stronger cytotoxicity on MCF7 cells compared to HUVECs (14.8 and 32.6 μ M); similarly, **15** had higher cytotoxicity on MCF7 and PC3 cell lines (IC₅₀: 5.9 and 6.4 μ M, respectively) in comparison with HUVECs (11.4 μ M), indicating slight selectivity. It was previously reported that even if healthy cell lines are used as control groups to define the specificity of a compound, these healthy cells are either murine cell lines (280,281) which do not reflect human cells, or human fibroblasts (282), which are also present in tumors. Herein, HUVECs are used to represent a healthy cell line, but ideally, healthy cells of the respective cancer cells should be included in cytotoxicity evaluations to state the exact specificity. Accordingly, the inclusion of healthy cells of respective cancer cell lines instead of HUVECs may give more accurate information about the selectivity of compounds. For instance, the selectivity of some of the aforementioned compounds was reported in the literature. Tian et al. used MCF10-A (healthy human breast epithelial cells) cells to test the selectivity of valtrate against four breast cancer cells (three MDA-MB series and MCF7). The authors indicated that valtrate was a relatively selective

cytotoxic compound against the tested breast cancer cells (IC_{50} : 30.55 μ M for MCF10-A; IC_{50} : 7.81-9.21 μ M for breast cancer cells) (107). In another study, Chen and co-workers demonstrated the selectivity of valtrate against a panel of pancreatic cancer cells (IC_{50} : 1.98 - 12.13 μ M), compared to healthy pancreas epithelial cells, namely HDPE (IC_{50} : >200 μ M) (26).

Another aim of this thesis was to elucidate the mechanisms of cancer cell deaths induced by most active compounds possessing IC_{50} values close to or lower than 10 μ M on relevant cancer cell lines. Thus, IVHD-valtrate (**2**), valtrate (**3**), 1- β -acevaltrate (**4**), senecioivaltrate (**5**), valtrate hydrine B2 (**6**), 1- α -acevaltrate (**15**), 7-homovaltrate (**16**) and sisymbriifolivaltrate (**17**) were selected for further bioassays. The below Figure shows the investigated compounds and the relevant cell lines studied (Figure 38).

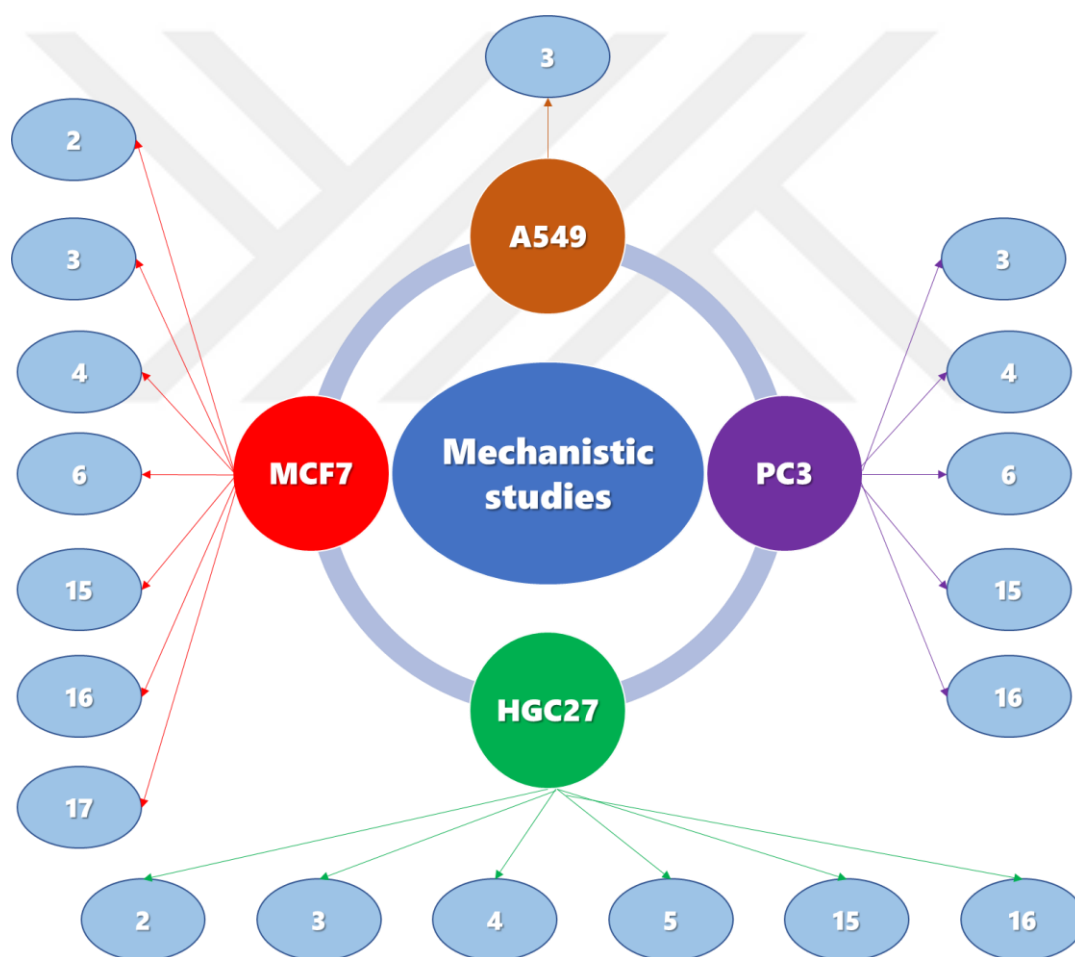


Figure 38. The investigated compounds and the relevant cell lines used in mechanistic studies.

Natural products have gained importance in cancer treatment due to their relatively safe potential and activation of multiple cell death pathways in tumor cells (10). Furthermore, recent studies conducted on the mechanistic pathways of different cancers revealed that programmed cell deaths are important target in the treatment of cancer by activating the cell death mechanism

to kill cancerous cells (6). In this thesis, the most potent compounds (**2-6**, **15-17**) as mentioned above were chosen for further analysis in order to unravel their cell death mechanisms including apoptosis, necrosis and autophagy by flow cytometry.

The effects of compounds on viability, apoptosis and necrosis were investigated with Annexin V/PI staining, that is known as the gold standard for the determination of apoptosis (283). Mechanistically, Annexin V attaches to phosphatidylserine (PS) parts, which are mostly found inner leaflet of the cell membrane. PS residues migrate to the outer leaflet of the cell membrane upon activation of apoptosis, allowing Annexin V to connect and mark early apoptotic cells. Similarly, Annexin V can also penetrate cells with damaged membranes and attaches PS found in the inner leaflet, marking the cells while cells are in the last apoptotic stages. Conversely, PI, a cell-impermeable intercalating agent, is used to differentiate apoptotic cells from necrotic and living cells (284). In addition to the evaluation of necrosis with Annexin V/PI staining, p-MLKL (phosphorylated mixed lineage kinase like)/MLKL (mixed lineage kinase like) ratios of the tested compounds were also utilized for assessment of necrosis, which is obtained by dividing the p-MLKL levels by the MLKL levels. Necrosis is activated by the interaction of Receptor Interacting Serine/Threonine Kinase 3 (RIPK3) and MLKL. Briefly, the phosphorylation of MLKL is accomplished by RIPK3 and then, this situation leads to activation of necrosis by some factors such as the genesis of oligomers, inducing plasma membrane migration, disrupting membrane integrity by binding to phosphatidylinositol (285). According to results obtained from flow cytometry, all tested compounds reduced viability of all cancer cell lines used in the study. Furthermore, Annexin V/PI analysis along with increased p-MLKL/MLKL protein ratios demonstrated that all tested compounds also promoted both apoptotic and necrotic pathways on related cells, except for IVHD-valtrate (**2**) in MCF7 cell line. Further, **2** led to an increase in the level of necrosis in MCF7 cells without increasing p-MLKL/MLKL protein ratios. This result indicated that compound **2** may activate the other cell death mechanism such as pyroptosis due to increasing numbers of Annexin V/PI⁺ staining cells. Compactly, pyroptosis, inflammation-based cell death mechanism, is triggered by caspases particularly caspase-1, -4, -5 and -11 and then, different inflammasomes are released to initiate to cell lysis (286). However, it is hard to make an interpretation about the exact effects of **2** on pyroptosis, because these aforementioned caspases were not included in the mechanistic studies carried out within the framework of this thesis. Thus, further protein analyses especially caspases need be performed in future studies to elucidate the possible mechanism of **2** on pyroptosis in MCF7 cells.

Cell cycle consists of four phases including G₁, S, G₂ and M. During the cell cycle, cycle or regulatory systems cause some mistakes that led to uncontrolled cell proliferation, and this uncontrolled cell proliferation, a hallmark of cancer, induced the formation of cancers (287). Thus, cell cycle phases have become an important target for cancer chemotherapeutic agents. The effects of potent compounds (**2-6**, **15-17**) on cell cycle were analyzed with flow cytometry. The results showed that all of the tested compounds except for IVHD-valtrate (**2**) caused the accumulation of cells in SubG₁ phase significantly ($p < 0.05$) in A549, MCF7 and PC3 cancer cells. In HGC27 cells, seneciovaltrate (**5**), 1- α -acevaltrate (**15**) and 7-homovaltrate (**16**) increased the number of cells at SubG₁ phase. Also, increases in cell population at the G₂/M phase of tested cell lines, was observed in A549 cells treated with **3**; MCF7 cells treated separately with **15**, **16** and **17**; HGC27 cells separately treated with **2** and **15**; PC3 cells treated with **15**. Besides, **3** and **6** induced the cell cycle arrest at G₀/G₁ in MCF7 cells, whereas **3** and **5** led to G₀/G₁ accumulation in HGC27 cells. Conversely, none of the tested isolates significantly caused the S phase accumulation in tested cancer cell lines. The impacts of tested compounds on the cell cycle phases were summarized in the Table 113.

Table 113. Effects of tested compounds on the cell cycle phases.

	SubG₁	G₀/G₁	S	G₂/M
A549	3 ↑	-	-	3 ↑
MCF7	3, 4, 6, 15, 16 and 17 ↑	3 and 6 ↑	-	15, 16 and 17 ↑
HGC27	5, 15 and 16 ↑	3 and 5 ↑	-	2 and 15 ↑
PC3	3, 4, 6, 15 and 16 ↑	-	-	15 ↑

In order to deeply investigate the effects of compounds on cell cycle progression, cell cycle proteins including p21, p53, cyclin D1, CDK4 and CDK6 were also investigated through flow cytometry. Cyclins and cyclin-dependent kinases (CDKs) play an important role in the initiation of phases and transitions between them by strictly controlling the cell cycle progression. Also, CDKs particularly CDK4 and CDK6 are activated by Cyclin D series (Cyclin D1, D2 and D3) to direct cells from G₁ to S phase in cell cycle (288). On the other hand, the p53 gene, DNA-binding phosphoproteins and known as “gatekeeper of the cell”, is a kind of tumor suppressor gene that plays a critical role in preventing uncontrolled cell proliferation.

Also, it is well-known that p53 gene activated and regulated the expression of p21 gene (289). P21, a CDK inhibitor gene, causes cell cycle arrest in the progression of cells at G₁/S and G₂/M transitions by interrupting cyclin D-CDK4/CDK6 complexes as well as CDK2/cyclin E complex, respectively (290). According to the results, all tested compounds increased the expression of p21 genes in the tested cancer cell lines. Except for the valtrate hydrine B2 (**6**) tested against PC3 cells, the p53 levels were increased with individual treatment of all tested cell lines. The increase in the p21 and p53 protein levels proved the cell cycle-arresting effect of the tested compounds. Besides, compounds **2**, **6**, **15-17** in MCF7 cells, compounds **2-5**, **15** in HGC27 cells, and **6** in PC3 led to a decrease in the levels of CDK4, CDK6 and Cyclin D1. Thus, this finding indicated that mentioned compounds caused cell accumulation at the G₁ phase. Also, effects of the compounds on cell cycle proteins were represented in Figure 39.

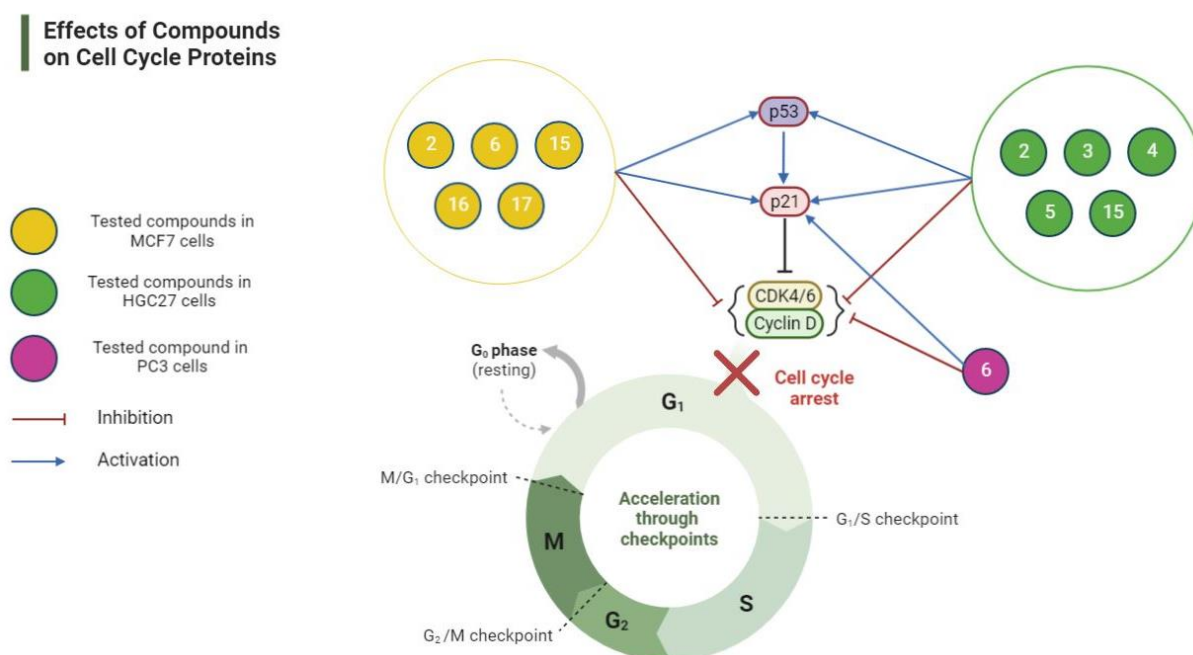


Figure 39. Effects of compounds (**2-6**, **15-17**) on cell cycle proteins

Autophagy has been likened to a double-edged sword in tumorigenesis due to its role in tumor suppression and cancer promotion. Many genes are involved in the initiation and regulation of this process (231). Amongst, beclin-1 and LC3B proteins are the well-known biomarkers of autophagy. The levels of both proteins are increased in case of induction of apoptosis (291). In this study, the expression levels of beclin-1 and LC3B were evaluated by

flow cytometry. Experimental results indicated that valtrate (**3**), 1- α -acevaltrate (**15**) and 7-homovaltrate (**16**) increased the expression of beclin-1 and LC3B levels in all tested cancer cell lines. Thus, it can be interpreted that these compounds induced the autophagy-related cell death mechanism to kill the cancer cells. Similarly, 1- β -acevaltrate (**4**) also caused an elevation of Beclin-1 and LC3B levels in all tested cell lines, except for HGC27. On the other hand, several studies have shown that increased p53 expression may lead to the initiation of the autophagic pathway (292). When these results were evaluated in conjunction with p53 levels, it is obvious that compounds **3**, **4**, **15** and **16** induced autophagy.

Among the tested compounds (**2-6** and **15-17**) for mechanistic studies, the mechanisms behind the cytotoxicity of all these mentioned compounds except for IVHD-valtrate (**2**) and valtrate (**3**) were evaluated for the first time within the scope of this thesis. Tian et al. investigated the cytotoxic potential and the underlying mechanism of valtrate against breast cancer cell lines (MDA-MB cells and MCF7). According to their findings, MCF7 cells treated with valtrate (5 μ M) led to cell cycle arrest at G₀/G₁, while treatment with 10 μ M concentration of valtrate caused G₂/M cell cycle arrest. Also, valtrate increased the expression of p21 levels in MCF7 cells without no change in cyclin B1 levels. The authors concluded that cell cycle arrest was not the main underlying mechanism for the cytotoxicity of valtrate on MCF7 cells (107). Our results were consistent with the findings of Tian et al. on the induction of G₀/G₁ cycle arrest treatment upon with valtrate (**3**) (2.5 μ M) in MCF7 cells. However, **3** led to the upregulation of p21, and downregulation of CDK4, CDK6 and Cyclin D1 levels in MCF7 cells. Through the activities of valtrate (**3**) on these cell cycle proteins, it can be interpreted that cell cycle arrest played a crucial role for inhibition of MCF7 cell proliferation treated with **3**. In addition, the inhibitory effects of valtrate and IVHD-valtrate on cell cycle at G₂/M stage have also been reported in previous mechanistic studies using ovarian and pancreatic cancer cell lines, respectively (26,27). It seems not appropriate to compare our results with theirs, because the selected cancer cell lines and tested concentrations are quite different from each other. However, cell cycle arrests at G₂/M stage were observed in the IVHD-valtrate (**2**) treated HGC27 cells and valtrate (**3**) treated A549 cells in this study.

To conclude, totally 17 secondary metabolites were obtained from *V. alliariifolia*, 14 (**1-14**) of which were from active subextracts (CHCl₃ and EtOAc). Among the isolated compounds from active subextracts, didrovaltrate (**1**), IVHD-valtrate (**2**), valtrate (**3**), 1- β -acevaltrate (**4**), seneciovaltrate (**5**), valtrate hydrine B2 (**6**) and isovaleroxyvaltrate hydrine (**7**) were assessed to be responsible compounds for the *in vitro* cytotoxic activities of *V. alliariifolia*

against A549, MCF7, HGC27 and PC3 cancerous cell lines. Moreover, cell death mechanisms underlying the cytotoxicity of the most potent isolates against respective cell lines were elucidated with Annexin V/PI staining, DNA content analysis, and protein levels analysis by using flow cytometry. Thus, compounds **2-6** caused cell death by inducing apoptosis, necrosis and autophagy showing some variation depending on the tested cancer cell lines. Activation of the apoptotic pathway was further confirmed with the increased SubG₁ phase in DNA content analysis. The accumulation of cells at the SubG₁ phase in DNA content analysis confirmed the induction of the apoptotic mechanism. In addition, these potent compounds also caused the cell cycle arrest at different phases through activating upregulation of p21 and p53 genes and downregulation of CDK4, CDK6 and Cyclin D1 proteins. Our findings on *V. alliariifolia* have recently been published (271).

Cytotoxicity-guided fractionation studies on the underground parts of *V. sisymbriifolia* led to the purification of 13 compounds (**1, 3, 9, 15-24**) including four new ones, named sisymbriifolivaltrate (**17**), sisymbriifolioside (**18**), sisymbriifolins A (**22**) and B (**23**). Among the isolated compounds, didrovaltrate (**1**), valtrate (**3**), 1- α -acevaltrate (**15**) and 7-homovaltrate (**16**) and sisymbriifolivatrate (**17**) could be attributed as accountable compounds for the potential cytotoxic effects of *V. sisymbriifolia* on A549, MCF7, HGC27 and PC3 cells. Compounds **3** and **15-17** were selected for further analysis to understand their possible cell death mechanisms, including apoptosis, necrosis, autophagy, and cell cycle assays by using flow cytometry. 1- α -acevaltrate (**15**), 7-homovaltrate (**16**) and sisymbriifolivatrate (**17**) promoted both apoptotic and necrotic pathways in the tested cell lines based on the Annexin V/PI analysis along with the increased p-MLKL/MLKL ratio. When evaluating DNA content analysis together with levels of cell cycle proteins, compounds **15-17** were found to arrest the cell cycle at different phases including subG₁, G₀/G₁ and G₂/M. In addition, **15** and **16** also activated autophagy via increasing the expression of both autophagy proteins namely beclin-1 and active LC3B. Taken together, valtrate (**3**), 1- α -acevaltrate (**15**) and 7-homovaltrate (**16**) and sisymbriifolivatrate (**17**) were determined to possess significant cytotoxic effects against respective cell lines by activating multiple cell death mechanisms.

The *in vitro* cytotoxic activity-guided isolation studies were also performed on *V. speluncaria*, an endemic species to flora of Türkiye. In consequence of successive chromatographic separations on active subextracts (CHCl₃ and EtOAc), two new compounds, named speluncarins A (**30**) and B (**31**) together with seven known compounds (**11, 25-29** and **32**) were isolated and also, their chemical structures were elucidated. The cytotoxic effects of

compounds **11**, **25-32** were tested against U87 and MCF7 cancer cells. However, such activities observed in main fractions and extracts, were not observed in any of the tested compounds at the tested concentrations. This situation can be explained by the fact that the compounds in the extracts and fractions interact synergistically with each other and create a cytotoxic effect. Besides the activity-guided study, five lignans (**25**, **35-38**) were isolated from *n*-BuOH subextract of this species. Our findings on *V. speluncaria* have recently been published (272).

Consequently, compounds **2-6** and **15-17** led to the activation of multiple-cell death mechanisms, including apoptosis, necrosis, autophagy as well as cell cycle arrest. Thus, these compounds could be considered as potential anticancer drug leads that entitle further *in vitro* and *in vivo* studies on the way to discovering new natural anticancer drug candidates. When our findings are evaluated together with the findings from previous cytotoxic and anticancer studies on different *Valeriana* species, it can be concluded that other members of the *Valeriana* genus growing in the flora of Türkiye deserve further investigation in terms of their potential bioactive secondary metabolites.

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7. CURRICULUM VITAE

Personal Informations

Name	Murat	Surname	Erdoğan
Place of Birth	-	Date of Birth	-
Nationality	TR	TR ID Number	-
E-mail	-	Phone number	-

Education

Degree	Department	The name of the Institution Graduated From	Graduation year
Ph.D.	Pharmacognosy	Yeditepe University	2022
B.A.	Faculty of Pharmacy	Yeditepe University	2018
High school	-	Kemer Anatolian High School	2011

Language Skill

Language	Exam Degree
English	YÖKDİL (17/03/2018): 85.00

Work Experience

Position	Institution	Dates
Research and Teaching Asistant	Yeditepe University, Faculty of Pharmacy, Department of Pharmacognosy	2018-

Computer Skills

Program	Level
Microsoft Office	Very good
MestReNova	Very good
Jasc Paintshop Pro	Very good
Mendeley	Very good
GraphPad Prism	Very good
Adobe Photoshop	Good

Scientific works

The articles published in the journals indexed by SCI, SSCI, AHCI

Kırmızıbekmez, H., Erdoğan, M., Kúsz, N., Karaca, N., Erdem, U., Demirci, F., Hohmann, J. (2021). Secondary metabolites from the aerial parts of <i>Sideritis germanicopolitana</i> and their <i>in vitro</i> enzyme inhibitory activities. <i>Natural Product Research</i> , 35(4), 655-658.
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Erdoğan, M., Aru, B., Öztürk, C., Özdemir, O. E., Yeşilada, E., Yanıkkaya-Demirel, G., & Kırmızıbekmez, H. (2022). <i>In vitro</i> cytotoxic activity-guided isolation studies on the underground parts of <i>Valeriana speluncaria</i> Boiss. <i>South African Journal of Botany</i> , 149, 315-319.
Erdoğan, M., Aru, B., Taygun, U. C., Şimşek, C., Yeşilada, E., Yanıkkaya-Demirel, G., Kırmızıbekmez, H. (2022). Activity-Guided Isolation of Cytotoxic Non-Glycosidic Ester Iridoids from <i>Valeriana alliariifolia</i> Adams and Unravelling Their Cell Death Mechanisms. <i>Chemistry & Biodiversity</i> , 19(10), e202200659.

International Conference Proceedings

Kırmızıbekmez, H., <u>Erdoğan, M.</u> , Kúsz, N., Karaca, N., Erdem, U., Demirci, F., & Hohmann, J. Secondary Metabolites from of <i>Sideritis germanicopolitana</i> . 30 th International Symposium on the Chemistry of Natural Products and the 10 th International Congress on Biodiversity. 2018, November 25-29. Athens, Greece (Poster Presentation)
<u>Erdoğan, M.</u> , Aru, B., Taygun, U. C., Şimşek, C., Yeşilada, E., Yanıkkaya-Demirel, G., Kırmızıbekmez, H. Bioactivity-Guided Isolation of Cytotoxic Compounds from the Underground Parts of <i>Valeriana alliariifolia</i> Adams. 13 th International Symposium on Pharmaceutical Sciences. 2021, June 22-25. Ankara, Türkiye. (Oral Presentation)
<u>Erdoğan, M.</u> , Aru, B., Yılmaz, Ş. T., Yeşilada, E., Yanıkkaya-Demirel, G., Kırmızıbekmez, H. Isolation of Cytotoxic Compounds from the Underground Parts of <i>Valeriana sisymbriifolia</i> Vahl. Through Activity-Guided Fractionation. GA- 69 th Annual Meeting 2021, September 5-8. Bonn, Germany. (Oral Presentation)

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Erdoğan, M., Aru, B., Taygun, U. C., Şimşek, C., Yeşilada, E., Yanikkaya-Demirel, G., Kırmızıbekmez, H. *Valeriana alliariifolia*'dan Elde Edilen Non-Glikozidik Ester İridoitlerin *İn Vitro* Sitotoksik Etki Mekanizmalarının Araştırılması. XXIV. Bitkisel İlaç Hammeddeleri Toplantısı. 2022, June 23-26. Ankara, Türkiye. (Poster Presentation)

Projects

Scholarship in 1001 TÜBİTAK Project (119Z094) "Türkiye Bitki Örtüsünde Doğal Olarak Yetişen Bazı *Valeriana* Türlerinden Sitotoksik Etkili Sekonder Metabolitlerin İzolasyonu, Yapılarının Tayini ve Etki Mekanizmalarının İncelenmesi", 2019- 2021.

