NAPHTHOQUINONES FROM NATURAL SOURCES AND THEIR BIOACTIVITIES

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ABSTRACT

NAPHTHOQUINONES FROM NATURAL SOURCES AND THEIR BIOACTIVITIES

Onosma L. is a large heterogenic genus of Boraginaceae family includes about 230 species distributed mainly in Central Asia and the Mediterranean region. According to 'Flora of Turkey, *Onosma* genus is represented with 104 species and 108 taxa and 52% of which are endemic.

Phytochemical studies have revealed that *Onosma* species possess various constituents including alkaloids, naphthoquinones, polyphenols, phytosterols, terpenoids and fatty acids. Naphthoquinones are naturally widespread secondary metabolites deriving from some higher plants, fungi and bacteria, and have shown significant biological activities such as cytotoxic, antibacterial, antifungal and wound healing.

In this thesis, bioassay-guided isolation studies were performed on *Onosma aksoyii* and *Onosma isaurica* to obtain naphthoquinone type cytotoxic compounds and investigate their topoisomerase inhibitory properties. Isolation studies were guided by MTT assay using three human cancer cell lines (HeLa, HCC-1937, DU-145) and a non-tumor cell line (MRC-5). whereas the enzyme inhibition tests were against human topoisomerases IIα and IIβ. Six compounds, one of which was new (**OA-PE-D1**), were isolated using chromatographic methods and their structures were elucidated by spectral methods (1D, 2D NMR, and MS). The known compounds were acetylshikonin, β,β-dimethylacrylshikonin, arnebidin, arnebifuranone and shikonofuran E. The cytotoxicity screenings showed that these compounds had IC_{50} values ranging from 6.485 μM to 32 μM. According to topoisomerase inhibition studies, OA-PE-D1 and β,β-dimethylacrylshikonin showed promising inhibitory effects on topoisomerase IIβ at dose of 1 mg/mL.

ÖZET

DOĞAL KAYNAKLARDAN ELDE EDİLEN NAFTAKİNONLAR VE BİYOAKTİVİTELERİ

Onosma L. Boraginaceae familyasında yer alan, genel olarak Orta Asya ve Akdeniz ülkelerinde yayılış gösteren ve dünya üzerinde 230 türü bulunan bir cinstir. Türkiye florasında 104 tür ve 108 takson ile temsil edilen *Onosma* cinsi oldukça yüksek bir endemizm oranına (52%) sahiptir. Bitkinin yapısında bulunan temel bileşenler alkaloidler, naftakinonlar, polifenoller, fitosteroller, terpenoidler ve yağ asitleri olarak tanımlanmıştır. Naftakinonlar bitkilerden funguslara doğada geniş bir yayılım gösteren ve önemli biyolojik etkilere (sitotoksisite, antibakteriyel, antifungal, yara iyileştirme) sahip bir sekonder metabolit grubudur.

Bu çalışma kapsamında, naftakinon yapıdaki sitotoksik bileşiklerin elde edilmesi, karakterizasyonlarının yapılması ve topoizomerazlar üzerindeki inhibitör etkilerinin araştırılması amacıyla *Onosma aksoyii* ve *Onosma isaurica* türleri üzerinde biyoaktivite rehberli izolasyon çalışmaları gerçekleştirilmiştir. İzolasyon çalışmaları, sitotoksik ativite rehberliğinde, üç tümörojenik (HeLa, DU-145, HCC-1937) ve bir tümörojenik olmayan (MRC-5) hücre hattına karşı inhibisyonların MTT metodu ile taranması şeklinde yürütülmüş olup, ayrıca insan topoizomeraz Iiα ve IIβ enzimleri üzerinde enzim inhibisyon testleri gerçekleştirilmiştir.

Farklı analitik yöntemler kullanılarak bir tanesi yeni olmak üzere 6 bileşik elde edilmiş ve yapıları modern spektroskopik yöntemler (1D-, 2D NMR ve MS) kullanılarak aydınlatılmıştır. Elde edilen yeni bileşik OA-PE-D1; bilinen bileşikler ise asetilşikonin, arnebidin, arnebifuranon, şikonofuran E ve β,β-dimetilakrilşikonindir.

Sitotoksisite tarama sonuçlarına göre, elde edilen bileşiklerin IC_{50} değerlerinin 6.485 μM ile 32 μM arasında değiştiği görülmüştür. Topoizomeraz inhibisyon testi sonuçları ise, OA-PE-D1 ve OA-PE-D14 kodlu bileşiğin, topoizomeraz IIα enzimi üzerinde 1 mg/mL konsantrasyonda inhibisyon etkisine sahip olduğunu göstermiştir.

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ABBREVIATIONS

FA	Formic Acid
HCI	Hydrochloric Acid
TFA	Trifluoroacetic Acid
EtBr	Ethidium Bromide
KCI	Potassium Chloride
$MgCI_2$	Magnesium Chloride
dH_2O	Distilled Water
UPW	Ultrapure Water
IPA	Isopropyl Alcohol
EDTA	Ethylenediaminetetraacetic Acid
PTFE	Polytetrafluoroethylene
$CHCI_3$	Chloroform
EtOAc	Ethyl Acetate
MeOH	Methanol
$CDCI_3$	Deuterated chloroform
DMSO	Dimethyl sulfoxide
RP-C18	Reversed Phase C18
UV	Ultraviolet
CC	Column Chromatography
TLC	Thin Layer Chromatography
MPLC	Medium Pressure Liquid Chromatography
HPLC	High Performance Liquid Chromatography
MPLC	Medium Pressure Liquid Chromatography
PDA	Photometric Diode Array
DAD	Diode-Array Detector
MS	Mass Spectrometry
LC-MS	Liquid Chromatography-Mass Spectrometry
NMR	Nuclear Magnetic Resonance

NMR	Nuclear Magnetic Resonance
COSY	Correlation Spectroscopy
HMBC	Heteronuclear Multiple Bond Correlation
s	Singlet
d	Dublet
t	Triplet
m	Multiplet
td	Triplet of Dublet
dt	Dublet of Triplet
MIC	Minimum Inhibitory Concentration
IC_{50}	The Half Maximal Inhibitory Concentration
PBS	Phosphate Buffered Saline
DMEM	Dulbecco's Modified Eagle Medium
RPMI	Roswell Park Memorial Institute Medium
DU145	Metastatic Prostate Cancer
MCF-7	Human Breast Adenocarcinoma
A-549	Human Lung Carcinoma
MRC-5	Human Lung Fibroblast
HEP-G2	Human Hepatocellular Carcinoma
Capan-1	Human Pancreatic Ductal Adenocarcinoma
HCC-1937	Human Breast Carcinoma Cell
HeLa	Human Cervix Carcinoma

CHAPTER 1

INTRODUCTION

Boraginaceae is one of the widely distributed families worldwide, especially in tropical, subtropical and temperate regions. The highest diversity centers are Iran-Turanian, Mediterranean regions, Central, North and South America and South Asia. Boraginaceae family represents 2500 species belonging to 131 genera, including *Borago*, *Anchusa*, *Arnebia*, *Echium* and *Onosma*.[1, 2]

Members of Boraginaceae family consist of annual, biennial or perennial herbaceous plants. The family has different morphological types having characteristic stems and leaves which have glandular or eglandular hairs on their surfaces and intensity of these hairs could be changed according to the various habitats. The plants of the family are widely distributed in dry and sandy habitats and rocky areas.[3]

O*nosma* L. is a large heterogenic genus of Boraginaceae including about 230 species distributed mainly in Southwest Asia, Mediterranean region and temperate Europe[4]. According to 'Flora of Turkey,and the East Aegean Islands', *Onosma* genus is represented with 104 species and 108 taxa and the rate of endemism is 52 %.[3, 5] Therefore, Anatolia can be considered as the gene center of this genus. *Onosma* L., consists of biennial and perennial herbs. Morphological characterization of plants has been made by scorpioid cymes, linear or linear-lanceolate calyx lobes that are parted to base, corolla without ribs or deep furrows unappendaged corolla throat, sagittate anthers coherent at base, capitate stigma and ovate to triangular nutlets.[6]

Major constituents of *Onosma* species are alkaloids, naphthoquinones, polyphenols, phytosterols, terpenoids, and fatty acids.[7] Naphthoquinones are naturally widespread secondary metabolites deriving from some higher plants[8], fungi[9] and bacteria.[9] They exhibit significant biological activities such as cytotoxic, antibacterial, antifungal, analgesic and wound healing.[8, 9]

Traditionally, the genus *Onosma* L. plants are used in folk medicine for the treatment of various diseases such as rheumatism, kidney irritations and palpitation of the heart.[10] Moreover, they have been used as remedies for hypertension, fever and nervous system disorders in India. In Turkey, these plants are used to cure inflammatory disorders,

wounds[11], burns[11] and infectious diseases.[12] In the Middle East, *Onosma bracteatum* Wall. is traditionally used in the treatment of respiratory tract diseases such as asthma and bronchitis. Also this plant is used for its sedative, diuretic, immune system activator[13] and antispasmodic activities.[14]

It is well known that, *Onosma* species has high endemism ratio and contain high levels of naphthoquinone derivatives, responsible for cytotoxic activity.[5]

Since there are numerous phytochemical and cytotoxicity screening studies on *Onosma* species, we aimed to isolate naphthoquinone derivatives from various *Onosma* species harvested from different locations of Southwest Anatolia. Within this scope, HPLC fingerprinting analysis and cytotoxicity screenings were performed on 12 *Onosma* species to determine the ones with remarkable cytotoxic activity and select for the isolation studies.

1.1. *Onosma aksoyii* Aytaç & Turkmen

Figure 1. 1. *Onosma aksoyii*

Onosma aksoyii is defined as a perennial herb by Aytaç & Türkmen (See Figure 1.1.). Stems numerous, 5-15 cm tall, ascending, simple to branched in the flowering part; brownish later greenish and leafy; with antrorse bristles arising from small white and glabrous tubercles, adpressed silvery and simple hairy. Leaves crowded at base. Basal and lower cauline leaves 18-50 × 1.2-1.5 mm, oblanceolate to spathulate; with strongly revolute margins, acute; covered with densely adpressed setae with glabrous tubercles (haplotrichous). Middle and upper cauline leaves similar, but smaller. Inflorescence with

3-10 flowers with scorpioid cymes. Bracts linear-lanceolate to narrowly linear, as long as or longer than pedicels and indumentum similar to leaves. Pedicels 5-8 mm. Calyx 7-10 mm in flower, 10-14 mm in fruit with linear-lanceolate lobes, acute. Corolla yellow, 12-16 mm, cylindrical, slightly lobed; lobes acuminate and revolute; annulus glabrous. Stamens 5, as long as or shorter than corolla. Anthers linear, with a sterile apex and bilobed at the base; lobes longer than wide, 4-5 mm, as long as fi laments.
Filaments inserted below the middle of the corolla tubes. Style 15-17 mm, 1-2 mm; longer than the corolla. Stigma very small and distinctly bilobed. Nutlets 5 × 2 mm, ovoid-pyramidal with an obtuse beak, smooth and chestnut-brown.[15]

Type: [Turkey C3] in Anatolia *Aytaç* & *Türkmen*
C3 Konya: Derebucak, Çamlık, *Pinus nigra* forest, 1350-1400 m, *Z. Aytaç* (8713). Konya: Derebucak, Çalık, Kızıldağ, 1960 m. *Hamzaoğlu* (4536). Konya: Derebucak, Çamlık, Kızıldağ, *Pinus nigra* forest, 1450-1600 m. *Z. Aytaç* (7833).

1.2. *Onosma isaurica* Boiss & Heldr

Figure 1. 2. *Onosma isaurica*

In the "*Flora of Turkey and The East Aegean Islands*", *O. isaurica* is defined as perennial herb. *O. isaurica* can grow from 10 to 20 cm. Stem is straight or riser and simple. The base leaves are 50 to 80 mm long, oblanceolate, obtuse, attenuate at the base, stem leaves are 20 to 35 mm long, oblong, acute and stemless. The floral board is in the form of 2 to 3 terminal clusters, the flowering time is capitate, the fruit time is longitidunal (See Figure 1.2.). Bractea ovate is lanceolate and acute. The calyx is almost stemless, 3

to 4 mm long and acute lobes; fruit time calyx pedicellate briefly and 6 to 8 mm. Corolla dark blue, 10 to 12 mm long. It blooms in May, June, July, August, and September. *O. isaurica* is seen in sunny or semi-shaded areas, rocky permeable, moist and sandy soil.[16]

Type: Crimea, Anatolia, Boiss & Heldr

B5 Nevşehir: Nevşehir to Avanos, 1150 m, *Vural et. al.* 5026. **A4** Çankırı: Ilgaz Gateway, 1775 m, *Aytaç et. al*., 5610. Kastamonu: Behind the Alçıcılar Village, 900 m, *Dönmez et. al*., 3622. Ankara: 10 km from Irmak to Kalecik, 640-750 m. *Dönmez et. al*., 3530. Kırıkkale: Delice, Büyükavşar Village, Büyükhamut Location, 1150 m. *C. Birden* 1109. **A8** Artvin: Yusufeli, Yusufeli-Turhal road (UTM 712069 E, 4531138 N), 730 m, H. Duman 9395. **C4** Karaman: Ermenek, Kazancı Town, Değirmen alanı Location, 600 m, H. Sümbül 2015. Karaman:Ermenek, West to Damlaçal, 1600 m, *Vural et. al.* 1752. Ankara: Ayaş,10 km from Ayaş to Beypazarı, 650 m, *Vural et. al.* 4124. **B5** Nevşehir: Ürgüp, Göreme, Arılıburun Brook, 1100-1250 m, *Vural et. al.* 4707. Nevşehir: Zelve, Akdağ, 1250-1350 m, *Vural et. al*. 4862. Nevşehir: Nevşehir to Ortahisar, 1250 m, *Vural et. al*. 4920. **A4** Ankara: Hasanoğlan, İsris Mountain, 1400-1700 m. *M. Koyuncu* 8061. Kastamonu: Kastamonu to Pınarbaşı, Kurtgirmez Mountain, 1200 m M. *V. Özbek* 1676. Ankara: Kızılcahamam, Soğuksu National Park, Keltepe Location, 1350-1400 m, *Ö. Eyüboğlu* 1483. Ankara: Kızılcahamam, Soğuksu National Park, Göllü Location, 1630 m, *Ö. Eyüboğlu* 1793.

1.3. Naphthoquinone Type Compounds

The genus *Onosma* is one of the major sources of naphthoquinones that are important bioactive secondary metabolites. Naphthoquinones are widespread in nature and produced by various organisms such as plants, fungi, lichens, bacteria, and algae.[12] They are derived from naphthalene skeleton and the presence of two carbonyl groups at C1 and C4 (1,4-naphthoquinones) or C1 and C2 (1,2-naphthoquinones) plays a general role in discrimination of naphthoquinone type compounds.. In general, naphthoquinones consist mostly monomeric and dimeric structures as well as trimeric and tetrameric forms. Besides, there is a wide variety of substituent groups linked to the main skeleton.[17]

There are various pathways for biosynthesis of naphthoquinones, including *O*-succinylbenzoate (OSB) pathway, the 4-hydroxybenzoic acid (4HBA)/geranyl

diphosphate (GPP) pathway; the acetate-polymalonate pathway, the homogentisate (HGA)/mevalonic acid (MVA) pathway and futasoline pathway.[18]

Naphthoquinones are well known organic compounds in pharmaceutical industry due to their considerable structural diversity and broad spectrum of pharmacological activities such as antimicrobial, antiviral, antiparasitic, antioxidant, antitumoral, antithrombotic and wound healing.[17]

1.3.1. Antimicrobial Activity

Naphthoquinones have considerable inhibitory activities against a group of microorganisms, including bacteria, fungi, parasites, and viruses.[17]

Tabata et. al. reported that the *n*-hexane extracts and naphthoquinone derivatives obtained from callus culture of *Lithospermum erythrorhizon* (Boraginaceae) exhibited strong antimicrobial activity. Moreover naphthoquinone derivatives showed antibacterial activity against gram-positive bacteria including *Staphylococcus aureus*, *Enterococcus faecium* and *Bacillus subtilis*, whereas the minimum inhibitory concentration (MIC) of these compounds were ranging from 0.3 mg/mL to 6.25 mg/mL.[19]

Sasaki et. al. focused on antifungal activity of methanol extracts of *Arnebia nobilis* (Boraginaceae) versus *Candida albicans*-infected mouse, and 5% solution of arnebin was applied topically every other day resulted in complete healing within six days.[20]

Rahmoun et. al. explored the antibacterial and antifungal activities of lawsone and some other novel naphthoquinone derivatives by disk diffusion and broth microdilution methods against seven pathogenic bacteria and three *Candida* species. As a result, two of the naphthoquinone derivatives showed significant activity against two gram-positive bacteria, *Staphylococcus aureus* and *Enterococcus faecalis,* with MIC values ranging from 16 μg/ml to 32 μg/ml respectively.[21]

Chen et. al. found that the crude extracts of *Lithospermum* species were 200 times more active than other commonly used herbal medicines in terms of the antiHIV-1 activity.[22]

The studies on shikonin, a common naphthoquinone derivative, showed significant antiamoebic activity against *Entamoeba histolytica*. Besides, shikonin has antiparasitic activity against *Aedes aegypti* larvaes.[23]

1.3.2. Antitumor Activity

Biological activity studies revealed that naphthoquinones had antitumor effects by blocking cancer cell growth, inducinh apoptosis, inhibiting DNA topoisomerases and decreasing angiogenesis.[24]

Bioactivity guided fractionation of *n*-hexane extract of *Arnebia densiflora* led to the isolation of isovalerylalkannin, α-methyl-n-butylalkannin, acetylalkannin, β-acetoxy isovalerylalkannin, alkannin, and 4-hydroxy-4-methylvalerylalkannin. The crude extract of *A. densiflora* exhibited higher cytotoxic activity on the tumorigenic HEp-2 cell line (IC_{50} value is 4.96 μM) compared to pure compounds (IC_{50} values varied between 14.35 μM and 34.33 μM). Isovalerylalkannin and acetylalkannin showed moderate to strong cytotoxic effect with IC_{50} values of 14.35 and 14.82 μM on HeLa cell lines. Besides, further studies for exploring mechanisms of the cytotoxicity showed that there was a significant association between cell death and induction of apoptosis.[24]

Sevimli Gür et. al. performed a bioactivity guided isolation study on *Alkanna cappadocica* (Boraginaceae) to obtain deoxyalkannin, β,β-dimethylacrylalkannin, 11-*O*-acetylalkannin, alkannin, 5-*O*-methyl-11-deoxyalkannin, 5-*O*-methyl-β,β-dimethylacrylalkannin. Compounds were tested versus cancer cell lines using MTT assay and it was found that all of the isolates were active againsts the tested cell lines. Deoxyalkannin, β,β-dimethylacrylalkannin, and 5-*O*-methyl-11-deoxyalkannin demonstrated superior activity compared to other compounds (IC_{50} values ranging between 0.09 μM and 14.83 μM). Deoxyalkannin showed high inhibitory activity toward MCF-7 and DU-145 cell lines while β,β-dimethylacrylalkannin was the most active compound on Saos-2 cell line.[25]

1.3.3. Wound Healing Activity

Naphthoquinone containing plants have been used for the treatment of wounds and burns in folk medicine for centuries.

Papageorgiou et. al. reported that *n*-hexane extract of *Alkanna tinctoria* root have wound healing activity on test animals with deep skin ulcers. The compounds responsible for activity were obtained using isolation and purification techniques. Besides, the chemical structures of compounds, which are the esters of alkannin such as β,β-

dimethylacrylate, isovalerate, angelate and β-acetoxyisovalerate, were elucidated by spectroscopic methods.[26]

Hayashi et. al. investigated the effects of *Lithospermum erythrorhizon* root extract and some naphthoquinone derivatives on *in vivo* wound modeling. The root extract and compounds were applied topically on test animals. Results showed that the formation of granuloma tissue was induced and wound healing was accelerated. It was concluded that naphthoquinone derivatives which had anti-inflammatory, antipyretic and analgesic effects were quite effective for the treatment of skin damage.[27]

Sidhu et. al. have investigated wound healing effects of Arnebin-1 (β,β-dimethylacrylshikonin) and shikonin in the subcutaneous tissue. For testing the bioactivity of these compounds, a punch wound model was created and compounds were administrated with doses ranging from 50 μg to 1000 μg. Between the fifth and seventh days of the treatment, healing of the wound treated with shikonin was observed. The expression of vascular endothelial growth factor (VEGF) increased dramatically from the first day of treatment, moreover, neovascularization was observed. Arnebin-1 administration supported the migration, vascularization and cell proliferation, for re-epithelialization and granulation tissue formation. Arnebin 1 increased the synthesis of transforming growth factor beta-1 (TGFβ-1), collagen and fibronectin comparing the control group. Increasing the expression of TGFβ-1 at both transcription and translation level with arnebin-1 administration might be responsible for accelerated wound healing.[28]

1.3.4. Immune System and Inflammatory Process

Naphthoquinones have an important role in the treatment of immune system disorders such as inflammatory, autoimmune and vascular diseases.[17]

Checker et. al. investigated the immunomodulatory effects of plumbagin, a naphthoquinone derivative extracted from the roots of *Plumbago* zeylanica (Plumbaginaceae). Suppression of NF-κB activation pathway have therapeutic potential for prevention from acute graft rejection.[29]

In another study, plumbagin exhibited immunosuppressive effects in mice after tissue transplantation by inhibiting the T cell activation, proliferation, and cytokine production.[17]

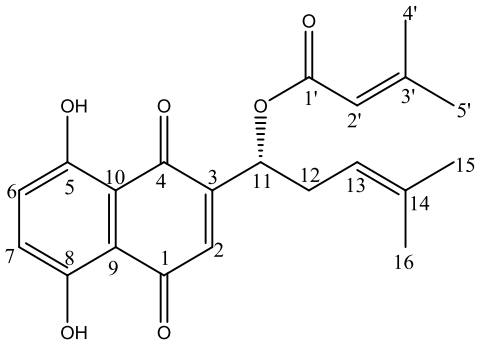
Except for the immunosuppressive actions, there are numerous in vivo studies on anti-inflammatory effects of naphthoquinones. The topical application of alkannin and shikonin with doses ranging from 50 μg to 100 μg suppressed the capillary permeability on rats with heat-induced oedema on their epidermis.[27] The pomade made from the petroleum ether extract of *Arnebia euchroma* roots had similar effects. It was applied topically with a dose 50 mg/area on test animals having acute oedema and local erythema induced by histamine serotonin and bromelain. As a result, the pomade suppressed the capillary permeability, acute oedema and local erythema on rats.[30]

Recent studies have shown that the anti-inflammatory effects of naphthoquinones are mainly due to inhibition of leukotriene B4 (LTB4), neutrophil inhibition, the suppression of mast cell granulation, blockade of chemokine ligands, and deterioration of phosphatidylinositol signaling pathway.[31]

1.4. Naphthoquinone Derivatives From Boraginaceae

Structure of frequently encountered naphthoquinone/benzoquinone derivatives are given below with their physical and ^{13}C-NMR spectral data.

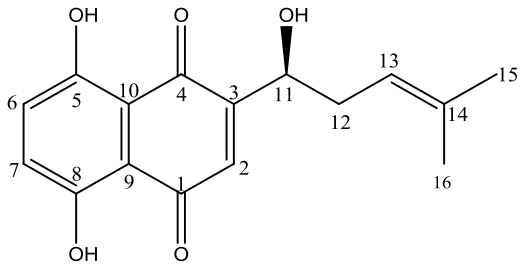
β,β-Dimethylacrylshikonin[32]



Plant Material	: *Onosma paniculatum*
Molecular Formula	: $C_{21}H_{22}O_6$
Molecular Weight	: 370
Optical Rotation	: $[\alpha]_D^{25}$ +120 (c 0.1, MeOH)
^{13}C-NMR Solvent	: $CDCI_3$
Name	: (R)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-en-1-yl 3-methylbut-2-enoate
MP	: 103 ^{0}C

C-1	179.2	C-13	68.8
2	149.2	14	131.7
3	136.0	15	27.7
4	177.7	16	20.5
5	166.9	1’	166.4
6	132.7	2’	115.4
7	132.6	3’	159.1
8	165.4	4’	25.9
9	112.0	5’	18.1
10	111.7		
11	118.2		
12	33.0		

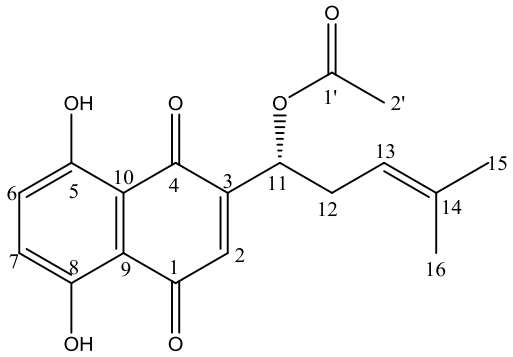
Shikonin [33]



Plant Material	: *Onosma sericium*
Molecular Formula	: $C_{16}H_{16}O_5$
Molecular Weight	: 288
Optical Rotation	: $[\alpha]_D^{20}$ +140 (c, 0.1, $CHCl_3$)
^{13}C-NMR Solvent	: CD_3OD
Name	: (R)-5,8-dihydroxy-2-(1-hydroxy-4-methylpent-3-en-1-yl)naphthalene-1,4-dione
MP	: 147 ^{0}C

C-1	182.5	C-13	121.1
2	133.5	14	135.8
3	154.8	15	26.3
4	181.5	16	18.3
5	166.5		
6	133.3		
7	133.0		
8	165.9		
9	113.5		
10	113.1		
11	68.8		
12	36.9		

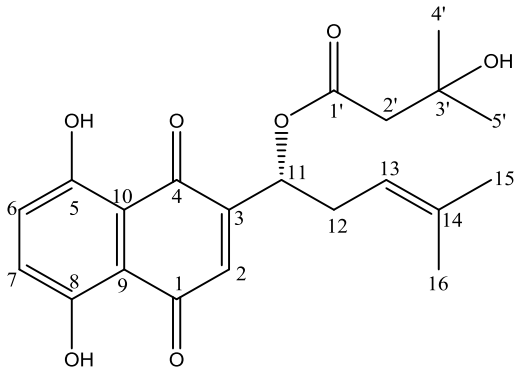
Acetylshikonin [34]



Plant Material	: *Onosma paniculatum*
Molecular Formula	: $C_{18}H_{18}O_6$
Molecular Weight	: 330
Optical Rotation	: $[\alpha]_D^{20}$ +456 (c, 0.1, $CHCl_3$)
^{13}C-NMR Solvent	: $CDCI_3$
Name	: (R)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-en-1-yl acetate
MP	: 86 ^{0}C

C-1	178.3	C-13	117.8
2	148.3	14	136.2
3	131.5	15	18.0
4	176.8	16	25.9
5	167.6	1’	169.9
6	133.0	2’	21.1
7	132.8		
8	167.0		
9	111.7		
10	111.9		
11	59.6		
12	32.9		

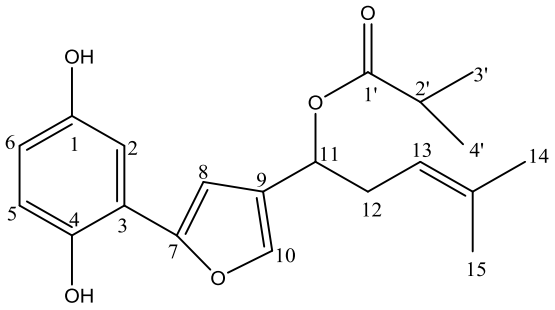
β-Hydoxyisovalerylshikonin[35]



Plant Material	: *Onosma visianii*
Molecular Formula	: $C_{21}H_{24}O_7$
Molecular Weight	: 388
Optical Rotation	: $[\alpha]_D^{20}$ +102 (c, 0.5, $CHCl_3$)
^{13}C-NMR Solvent	: $CDCI_3$
Name	: (R)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-en-1-yl 3-hydroxy-3-methylbutanoate
MP	: 95 ^{0}C

C-1	177.2	C-13	117.9
2	147.7	14	135.5
3	130.8	15	26.4
4	177.2	16	17.9
5	167.5	1’	170.3
6	132.6	2’	19.4
7	132.8	3’	69.1
8	168.0	4’	29.3
9	111.4	5’	29.3
10	111.7		
11	69.1		
12	41.1		

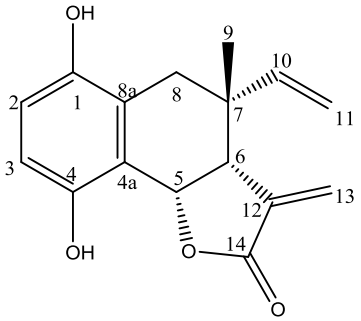
Shikonofuran D[36]



Plant Material	: *Lithospermum erythrorhizon*
Molecular Formula	: $C_{20}H_{24}O_5$
Molecular Weight	: 344
Optical Rotation	: $[\alpha]_D^{20}$ +56 (c, 0.1, $CDCI_3$)
^{13}C-NMR Solvent	: $CDCI_3$
Name	: 1-(5-(2,5-dihydroxyphenyl)furan-3-yl)-4-methylpent-3-en-1-yl isobutyrate
MP	: 103 ^{0}C

C-1	149.3	C-13	118.6
2	112.1	14	135.2
3	117.0	15	25.8
4	146.6	16	18.1
5	118.1	1’	176.8
6	116.6	2’	34.3
7	152.4	3’	19.0
8	106.7	4’	18.9
9	127.2		
10	138.8		
11	68.2		
12	33.6		

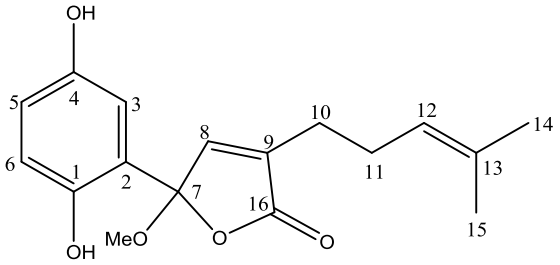
Arnebinol C[37]



Plant Material	: *Arnebia euchroma*
Molecular Formula	: $C_{16}H_{16}O_4$
Molecular Weight	: 272
Optical Rotation	: $[\alpha]_D^{20}$ +106.5 (c, 0.07, MeOH)
^{13}C-NMR Solvent	: CD_3OD
Name	: (3aR,4R,9bS)-6,9-dihydroxy-4-methyl-3-methylene-4-vinyl-3a,4,5,9b-tetrahydronaphtho[1,2-b]furan-2(3H)-one
MP	: 103 ^{0}C

C-1	148.4	C-11	115.1
2	117.4	12	140.0
3	114.1	13	123.4
4	151.2	14	173.1
4a	119.7		
5	75.7		
6	50.1		
7	38.5		
8	34.8		
8a	125.0		
9	24.7		
10	142.0		

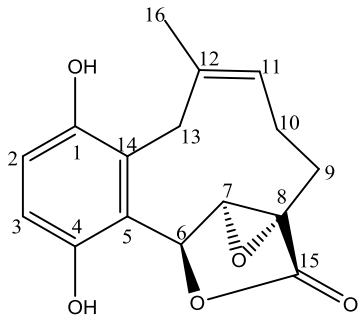
Arnebinol D[38]



Plant Material	: *Arnebia euchroma*
Molecular Formula	: $C_{17}H_{20}O_5$
Molecular Weight	: 304
Optical Rotation	: $[\alpha]_D^{20}$ 0 (c, 0.1, MeOH)
^{13}C-NMR Solvent	: CD_3OD
Name	: 5-(2,5-dihydroxyphenyl)-5-methoxy-3-(4-methylpent-3-en-1-yl)furan-2(5H)-one
MP	: 103 ^{0}C

C-1	149.3	C-13	134.1
2	123.6	14	17.7
3	114.6	15	25.7
4	151.2	16	173.4
5	118.2	1’	52.2
6	118.4		
7	108.6		
8	148.2		
9	135.9		
10	25.9		
11	26.9		
12	123.8		

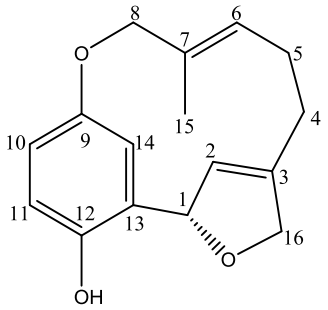
Clavilactone A[39]



Plant Material	: *Arnebia euchroma*
Molecular Formula	: $C_{16}H_{16}O_5$
Molecular Weight	: 288
Optical Rotation	: $[\alpha]_D^{20}$ 0 (c, 0.1, MeOH)
^{13}C-NMR Solvent	: CD_3OD
Name	: (11S,11aS,Z)-7,10-dihydroxy-5-methyl-3,6,11,11a-tetrahydro-2H-11,1a-(epoxymethano)benzo[4,5]cyclodeca[1,2-b]oxiren-13-one
MP	: 103 ^{0}C

C-1	150.3	C-13	28.0
2	118.4	14	127.8
3	115.3	15	173.1
4	150.3	16	21.6
5	120.9		
6	75.9		
7	64.3		
8	62.1		
9	26.0		
10	23.1		
11	122.4		
12	138.9		

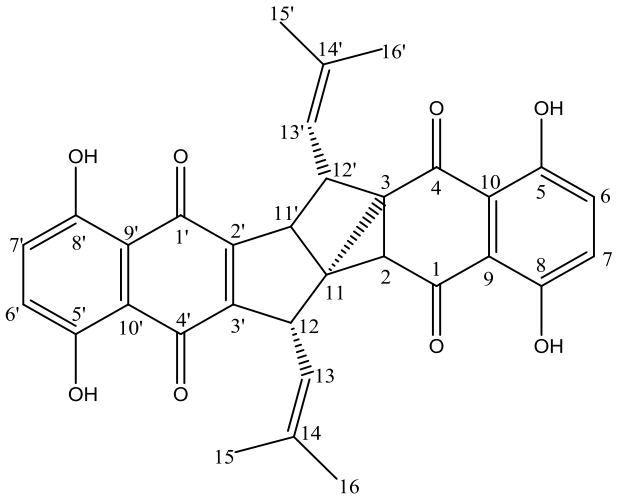
9,17-Epoxyarnebinol[40]



Plant Material	: *Arnebia euchroma*
Molecular Formula	: $C_{16}H_{18}O_3$
Molecular Weight	: 258
Optical Rotation	: $[\alpha]_D^{26}$ -576.8 (c, 0.16, acetone)
^{13}C-NMR Solvent	: $(CD_3)_2CO$
Name	: (S,13Z,5E)-5-methyl-12,15-dihydro-3-oxa-1(2,4)-furana-2(1,3)-benzenacyclooctaphan-5-en-26-ol
MP	: 103 ^{0}C

C-1	78.7	C-13	119.1
2	135.4	14	129.0
3	133.4	15	13.2
4	26.6	16	75.0
5	27.5		
6	141.3		
7	127.9		
8	81.6		
9	149.0		
10	116.8		
11	118.5		
12	152.6		

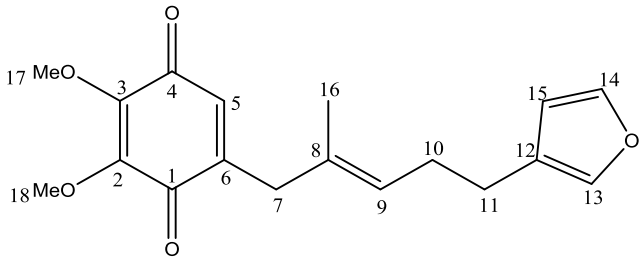
Arnebidin[41]



Plant Material	: *Arnebia hispidissima*
Molecular Formula	: $C_{32}H_{26}O_8$
Molecular Weight	: 538
Optical Rotation	: $[\alpha]_D^{26}$ -576.8 (c, 0.16, acetone)
^{13}C-NMR Solvent	: $CDCI_3$

C-1	193.1	C-13	116.9	9’	112.5
2	45.3	14	138.6	10’	111.4
3	49.2	15	18.6	11’	38.1
4	195.5	16	26.0	12’	37.6
5	156.3	1’	182.2	13’	117.4
6	129.1	2’	144.3	14’	137.8
7	128.9	3’	140.0	15’	18.7
8	157.4	4’	182.6	16’	25.8
9	111.7	5’	159.1		
10	112.7	6’	129.3		
11	38.3	7’	129.9		
12	40.7	8’	158.6		

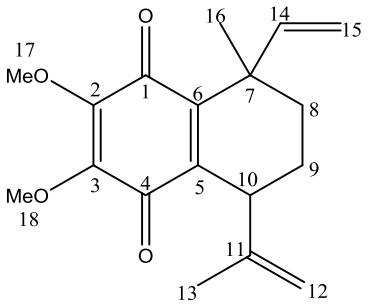
Arnebifuranone[42]



Plant Material	: *Arnebia euchroma*
Molecular Formula	: $C_{18}H_{20}O_5$
Molecular Weight	: 316
^{13}C-NMR Solvent	: $CDCI_3$
Name	: (E)-5-(5-(furan-3-yl)-2-methylpent-2-en-1-yl)-2,3-dimethoxycyclohexa-2,5-diene-1,4-dione

C-1	184.3	C-13	139.1
2	144.9	14	142.9
3	145.3	15	111.1
4	184.4	16	23.7
5	130.5	17	61.4
6	145.2	18	61.3
7	30.9		
8	130.2		
9	129.1		
10	29.8		
11	24.8		
12	124.6		

Arnebinone[42]



Plant Material	: *Arnebia euchroma*
Molecular Formula	: $C_{18}H_{22}O_4$
Molecular Weight	: 302
Optical Rotation	: $[\alpha]_D^{20}$ -4 (c, 0.01, dioxane)
^{13}C-NMR Solvent	: $CDCI_3$
Name	: 2,3-dimethoxy-5-methyl-8-(prop-1-en-2-yl)-5-vinyl-5,6,7,8-tetrahydronaphthalene-1,4-dione

C-1	183.5	C-13	24.6
2	146.1	14	145.5
3	145.5	15	111.5
4	183.6	16	20.1
5	144.4	17	61.1
6	144.3	18	61.1
7	47.5		
8	26.3		
9	34.5		
10	38.1		
11	146.1		
12	113.2		

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Materials

To pinpoint locations of different *Onosma* species, various academic information sources, mainly "*Flora of Turkey and the East Aegean Islands*" articles, herbarium records, and related graduate thesis were utilized.

The field studies to collect chosen *Onosma* species were carried out in the flowering period, particularly between April and June. Collected species were identified by Dr. Ademi Fahri Pirhan (Department of Biology, Faculty of Sciences, Ege University, Izmir, Turkey). Voucher specimens were deposited in the Herbarium of Ege University, Izmir, Turkey (Table 2.1).

The collected plants were dried in the shade according to the herbarium rules and the roots were separated from aerial parts. The dried samples were powdered using a grinder and stored in opaque plastic bottles at room temperature (Figure 2.1).

Table 2. 1. Collected *Onosma* species and their locations

Name	Analysis Code	Herbarium Code	Location	Date
***O. aksoyii**	G4-09	42718	Sütçüler-Beyşehir, Ayvalıpınar,1146 m 37º41.614'K 31º01.340'D	16.06.2017
O. strigossima	G5-01	3661	Kemer-Alacasu Cove, 109 m 36º32.086'K 30º33.464'D	27.04.2018
O. nydeggeri	G5-02	3662	Antalya-Kumluca/Altınyaka Neighborhood, 994m 36º33.825'K 30º20.843'D	27.04.2018
O. frutescens	G5-03	3663	Fethiye-Korkuteli road, 888 m 36º43.235'K 29º26.586'D	28.04.2018

(cont. on next page)

Table 2.1. (Cont.)

Name	Analysis Code	Herbarium Code	Location	Date
O. taurica var. *taurica*	G5-6	3666	Fethiye-Korkuteli road/Dirmil, 1245 m 36º53.963'K 29º41.020'D	28.04.2018
O. albo-roseum subsp. *songuinolentum*	G5-10	3670	E87 highway, Antalya-Denizli road 1412 m 36º45.93'K 28º53.76'D	28.04.2018
O. inexpectata	G6-01	3671	Kızılcadağ-Çığlık plateau, 1576 m 37º2.080'K 29º59.941'D	05.06.2018
O. giganteum	G6-02	3672	Osmaniye/Hasanbey, 1005 m 37º6.888'K 36º35.789'D	05.06.2018
O. alboroseum subsp. *albo-rosea* var. *albo-rosea*	G6-03	3673	Mersin, Cemilli Village, 412 m 36º47.814'K 34º27.441'D	06.06.2018
O. roussai	G6-04	3675	Gülnar-Ermenek road, Bereket Neighborhood 1227 m 36º21.318'K 33º13.687'D	06.06.2018
***O. isaurica**	G6-05	3676	Gülnar-Ermenek road 24. km, 1389 m 36º24.577'K 33º10.337'D	06.06.2018
O. mite	G7-02	3678	Termessos National Park, 395 m 37º1.004'K 30º30.306'D	20.07.2018

2.1.2. Technical Devices and Chemicals Used for Extraction and Isolation Studies

2.1.2.1. Chemicals

- Acetone : Macron Fine Chemicals
- Acetonitrile : Macron Fine Chemicals
- Chloroform : VWR
- CycloHexane : Macron
- Dichloromethane : Baker
- Dimethyl Sulfoxide : Sigma Aldrich
- EDTA : Sigma-Aldrich

- Ethanol : Carlo Erba
- Ethyl Acetate : Fisher
- Formic Acid : Merck
- HCI : Sigma-Aldrich
- Methanol : Fisher
- *n*-hexane : VWR
- *n*-butanol : Carlo Erba
- Petroleum Ether : ChemLab
- RP-C18 : Merck, 9303
- Sephadex LH-20 : GE
- Silica gel 60 : Merck, 7734

2.1.2.2. Technical Devices

- Rotary Evaporator : Heidolph Laborata 4001
- Ultrasonic Bath : Ultrasonic LC30
- UV Lamp : Vilber Lourmat
- Precision Scale : Radwag
- Vacuum pump : Labnet
- Peristaltic Pump : Thermo
- Nuclear Magnetic Resonance Spectrometry : Varian AS400 (400 MHz)
- High Pressure Liquid Chromatography : Thermo Scientific-Dionex Ultimate 3000
- Optical Rotation : Rudolph Research Analytical
- High Resolution Mass Spectrometry : Agilent 1200/6530 Instrument

2.1.3. Materials in Cytotoxicity Screening Studies

2.1.3.1. Cell Lines

1. A-549 (Human lung carcinoma)
2. Capan-1 (Human Pancreatic Ductal Adenocarcinoma)
3. DU-145 (Metastatic Prostate Carsinoma) (Cat no (ATCC® HTB-81™))

4. HCC-1937 (Human Breast Carcinoma) (Cat No: ATCC® CRL-2336™)
5. HeLa (Human endometrial carcinoma cell line (ATCC® CCL-2™)
6. HEP-G2 (Human Hepatocellular Carcinoma)
7. MRC-5 (Human Lung Fibroblast cell line (ATCC® CCL-171™))
8. MCF-7 (Human Breast Adenocarcinoma)

2.1.3.2. Cell Culture Equipment and Supplies

1. Sterile pipettes
2. Sterile growth medium (DMEM or RPMI, 10 % fetal bovine serum (Gibco, USA)., 1% L-glutamine,
3. Sterile PBS (Ca^{+2} and Mg^{+2} free phosphate buffer saline)
4. Sterile enzyme solution at 37°C (0.25 % trypsin in PBS)
5. Cell culture flasks : Sigma-Aldrich
6. Centrifuge : Thermo
7. Incubator : Nüve
8. Laminar Flow Cabinet : Thermo
9. 96 well plate : Sigma-Aldrich
10. Microplate Reader : Varioskan microplate reader

2.1.4. Materials in DNA Topoisomerase Studies

2.1.4.1. Chemicals

1. Supercoiled DNA prB322 : Inspiralis
2. Kinetoplast DNA : Inspiralis
3. DNA Topoisomerase IIα, IIβ : Inspiralis
4. DNA Assay Buffer : Inspiralis
5. DNA Enzyme Buffer : Inspiralis
6. ATP : Inspiralis
7. Agarose : Basica LE
8. EtBr : Sigma-Aldrich
9. Methanol : Merck
10. DMSO : Sigma-Aldrich

11. EDTA : Sigma-Aldrich
12. 6X DNA Loading Dye : Thermo Fisher, R0611

2.1.4.2. Technical Instruments

1. Electrophoresis Power Supply : Thermo
2. UV Imaging System : Vilber Lourmat
3. Horizontal Electrophoresis Device : Thermo EC250 (5 V/cm)
4. Centrifuge : Labnet-Spectrafuge- Hettich Zentrifugen
5. Shaker :Janke-Kunkal KS 501D
6. Heater :Lab- Line Multiblok Heater 30 C
7. Oven :Profilo
8. Micropipette :Thermoelectron Finnpipet
9. Centrifuge tubes (1.5 ml) :Thermo

2.2. Methods

2.2.1. Preliminary Studies

Preliminary experiments were carried out to select bioactive *Onosma* species for bioassay-guided isolation studies.

The dried and powdered roots of *Onosma* species (10 g each) were sequentially extracted with petroleum ether, dichloromethane and methanol (100 ml each) using an ultrasonic bath for 30 minutes at room temperature and this process was repeated thrice. Residues obtained from the extracts were separated using filter paper and filtrates were evaporated by rotary evaporator at 40 °C. The obtained crude extracts were subjected to cytotoxicity screening assays (Method 2.3) and HPLC studies (Method 2.2.1.1). Considering test results, *Onosma* species with higher chemical diversity and stronger cytotoxic effect were selected for further isolation studies.

2.2.1.1. HPLC Analysis

The petroleum ether and dichloromethane extracts of the roots were weighed, and the samples were adjusted to a final concentrations of 1000 ppm with 750 µl $CHCI_3$ and 1250 µl of MeOH solution (37.5% $CHCI_3$-62.5% MeOH). The samples were filtered (through a 0.22 µm PTFE filter) and then analyzed.

Thermo Scientific-Dionex Ultimate 3000 system was used for analysis. The system consists of an automatic sample injection section, quadruple pump, column furnace and sequential diode array detector (DAD) equipment. Analysis condition is given in Table 2.2.

Flow rate : 1 mL/min

Column : Synergi 4µm Max-RP 80A 150x4.6ID

Temperature : 35°C

UV : 520 nm

Sample volume : 20 µl

Solvent system : TFA aqueous solution (0.025%, v/v) and TFA solution in ACN (0.025%, v/v)

Table 2. 2. HPLC Analysis Condition

Time **(min)**	**Solvent A** (TFA aqueous solution, 0.025%, v/v) **(%)**	**Solvent B** (TFA solution in ACN, 0.025%, v/v) **(%)**
0.00	40	60
10	27	73
13	25	75
20	20	80
25	0	100
30	0	100
31	40	60
35	40	60

2.2.2. Bioactivity Guided Extraction, Isolation and Purification Studies

According to the results of preliminary studies (HPLC analysis and cytotoxicity screening assay), *O. aksoyii* and *O. isaurica* were chosen for further isolation and purification studies.

2.2.2.1. Bioactivity Guided Extraction, Isolation and Purification Studies on *Onosma aksoyii*

Onosma aksoyii was selected for the bioassay-guided isolation studies, due to its phytochemical content and promising cytotoxic activity (IC_{50}<8 μM) results against tumorigenic cell lines (A-549, Capan-1, HeLa, HCC-1937, HEP-G2, MCF-7 and DU-145).

The powdered roots of *O. aksoyii* (1050 g) was sequentially extracted with petroleum ether, dichlorometane and methanol by maceration technique. The extraction was performed using 10 liters of each solvent until the reddish-pink color (which proves the presence of quinonoid pigments) disappeared. After filtration, solvents were evaporated by rotary evaporator at 35°C and yielded 7.10 g petroleum ether, 4.03 g dichlorometane and 18.7 g methanol extracts. Considering TLC chromatogram profiles of the extracts, the petroleum ether extract was selected for further studies.

At the first step of isolation studies, the petroleum ether extract was applied to a medium pressure liquid chromatography prepared with 250 g RP-C18 and 200 g Sephadex LH-20 (Two separate layers on the same column, respectively). Elution was started with H_2O (500 mL) and followed by H_2O:MeOH (5:5, 2000 mL; 4:6, 1500 mL; 3:7, 1750 mL; 1:9, 1000 mL), MeOH:IPA (9:1, 500 mL; 8:2, 500 mL; 5:3, 350 mL) and terminated with 100% IPA (500 mL). The fractions, which had similar TLC profiles, were combined and 19 main fractions were obtained. Based on the cytotoxicity screenings and DNA topoisomerase inhibition tests, **ES-1**, **ES-2**, **ES-3** and **ES-6** encoded fractions were chosen to carry out further studies. Bioactivity guided isolation of these main fractions afforded a new monoterpenylbenzoquinone derivative together with five known compounds.

The TLC analysis of fraction ES-02 revealed two major spots. Thus, it was fractionated by silica gel column chromatography with Cyclohexane: EtOAc: FA (95:5:0.5 to 80:20:0.5) solvent system to afford six subfractions. The subfraction ES-02_{4-}

$_{12}$ and ES-2$_{13\text{-}24}$ were purified individually on silica gel column chromatography using the same solvent system to obtain **OA-PE-D1** (4.1 mg) and **OA-PE-D2** (3.7mg).

The other major fraction ES-03 was subjected to a silica gel column, and eluted with cyclohexane: EtOAc: FA (95:5:0.5 to 50:50:0.5) yielding 15 fractions. The subfraction ES-03$_{243\text{-}247}$ was subjected to a preparative silica TLC employing with a solvent system of cyclohexane: EtOAc: MeOH: FA (60:40:5:2) to obtain **OA-PE-D3** (2.4 mg).

Consecutive silica gel columns were performed to isolate 2 major compounds of fraction ES-06. Both of the subfractions ES-06$_{2\text{-}3}$ and ES-06$_{9\text{-}26}$ were applied to RP-C18 columns eluted with MeOH: ACN (70:30) to obtain **OA-PE-D10** (1.4 mg) and **OA-PE-D14** (1.7 mg), respectively. The isolation steps were illustrated in Figure 2.2. and structure elucidations of pure compounds were discussed in Section 3.

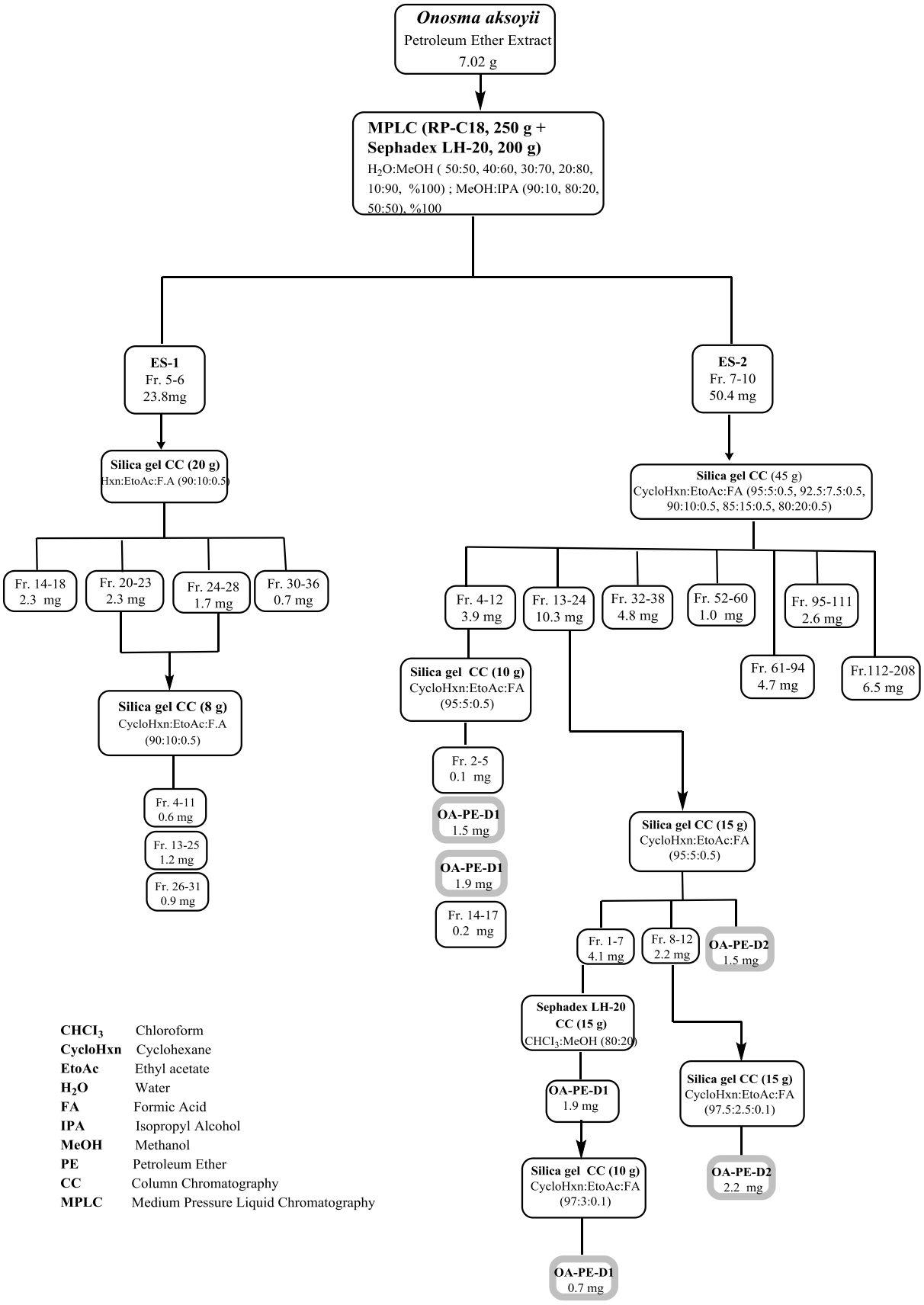


Figure 2. 1. Isolation scheme of *O. aksoyii*

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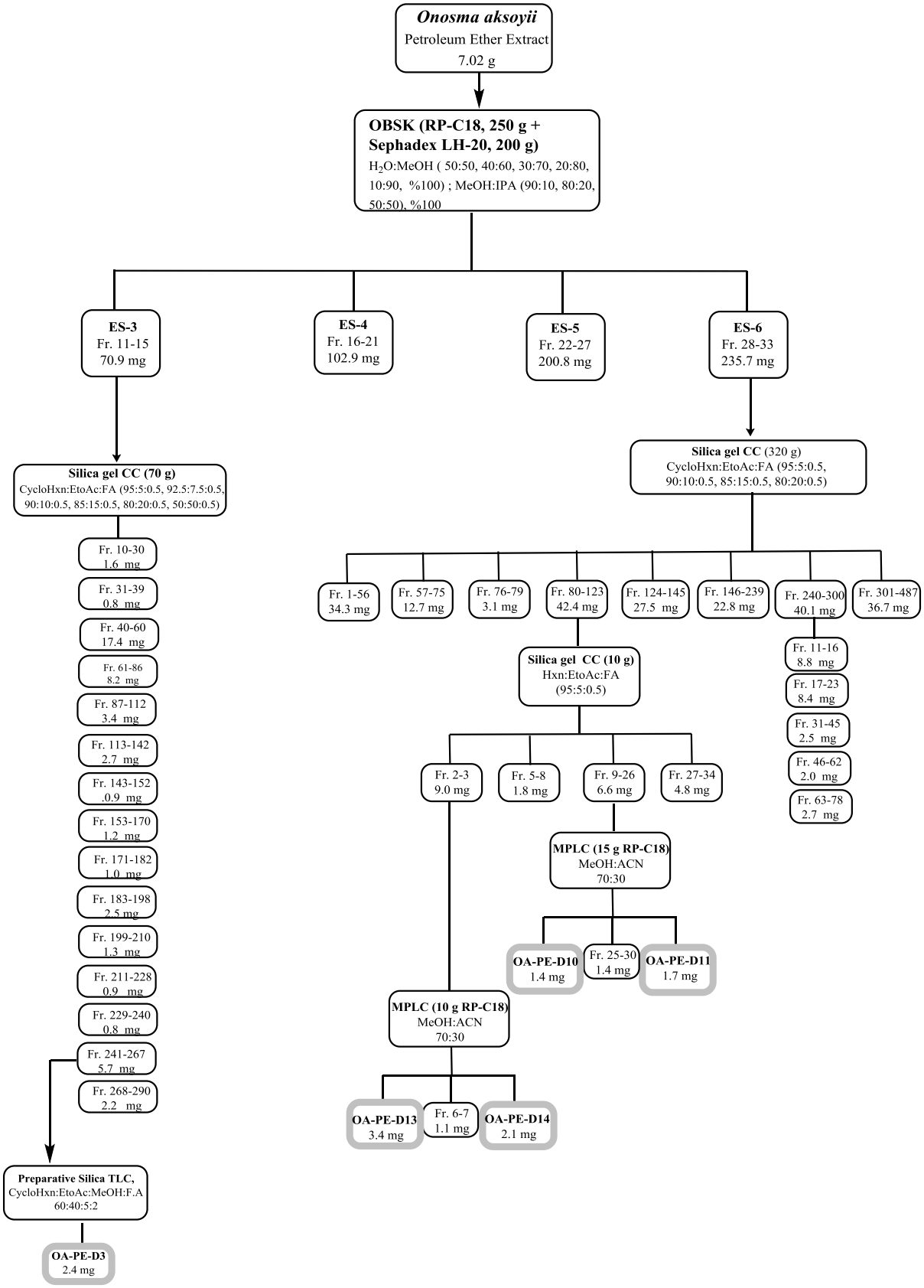

Figure 2. 2. Isolation scheme of *O. aksoyii*

2.2.2.2. Bioactivity Guided Extraction, Isolation and Purification Studies on *Onosma isaurica*

Onosma isaurica was another species with significant cytotoxic activity (IC_{50}<8 μM) and prominent chemical diversity, thus it was selected for the bioassay-guided isolation studies.

The powdered roots of *O. isaurica* (1200 g) was extracted sequentially with petroleum ether and dichloromethane using 10 liters of each solvent until the reddish-pink color disappeared. After filtration, solvents were evaporated by rotary evaporator at 35°C (Petroleum ether extract:9.10 g; dichloromethane extract: 5.33 g). According to the TLC profiles and cytotoxicity screening results, we decided to move forward further purification studies with the extract of petroleum ether.

The petroleum ether extract was applied to a silica gel (800 g) column and elution was started with *n*-Hxn: EtOAc (97: 3 → 60: 40) and terminated with 100% MeOH (1000 mL) to give 21 main fractions. Isolation studies were carried out on IS-02, IS-10, IS-15 and IS-18 fractions which showed promising cytotoxic activity and chemical diversity. Active fractions were subjected to consecutive RP-C18 columns using the solvent systems MeOH: H_2O (9:1, 8:2, 7:3, 6:4) respectively, to obtain pure compounds as **OIS-5**, **OIS-14**, **OIS-15**, **OIS-23** and **OIS-26**.

Elaborations of the isolation studies were illustrated in Figure 2.4, 2.5 and 2.6, and structure elucidations of pure compounds were discussed in Section 3.4.

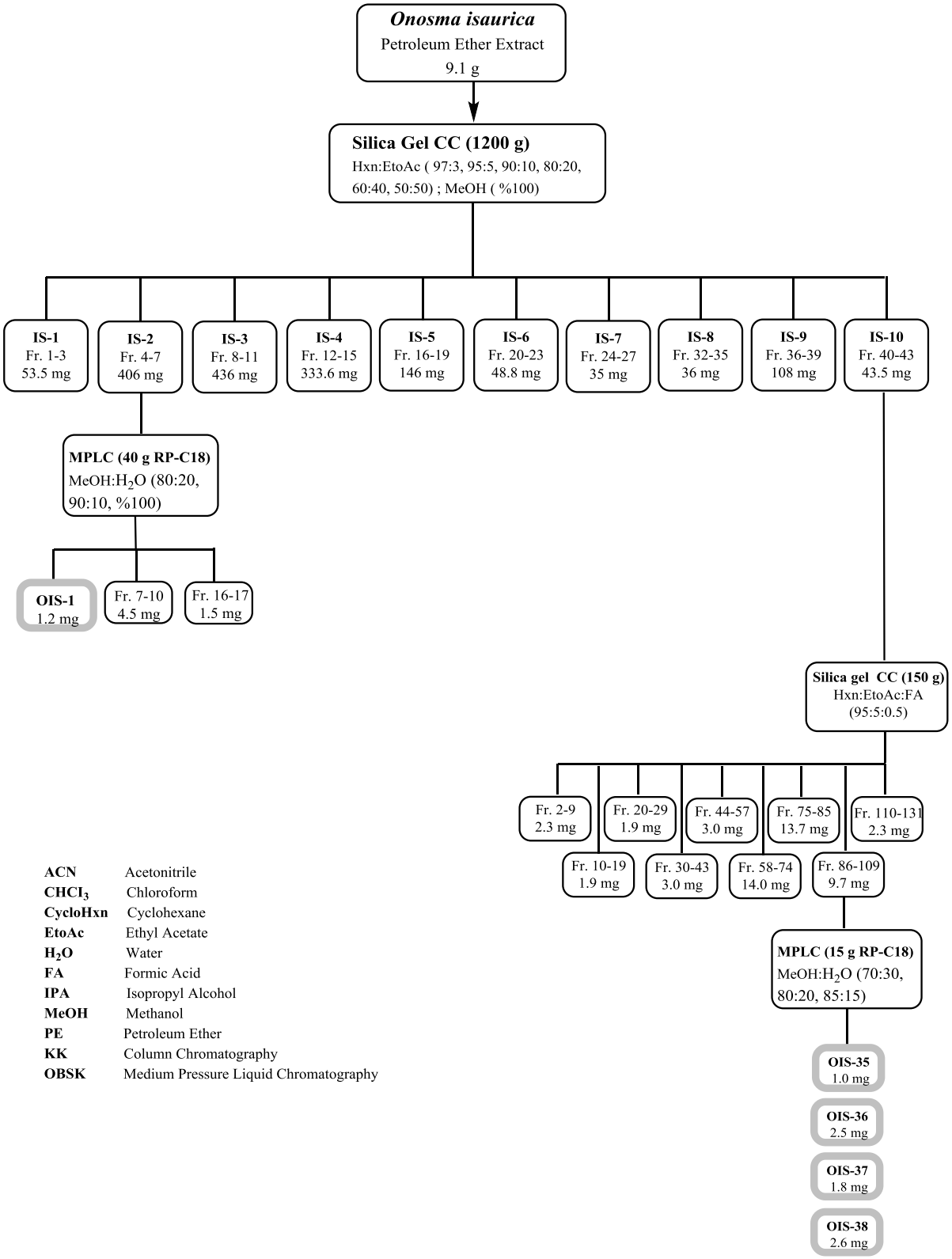

Figure 2. 3. Isolation studies on *O. isaurica*

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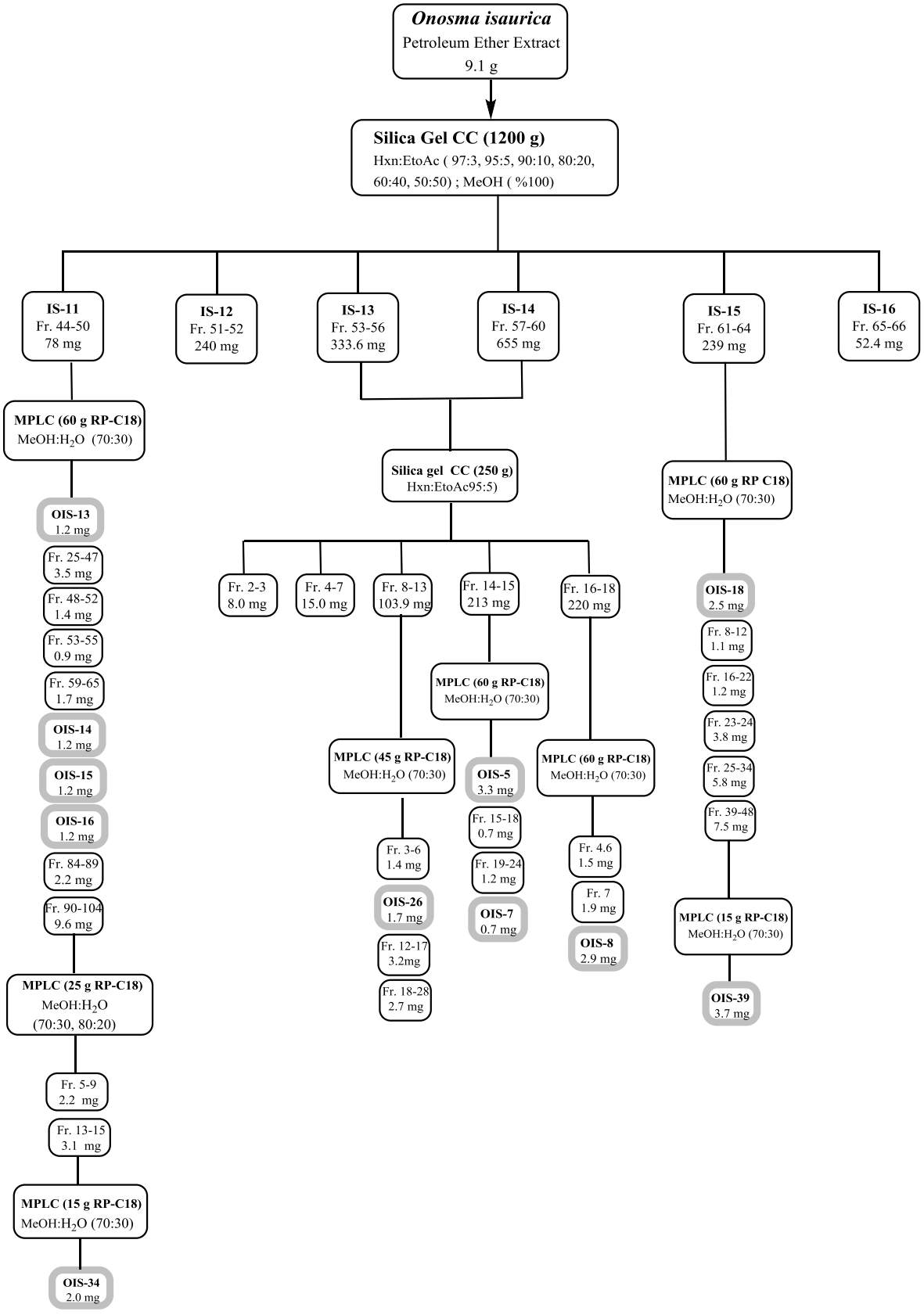

Figure 2. 4. Isolation studies on *O. isaurica*

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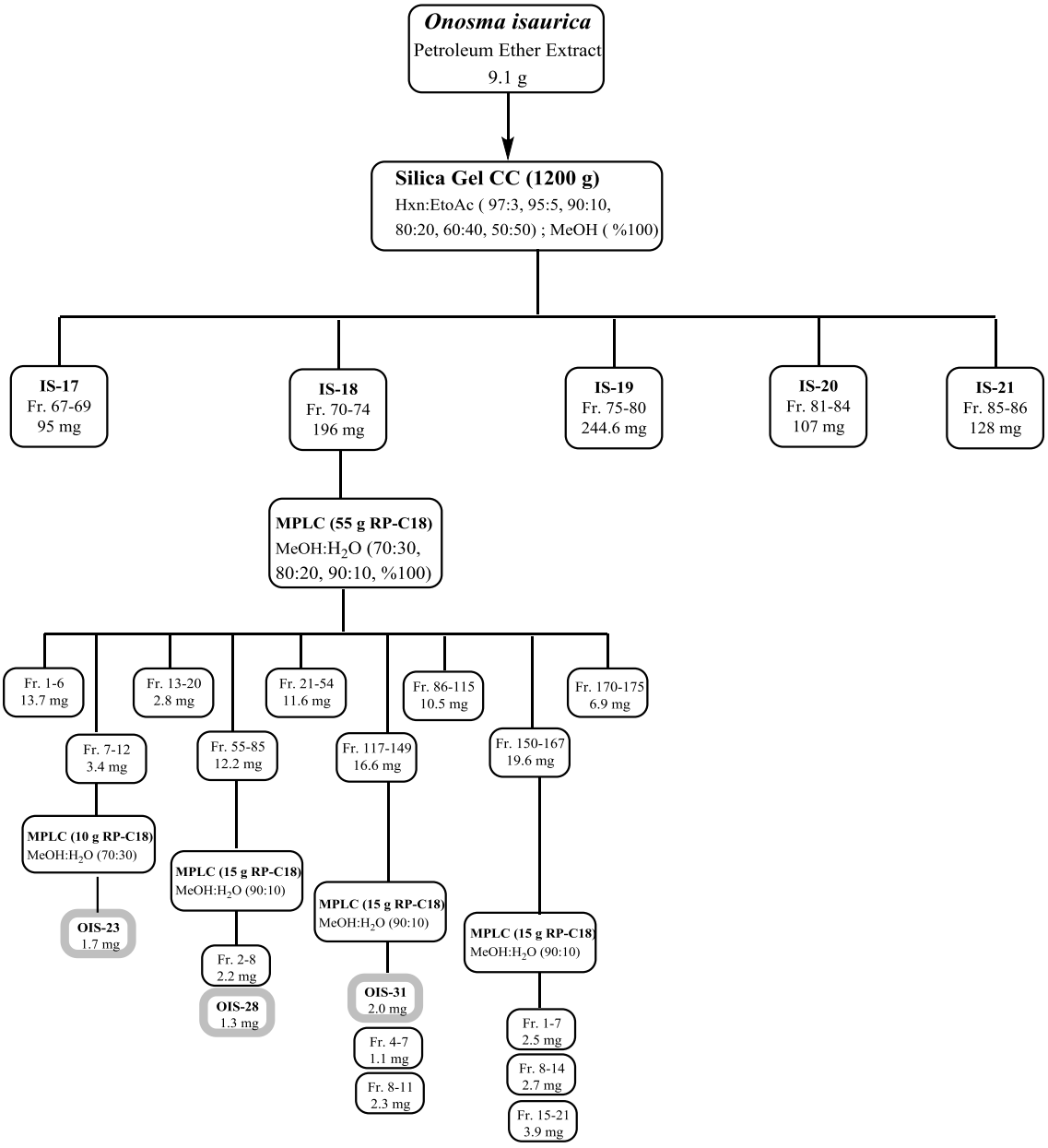


Figure 2. 5. Isolation studies on *O. isaurica*

2.3. Cytotoxicity Studies

MTT cell viability assay is a colorimetric method based on the conversion of a tetrazolium salt, 3-(4,5 dimethyl thiazol-2-yl)-2,5 diphenyl tetrazolium bromide, into blue formazan product by mitochondrial dehydrogenase.

Cytotoxic activity of the crude extracts, fractions and pure compounds were examined on various cancer cell lines such as A-549, Capan-1, DU-145, HCC-1937, HeLa, HEP-G2, MCF-7 and a non-tumorigenic cell line MRC-5 using MTT method. The cells were incubated in 96 well plates with RPMI-1640 (Sigma, USA) and DMEM (Invitrogen, USA) medium containing 10% fetal bovine serum (5% CO_2 in air) at 37°C. After incubation, test samples with concentrations ranging from 8 to 32 µg/mL were added to each well and incubated for 48 h. Later on, the medium was removed from the plates, 100 µL/well MTT working solution was added and incubated at 37°C in 5% CO_2 environment for 4 hours. The solution was decanted and 150 µL of DMSO added per well to dissolve the formazan crystals. The amount of blue formazan crystals was read on microplate reader at 570-690 nm. Formazan production is directly proportional to cell number and can be used to detect the presence of very small numbers of living cells. Considering to percent of cell viability, cytotoxicity levels of samples were determined.

2.4. DNA Topoisomerase Inhibition Studies

DNA topoisomerase inhibition tests were performed to state the activity of fractions and pure compounds using commercially obtained enzymes as explained below.

2.4.1. DNA Topoisomerase IIα and IIβ

A decatenation assay was carried out to determine the effects of the test samples on hTopo IIα and IIβ enzymes. This method based on the separation of open circular DNA monomers (OC-DNA) from covalently closed circular DNA molecules (CCC-DNA).

The unit activity of topoisomerases II is described as the decatenation activity of 200 ng kinetoplast DNA (kDNA) after incubation at 37°C for 30 min. Firstly, test

samples dissolved in DMSO at a 2 mg/ml were diluted with dH_2O in a ratio of 1:2, 1:10, 1:100.

The reaction mixture, which had a total volume of 20 µL, contained 17 µL of substrate solution, 2 µL of test sample and 1 µL of enzyme mixture. Substrate solution were comprised of 2 µL 10X Topo II buffer (50 mM Tris-HCI, (pH:8.0), 120 mM KCI, 10 mM $MgCI_2$, 5 mM DTT, 5 mM spermidine), 2 µL 0.1% ATP, 12 µL dH_2O and 1 µL (0.2 µg) supercoiled pBR322 (Inspiralis). Following incubation at 37°C and 30 minutes, 6 µL of bromophenol blue (6X DNA Loading Dye Solution, Thermo R0611) was added to each of the reaction tubes and centrifuged at 1000 rpm. Finally 20 µL of the reaction mixture were loaded onto gel electrophoresis (5 V/cm) prepared with 1.2% agarose.

For the preparation and running of agarose gel, TAE buffer (40 mm Tris-acetate (pH:8.0), 2 mM EDTA) was used. With the purpose of staining relaxation products of gel, 6 µL ethidium bromide (EtBr) was used. Then DNA bands in the gel was visualized under UV light.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Collection and Preliminary Processing of *Onosma* Species

Twelve different *Onosma* species were collected from several locations in Southwest Anatolia in May, June and July. Collected species including *O. strigossima, O. nydeggeri*, *O. frutescens*, *O. taurica* var. *taurica*, *O. alboroseum* subsp. *songiunolentum, O. inexpectata*, *O. giganteum*, *O. alboroseum* subsp. *albo-rosea* var. *albo-rosea*, *O. roussai*, *O. isaurica,* O. *mite* were identified by Dr. Ademi Fahri Pirhan and samples were stored at the Ege University Herbarium, Izmir, Turkey (Table3.1).

Table 3. 1. Collected *Onosma* species and their locations

Name	Analysis Code	Herbarium Code	Location	Date
**O. aksoyii*	G4-09	42718	Sütçüler-Beyşehir, Ayvalıpınar Village 1146 m 37º41.614'K 31º01.340'D	16.06.2017
O. strigossima	G5-01	3661	Kemer-Alacasu Cove, 109 m 36º32.086'K 30º33.464'D	27.04.2018
O. nydeggeri	G5-02	3662	Antalya-Kumluca/Altınyaka Neighborhood, 994m 36º33.825'K 30º20.843'D	27.04.2018
O. frutescens	G5-03	3663	Fethiye-Korkuteli road, 888 m 36º43.235'K 29º26.586'D	28.04.2018
O. taurica var. *taurica*	G5-6	3666	Fethiye-Korkuteli road/Dirmil, 1245 m 36º53.963'K 29º41.020'D	28.04.2018
O. albo-roseum subsp. *songuinolentum*	G5-10	3670	E87 highway, Antalya-Denizli road 1412 m 36º45.93'K 28º53.76'D	28.04.2018
O. inexpectata	G6-01	3671	Kızılcadağ-Çığlık plateau, 1576 m 37º2.080'K 29º59.941'D	05.06.2018

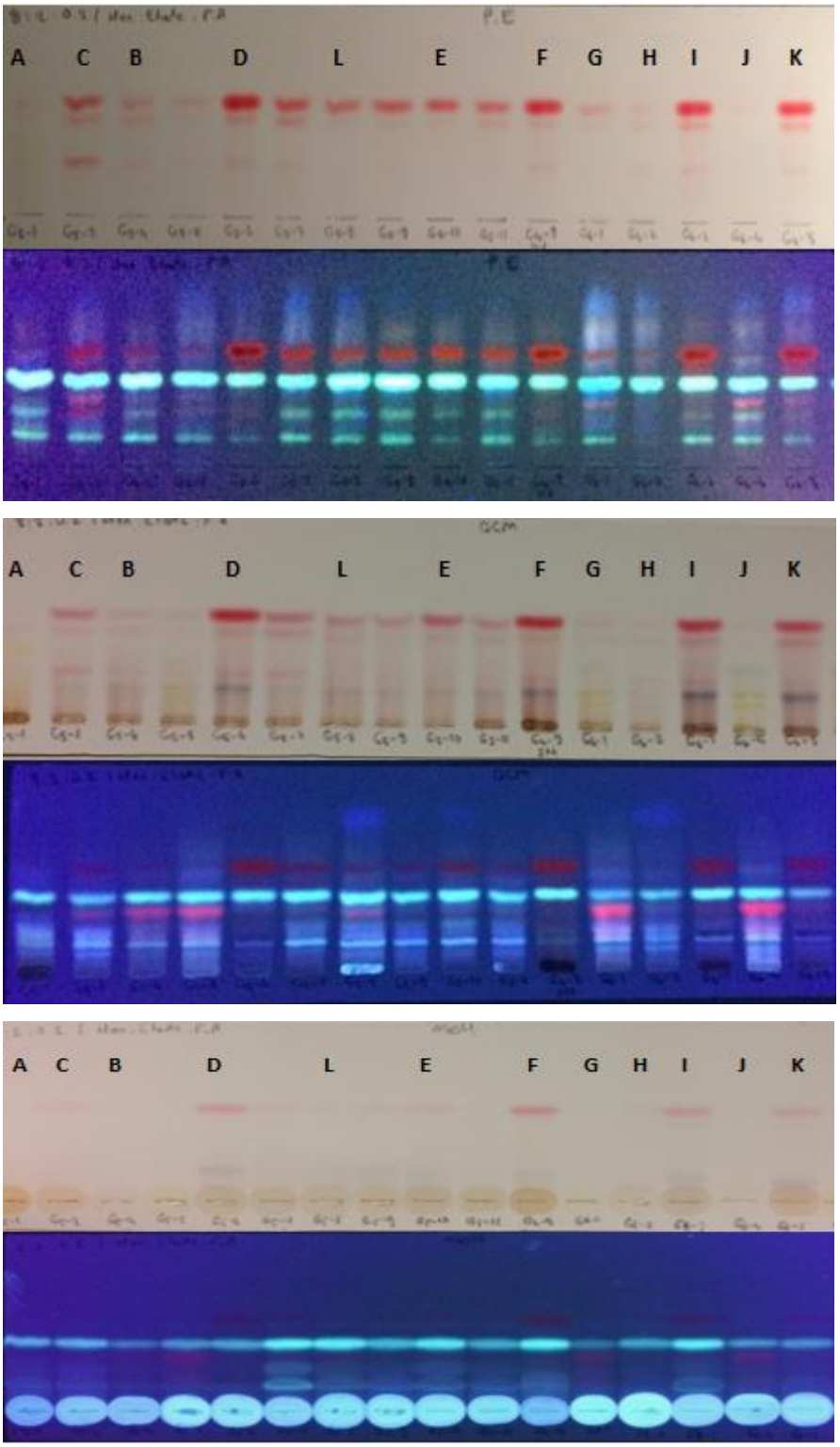
(cont. on next page)

Table 3.1. (Cont)

Name	Analysis Code	Herbarium Code	Location	Date
O. giganteum	G6-02	3672	Osmaniye/Hasanbey 1005 m 37°6.888'K 36°35.789'D	05.06.2018
O. alboroseum subsp. *albo-rosea* var. *albo-rosea*	G6-03	3673	Mersin, Cemilli Village 412 m 36°47.814'K 34°27.441'D	06.06.2018
O. roussai	G6-04	3675	Gülnar-Ermenek road, Bereket Neighborhood 1227 m 36°21.318'K 33°13.687'D	06.06.2018
**O. isaurica*	G6-05	3676	Gülnar-Ermenek road 24. km 1389 m 36°24.577'K 33°10.337'D	06.06.2018
O. mite	G7-02	3678	Termessos National Park 395 m 37°1.004'K 30°30.306'D	20.07.2018

3.2. TLC Profiles of Extracts

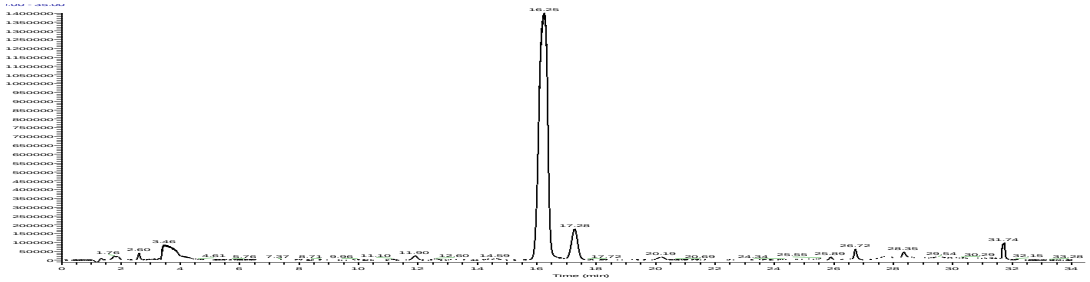
The TLC method was used to monitor the phytochemical profiles of *Onosma* species extracted with several solvents. The solvent system of hexane: ethyl acetate: formic acid (8:2:0.2) was used as a mobile phase (Chromatogram 2). In TLC plate, relatively non-polar compounds showed pink-reddish colored spots (which were presumably coming from naphthoquinone type compounds) under visible and 365 nm UV light. Depending on the phytochemical profiles of the extracts obtained from the powdered roots of *Onosma* species, it was concluded that naphthoquinone contents of the petroleum ether and dichloromethane extracts were richer than the methanol extracts. Besides, *O. taurica* var. *taurica*, *O. aksoyii*, *O. alboroseum* subsp. *albo-rosea* and *O. isaurica* were found to be richer in terms of naphthoquinone content compared to the other species. Based on the TLC profiles, the cytotoxic activity results and the amount of root materials available, two species were selected for further chromatographic studies.



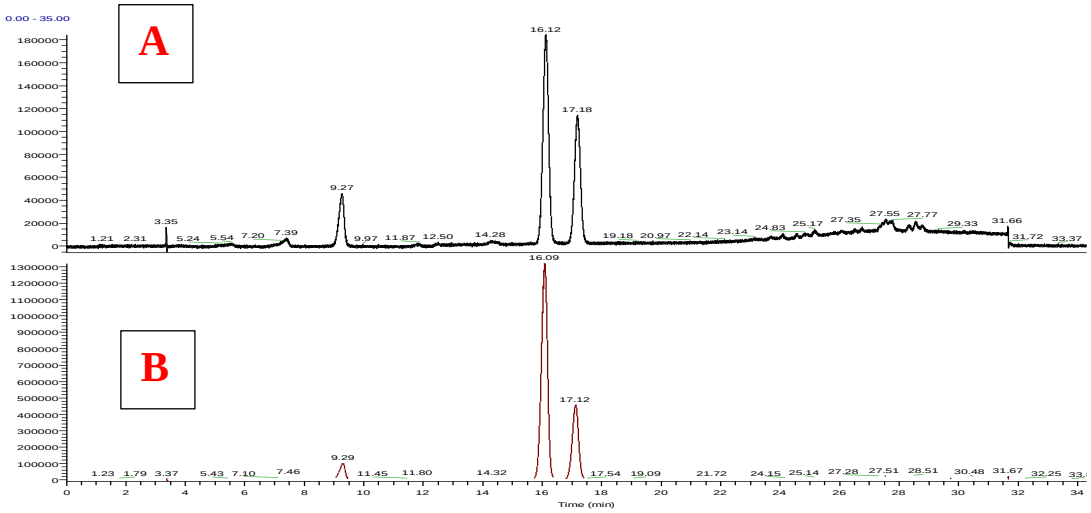
Chromatogram 3. 1. TLC chromatogram of the petroleum ether, dichlorometane and methanol extracts of different *Onosma* species (**A.** *O. strigossima*, **B.** *O. nydeggeri*, **C.** *O. frutescens*, **D.** *O. taurica* var. *taurica*, **E.** *O. alboroseum* subsp. *songiunolentum*, **F.** *O. aksoyii*, **G.** *O. inexpectata*, **H.** *O. giganteum*, **I.** *O. alboroseum* subsp. *albo-rosea* var. *albo-rosea*, **J.** *O. roussai*, **K.** *O. isaurica*, **L.** *O.mite*), (Silica gel, *n*-Hexane: Ethyl acetate: Formic acid; 8: 2: 0.2)

3.3. HPLC Fingerprinting Analysis

In the present study, HPLC fingerprinting analysis was employed to screen constituents of the crude extracts of twelve *Onosma* species. We aimed to detect the pigments, mainly the alkannin/shikonin derivatives, in the root extracts. Consequently, the absorbtion spectra were monitored at 520 nm a characteristic wavelength for naphthoquinones[43]. The relative retention time and relative peak area of each sample constituents were monitored with the standard (β,β-dimethylacrylshikonin) spectrum (See in chromatogram 3.1). The retention time of the most common peak was ca. 16-17 min in each sample as same reference compound.

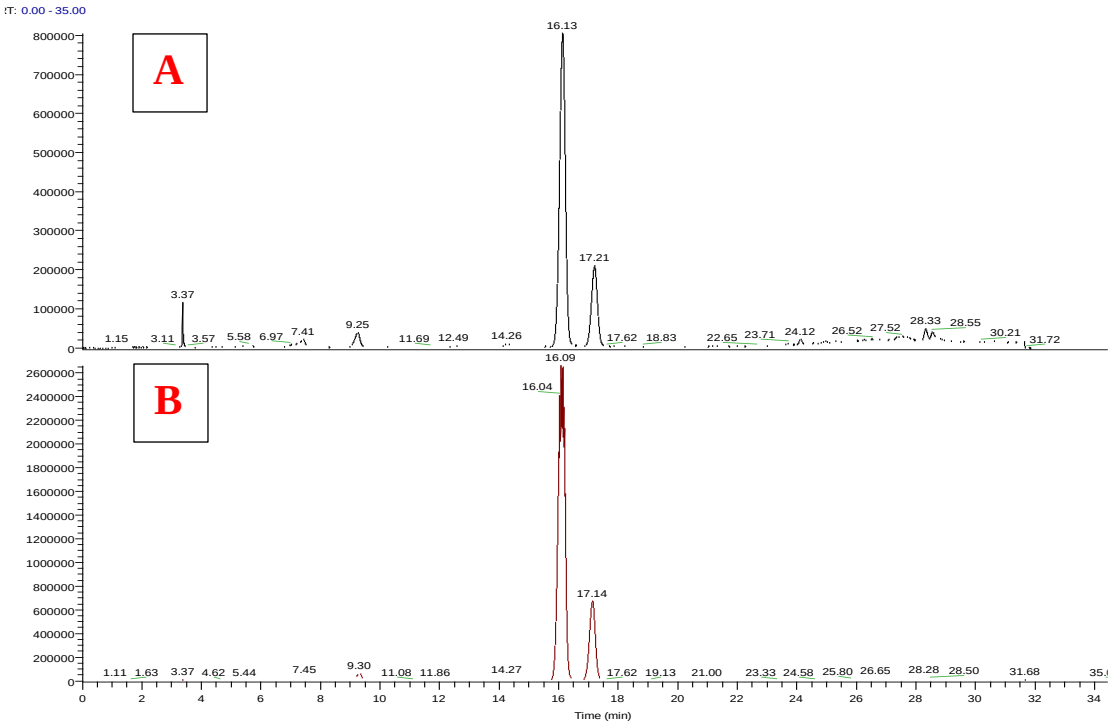


Chromatogram 3. 2. HPLC chromatogram of β,β-dimethylacrylshikonin (reference compound)

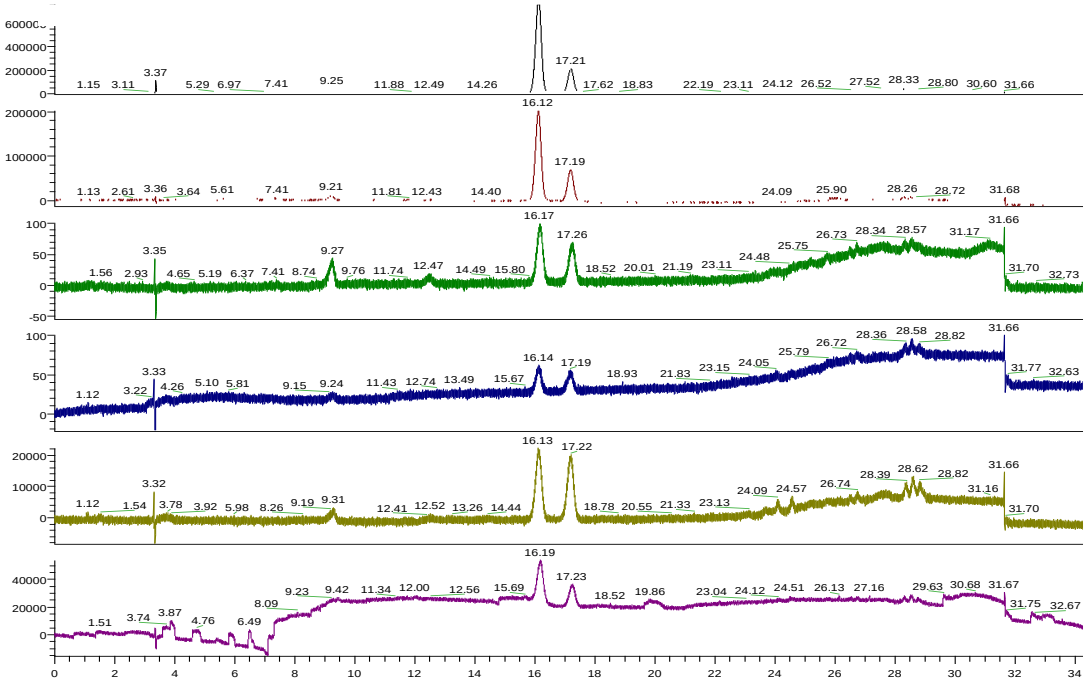


Chromatogram 3. 3. HPLC chromatograms of *O. isaurica* extracts at 520 nm

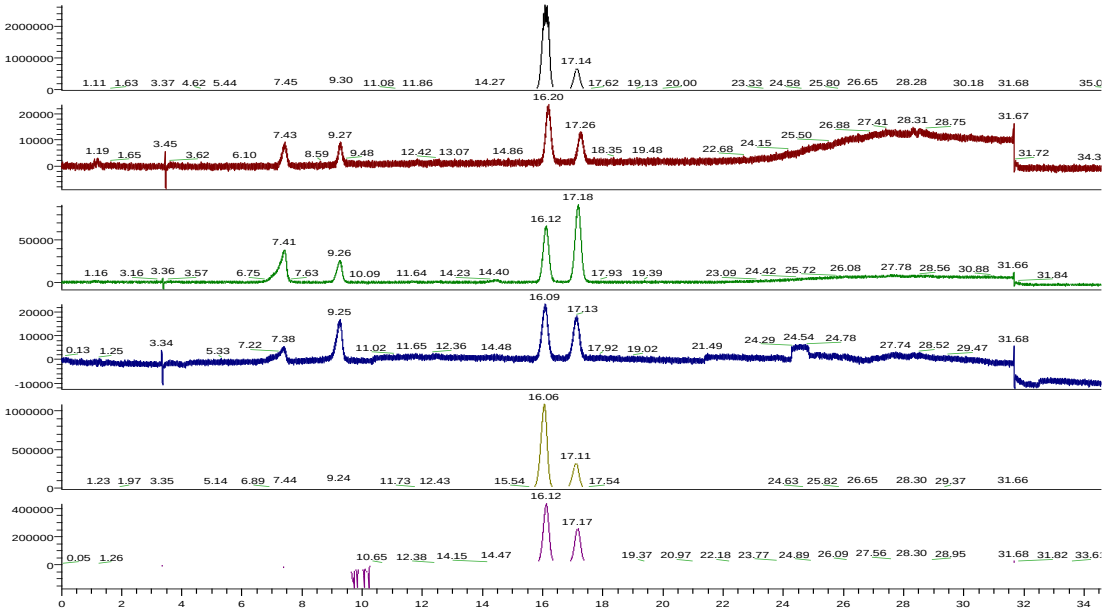
A. Dichloromethane **B.** Petroleum ether



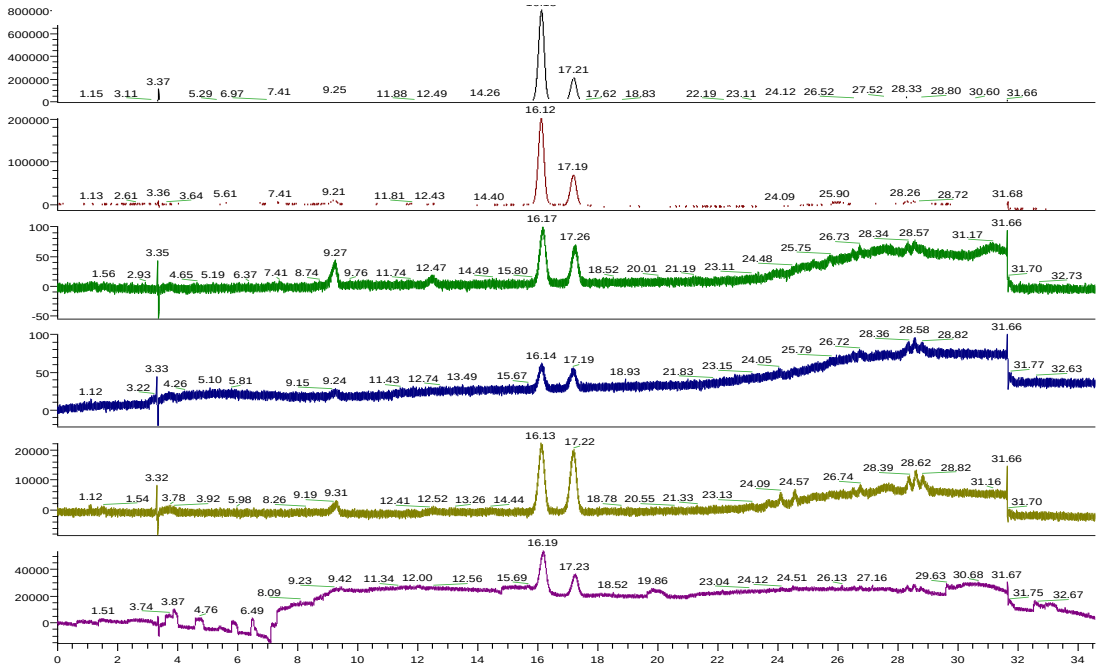
Chromatogram 3. 4. HPLC chromatograms of *O. aksoyii* extracts at 520 nm



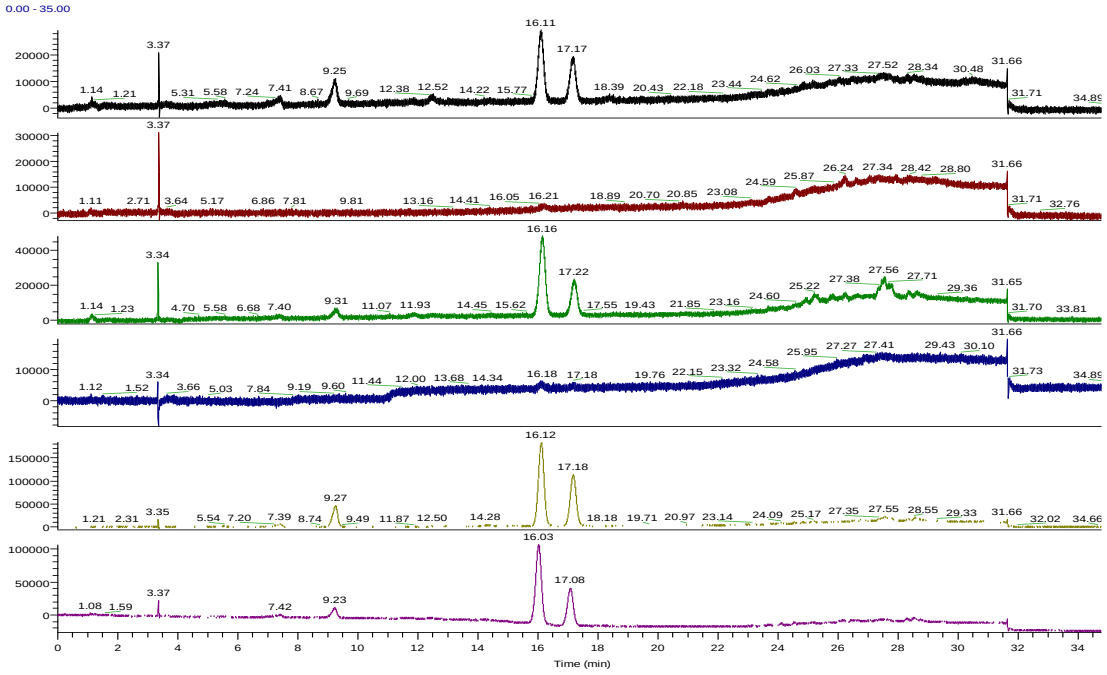
Chromatogram 3. 5. HPLC chromatograms of the petroleum ether (PE) extracts from different *Onosma* species at 520 nm. **1.** *O. albo-roseum* subsp. *albo-rosea* var. *albo rosea* (G6-3), **2.** *O. roussai* (G6-04), **3.** *O. isaurica* (G6-5), **4.** *O. mite* (G7-02), **5.***O. inexpectata* (G6-01) **6.** *O. giganteum* (G6-02).



Chromatogram 3. 6. HPLC chromatograms of the petroleum ether (PE) extracts from different *Onosma* species at 520 nm. **1.** *O. aksoyii* (G4-09), **2.** *O. strigossima* (G5-01),**3.** *O. nydeggeri* (G5-02), **4.** *O. frutescens* (G5-03), **5.** *O. taurica* var. *taurica* (G5-06),**6.** *O. albo-roseum* subsp. *songuinolentum* (G5-10)



Chromatogram 3. 7. HPLC chromatograms of the dichloromethane (DCM) extracts from different *Onosma* species at 520 nm. **1.** *O. aksoyii* (G4-09), **2.** *O.taurica* var. *taurica*(G5-06), **3.** *O. nydeggeri* (G5-02), **4.** *O. frutescens* (G5-03), **5.** *O. inexpectata*(G6-01)**6.** *O. albo-roseum* subsp. *songuinolentum* (G5-10).



Chromatogram 3. 8. HPLC chromatograms of the dichloromethane (DCM) extracts from different *Onosma* species at 520 nm. **1.** *O. mite* (G7-02), **2.** *O. strigossima* (G5-01),**3.** *O. albo-roseum* subsp. *albo-rosea* var. *albo rosea* (G6-3), **4.** *O. roussai* (G6-04),**5.** *O. isaurica* (G6-5), **6.** *O. giganteum* (G6-02)

When the literature was examined, it was seen that some fingerprinting studies had been carried out on the members of Boraginеceae family. A summerized explanation of those previous researches is given below.

Assimopoulou et al. investigated the root extracts of ten different *Alkanna* species to determine their hydroxynaphthoquinone contents by HPLC/PDA/MS. Samples collected from several regions of Greek flora were analyzed and the main naphthoquinone derivatives were determined as alkannin, acetylalkannin, propionylalkannin, isobutylalkannin, angelyalkannin, β,β-dimethylacrylalkannin, isovalerylalkannin, α-methyl-*n*-butylalkannin, teracrylalkannin and β-hydroxyisovalerylalkannin.[44]

In another study, naphthoquinone content and the cytotoxic activity of *O. visianii* root extracts were examined. As a result of HPLC analysis, seven shikonin derivatives were detected. Acetylshikonin (16.027 mg/g) was identified as the major compound along with notable amounts of isobutyrylshikonin (8.403 mg/g) and α-methylbutyrylshikonin (6.150 mg/g). On the other hand, 5,8-*O*-dimethyldeoxyshikonin, 5,8-*O*-dimethyl

isobutyrylshikonin and deoxyshikonin were found in small amounts (0.988, 1.398 and 2.953 mg/g, respectively).[45]

Özgen et. al. carried out a quantitative analysis on *n*-hexane-dichloromethane (1:1) extract of *O. armeniacum* using HPLC-DAD. Fingerprinting analysis was performed on RP-C18 column which was eluted with the isocratic system of acetonitrile:methanol:water (60:20:20; containing 2% acetic acid v/v) and analysis were monitored at 525 nm. The major compounds were detected as acetylshikonin, deoxyshikonin, 3-hydroxy-isovalerylshikonin and relative contents were 23.82 %, 3.63% and 2.53%, respectively.[46]

Based on the previous studies, the ideal HPLC method (stationary phase: RP-C18; mobile phase: a gradient system of ACN:H_2O) was chosen and adapted to our experiment[47]. Petroleum ether and dichloromethane extracts of 12 *Onosma* species were analyzed via HPLC-DAD method. With a few exceptions, the major constituents in all extracts tested here were determined as β,β-dimethylacrylshikonin due to the retention time.

When the chromatograms were examined in detail, *O. isaurica*, *O. aksoyii*, *O. frutescens* and *O. taurica* var. *taurica* were found to be richer in terms of naphthoquinone derivatives comparing to the other species. In the petroleum ether extract of *O. aksoyii*, nine signals implying the presence of naphthoquinones, were observed. Furthermore, eight naphthoquinones were determined in each petroleum ether extracts of *O. isaurica*, *O. frutescens* and *O. taurica* var. *taurica.* The chromatographic analysis showed that the naphthoquinone contents of petroleum ether extracts were greater than the dichloromethane extracts, which were richer in relatively polar compounds.

3.4. Structure Identification of Pure Compounds Isolated from *O. aksoyii* and *O. isaurica*

The research reported here focused on the isolation and characterization of naphthoquinone type compounds from *Onosma* species, phytohemical content of which had not been studied before. After preliminary HPLC screenings, we selected two *Onosma* species. As a result of our bioactivity guided isolation studies on selected plants (*O. aksoyii* and *O. isaurica*), six compunds were obtained.

The structures of the five known compounds were elucidated as arnebifuranone, arnebidin, shikonofuran E, β,β-dimethylacrylshikonin and acetylshikonin via modern spectroscopic methods.

This is the first isolation and structural elucidation report on these species. A previously undescribed monoterpenylbenzoquinone derivative was isolated from *O. aksoyii* within the concept of this thesis.

On the other hand, several minor signals implying to naphthoquinones were also detected in the HPLC chromatograms. But we were not able to isolate those minor compounds owing to the insufficient amount of the root materials available.

3.4.1. Structure Identification of OA-PE-D1

In the HR-ESI-MS spectrum (negative mode) of **OA-PE-D1**, a major ion peak $[M]^{-}$. was observed at *m/z* 314.09883. When mass spectral data was evaluated together with ^{1}H and ^{13}C NMR data, it was determined that **OA-PE-D1** had a molecular formula of $C_{18}H_{18}O_5$ (See Fig. 3.1). **Optical Rotation:** $[\alpha]_D^{24} = -8°$ (MeOH, c = 0.32).

The ^{1}H- and ^{13}C-NMR and ^{1}H-^{1}H COSY ($CDCI_3$) spectra of **OA-PE-D1** partially suggested a benzoquinoid structure, displaying characteristic signals of two carbonyl carbons (δ 184.7 and δ 183.8) and a proton signals shifted downfield (6.21 d, *J*=2.8 Hz). Two methoxy methyl signals were deduced from δ 4.01 (s, 3H, C-2-O*Me*) and 3.99 (s, 3H, C-3- O*Me*) signals from the ^{1}H-NMR spectrum, which showed HMBC correlations (See Fig 3.1.b) with double bond carbon signals at δ_C 144.6 (C-3) and 145.4 (C-2) in the HMBC spectrum. The deshielded proton observed at δ 6.21 (H-5) showed $^{3}J_{H\text{-}C}$ correlations with C-3 (δ 144.6) and C-1 (δ 183.8), and the absence of any correlation from H-5 to other carbons in the benzoquinone ring suggested that the methoxy groups were *ortho* positioned in 1,4-benzoquinone skeleton. Thus the first structure inferred from the NMR spectra was 2,3-dimethoxy-1,4 benzoquinone fragment. When the mass of the quinone unit was subtracted from the molecular formula, the remaining section of the molecule was consistent with $C_{10}H_{11}O$ indicating a hydrogen deficiency number of 5. The presence of ten carbon atoms in the second fragment revealed a monoterpene moiety. A spin system including two methylene group and an olefinic proton was deduced from the COSY spectrum (δ 2.08 m, 2H, H_2-12; δ 2.48 m and 2.57 m, H_2-13; δ 5.76 m, H-14). Based on the HSQC spectrum, the carbon resonances of these protons were assigned to

be 24.9 (C-12), 24.1 (C13) and 129.1 (C-14), respectively. A methyl resonance was observed at δ 1.90 (s, 3H, H-16) in the ^{1}H-NMR spectrum revealing its close proximity to a double bond system. Indeed, this proton showed strong long-range correlations with C-14 (δ 129.1) and another olefinic proton at δ 135.5 (C-15), helping us to establish the first substructure of the monoterpenoid unit as -CH_2-CH_2-CH=C(CH_3)- and accounted for one of 5 unsaturations. Additionally, two key correlations from a methine carbon, deduced from the HSQC spectrum (δ 42.7 and δ 4.60 s, CH-7), to the methyl proton (δ 1.90, CH_3-16) and H-5 verified that this carbon was at the intersection of aforementioned spin system, quinone ring and the remaining section of **OA-PE-D1**. When further molecular formula deduction was made, the last fragment of the compound was found to be C_4H_2O. Since all of the remaining carbon resonances were observed in the aromatic region ((δ 145.0, 140.6, 122.8, 112.7), two of the carbons were deduced to be methine based on the HSQC spectrum and two of the downfield shifted resonances were neighboring an electron withdrawing oxygen atom, a disubstituted furan ring was readily inferred. Inspection of the HMBC spectrum showed that H-7 was correlating with C-8 of the furan ring, whereas H_2-13 displayed a cross peak with C-10, indicating an unusual ring closure and explaining hydrogen deficiency number of 5 for the monoterpenoid unit.

Consequently, the structure of **OA-PE-D1** was determined to be 2,3-dimethoxy-5((4Z,7Z),5-methyl-8-oxabicyclo[5.2.1]deca-1(9),4,7(10)-trien-6-yl)cyclohexa-2,5-diene-1,4-dione. A literature research revealed that **OA-PE-D1** was a previously undescribed compound.

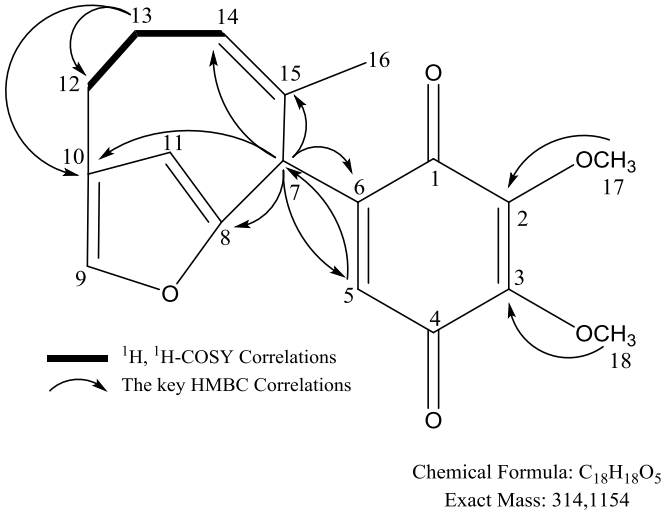
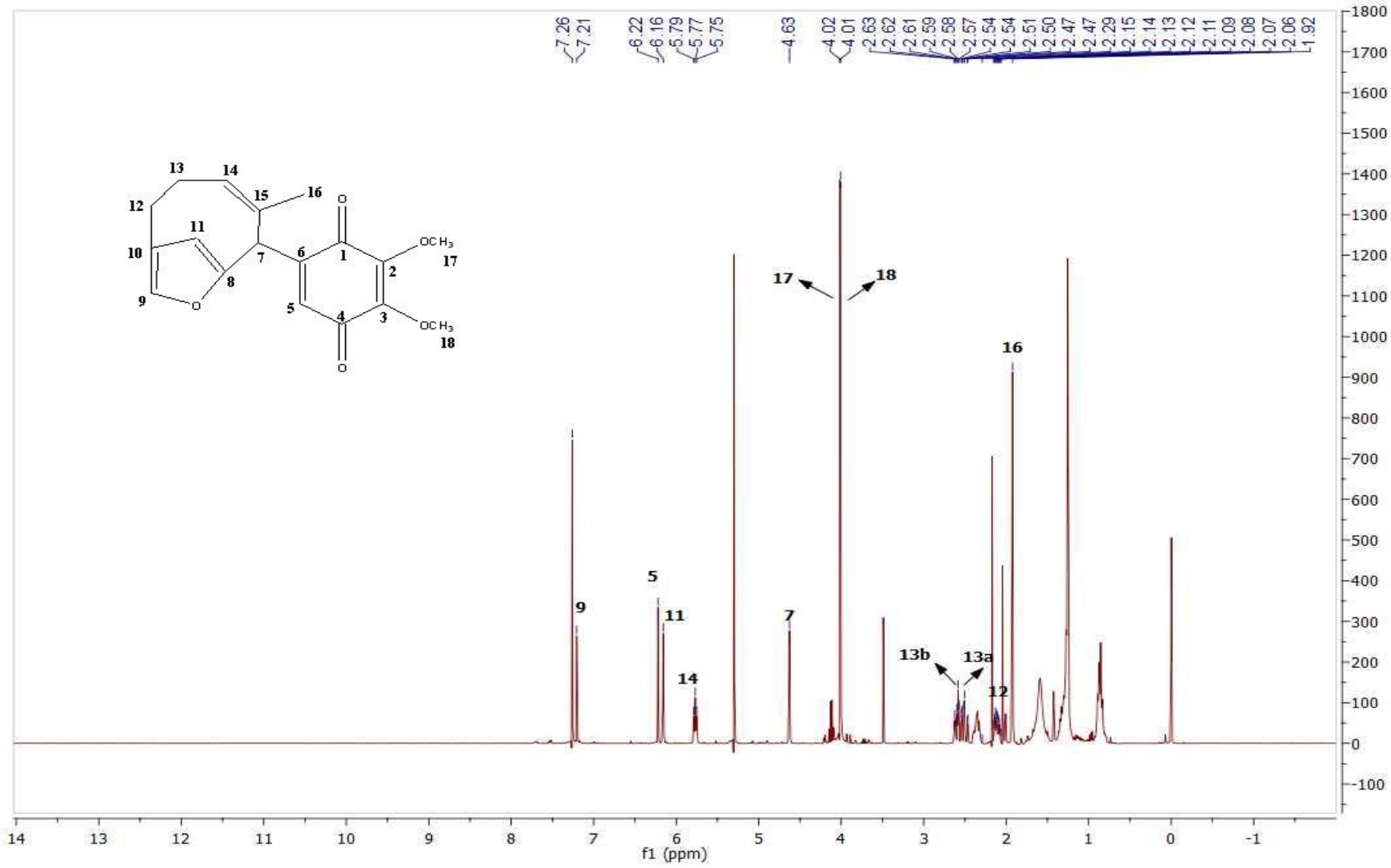


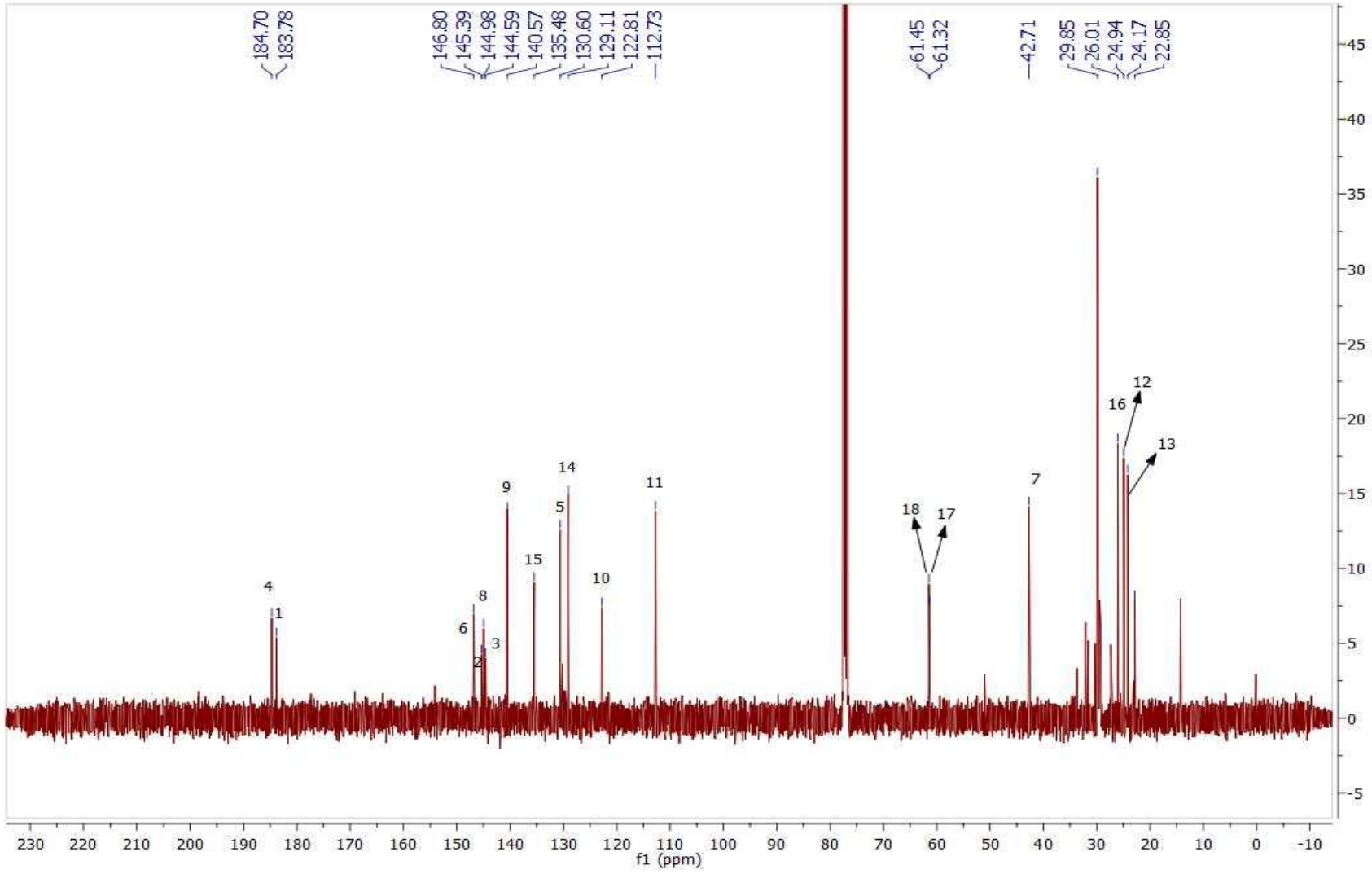
Figure 3. 1. Chemical structure of OA-PE-D1 and related ^{1}H-^{1}H COSY and HMBC correlations

Table 3. 2. ^{1}H and ^{13}C NMR Data of **OA-PE-D1** (400 MHz, δ ppm, in $CDCI_3$)

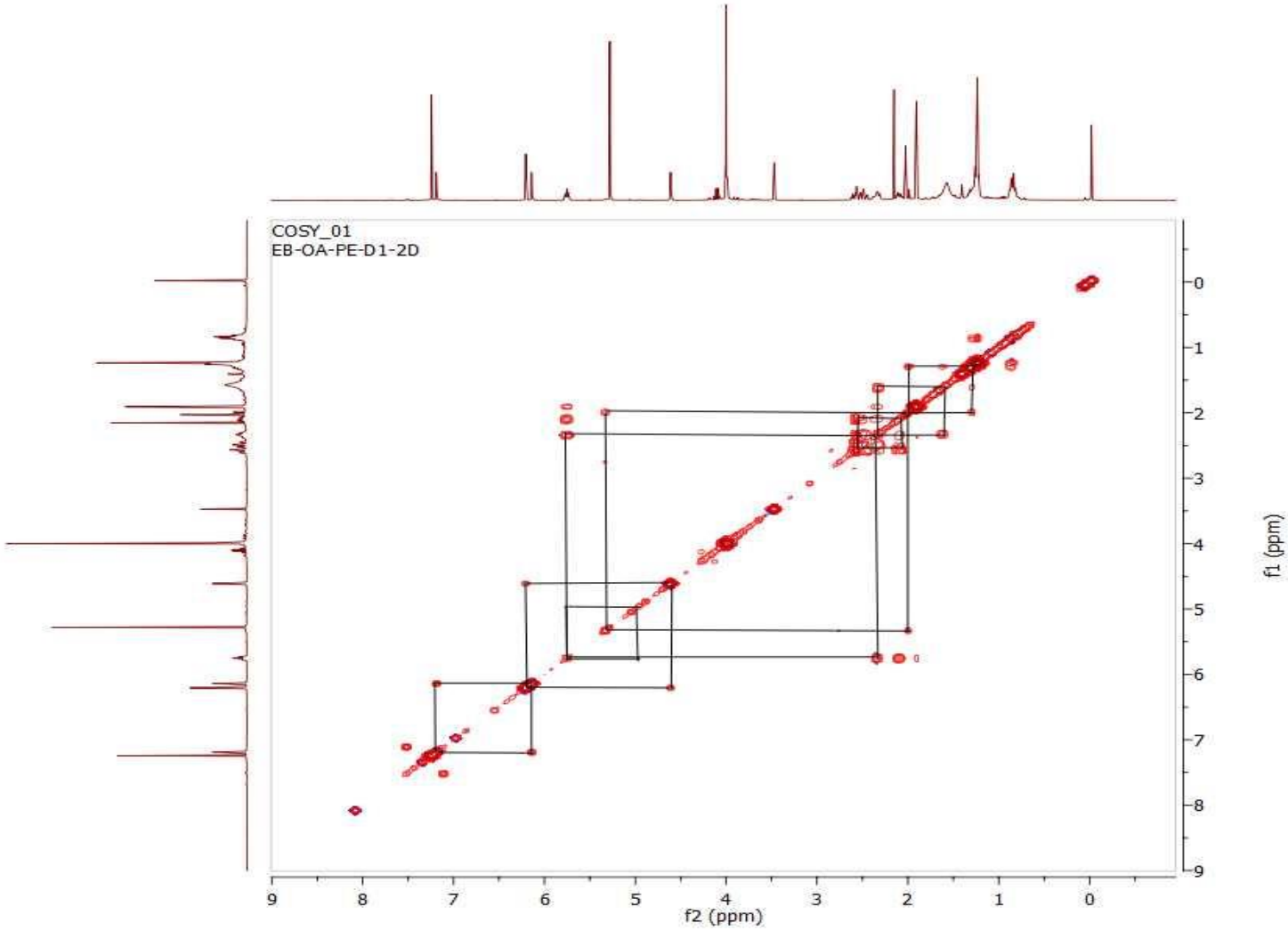
H/C	**Mult.**	**δ_H (ppm)**	***J* (Hz)**	**δ_C (ppm)**
1	C			183.8
2	C			145.4
3	C			144.6
4	C			184.7
5	CH	6.21	*d* (2.8)	130.6
6	C			146.8
7	CH	4.60	*s*	42.7
8	C			145.0
9	CH	7.19	*d* (2.8)	140.6
10	C			122.8
11	CH	6.15	*s*	112.7
12	CH_2	2.08	*m*	24.9
13	CH_2	2.48 2.57	*m* *m*	24.1
14	CH	5.76	*m*	129.1
15	C			135.5
16	CH_3	1.90	*s*	29.9
17	CH_3	4.01	*s*	61.5
18	CH_3	3.99	*s*	61.3



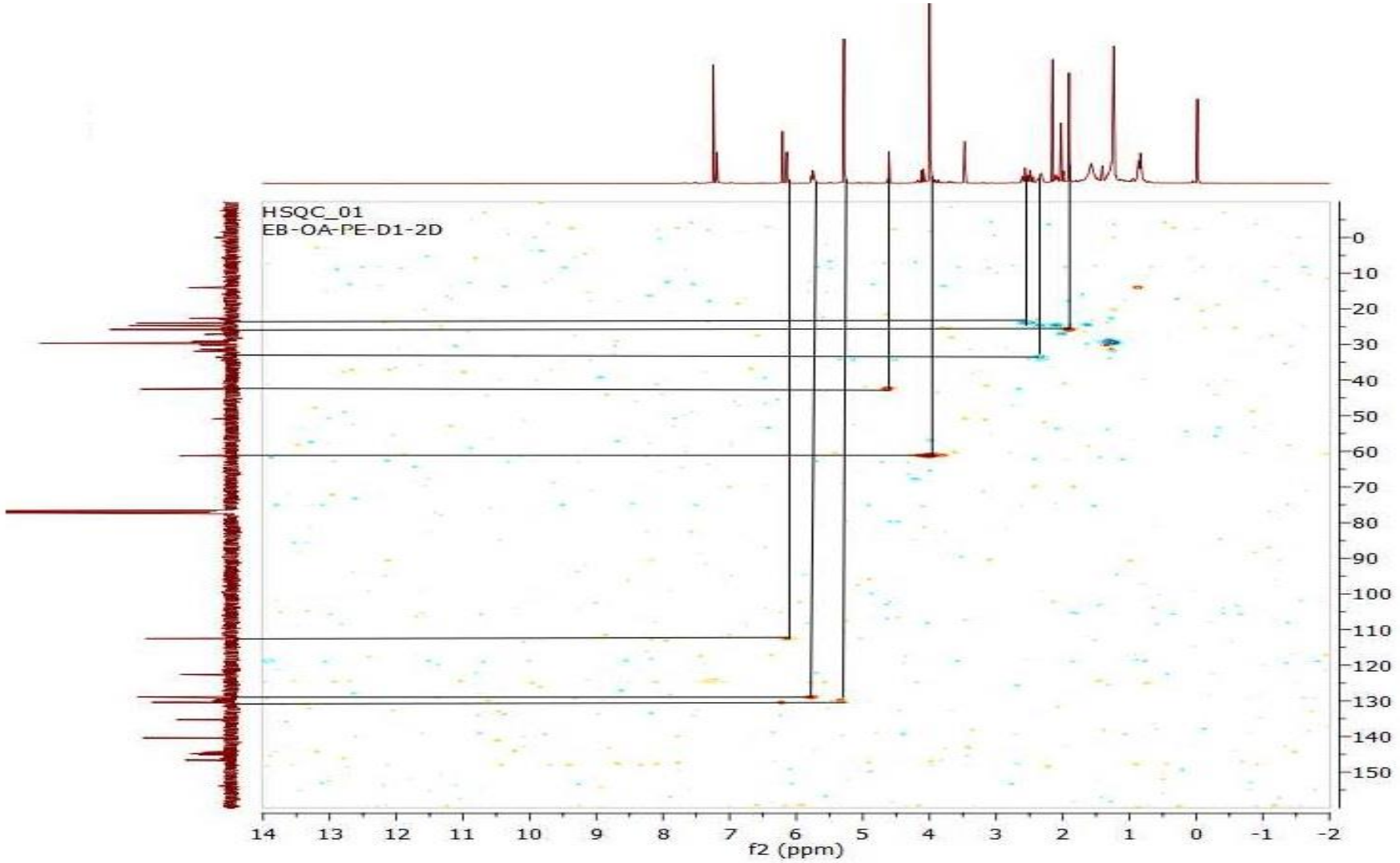
Spectrum 3. 1. ^{1}H NMR Spectrum of **OA-PE-D1**



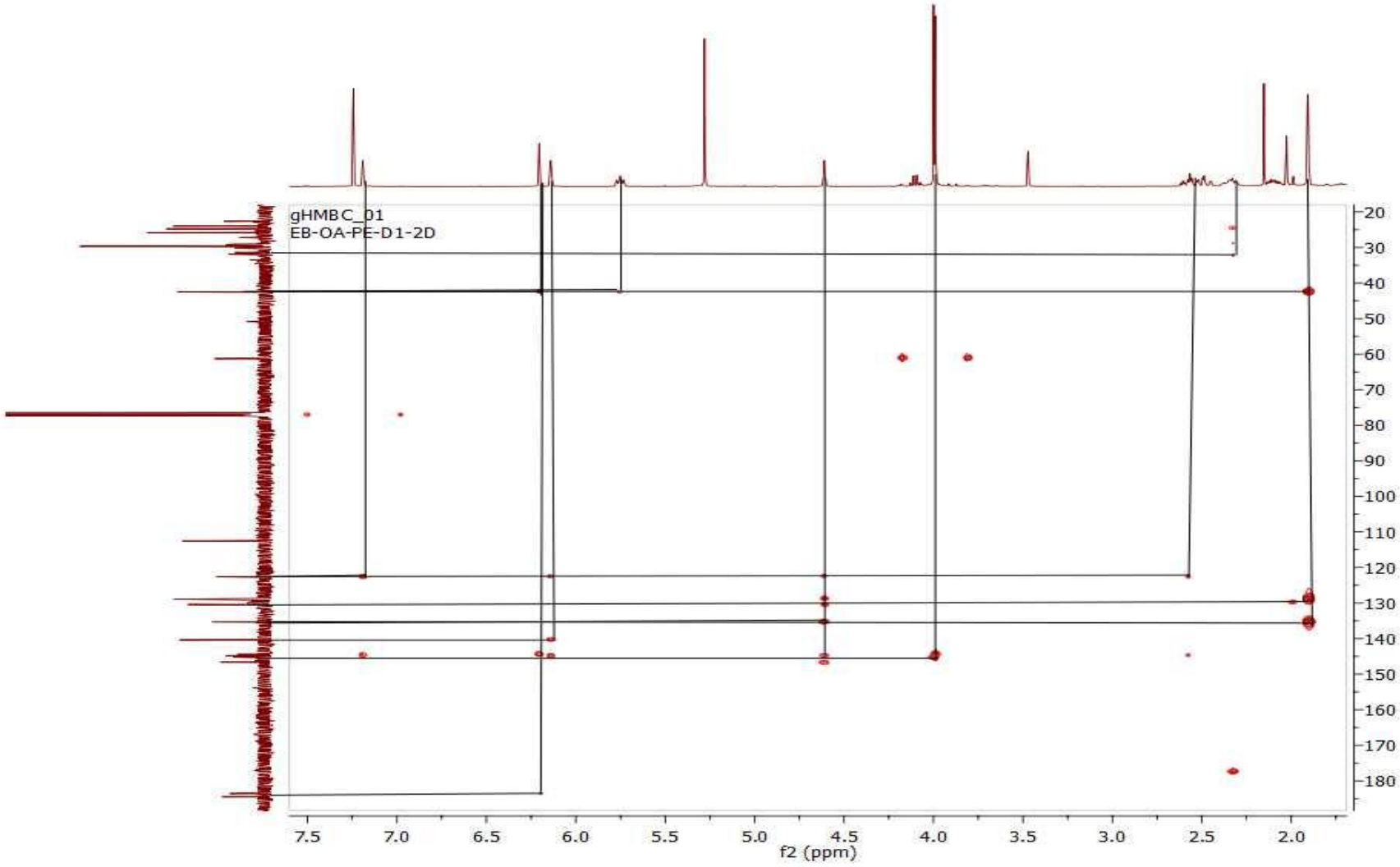
Spectrum 3. 2. ^{13}C NMR Spectrum of **OA-PE-D1**



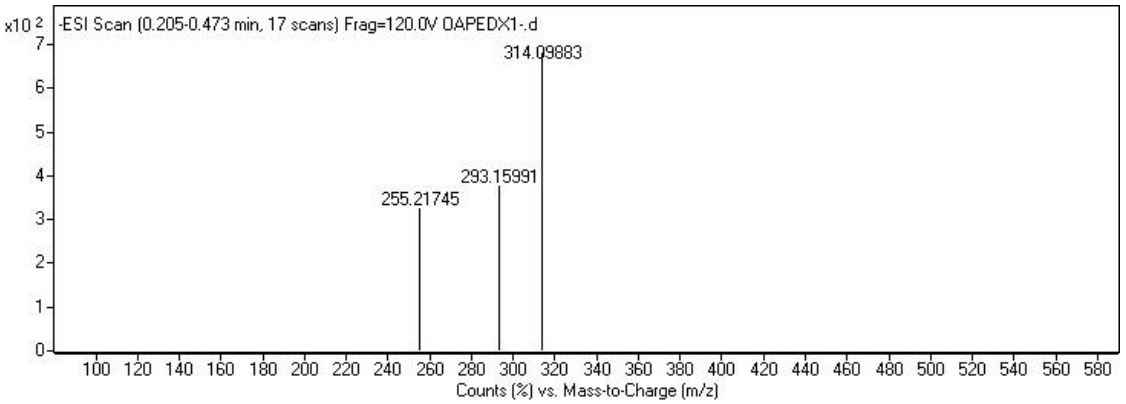
Spectrum 3. 3. COSY NMR Spectrum of **OA-PE-D1**



Spectrum 3. 4. HSQC NMR Spectrum of **OA-PE-D1**



Spectrum 3. 5. HMBC NMR Spectrum of **OA-PE-D1**



Spectrum 3. 6. HR-ESI-MS Spectrum of **OA-PE-D1** (negative mode)

Meroterpenoids could be explained as secondary metabolites bearing a quinonoid ring and a terpenoid subunit. This chemical group is originated from a collective biosynthetic pathway of polyketides and terpenoids[40].

Previously, a number of meroterpenoids have been isolated form higher plants, fungi and some marine orgaisms[48]. Reyes et. al. reported twelve meroterpenoids obtained from a brown alga, *Cystoseira usneoides*. According to their bioactivity results performed on the isolates; cystodiones A and B exhibited strong antioxidant activity. Moreover, *E* and *Z* isomer of usneoidone showed remarkable antiinflamatory activity, inhibiting TNF-α in LPS-stimulated THP-1 human macrophages[48].

Most recently, Wang et. al. carried out a deep research on *Arnebia euchroma* (Royle) Johnst. and they isolated 13 meroterpenoid with cytotoxic activity tested against human hepatocellular carcinoma cells[49].

Wang et. al. have also reported very promising anticancer bioactivity results for another new meroterpenylbenzoquinone derivative, isolated from the roots of *Lithospermum erythrorhizon*[49]. This compound (JNU-144), is structurally related to our new pigment OA-PE-D1. They revealed the cytotoxicity of JNU-144 on hepatoma cells *in vitro* and *in vivo*. The mechanism of cytotoxic effect of JNU-144 was explained via downregulating mTOR activation. As stated in the literature, the cytotoxic effect of JNU-144 is caused by the activation of the intrinsic apoptosis pathway.

All these reports mentioned above simply therapeutic potential of our new meroterpenoid isolate warranting for further bioactivity studies.

Additionally, to our knowledge, a meroterpenoid derivative is encountered for the first time *Onosma* genus.

3.4.2. Structure Identification of OA-PE-D2 (Arnebifuranone)

OA-PE-D2 was obtained as a dark yellow oil, and a yellow/orange band was seen on the TLC plate under visible light andUV was implied that the compound might be a benzoquinone derivative as **OA-PE-D1**. In the HR-ESI-MS spectrum (negative mode) of **OA-PE-D2**, a major ion peak was observed at *m/z* 331.1014 $[M-X]^-$. When the MS data was evaluated together with ^1H and ^{13}C NMR spectra, it was determined that **OA-PE-D2** had molecular formula of $C_{18}H_{20}O_5$.

By investigation of the overall ^1H NMR spectrum of **OA-PE-D2**, it was realized that the compound has a quite different structure from classical naphthoquinones. In the low field of ^1H NMR spectrum, three proton signals at δ 6.22 (*s*), 7.20 (*s*) and 7.33 (*s*) were assigned to the protons of a furan ring also verifying monosubstitution. A signal of methylene group at δ 2.44 (*t*, *J*=7.5 Hz, 2H) showed long range couplings with furan protons δ 7.20 (*s*) and 6.22 (*s*), indicating that the furan ring linked to through a methylene group to the rest of the molecule. In the ^{13}C NMR spectrum, the presence of the signals indicating typical dimethoxy-benzoquinone carbons as in **OA-PE-D1** [δ: 184.3 (C-1), 144.4 (C-2), 145.3 (C-3), 184.4 (C-4), 130.5 (C-5), 145.2 (C-6)] and the resonances of a monosubstituted furan ring [δ: 124.6 (C-12), 139.1 (C-13), 142.9 (C-14), 111.1 (C-15)] as part of a monoterpenoid fragment were evident. ^1H and ^{13}C NMR data is given in Table 3.3. As a result, the structure of **OA-PE-D2** was identified as another monoterpenylbenzoquinone derivative, arnebifuranone.[50]

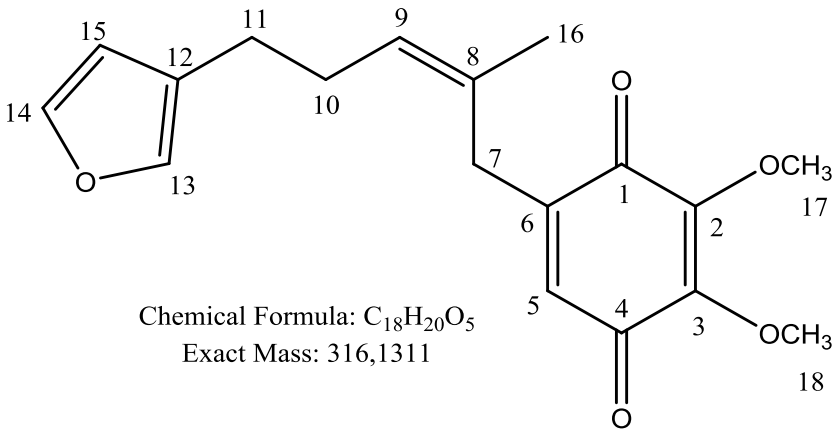
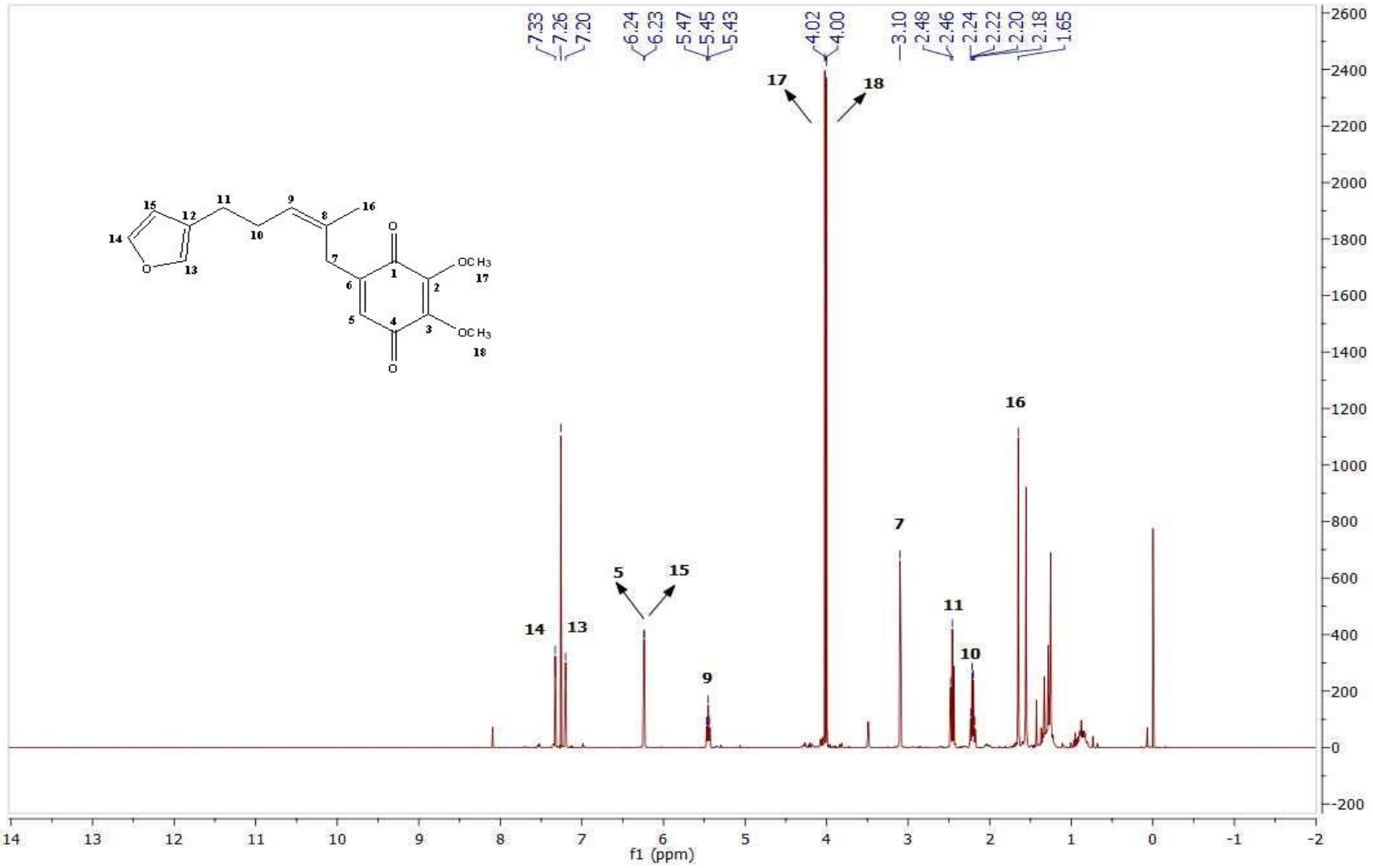


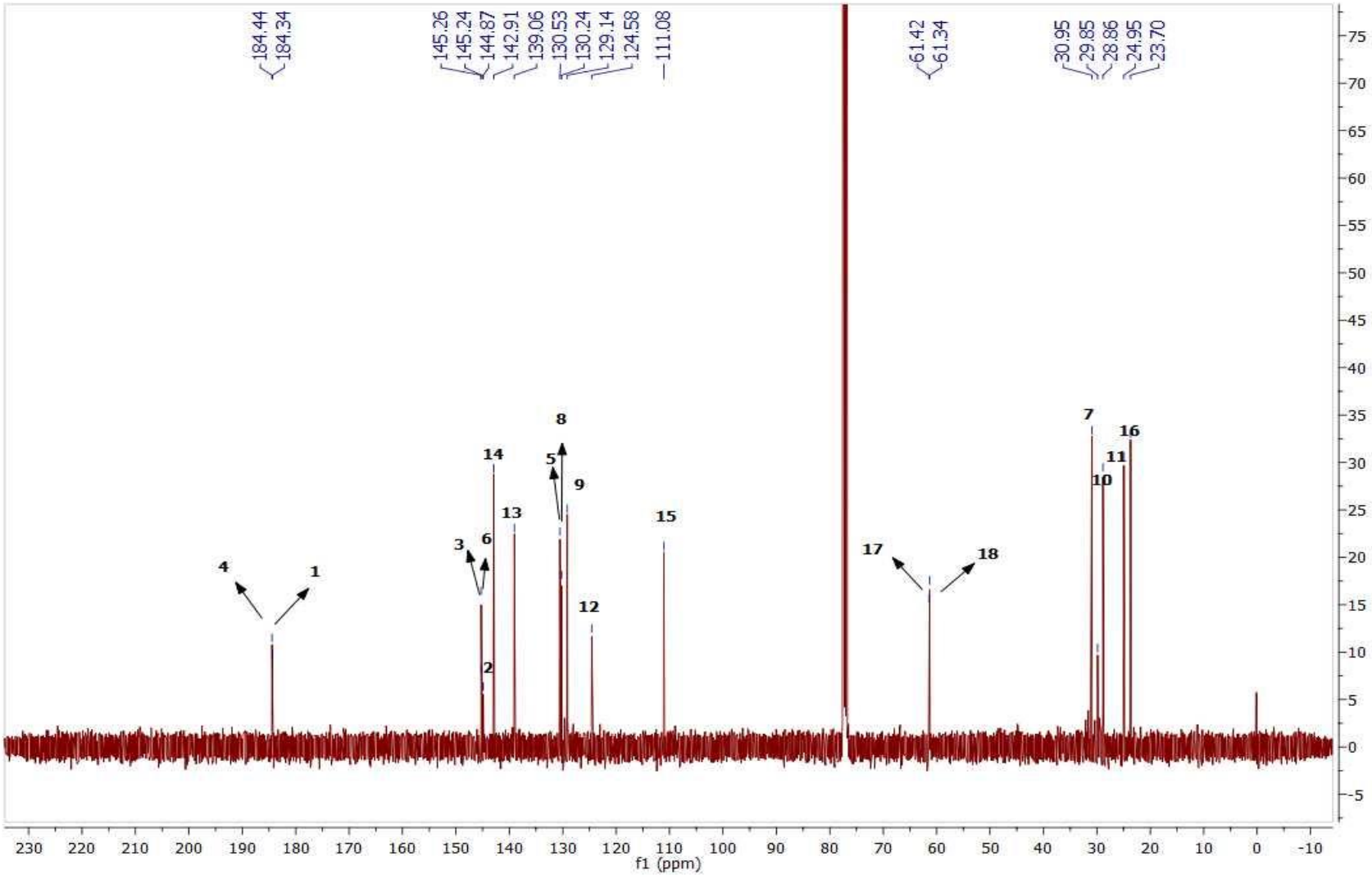
Figure 3. 2. Chemical structure of OA-PE-D2

Table 3. 3. ^{1}H and ^{13}C NMR Data of **OA-PE-D2** (400 MHz, δ ppm, in $CDCI_3$)

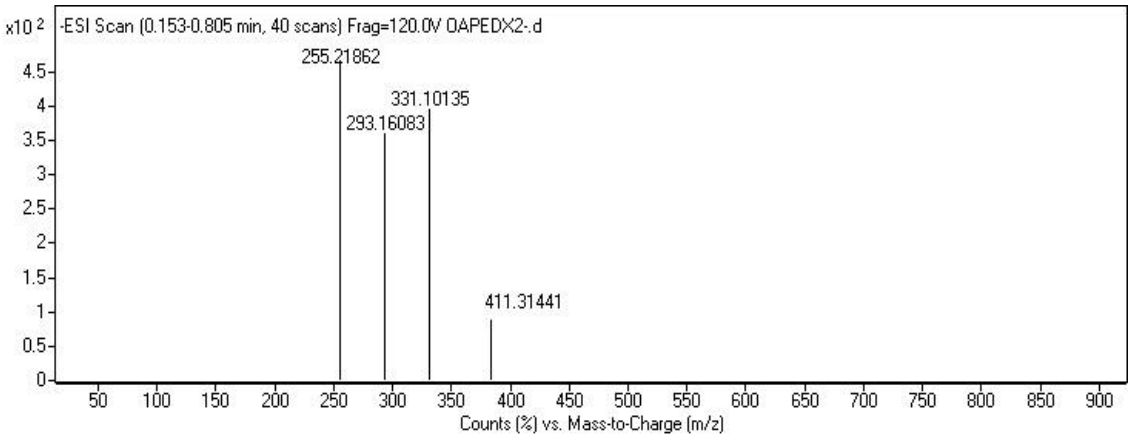
H/C	**Mult.**	**δ_H (ppm)**	***J* (Hz)**	**δ_C (ppm)**
1	C			184.3
2	C			144.4
3	C			145.3
4	C			184.4
5	CH	6.23	*s*	130.5
6	C			145.2
7	CH_2	3.08	*d* (1.4)	30.9
8	C			130.2
9	CH	5.44	*t* (7.0)	129.1
10	CH_2	2.19	*q* (7.4)	29.8
11	CH_2	2.44	*t* (7.5)	24.8
12	C			124.6
13	CH	7.20	*s*	139.1
14	CH	7.33	*s*	142.9
15	CH	6.22	*s*	111.1
16	CH_3	1.63	*s*	23.7
17	CH_3	4.02	*s*	61.4
18	CH_3	3.99	*s*	61.3



Spectrum 3. 7. ^{1}H NMR Spectrum of **OA-PE-D2**



Spectrum 3. 8. ^{13}C NMR Spectrum of **OA-PE-D2**



Spectrum 3. 9. HR-ESI-MS Spectrum of **OA-PE-D2** (negative mode)

Arnebifuranone, another meroterpenoid, was firstly isolated from *Arnebia euchroma* (Boraginaceae)[42, 51]. Later on, a retrosynthetic analysis on isoarnebifuranone was reported together with a detailed structural elucidation of arnebifuranone[52].

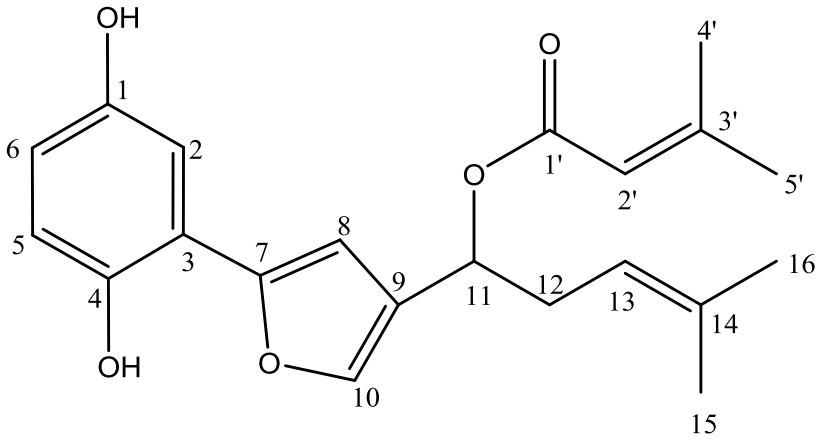
Also, *Arnebia euchroma* was subjected to another isolation study by Wang and coworkers, recently. They were able to obtain 12 meroterpenoids together with arnebifuranone[37]. Afterwards they presented the associated NMR data for those arnebifuranone derivatives[53].More recently, Shukla and coworkers have isolated the pigment from *Arnebia hispidissima*[54]. This is the first report on the isolation of arnebifuranone from the genus *Onosma* L.

3.4.3. Structure Identification of OA-PE-D3 (Shikonofuran E)

OA-PE-D3 was obtained as a pale yellow oil that acts more polar than **OA-PE-D2** (arnebifuranone) on the thin-layer chromatogram (stationary phase: silica gel; mobile phase: cyclohexane: EtOAc: F.A). In the HR-ESI-MS spectrum (positive mode) of **OA-PE-D3**, a major ion peak was observed at *m/z* 379.1583 $[M+Na]^+$ indicating the molecular formula of $C_{21}H_{24}O_5$. **Optical Rotation:** $[\alpha]^{25}$: -36° (*c* 0.1, MeOH).

By detailed investigation of the ^{1}H NMR spectrum of **OA-PE-D3**, the presence of three protons assignable to a 1,3,4-tri-substituted benzene ring [δ: 7.01 (*d*, *J*=3 Hz, 1H), 6.81 (*d*, *J*=8.7 Hz, 1H), 6.68 (*dd*, *J*=8.7, 3.0 Hz, 1H)], two aromatic protons characteristic for a furan ring [δ: 7.43 (*s*, 1H) ; 6.71 (*s*, 1H)], a vinyl proton δ 5.10 (*td*, *J*=7.5, 3.9 Hz, 1H) and two methyl groups [δ: 1.69 (*s*, 3H) and 1.62 (*s*, 3H)] indicated that it was a

shikonofuran derivative. In addition, the proton signals observed at δ 5.70 (*dd*, *J*=2.6, 1.3 Hz, 1H), 2.17 (*d*, *J*=1.2 Hz, 3H) and 1.89 (*s*, 3H) pointed out the presence of a dimethylacryl moiety bonded to C-11. Eventually, the structure of **OA-PE-D3** was determined as shikonofuran E, based on the spectral data.[55]

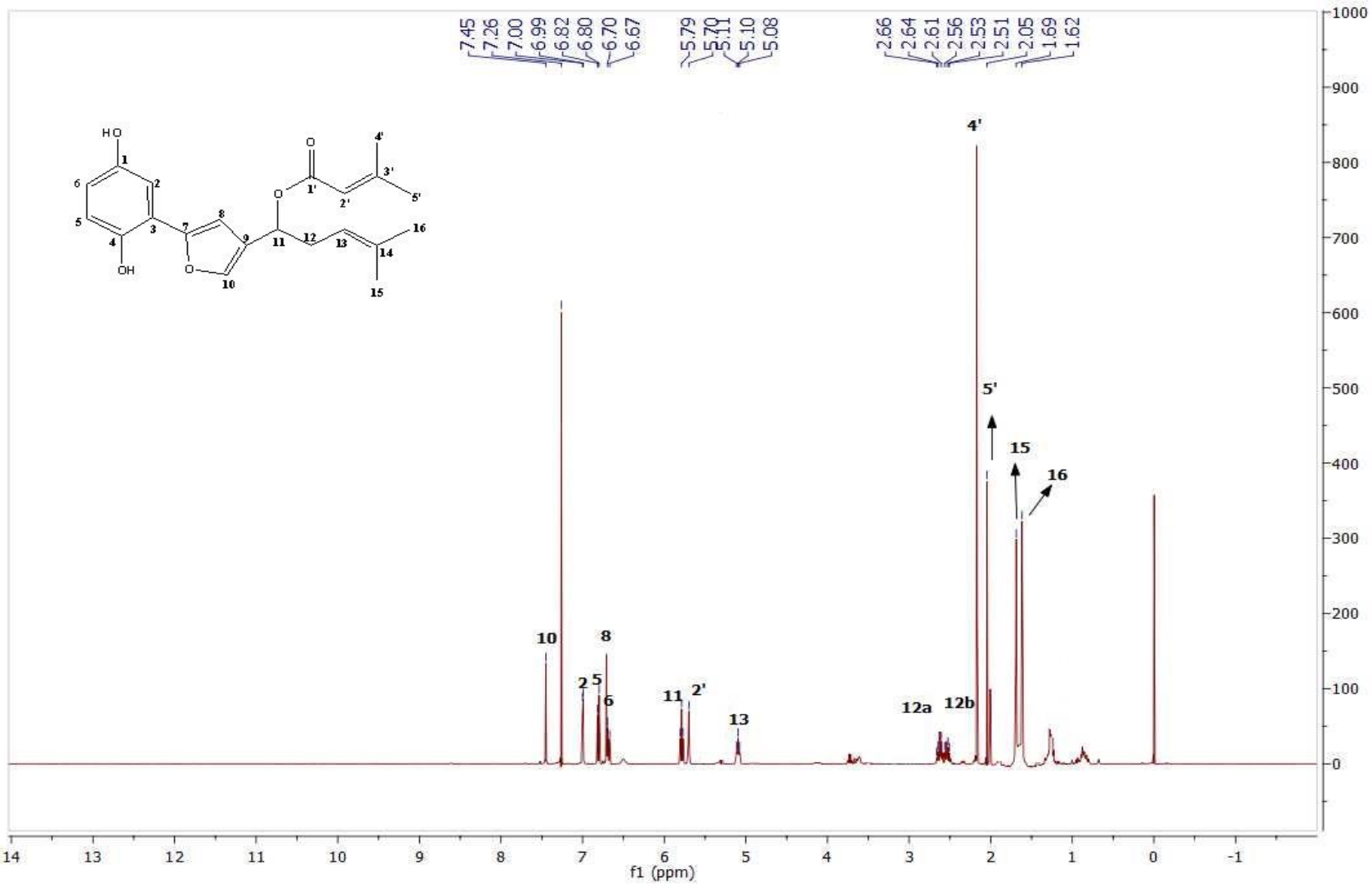


Chemical Formula: $C_{21}H_{24}O_5$
Exact Mass: 356,1624

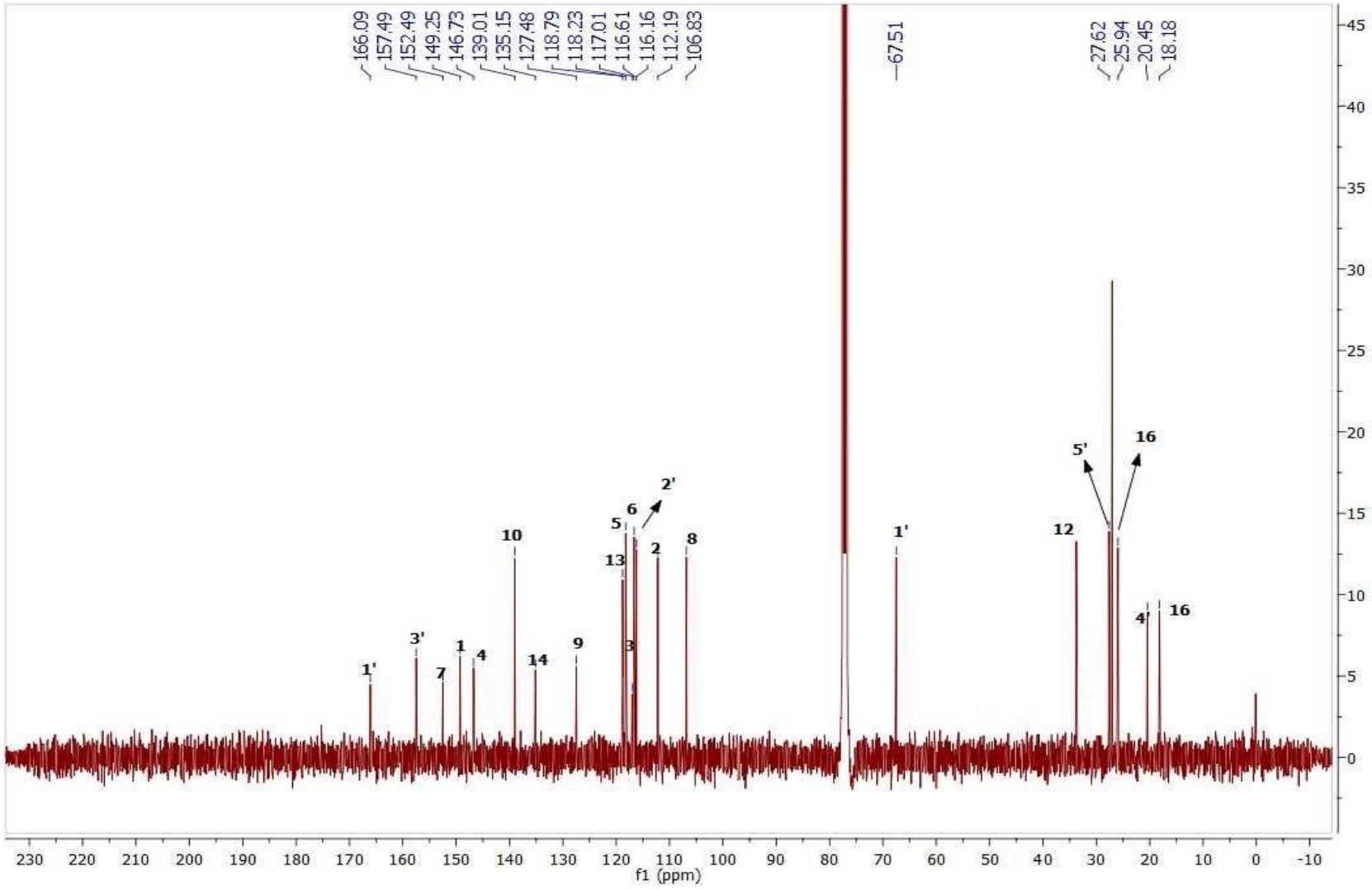
Figure 3. 3. Chemical structure of OA-PE-D3

Table 3. 4. ^{1}H and ^{13}C NMR Data of **OA-PE-D3** (400 MHz, δ ppm, in $CDCI_3$)

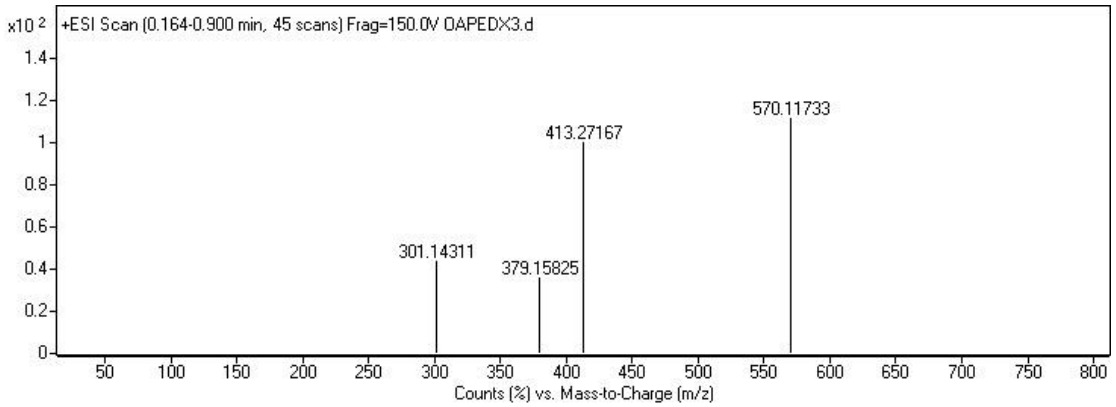
H/C	Mult.	δ_H (ppm)	*J* (Hz)	δ_C (ppm)
1	C			149.3
2	CH	7.01	*d* (3.0)	112.2
3	C			117.0
4	C			146.7
5	CH	6.81	*d* (8.7)	118.2
6	CH	6.68	*dd* (8.7, 3.0)	116.6
7	C			152.5
8	CH	6.71	*s*	106.8
9	C			127.5
10	CH	7.43	*s*	139.0
11	CH	5.79	*t* (6.8)	67.5
12	CH_2	2.71 2.56	*m* *m*	33.8
13	CH	5.10	*td* (7.5, 3.9)	118.8
14	C			132.5
15	CH_3	1.69	*s*	25.9
16	CH_3	1.62	*s*	18.2
1'	C			166.1
2'	CH	5.70	*dd* (2.6, 1.3)	116.2
3'	C			157.5
4'	CH_3	2.17	*d* (1.2)	20.5
5'	CH_3	1.89	*s*	27.6



Spectrum 3. 10. ^{1}H NMR Spectrum of **OA-PE-D3**



Spectrum 3. 11. ^{13}C NMR Spectrum of **OA-PE-D3**



Spectrum 3. 12. HR-ESI-MS Spectrum of **OA-PE-D3** (positive mode)

Shikonofuran E was isolated from various members of Boraginaceae such as *Arnebia euchroma*[37], *A. guttata*[56], *Lithospermum erythrorhizon*[57] and *O. paniculata*[58]. This is the firs report of the isolation of shikonofuran E, from *Onosma aksoyii*.

3.4.4. Structure Identification of OA-PE-D10 (Arnebidin)

OA-PE-D10 was isolated as an orange-red amorphous powder and its thin layer chromatogram illustrated a typical naphthoquinone profile. In the HR-ESI-MS spectrum (negative mode), the observed molecular ion peak was *m/z* 538.1442 $[M]^+$ (Calculated for $C_{32}H_{26}O_8$: 538.1628). When the MS spectrum was evaluated together with ^1H and ^{13}C NMR data, **OA-PE-D10** indicated the molecular formula of $C_{32}H_{26}O_8$.

In the low field of the ^1H NMR spectrum, resonances at δ 12.50, 12.39, 12.30 and 12.08 implied the presence of four aromatic hydroxy exchangeable protons while the signals at δ 7.29 (*s*, 1H), 7.28 (*s*, 1H), 7.26 (*s*, 1H) and 7.25 (*s*, 1H) indicated a couple of phenyl group protons. Apart from aromatic protons, there were four methine protons resonating at δ 3.88 (*t*, *J*=4.7 Hz, 1H), 3.81 (*dd*, *J*=8.9, 4.5 Hz, 1H), 3.35 (*m*, 1H) and 2.68 (*s*, 1H) which indicated the presence of a tricyclic ring attached to the skeleton at points of C-2, C-2', C-3 and C-3'. With the examination of side chain that extended from this skeleton, two proton signals at δ 4.62 (*d*, *J*=8.9 Hz, 1H) and 4.38 (*d*, *J*=8.9 Hz, 1H) referred to an olefinic group. The proton signals at δ 1.83 (s), 1.72 (s), 1.60 (s) and 1.54 (s) were assigned to the methyl groups which are adjacent to the olefinic systems. By comparison of the ^1H and ^{13}C NMR spectra and MS data of **OA-PE-D10** with those of previously published analogs, the structure was identified as **arnebidin**.[41]

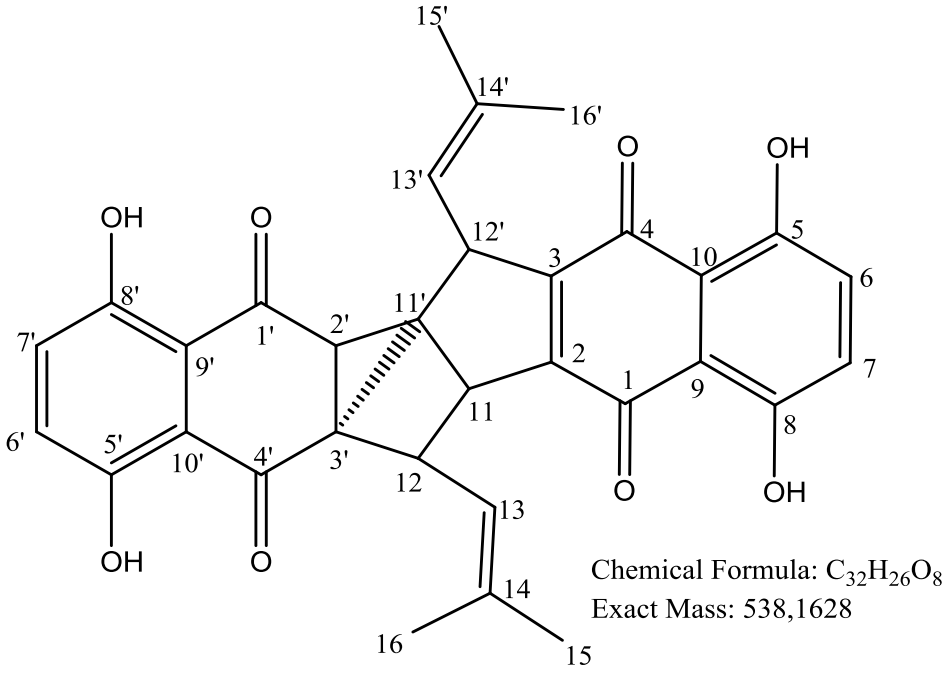
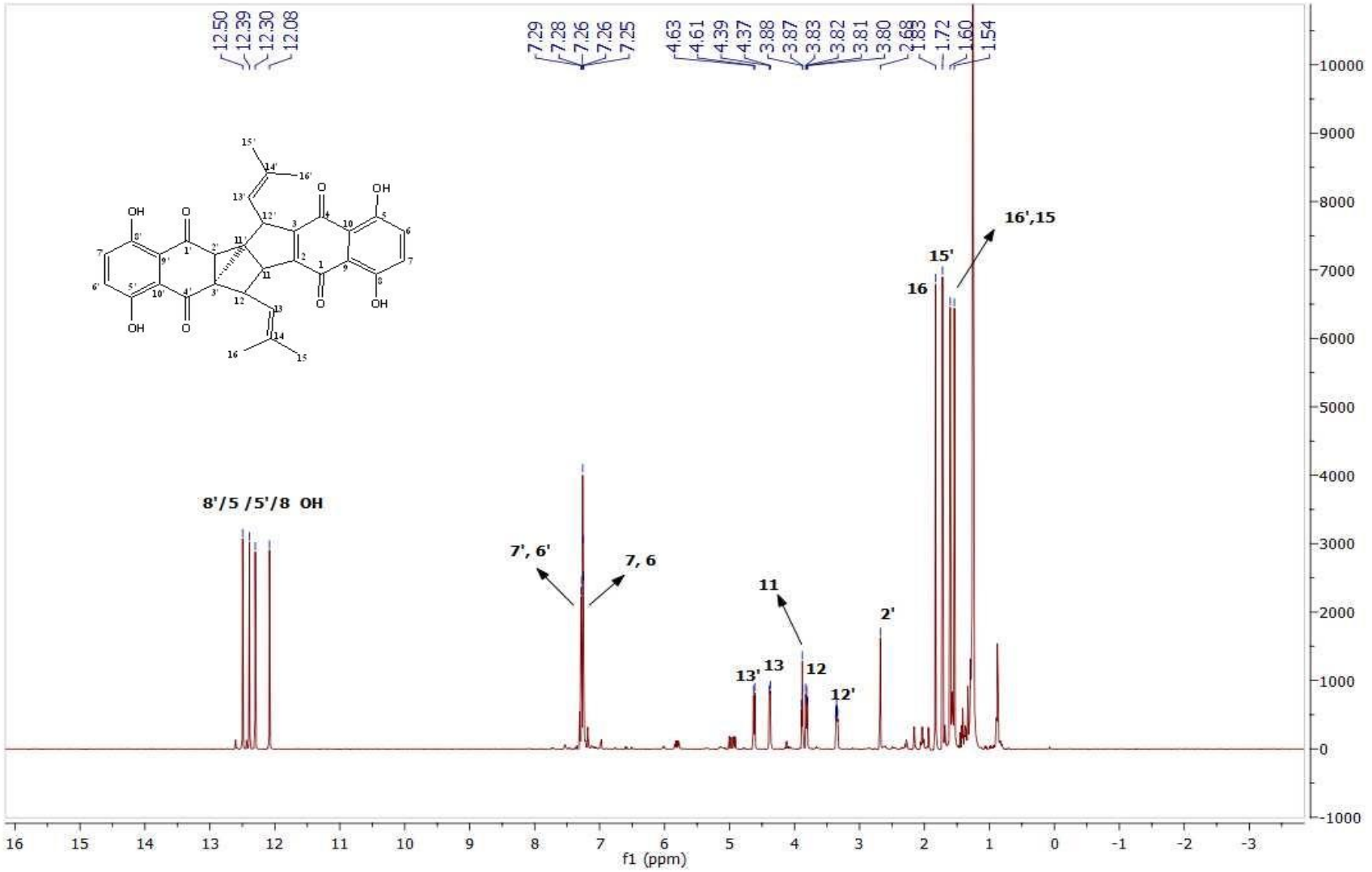


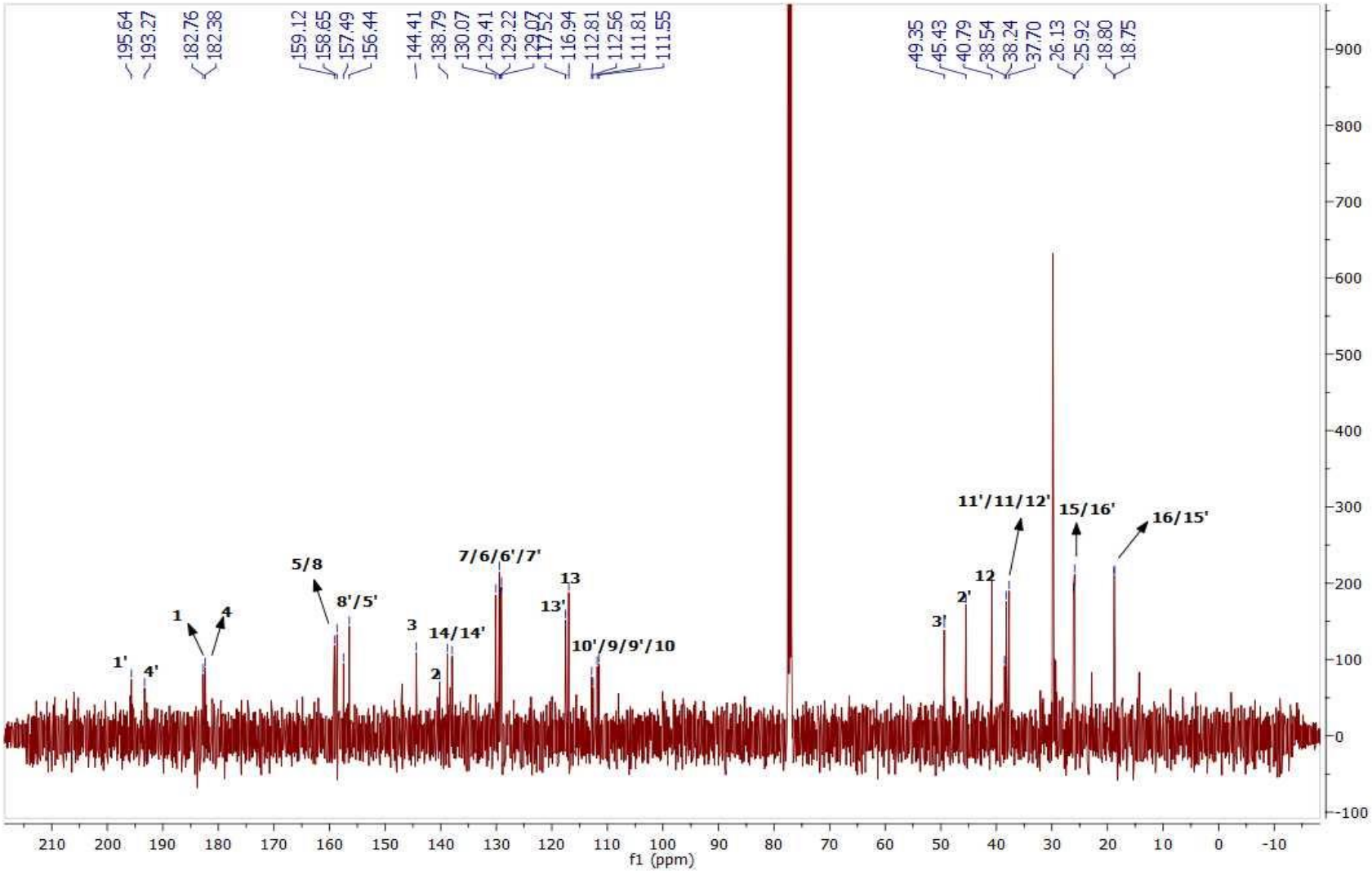
Figure 3.4. Chemical structure of OA-PE-D10

Table 3. 5. ^{1}H and ^{13}C NMR Data of **OA-PE-D10** (400 MHz, δ ppm, in $CDCI_3$)

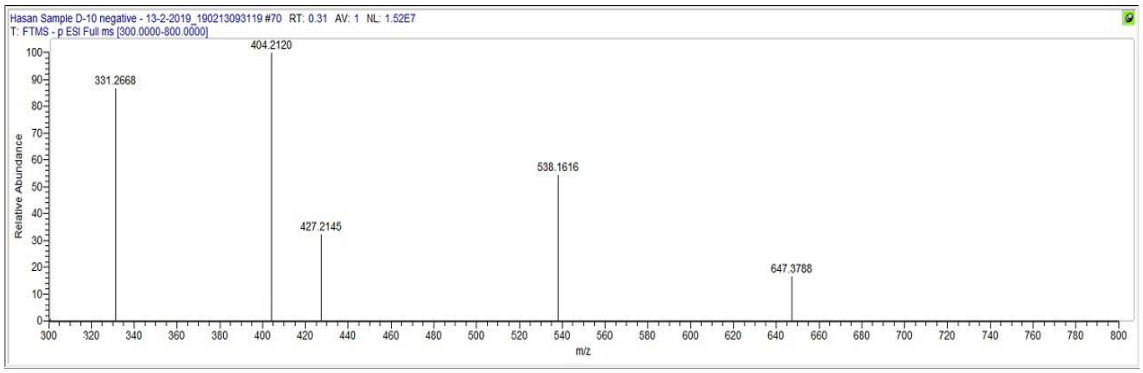
H/C	Mult.	δ_H (ppm)	*J* (Hz)	δ_C (ppm)
1	C			182.8
2	C			140.2
3	C			144.4
4	C			182.4
5	C			159.1
6	CH	7.25	*s*	129.4
7	CH	7.26	*s*	130.1
8	C			158.7
9	C			112.5
10	C			111.6
11	CH	3.88	*t* (4.7)	38.2
12	CH	3.81	*dd* (8.9, 4.5)	40.8
13	CH	4.38	*d* (8.9)	116.9
14	C			138.8
15	CH_3	1.54	*s*	26.2
16	CH_3	1.83	*s*	18.8
1'	C			195.6
2'	CH	2.68	*s*	45.4
3'	C			49.4
4'	C			193.3
5'	C			156.4
6'	CH	7.28	*s*	129.2
7'	CH	7.29	*s*	129.1
8'	C			157.5
9'	C			111.8
10'	C			112.8
11'	C			38.5
12'	CH	3.35	*m*	37.7
13'	CH	4.62	*d* (8.9)	117.5
14'	C			137.9
15'	CH_3	1.72	*s*	18.7
16'	CH_3	1.60	*s*	25.9



Spectrum 3. 13. ^{1}H NMR Spectrum of **OA-PE-D10**



Spectrum 3. 14. ^{13}C NMR Spectrum of **OA-PE-D10**



Spectrum 3. 15. HR-ESI-MS Spectrum of **OA-PE-D10** (negative mode)

Arnebidin, a naphthoquinone dimer, was previously isolated from *Arnebia hispidissima* alone[41]. This is the first report of the isolation of arnebidin from the genus *Onosma* L.

3.4.5. Structure Identification of OA-PE-D14 (β,β-dimethylacrylshikonin)

OA-PE-D14 was obtained as a red crystal and it was considered to be a naphthoquinone derivative, based on the TLC chromatogram under visible light. In the HR-ESI-MS spectrum (negative mode) of **OA-PE-D14,** molecular ion peak was observed as *m/z* 369.1318 (calculated value for $C_{21}H_{22}O_6^-$: *m/z* 369.1338). When MS data was evaluated together with ^1H and ^{13}C NMR spectra, it was determined that the compound holds the molecular formula of $C_{21}H_{22}O_6$. **Optical Rotation:** $[\alpha]^{25}$: +120°, (*c* 0.1, MeOH)[59].

In the low field of the ^1H NMR spectrum, the resonances at δ 12.60 (*s*) and 12.43 (*s*) belonging to the exchangeable protons of two aromatic hydroxy groups, and the signals at δ 6.97 (*s*, H; H-3) and 7.18 (s, 2H; H-6 and H-7) suggested that **OA-PE-D14** was a mono-substituted naphthoquinone derivative. The signal which observed at δ 6.01 (*dd*, *J*=7.1, 4.5 Hz) was characteristic for the oxymethine (H-11) protons and signal at δ 5.79 (*t*, *J*=7.1 Hz) indicated the presence of a tri-substituted double bond (H-13) proton. The chemical shift values of the methyl signals observed at 1.68 and 1.57 ppm showed that the methyl groups were adjacent to an olefinic group. However, the signals observed

at 2.61 and 2.48 ppm confirmed that there was also a methylene group close to the olefinic system. The additional carbon signals observed at 166.4, 115.4, 159.1, 25.9 and 18.1 ppm confirmed the presence of a dimethylacryl moiety on the naphthoquinone.

The ^{1}H and ^{13}C NMR data of the compound was identical with those of β,β-dimethylacrylalkannin (shikonin).[60] The positive optical rotation value and NMR results were evaluated together and the structure of **OA-PE-D14** was elucidated as a **β,β-dimethylacrylshikonin.**[61]

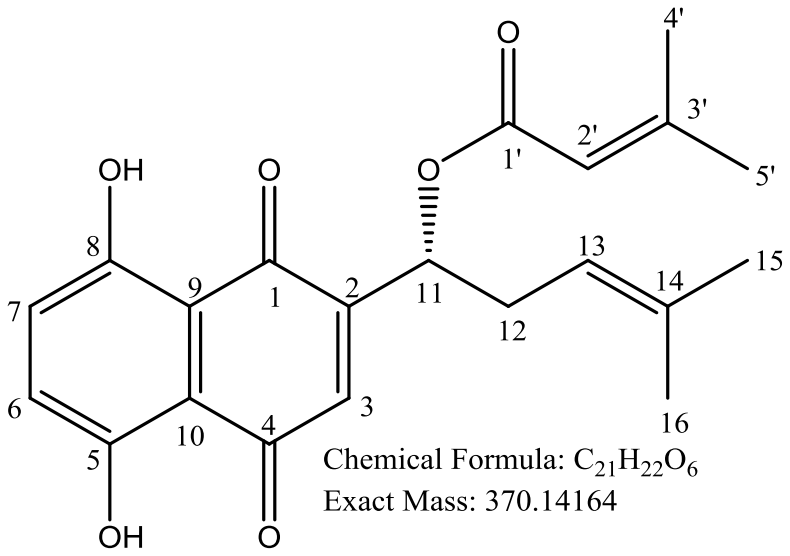
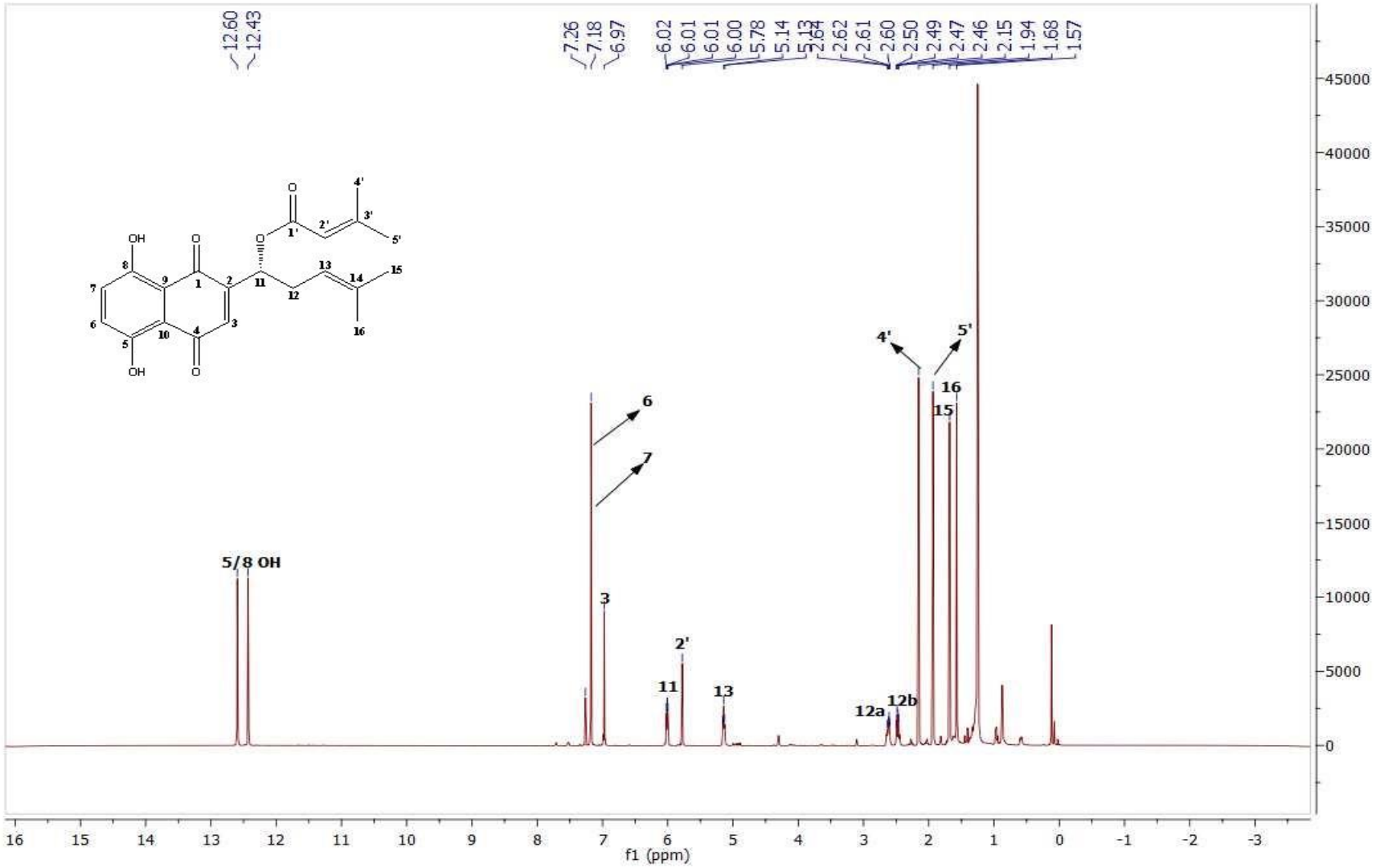


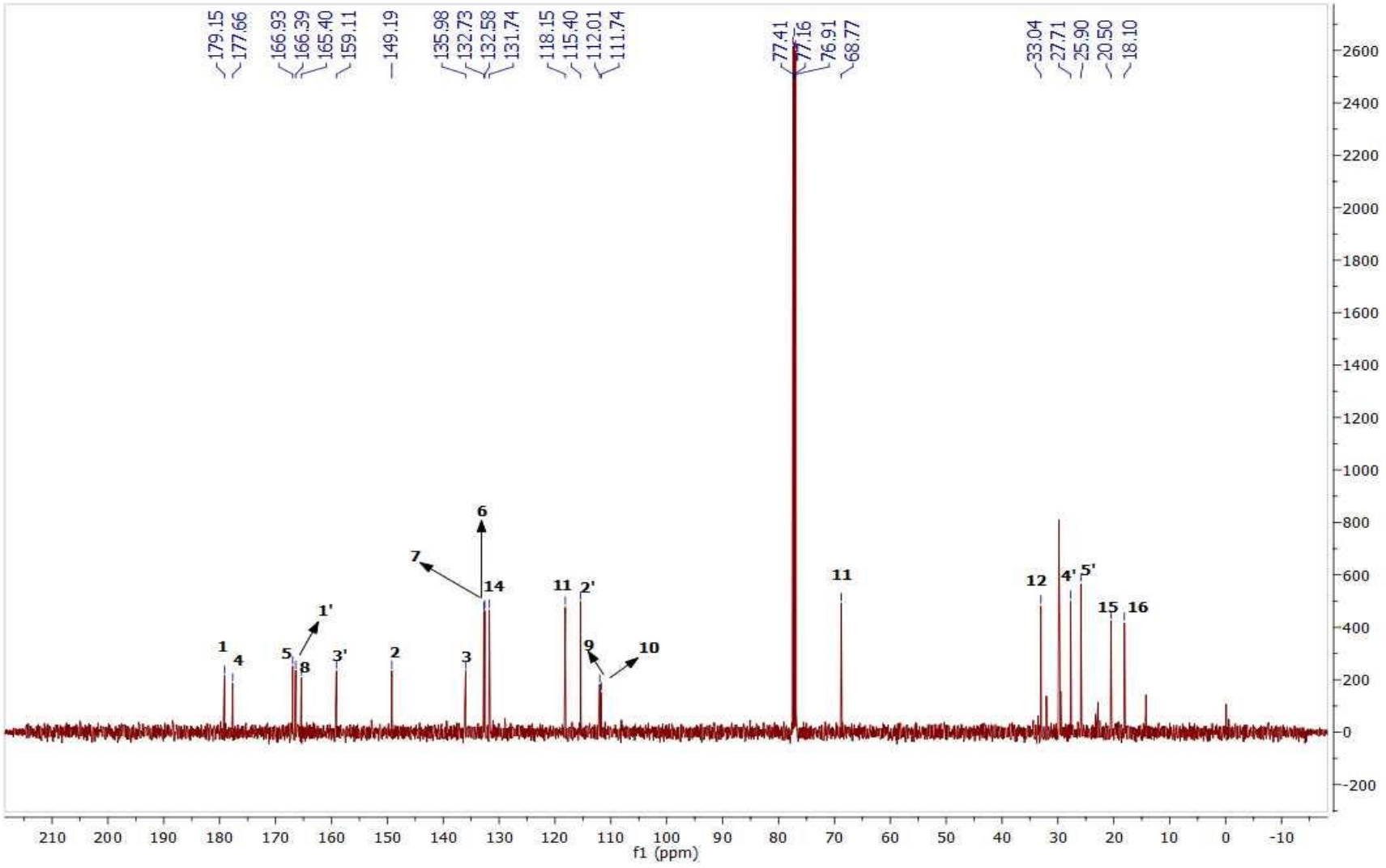
Figure 3. 5. Chemical structure of OA-PE-D14

Table 3. 6. ^{1}H and ^{13}C NMR Data of **OA-PE-D14** (400 MHz, δ ppm, in $CDCI_3$)

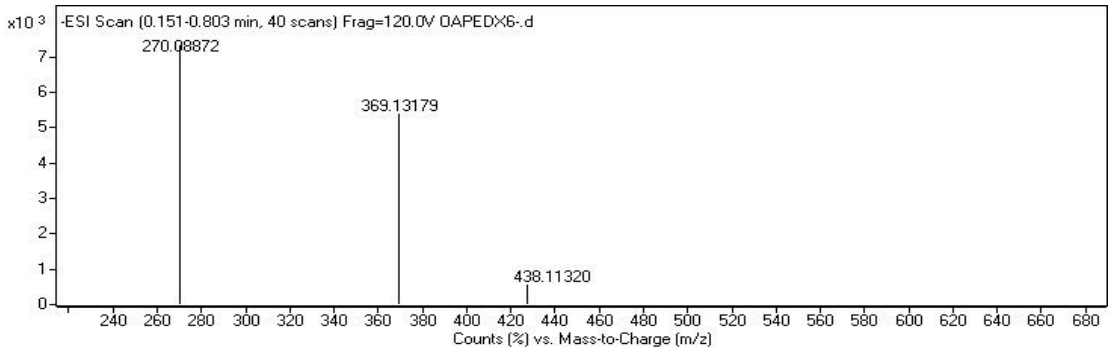
H/C	**Mult.**	**δ_H (ppm)**	***J* (Hz)**	**δ_C (ppm)**
1	C			179.2
2	C			149.2
3	CH	6.97	*s*	136.0
4	C			177.7
5	C			166.9
6	CH	7.18	*s*	132.7
7	CH	7.18	*s*	132.6
8	C			165.4
9	C			112.0
10	C			111.7
11	CH	5.79	*t* (7.1)	118.2
12	CH_2	2.61 2.48	*dd* (13.2, 7.3) *dd* (14.8, 7.4)	33.0
13	CH	6.01	*dd* (7.1, 4.5)	68.8
14	C			131.7
15	CH_3	1.68	*s*	27.7
16	CH_3	1.57	*s*	20.5
1'	C			166.4
2'	CH	5.78	*s*	115.4
3'	C			159.1
4'	CH_3	1.94	*s*	25.9
5'	CH_3	2.15	*s*	18.1



Spectrum 3. 16. ^{1}H NMR Spectrum of **OA-PE-D14**



Spectrum 3. 17. ^{13}C NMR Spectrum of **OA-PE-D14**



Spectrum 3. 18. HR-ESI-MS Spectrum of **OA-PE-D14** (negative mode)

β,β-dimethylacrylshikonin, one of the major components of Boraginaceae family, has been isolated from various *Onosma* species such as *O. nigricaule*[62], *O. dichroantha*[63], *O. paniculata*[64], *O. leptentha*[65], *O.zerizaminum*[12] *and O. echioides* var. *hispidum*[66]. It is also reported from other members of family including *L. erythrorhizon*[67], *Arnebia euchroma*[61].

This pigment is well known with its anti-microbial, anti-inflammatory, anti-tumor, immune regulation and anti-HIV properties[63]. This is the first report of the isolation of β,β-dimethylacrylshikonin from *O. aksoyii*.

3.4.6. Structure Identification of OIS-5

As a result of structure identification studies, the structure of **OIS-5** isolated from *O. isaurica* was defined to be same as **OA-PE-D2** (**arnebifuranone**)[68]. This is the first report of the isolation of arnebifuranone from *O. isaurica.*

3.4.7. Structure Identification of OIS-14

By the inspection NMR spectra of OIS-14, it was confirmed that OIS-14 has the identical structure with OA-PE-D14 which identified as **β,β-dimethylacrylshikonin**[61]. This is the first report of the isolation of β,β-dimethylacrylshikonin from *O. isaurica.*

3.4.8. Structure Identification of OIS-15

Spectral characterization studies on **OIS-15** obtained from *O. isaurica* confirmed that the compound had the same structure with **OA-PE-D10** (**arnebidin**)[41].

3.4.9. Structure Identification of OIS-23

When the ^{13}C and ^{1}H NMR spectra of **OIS-23** evaluated together, structure of compound was found to be the same as **OA-PE-D3** (**shikonofuran E**)[36]. This is the first report of the isolation of shikonofuran E from *O. isaurica.*

3.4.10. Structure Identification of OIS-26 (Acetylshikonin)

In the HR-ESI-MS spectrum (negative mode) of **OIS-26**, a major ion peak was observed at *m/z* 329.0103 $[M-H]^-$. Evaluating the MS spectra together with ^{1}H and ^{13}C NMR data, it was determined that **OIS-26** had the molecular formula of $C_{21}H_{22}O_6$. **Optical Rotation:** +456 (c 0.1, $CHCl_3$)[69]

Although the ^{1}H NMR spectrum of **OIS-26** showed great similarity with that of β,β- dimethylacrylshikonin (**OA-PE-D14**), the side chain of OIS-26 were different comprising an acyl group extending from C-11 position. By comparing to shikonin, two extra carbon signals were observed at 169.9 and 25.9 ppm in the ^{13}C NMR spectrum. In the light of the ^{1}H and ^{13}C NMR data, the presence of an acetyl group attached to the quinonoid moiety from C-11 position was revealed. Consequently, the structure of **OIS-26** was identified as acetylshikonin.[70]

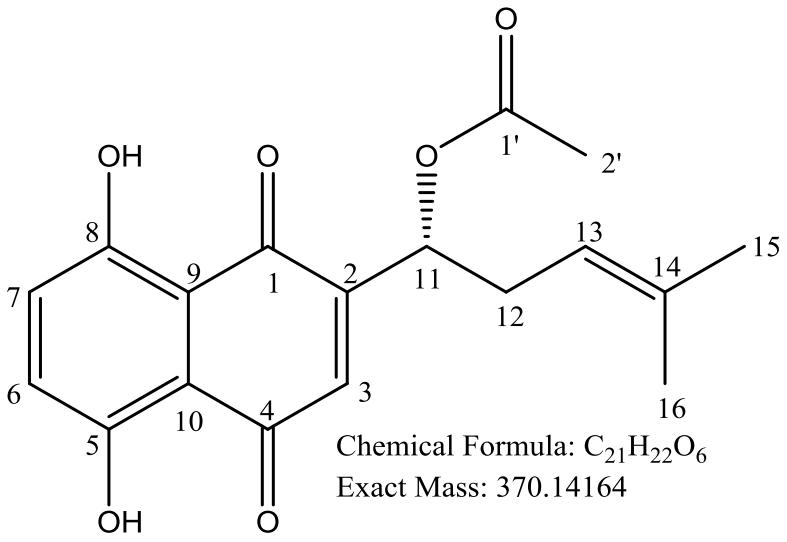
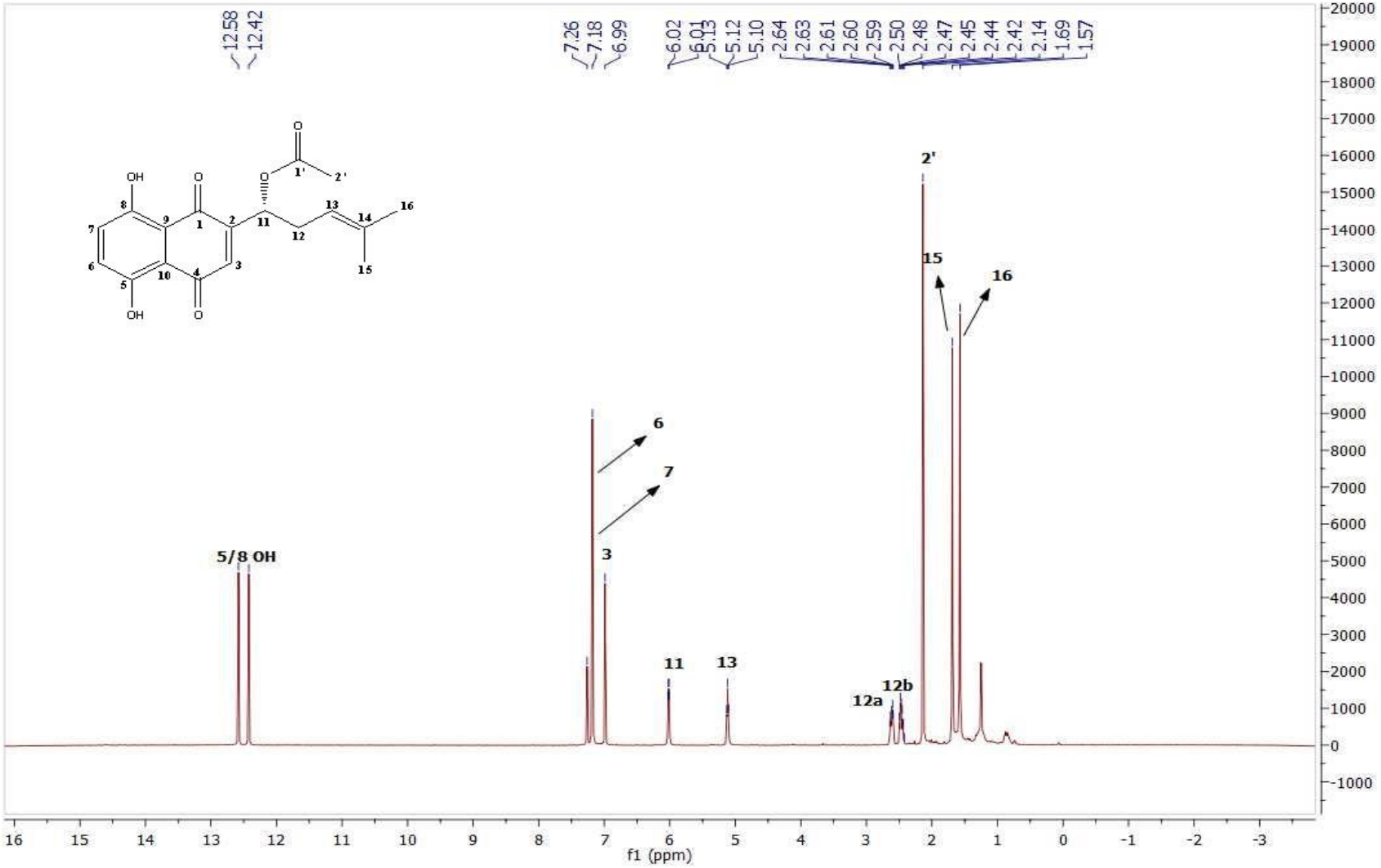


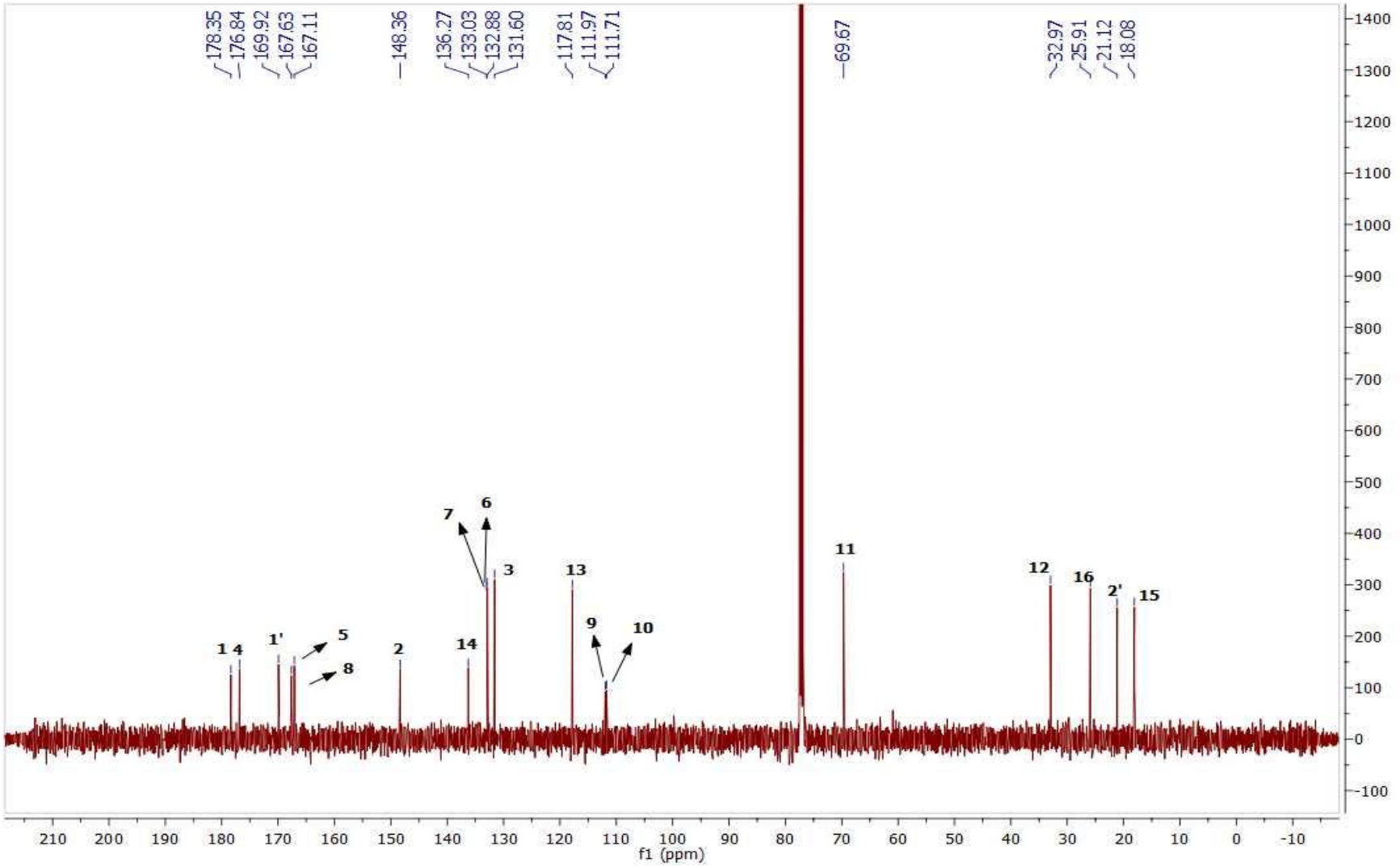
Figure 3. 6. Chemical structure of OIS-26

Table 3. 7. ^{1}H and ^{13}C NMR Data of **OIS-26** (400 MHz, δ ppm, in $CDCI_3$)

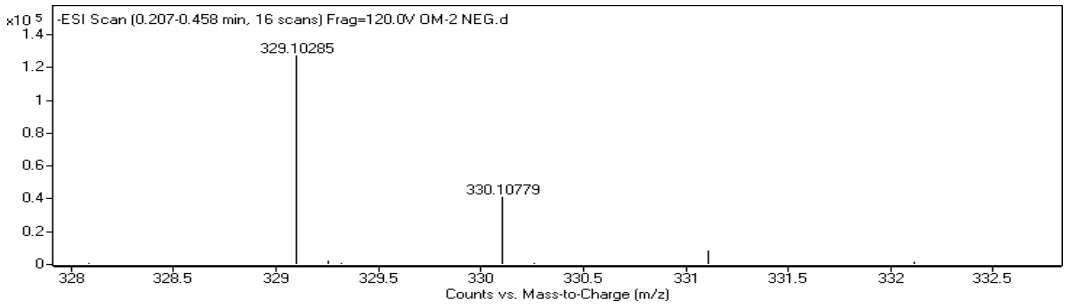
H/C	**Mult.**	**δ_H (ppm)**	***J* (Hz)**	**δ_C (ppm)**
1	C			178.4
2	C			148.4
3	CH	6.99	*s*	131.6
4	C			176.8
5	C			167.1
6	CH	7.18	*s*	132.9
7	CH	7.18		133.0
8	C			167.6
9	C			112.0
10	C			111.7
11	CH	6.02	*dd* (6.7, 4.7)	69.8
12	CH_2	2.62 2.47	*m* *dt* (14.7, 7.3)	33.0
13	CH	5.20	*t* (6.8)	117.8
14	C			131.6
15	CH_3	1.57	*s*	18.1
16	CH_3	2.14	*s*	21.1
1'	C			169.9
2'	CH_3	1.69	*s*	25.9



Spectrum 3. 19. ^{1}H NMR Spectrum of **OIS-26**



Spectrum 3. 20. ^{13}C NMR Spectrum of **OIS-26**



Spectrum 3. 21. HR-ESI-MS Spectrum of **OIS-26** (negative mode)

Acetylshikonin, another major compound produced by *Onosma* species was previously reported from *O. argentatum*[71], *O. visianii*[45], *O. armeniacum*[72], *O. paniculatum*[64], *O. echioides* var. *hispidum*[66], *O. hookeri*.[12]

This is the first report of the isolation of acetylshikonin from *O. isaurica* .

3.5. Cytotoxicity Studies

Cytotoxicity screening tests were not only performed on the root extracts of the collected *Onosma* species, but also on the fractions and pure compounds obtained from *O. aksoyii* and *O. isaurica*. Samples were tested against cancer cell lines (MCF-7, HeLa, Hepg-2, A-549, Capan-1, HCC-1937 and DU-145) and a non-cancerous cell line (MRC-5) using MTT method.

3.5.1. Cytotoxicity Studies on *Onosma* Species

Preliminary cytotoxicity screening studies were performed on the root extracts of all *Onosma* species against cancer cell lines (See Table 3.8). According to the results, two *Onosma* species were chosen for the bioactivity guided isolation studies.

Table 3. 8. Cytotoxicity screening results of the root extracts of *Onosma* species

IC_{50} VALUES (µg/mL) ±sd @ 48 h								
Extracts	**MRC-5**	**MCF-7**	**HeLa**	**Hepg-2**	**A-549**	**Capan-1**	**HCC-1937**	**DU-145**
***O. aksoyii* (P)**	<8	<8	<8	<8	<8	<8	<8	<8
***O. aksoyii* (D)**	<8	<8	<8	15	14	<8	<8	<8

(cont. on next page)

Table 3.8. (Cont.)

IC_{50} VALUES (µg/mL) ±sd @ 48 h								
Extracts	**MRC-5**	**MCF-7**	**HeLa**	**Hepg-2**	**A-549**	**Capan-1**	**HCC-1937**	**DU-145**
***O. aksoyii* (M)**	>32	>32	>32	>32	>32	>32	>32	>32
***O. strigossima* (P)**	>32	>32	>32	>32	>32	>32	>32	>32
***O. strigossima* (D)**	18.8	19.05	≈32	>32	>32	17	18.9	16.6
***O. strigossima* (M)**	>32	>32	>32	>32	>32	>32	>32	>32
***O. nydeggeri* (P)**	16.6	≈32	>32	>32	>32	>32	>32	>32
***O. nydeggeri*(D)**	>32	>32	>32	>32	>32	>32	>32	>32
***O. nydeggeri* (M)**	>16	>16	>16	>16	>16	>16	>16	>16
***O. frutescens* (P)**	>8	>8	>8	>8	>8	>8	>8	>8
***O. frutescens* (D)**	>8	>8	>8	>8	>8	>8	>8	>8
***O. frutescens* (M)**	>16	>16	>16	>16	>16	>16	>16	>16
***O. taurica* var. *tarica* (P)**	4.46	<4	≥16	>16	>16	5.4	6.8	4.8
***O. taurica* var. *tarica* (D)**	<8	<8	28.75	31.75	>8	<8	<8	<8
***O. taurica* var. *tarica* (M)**	>16	>16	>16	>16	>16	>16	>16	>16
***Undefined species* (P)**	≈16	>16	>16	>16	≈16	≈16	≈16	≈16
***Undefined species* (D)**	>16	>16	>16	>16	>16	>16	>16	>16
***Undefined species* (M)**	>32	>32	>32	>32	>32	>32	>32	>32
***O. albo-roseum* subsp. *songuinolentum* (P)**	7.28	15.9	>16	>16	>16	8.4	8.82	13.3
***O. albo-roseum* subsp. *songuinolentum* (D)**	>32	>32	>32	>32	>32	>32	>32	>32
***O. albo-roseum* subsp. *songuinolentum* (M)**	>16	>16	>16	>16	>16	>16	>16	>16
***O. roussai* (P)**	>16	>16	>16	>16	>16	>16	>16	>16
***O. roussai* (D)**	>16	>16	>16	>16	>16	≈16	>16	>16
***O. roussai* (M)**	>32	>32	>32	>32	>32	>32	>32	>32
***O. isaurica* (P)**	<4	<4	6.82	12.35	<4	<4	<4	10
***O. isaurica* (D)**	>32	>32	>32	>32	>32	>32	>32	>32

(cont. on next page)

Table 3.8. (Cont.)

IC_{50} VALUES (µg/mL) ±sd @ 48 h								
Extracts	**MRC-5**	**MCF-7**	**HeLa**	**Hepg-2**	**A-549**	**Capan-1**	**HCC-1937**	**DU-145**
***O. isaurica* (M)**	>32	>32	>32	>32	>32	>32	>32	>32
***O. inexpectata* (P)**	>16	>16	>16	>16	>16	≈16	≈16	≈16
***O. inexpectata* (D)**	>16	>16	>16	>16	>16	>16	>16	>16
***O. inexpectata* (M)**	>16	>16	>16	>16	>16	>16	>16	>16
***O. giganteum* (P)**	>16	>16	>16	>16	>16	>16	>16	>16
***O. giganteum* (D)**	>16	>16	>16	>16	>16	>16	>16	>16
***O. giganteum* (M)**	>32	>32	>32	>32	>32	>32	>32	>32
***O. albo-roseum* subsp.*albo-rosea* var. *albo-rosea* (P)**	<4	5	5.63	≈16	8.88	<4	<4	<4
***O. albo-roseum* subsp.*albo-rosea* var. *albo-rosea* (D)**	>16	>16	>16	>16	>16	>16	>16	>16
***O. albo-roseum* subsp.*albo-rosea* var. *albo-rosea* (M)**	>32	>32	>32	>32	>32	>32	>32	>32
***O. mite* (P)**	>16	>16	>16	>16	≈16	≈16	≈16	15
***O. mite* (D)**	>16	>16	>16	>16	>16	>16	>16	>16
***O. mite* (M)**	>32	>32	>32	>32	>32	>32	>32	>32
Doxorubicin	$9.1x10^{-2}$	$0.47x10^{-2}$	$0.8x10^{-3}$	$0.79x10^{-3}$	$0.41x10^{-3}$	$0.82x10^{-3}$	$0.53x10^{-3}$	$0.71x10^{-3}$

Petroleum ether, **D.** Dichloromethane, **M.** Methanol

A literature research revealed that there was no study on cytotoxicity screening of the extracts obtained from ***O. aksoyii*** and ***O. isaurica***.

The petroleum ether and dichloromethane extracts of *Onosma* species were more cytotoxic than the methanol extracts in a dose range of 4 to 32 µg/mL. Specificially, the petroleum ether and dichloromethane extracts of *O. isaurica*, *O. albo-roseum* subsp. *albo-rosea* var. *albo-rosea*, *O. aksoyii* and *O. frutescens* exhibited significant cytotoxic activity compared to the other extracts (4 µg/mL≤ IC_{50} ≤16 µg/mL). The petroleum ether extract of *O. isaurica* showed prominent toxicity towards MCF-7, A-549, Capan-1 and HCC-1937 while *O. albo-roseum* subsp. *albo-rosea* var. *albo-rosea* showed selective

inhibition on MCF-7, HeLa, Capan-1 and HCC-1937 and DU-145. Besides, *O. taurica* var. *taurica* exhibited cytotoxicity towards MCF-7, Capan-1 and HCC-1937 and DU-145 cell lines with IC_{50} values ranging between 4.0 μg/mL and 6.8 μg/mL.

Consequently, the petroleum ether extracts of *O. isaurica*, *O. albo-roseum* subsp. *albo-rosea* var. *albo-rosea*, and *O. aksoyii* and the petroleum ether-dichloromethane extracts of *O. frutescens* were found promising for further studies to purify the natural quinonoid compounds that are predicted to be responsible for the activity. The petroleum ether extracts of ***O. aksoyii*** and ***O. isaurica*** were selected for the bioactivity guided isolation studies reported within this thesis.

3.5.2. Cytotoxic Activity of the Main Fractions from *O. aksoyii*

The petroleum ether extract of *O. aksoyii* (6.22 g) was fractionated by medium pressure liquid chromatography (MPLC) prepared with RP-18 and Sephadex LH-20 to give 19 main fractions (from ES-01 to ES-19). These fractions were examined on seven human cancer cell lines, namely DU-145, Capan-1, HCC-1937, MCF-7, HeLa, HEP-G2, A-549, and a non-cancerous cell line (MRC-5) for determination of active fractions (See in Table 3.9).

As a result of cytotoxicity screening studies, fractions with strong bioactivity, encoded as **ES-06**, **ES-07**, **ES-08**, and **ES- 09,** were selected for further bioactivity guided isolation.

Table 3. 9. Cytotoxicity screening results of the main fractions of the petroleum extract of *O. aksoyii*

IC_{50} VALUES (μg/mL @48 h								
Extracts	**MRC-5**	**MCF-7**	**HeLa**	**Hepg-2**	**A-549**	**Capan-1**	**HCC-1937**	**DU-145**
ES-1	20,83	22,4	23,95	20,1	18,75	19,4	12,75	29,54
ES-2	5,9	8,4	14	15	12,8	12,2	<4	15
ES-3	5,37	13,4	12,5	12,1	14,25	12,9	<4	≈16
ES-4	11,8	≈16	≈16	15,8	≈16	14,6	>16	>16
ES-5	>8	>8	>8	>8	>8	>8	>8	>8

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Table 3.9. (Cont.)

IC_{50} VALUES (µg/mL @48 h								
Extracts	**MRC-5**	**MCF-7**	**HeLa**	**Hepg-2**	**A-549**	**Capan-1**	**HCC-1937**	**DU-145**
ES-6	10	≈16	14,4	≈16	≈16	≈16	9,44	>16
ES-7	13,45	>16	>16	>16	>16	>16	14,85	>16
ES-8	5,73	≈16	≈16	12,4	13,5	14,6	5,8	>16
ES-9	>16	>16	>16	>16	>16	>16	>16	>16
ES-10	<4	7,8	4,25	6,7	4,75	4,8	<4	5,54
ES-11	<4	7,3	6,69	6,2	8,8	5,1	4,85	9,2
ES-12	>16	>16	>16	>16	>16	>16	>16	>16
ES-13	>16	>16	>16	>16	>16	>16	>16	>16
ES-14	>16	>16	>16	>16	>16	>16	>16	>16
ES-15	>16	>16	>16	>16	>16	>16	>16	>16
ES-16	>16	>16	>16	>16	>16	>16	>16	>16
ES-17	>16	>16	>16	>16	>16	>16	>16	>16
ES-18	>16	>16	>16	>16	>16	>16	>16	>16
ES-19	>16	>16	>16	>16	≥16	>16	>16	>16
Doxorubicin	4.85	2.25	1.69	1.89	1.0	1.35	2.4	1.4

3.5.3. Cytotoxic Activity of the Main Fractions from *O. isaurica*

The petroleum ether extract of *O. isaurica* (9.10 g) was subjected to silica gel column chromatography to obtain 22 main fractions (IS-1→IS-22); they were tested towards seven human cancer cell lines, namely DU-145, Capan-1, HCC- 1937, MCF-7, HeLa, HEP-G2, A-549 and a non-cancerous cell line, MRC-5, for determination of active fractions. As a result of cytotoxicity studies on the main fractions obtained via chromatographic techniques, 13 of them (**IS-01→IS-13**) were found to be more active than the others. Moreover, cytotoxic activity results were evaluated along with TLC profiles, and it was concluded that non-polar fractions giving red-pink bands under visible light/365 nm UV light were more active due to their naphthoquinone contents. Considering both the naphthoquinone diversity and cytotoxic activity of the fractions,

promising ones (**IS-2**, **IS-10**, **IS-11**, **IS-13**, **IS-14**, **IS-15**, and **IS-18)** were selected for further bioactivity-guided isolation studies due to their IC_{50} values ranging between 4 μg/mL and 16 μg/mL.

Table 3. 10. Cytotoxicity screening results of the main fractions of the petroleum extract of *O. isaurica*

IC_{50} VALUES (μg/mL) ±sd (@ 48 h)								
Extracts	**MRC-5**	**MCF-7**	**HeLa**	**Hepg-2**	**A-549**	**Capan-1**	**HCC-1937**	**DU-145**
IS-1	<4	<4	<4	<4	≈4	<4	<4	<4
IS-2	<4	<4	<4	<4	<4	<4	<4	<4
IS-3	<4	<4	<4	<4	<4	<4	<4	<4
IS-4	<4	<4	<4	<4	<4	<4	<4	<4
IS-5	<4	<4	<4	<4	<4	<4	<4	<4
IS-6	<4	<4	<4	<4	<4	<4	<4	<4
IS-7	<4	<4	<4	<4	<4	<4	<4	<4
IS-8	<4	<4	<4	<4	<4	<4	<4	<4
IS-9	<4	<4	<4	<4	<4	<4	<4	<4
IS-10	<4	<4	<4	<4	4.42	<4	<4	<4
IS-11	<4	<4	<4	<4	5.23	<4	<4	<4
IS-12	<4	<4	<4	<4	4.74	<4	<4	<4
IS-13	≈4	4.35	≈4	≈4	7.46	≈4	4.345	≈4
IS-14	>16	>16	>16	>16	>16	>16	>16	>16
IS-15	≈16	≈16	13.1	12.22	≈16	15	≈16	≈16
IS-16	8.26	6.66	6.4	7.32	15.67	7.38	8.34	12.3
IS-17	9.38	6.44	10	7.64	13.85	7.11	6.88	10.78
IS-18	13.88	14.5	11.2	11.4	≈16	≈16	14	≈16
IS-19	10.38	6.08	7.94	6.64	13.6	6.85	5.26	10.6
IS-20	9.08	6.29	7.8	7.9	15.65	7.65	5.21	11.82
IS-21	12.27	9.72	15.75	≈16	15.92	≈16	9.14	13.12
IS-22	7.45	9.17	12.75	12.61	14.63	12.3	7.95	11.83
Doxorubicin	4.85	2.25	1.69	1.89	1.0	1.35	2.4	1.4

3.5.4. Cytotoxic Activity of Pure Compounds Obtained from *O. aksoyii* and *O. isaurica*

All isolates (β,β-dimethylacrylshikonin, arnebifuranone, shikonofuran E, arnebidin, acetylshikonin and OA-PE-D1) were tested against three different cancer cell lines (DU-145, HeLa and HCC-1937) and a non-cancerous cell line (MRC-5). Doxorubicin, a wide-spectrum anticancer antibiotic, was served as positive control. It was observed that compounds obtained from *O. aksoyii* and *O. isaurica* inhibited the cancer cells with IC_{50} values ranging between 2.65 and 32 μM (See Table 3.11).

Table 3. 11. Cytotoxicity screening results of the pure compounds isolated from *O. aksoyii* and *O. isaurica*

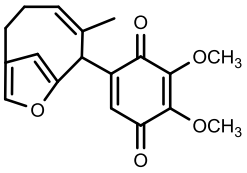
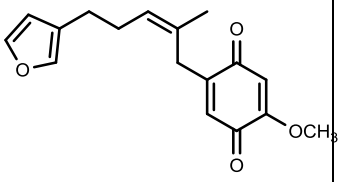
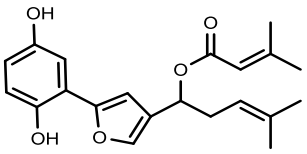
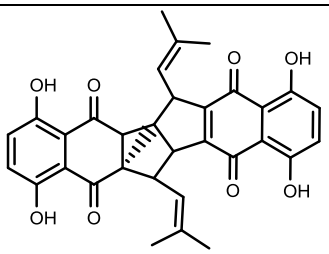
Compounds		**IC_{50} Values (μM) ±sd (@ 48 h)**			
		DU-145	**HCC-1937**	**HeLa**	**MRC-5**
OA-PE-D1		>25	>25	>25	>25
OA-PE- D2	 Arnebifuranone	11.05±0.32	11.7±0.24	12.2±0.25	7.01±0.12
OA-PE- D3	 Shikonofuran E	21.13±0.42	22.3±0.48	22.4±0.45	>25
OA-PED10	 Arnebidin	>25	>25	>25	>25

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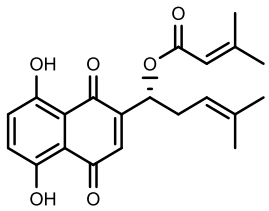
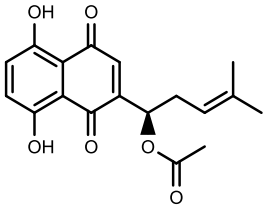
Table 3.11. (Cont.)

Compounds		IC_{50} Values (μM) ±sd (@ 48 h)			
		DU-145	**HCC-1937**	**HeLa**	**MRC-5**
OA-PE-D14	 β,β-Dimethylacrylshikonin	2.83±0.2	2.67±0.11	5.3±0.26	2.65±0.23
OIS-26	 Acetylshikonin	23.7	>25	>25	>25
Positive Control	Doxorubicin	1.375±0,35	1.93±0,19	1.69±0,22	4.85±0,28

In the literature, a number of cytotoxicity studies with the isolated compounds from different *Onosma* species were reported.

Wang et. al. studied antiproliferative effects and apoptotic properties of shikonofuran E on human osteosarcoma (MG-63) and hepatoma (SNU387) cell lines. While the growth of MG-63 cells was inhibited a dose dependent manner with an IC_{50} value of 11.50 μM, growth of SNU387 was inhibited with an IC_{50} value of 7.90 μM.[37]

Another study on the effects of shikonofuran E from *O. paniculatum* showed that, growth of mouse leukemic macrophage (RAW 264.7) were inhibited by shikonofuran E with doses ranging between 25 μM and 100 μM.[58]

Tung et.al. investigated the antiproliferative properties and cell cycle effects of arnebifuranone on human colorectal cancer cell lines (HCT-116 and SW-480). Arnebifuranone inhibited HCT-116 tumor cell growth with an IC_{50} value of 34.6 μM while inhibited the growth of SW-480 cells with IC_{50} values of 40 μM. Furthermore,

arnebifuranone decreased the percentage of cells in the G1 phase and increased cell proportions in both the S and G2/M phases.[26]

Acetylshikonin exhibited considerable inhibitory effect on human pancreatic cancer cells (PANC-1) in time and dose dependent manner with an IC_{50} value of 10 μM. In addition, acetylshikonin suppressed the production of a number of cytokine mRNAs such as IL-2, IL,4, IL-8, IL-12, IL-13 and it significantly inhibited the NF-κB luciferase activation, induced by PMA and TNF-α with IC_{50} values of 1.87 μM and 7.13 μM, respectively.[73]

Xiong et al. demonstrated in vivo and in vitro antitumor effects of acetylshikonin. According to this research, acetylshikonin inhibited the growth of A549, Bel-7402, MCF-7 and LLC cell lines in a dose dependent manner with IC_{50} values of 5.6 ± 0.86 μg/mL, and 6.82 ± 1.5 μg/ mL, 3.04 ± 0.44 μg/mL, 2.72 ± 0.38 μg/mL respectively. Also, in vivo tests on C57BL/6 mice demonstrated that, acetylshikonin suppressed the growth of LCC tumorigenic cells with a rate of 42.85% at 2 mg/kg dose.[74]

Wang et. al. investigated the mitochondria-dependent apoptosis properties of β,β dimethylacrylshikonin which suppressed the cell proliferation of A549 cells with IC_{50} values of 14.22 and 10.61 μM, respectively at 24 and 48 h. Apoptosis was induced dose dependently and downregulated cIAP-2 and XIAP expression, and up-regulated Bax and Bak expression in the cells. Moreover, β,β-dimethylacrylshikonin induced the loss of mitochondrial membrane potential, release of cytochrome c in the cells and activation of caspases 3,8 and 9.[75]

Kundakovic et.al. performed an isolation study on *O. lephantha* to obtain β,β dimethylacrylshikonin and acetylshikonin. The cytotoxic activities of compounds were tested on cancer cell lines, murine leukemia (L1210) and a human solid tumor (HT-1080). β,β-dimethylacrylshikonin showed the best activity against both cancer cell lines, with IC_{50} values of 0.39 μM and 2.0 μM, respectively. However, acetylshikonin showed moderate cytotoxic activity against tested cell lines.[65]

When our cytotoxic activity results presented in this thesis were evaluated, β,β-dimethylacrylshikonin (**OA-PE-D14**) was found to be the most active compound against DU-145, HCC-1937, HeLa and MRC- 5 cell lines with IC_{50} values of 2.83, 2.67, 5.30, 2.65 μM, respectively. Acetylshikonin (**OIS-26**) exhibited moderate cytotoxic activity versus MRC-5, HeLa, DU-145 and HCC-1937 cell lines ($IC_{50} \geq 23.7$ μM). Based

on these data, it can be concluded that the results of our experiments are consistent with the result of the study of Kundanovic et. al.[65]

Another significant bioactivity was observed with Arnebifuranone (**OA-PE-D2**) versus tested cancer cell lines with IC_{50} values ranging from 11.05 to 12.20 μM. The most promising IC_{50} value was observed with arnebifuranone against DU145 cell line (IC_{50} value: 11.05 μM). However, arnebifuranone demonstrated low selectivity versus cancer cells lines, in view of the inhibition on non-cancerous cells (See Table 3.11). Based on a literature search, the only cytotoxic activity test on arnebifuranone.was performed by Huu et. al. The results showed that arnebifuranone has lower inhibitory activity. versus two colorectal cancer cell lines. [76] Considering the colorectal cancer has high rate of drug resistance, it is clear that the results of our bioactivity studies coincide with the literature mentioned above.[77]

As the inhibition values on non-cancerous cell line (MRC-5) were evaluated, it can be deduced that the selectivity of our isolates is not convincing at this point. In order to overcome this limitation and reduce the toxicity, apart from the administration of naphthoquinones by conventional methods, various studies have been conducted on drug delivery systems such as liposomes, niosomes, polymeric micelles and metal nanoparticles.[78] Tiwari et.al. and Oommen et. al. managed to reduce toxicity and improve therapeutic efficacy of plumbagin, a natural naphthoquinone derivative, by loading it into liposomes and niosomes.[79, 80] In another study, Kontogiannopoulos et. al. used liposome-based drug delivery systems to develope and evaluate the therapeutic properties of alkannin/shikonin, and achieved significant progress in this field.[81] Targeting studies improve the therapeutic efficacy of naphthoquinone derivatives and provide them possibilities for their application in anticancer drug discovery.

3.6. DNA Topoisomerase Inhibitory Activity of the Main Fractions and Pure Compounds

Topoisomerase inhibition screening studies were performed on the root extracts of selected *Onosma* species, fractions and pure compounds obtained from *O. aksoyii* and *O. isaurica*. The extracts and fractions were tested on human topoisomerase IIα, whereas pure compounds were tested on human topoisomerase IIβ.

3.6.1. The Inhibitory Activity of Extracts on DNA Topoisomerases

The decatenation effects of the petroleum ether and dichloromethane extracts of the selected *Onosma* species (of *O. isaurica, O. albo-roseum* subsp. *albo-rosea* var. *albo-rosea*, *O. aksoyii* and *O. taurica* var. *taurica*; at concentrations of 1 mg/mL, each) were tested on DNA Topoisomerase IIα and reaction products visualized under UV were given in (Figure 3.7).

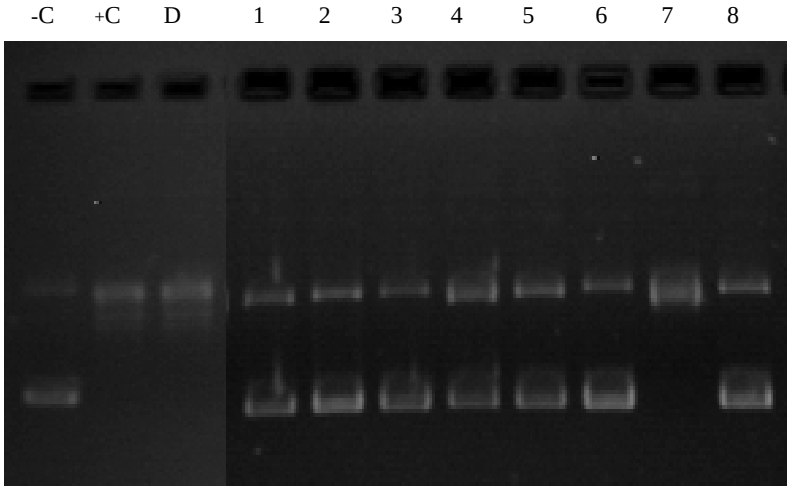


Figure 3. 7. Inhibitory effects of dichloromethane and petroleum ether extracts of selected species (**1-2.** *O. alboroseum* subsp. *albo-rosea* var. *albo-rosea,* **3-4.** *O. isaurica,* **5-6.** *O. taurica* var. *taurica,* **7-8.** *O. aksoyii*) with 1 mg/ml concentration were performed on hTopo IIα enzyme. **-C.** negative control, **+C** positive control at concentration of 2.0%), **D.** DMSO at a concentration of 2.0%.

hTopo IIα decatenation reactions illustrated that the petroleum ether and dichloromethane extracts of *O. isaurica, O. albo-roseum* subsp. *albo-rosea* var. *albo-rosea*, *O. aksoyii* and *O. taurica* var. *taurica*, except dichloromethane extracts of *O. aksoyii,* had strong inhibitory effects on the enzyme (Figure 3.7) at a working concentration of 1 mg/mL.

3.6.2. The Inhibitory Activity of *O. isaurica* on DNA Topoisomerases

The decatenation effects of the main fractions of *O. isaurica* (IS 1-22; at concentrations of 1 mg/mL, each) were tested on DNA topoisomerase IIα and reaction products visualized under UV were given in (Figure 3.8).

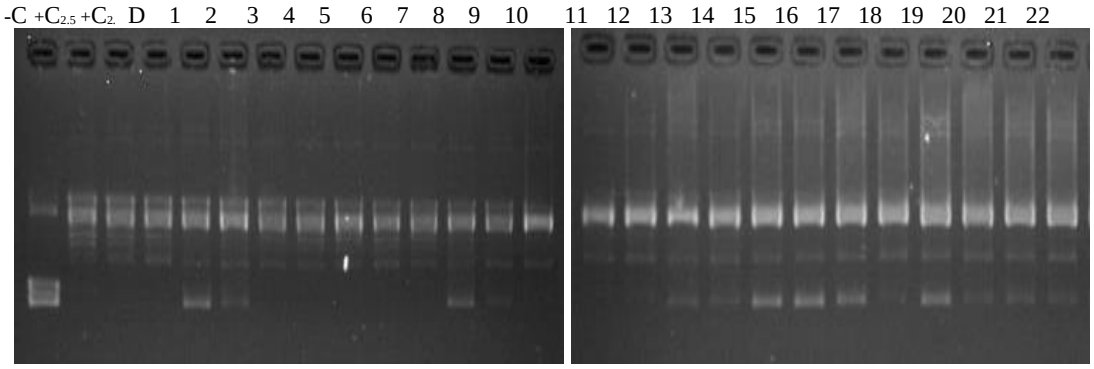


Figure 3. 8. Inhibitory effects of *O. isaurica* main fractions (1-22) with 1 mg/ml concentration were performed on hTopo IIα enzyme. **-C.** negative control, **+C2.5.** positive control (concentration of 2.5%), **+C2.0.** positive control (concentration of 2.0%), **D.** DMSO at a concentration of 2.0%.

The IS-1, IS-8, IS-15, IS-16, IS-17 and IS-19 coded main fractions from *O. isaurica* showed decatenation activity on hTopo IIα enzyme at 1 mg/mL concentration (Figure 3.8, encoded application) while fractions IS-2, IS-9, IS-13, IS-14, IS-20, IS-21, IS-22 were less effective than the other fractions.

3.6.3. The Inhibitory Activity of *O. aksoyii* on DNA Topoisomerases

Topoisomerase IIα decatenation analyses were carried on *O. aksoyii* main fractions (ES 1-16) at concentration of 1 mg/mL and visualized under UV (Figure 3.8).

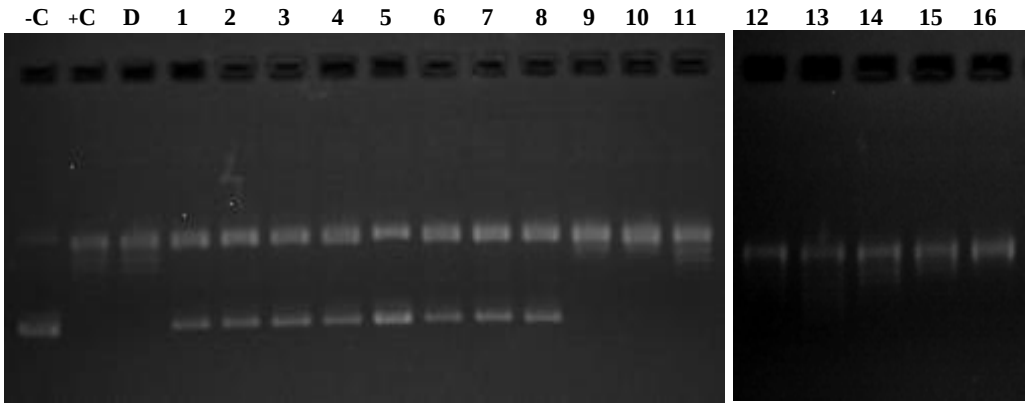


Figure 3. 9. Inhibitory effects of *O. aksoyii* main fractions (1-16) with 1 mg/ml concentration were performed on hTopo IIα enzyme. **-C.** negative control, **+C2.0.** positive control (concentration of 2.0%), **D.** DMSO at a concentration of 2.0%.

According to hTopo IIα decatenation reactions, the main fractions ES-1, ES-2, ES-3, ES-4, ES-5, ES-6, ES-7, ES-8 and ES-9 exhibited inhibitory effect on the hTopo IIα enzyme at concentration of 1 mg/mL. Other fractions did not showed any decatenation activity.

As a result, activity screening tests of the extracts and main fractions were completed consistently. The next part of the study, the inhibitory properties of the pure compounds on hTopo IIβ were investigated.

3.6.4. Inhibitory Activity of Pure Compounds on DNA Topoisomerase IIβ

Structure identification studies showed that **OA-PE-D2**, **OA-PE-D3**, **OA-PE-D10** and **OA-PE-D14** coded compounds obtained from *O. aksoyii* and **OIS-5**, **OIS-23**, **OIS-15** and **OIS-14** coded compounds obtained from *O. isaurica* were identical. The topoisomerase inhibition effects of pure compounds obtained from *O. aksoyii* and *O. isaurica* were tested on human topoisomerase IIβ (Figure 3.10).

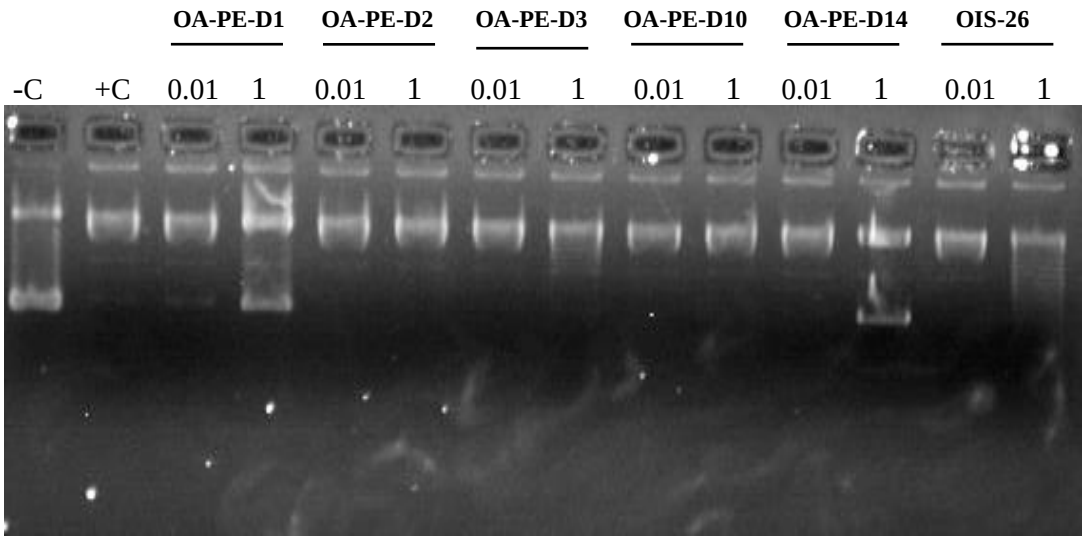


Figure 3. 10. Dose dependent inhibitory activity of pure compounds at final concentration of 1.0 and 0.01 mg/mL on hTopo IIα enzyme, **-C.** negative control, **+C** positive control (concentration of 2.0%), **OA-PE-D1.** New meroterpenylbenzoquinone derivative **OA-PE-D2.** Arnebifuranone **OA-PE-D3**. Shikonofuran E **OA-PE-D10.** Arnebidin **OA-PE-D14.** β,β-dimethylacrylshikonin **OIS-26** Acetylshikonin.

Topoisomerase inhibition tests showed that **OA-PE-D1** (new meroterpenylbenzoquinone derivative) and **OA-PE-D14** (β,β-dimethylacrylshikonin) had decatenation activity on hTopo IIβ enzyme at 1 mg/mL concentration (Figure 3.10). Moreover, these compounds lost their inhibitory effects on hTopo IIβ enzyme at 0.01 mg/mL concentration.

In the light of literature survey, a number of topoisomerase inhibition studies with the naphthoquinone derivatives were reported.

Fujii et. al. investigated the inhibitory properties of a group of naphthoquinone derivatives like shikonin, plumbagin, lawson and lapacol on topoisomerase II enzyme. Shikonin and plumbagin exhibited promising decatenation activity at dose of 0.5 and 12.5 μM, respectively, whereas lapacol and lawson showed weaker activity against topoisomerase II at concentration of 50 μM.

CHAPTER 4

CONCLUSION

In the scope of this study, 12 different *Onosma* species collected from different locations of Soutwest Anatolia were phytochemically investigated, particularly in terms of naphthoquinone contents using HPLC-DAD. Also, cytotoxic properties of the root extracts were examined by MTT assay versus various human cancer cell lines (HeLa, HCC-1937, DU-145); whereas DNA topoisomerase inhibition properties were tested on hTopo IIα and hTopo IIβ.

Following, all findings obtained from the chemical screening assays and cytotoxicity tests were evaluated together to determine the ideal *Onosma* species for further bioactivity guided isolation studies.

According to the results of cytotoxicity experiments, petroleum ether and dichloromethane extracts of the plants showed superior cytotoxic activities compared to the methanol extracts. Especially, the extracts of *O. aksoyii*, *O.isaurica* and *O. frutescens* with IC_{50} values less than 8 μg/mL, exhibited relatively outstanding cytotoxic activities than the other extracts. Therefore, we decided to continue further isolation studies with *O. aksoyii* and *O. isaurica* to obtain the pure compounds responsible for the bioactivity.

As a consequence of our bioactivity guided isolation studies on these two species, a new quinonoid pigment was isolated together with five known compounds, using chromatographic techniques. The structures of the isolates were elucidated as arnebidin (**OA-PE-D10**), acetylshikonin (**OIS-26**), arnebifuranone (**OA-PE-D2**), shikonofuran E (**OA-PE-D3**), β,β-dimethylacrylshikonin (**OA-PE-D14**) and the new compound **OA-PE-D1** using modern spectral methods (1D-2D NMR and MS).

The most important finding of the present study was the isolation of an undescribed meroterpenylbenzoquinone derivative, OA-PE-D1, from *O. aksoyii*. OA-PE-D1 differs from other meroterpenoids with its extraordinary structural rearrangement. Meroterpenoids are well-known with their biological activities, especially anticancer and antitumoral effects. Therefore, detailed investigation, particularly further cytotoxic screening studies, are needed for OA-PE-D1.

This is the first report of the isolation of arnebifuranone and arnebidin from the genus *Onosma* L. Those known compounds were previously isolated from *Alkanna* and *Arnebia* species as mentioned in the text. But still it is very important to report them particularly from the genus *Onosma* L., contributing to ongoing phytochemical studies on Boraginaceae plants.

Arnebifuranone is a member of monoterpenylbenzoquinone derivatives. Wang et al. called this quinonoid group of compounds as "meroterpenoids" due to their mixed biosynthetic origins. Structure of meroterpenoids which comprises of quinonoid and terpenoid subunits, are originated from both polyketide and terpenoid pathways[40].

Bioactivity screenings of the isolates revealed that, β,β-dimethylacrylshikonin has superior cytotoxic activity (2.67 μM $\leq IC_{50} \leq$ 5.30 μM) against cancer cell lines compared to the others. Arnebifuranone specifically inhibited DU-145 and HeLa cell lines with IC_{50} values of 10.75 and 10.50 μM, respectively. The remaining compounds, arnebidin, acetylshikonin, shikonofuran E and OA-PE-D1, exhibited moderate to strong cytotoxic activity with IC_{50} values ranging from 19.54 μM to 32.0 μM on tumorigenic cell lines.

As part of these bioactivity tests, using a sensitive cell line (MRC-5), provides more accurate calculation of therapeutic potential of the isolated compounds. Our findings have shown that the isolates inhibit the cancer lines with low selectivity, because they are also cytotoxic against MRC-5 with approximate IC_{50} values.

Since the bioactive compounds are not selective, it is hard to nominate them as lead molecules at this stage. Taking the importance of therapeutic index into consideration, it is clear that, our isolates require some molecular modifications (such as semi-synthesis or microbial biotransformation reactions) to be a potent candidate for cancer chemotherapy. Apart from these, drug delivery systems could be investigated as an alternative approach to reduce the cytotoxicity of naphthoquinones and increase their therapeutic efficacy.

Naphthoquinones have strong inhibitory effects on DNA topoisomerases by intercalation of DNA afterward stimulating DNA cleavage and apoptosis.[82] From this point of view, the inhibitory effects of the *Onosma* root extracts, fractions and isolated compounds were examined using *in vitro* plasmid relaxation and decatenation assays to state minimum inhibitory concentrations.

As a result of DNA topoisomerase inhibition test of OA-PE-D1, it was found to be the most potent inhibitory compound at 25 μM (1 mg/mL) concentration on

Topoisomerase IIβ. Likewise, β,β-dimetylacrylshikonin was also significantly potent at 25 μM (1 mg/mL) concentration on Topoisomerase IIβ. Thus, they both were taken into further analyses for Topoisomerase I mediated-DNA cleavage.

Unsurprisingly, there was no significant correlation between the findings of cytotoxic activity and topoisomerase inhibition. Because it is thought that naphthoquinones exhibit cytotoxic activity through some other mechanisms apart from topoisomerase inhibition. Based on the literature search, a mechanism which might be responsible for the cytotoxicity of naphthoquinones is oxidative stress due to the formation of quinone radicals that cause macromolecule damage within the cells.[83] Another proposed mechanism is the bioreductive alkylation mechanism in which naphthoquinones cause cell death and DNA damage as an effective alkylating agents.[84]

Moreover, the decrease in the alkylation capacity of naphthoquinones may facilitate the compounds to pass into the nucleus without disturbing the cytoplasm integrity and act on the topoisomerase enzymes present in the nucleus. Such a mechanism can explain why **OA-PE-D1,** a compound with low cytotoxic activity on tested cell lines, has the highest topoisomerase inhibition property.

Further bioactivity studies are required for **OA-PE-D1** according to our topoisomerase inhibition studies. Since two of closely related meroterpenoid derivatives were previously reported as very potent cytotoxic agents against human hepatocellular carcinoma cells[49], **OA-PE-D1** is still promising for anticancer drug discovery. Moreover, the findings of topoisomerase inhibition assays seem to be supportive for this inference.

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