THE EFFECTS OF NAPHTHOQUINONES ISOLATED FROM *ONOSMA* SPECIES ON DNA TOPOISOMERASES AND THEIR CYTOTOXIC PROPERTIES

A Thesis Submitted to
The Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfilment of the Requirements for the Degree of

MASTER OF SCIENCE

in Biotechnology

by

Özge GÜZEL

July, 2018

İZMİR

We approve the thesis of **Özge GÜZEL**

Examining Committee Members:

_____

Prof. Dr. Erdal BEDİR

Department of Bioengineering, İzmir Institute of Technology

_____

Prof. Dr. Zeki TOPÇU

Department of Pharmaceutical Biotechnology, Ege University

_____

Prof. Dr. Ali ÇAĞIR

Department of Chemistry, İzmir Institute of Technology

9 July 2018

_____

Prof. Dr. Erdal BEDİR
Supervisor, Department of Bioengineering
İzmir Institute of Technology

_____

Assoc. Prof. Dr. Sevil ZENCİR
Co-Supervisor, Department of Medical Biology
Pamukkale University

_____

Assoc. Prof. Dr. Engin ÖZÇİVİCİ
Head of the Department of Biotechnology
and Bioengineering

_____

Prof. Dr. Aysun SOFUOĞLU
Dean of the Graduate School of
Engineering and Sciences

ACKNOWLEDGEMENT

I am deeply grateful to Prof. Dr. Erdal BEDİR for his patience,

I would like to express my gratitude Assoc. Prof. Dr. Sevil ZENCİR and Prof. Dr. Zeki TOPÇU for their kind supervision, valuable suggestion and discussions through the whole study,

I am grateful to Asst. Prof. Dr. Ademi Fahri PİRHAN for the identification of *Onosma* species,

I also would like to thank to Seda DUMAN, who assisted me during study, for her support, valuable guidance and advice and friendship,

I want to thank Assoc. Prof. Dr. Petek BALLAR KIRMIZIBAYRAK and Sinem YILMAZ for their contribution to cytotoxicity studies,

I also want to thank Onur MUŞMULA for assisting HPLC studies,

I would like to thank to my friends, Dr. Özge ÖZÇINAR, Fadime AYDOĞAN, Melis KÜÇÜKSOLAK, for their encouragement, understanding and attitude throughout my study,

I also want to thank all Bedir Group members for their helps.

I would like to thank to Beste ELVEREN and Fazilet GÜRER for their support and motivation throughout my study,

I am deeply thankful to my family who always supporting me to go after my dreams.

This study was supported by the TUBITAK project "116Z463" and BAP Project "2017IYTE64"

İzmir, July 2018

Özge GÜZEL

"Look deep into nature, and then you will understand everything better."

Albert EINSTEIN

ABSTRACT

THE EFFECTS OF NAPHTHOQUINONES ISOLATED FROM *ONOSMA* SPECIES ON DNA TOPOISOMERASES AND THEIR CYTOTOXIC PROPERTIES

Onosma L. genus (Boraginaceae) comprises of 230 species that is represented by 102 species and 108 taxa in the flora of Turkey. As 50% of the genus is endemic, Anatolia can be considered as the gene center of *Onosma* species. Phytochemical investigations performed on *Onosma* genus have led to the identification of various naphtoquinones, alkaloids and phenolic constituents. Biological activity studies on naphthoquinones demonstrated antimicrobial and wound-healing properties as well as cytotoxicity.

In this thesis, *Onosma taurica* var. *taurica* and *O. mollis* were taken into cytotoxic activity-guided isolation studies, by MTT using seven human cancer cell lines (DU145, Capan-1, HCC-1937, MCF-7, HeLa, HEPG2, A-459) and a normal cell line (MRC-5), to isolate their bioactive compounds. Additionally, the isolation studies were guided by enzyme inhibition tests towards human Topoisomerases I and II. Six compounds were isolated using chromatographic methods, and the structures of the five of them were elucidated by spectral methods, (1D-, 2D NMR and HR-ESI-MS) as acetylshikonin, shikonin**,** β-hydroxyisovalerylshikonin, β,β-dimethylacrylshikonin and deoxyshikonin. The isolated compounds showed prominent cytotoxicity with IC_{50} values ranging from 1.83 to 25 μM. β,β-dimethylacrylshikonin was found to be the most cytotoxic compound (IC_{50}: 1.84 μM versus HCC-1937), whereas β-hydroxyisovalerylshikonin on Topoisomerase I and II (each at 25 μM dose) exhibited strong inhibitory effects.

ÖZET

ONOSMA TÜRLERİNDEN İZOLE EDİLEN NAFTOKİNONLARIN DNA TOPOİZOMERAZLAR ÜZERİNE ETKİLERİ VE SİTOTOKSİK ÖZELLİKLERİ

Onosma L. (Boraginaceae) dünya üzerinde 230 türü bulunan ve Türkiye florasında 102 tür ve 108 takson ile temsil edilen bir cinstir. Ülkemizdeki türlerin neredeyse %50'si endemiktir (48 tür), bu nedenle Anadolu'nun *Onosma* türlerinin gen merkezi olduğu söylenebilir. *Onosma* cinsi üzerinde gerçekleştirilen fitokimyasal araştırmalar çeşitli naftakinon, alkaloitler ve fenolik bileşiklerin tanımlanmasına olanak sağlamıştır. *Onosma* türlerinden elde edilen naftakinon türevleri için antimikrobiyal (Ozgen vd., 2004; Pavol vd., 2008) ve yara iyi edici etki ile birlikte sitotoksisite rapor edilmiştir. Bu çalışmada, *O. mollis* ve *O. taurica* var. *taurica* bitkileri MTT yöntemi ile 8 adet tümörjenik (DU145, Capan-1, HCC-1937, MCF-7, HeLa, HEPG2, A-459) ve 1 tümörjenik olmayan (MRC5) hücre hatlarına karşı sitotoksisiteleri yönünden taranarak biyoaktif moleküllerin elde edilmesi için sitotoksik aktivite rehberli izolasyon çalışmasına alınmıştır. Bu duruma ek olarak, DNA Topoizomerazları I ve II enzimleri üzerindeki inbitisyon etkileri incelenmiştir.

Farklı kromotografik yöntemlerle elde edilen altı bileşikten beş tanesinin yapıları spectral yöntemlerle (1D-, 2D NMR ve HR-ESI-MS) aydınlatılmış ve asetilşikonin, şikonin, β-hidroksiizovalerilşikonin, β, β-dimetilakrilşikonin ve deoksişikonin olduğu saptanmıştır.

İzole edilmiş bileşikler, 1.83 ile 25 μM arasında değişen IC_{50} değerleri ile belirgin sitotoksisite göstermiştir. β, β-dimetilakrilşikonin en aktif bileşik olarak (HCC-1937 üzerinde IC_{50}: 1.84 μM) bulunmuştur. Topoizomeraz inhibisyon testleri ile ilgili olarak, β-hidroksiizovalerilşikonin 25 μM konsantrasyonda Topoizomeraz I ve II enzimleri üzerinde güçlü inhibisyon etkisi göstermiştir.

To my family

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

LIST OF CHROMATOGRAMS

Chromatogram **Page**

LIST OF SPECTRA

ABBREVIATIONS

ACN	Acetonitrile
PE	Petroleum Ether
CH_2CI_2	Dichloromethane
H_2SO_4	Sulfuric Acid
FA	Formic Acid
kDNA	kinetoplast DNA
BuOH	Butanol
$CHCI_3$	Chloroform
EtOAc	Ethyl Acetate
MeOH	Methanol
H_2O	Water
$CDCI_3$	Deuterated chloroform
DMSO	Dimethyl sulfoxide
UV	Ultraviolet
CC	Column Chromatography
TLC	Thin Layer Chromatography
HPLC	High Performance Liquid Chromatography
MPLC	Medium Pressure Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
NMR	Nuclear Magnetic Resonance
1D-NMR	One-Dimensional Nuclear Magnetic Resonance
2D-NMR	Two-Dimensional Nuclear Magnetic Resonance
s	Singlet
m	Multiplet
bs	Broad singlet
d	Dublet
IC_{50}	The Half Maximal Inhibitory Concentration
DU145	Metastatic Prostate Cancer
HeLa	Human Cervix Carcinoma

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

CHAPTER 1

INTRODUCTION

Onosma L. genus (Boraginaceae) comprises of 230 species that is represented by 102 species and 108 taxa in the flora of Turkey.[1] Almost 50% of the genus is endemic (48 species)[2]; thus, Anatolia is considered as the gene center of *Onosma* species.

Phytochemical investigations performed on *Onosma* genus have led to the identification of various naphtoquinones[2], alkaloids[3] and phenolic constituents.[4] Biological activity studies on naphthoquinone derivatives obtained from *Onosma* species demonstrated antimicrobial[2] and wound-healing properties[5] as well as cytotoxicity.[6]

Onosma L. species have been used in folk medicine for the treatment of various diseases. Different species of *Onosma* are used for curing symptoms of rheumatism, kidney problems and heart attack. Plant roots are used as diuretic, bleeding inhibitor and sedative. Apart from these, they have been used in hypertension treatment, fever and nervous system disorders in India[4], and inflammatory disorders such as pain, hemorrhoids, and tonsillitis in Turkey.[2] In Turkey, the roots of *O. argentatum* and *O. armenicacum* are used for wounding and burn treatment in folk medicine, whereas the oily extract of *O. chlorotricum* roots, also known as Tashnehdary, is used topically for wound healing.[7]

From phytochemistry and cytotoxicity points of view, *Onosma* species possessing high endemism ratio were not examined thoroughly. This study was designed to focus on naphthoquinones, which most probably are secondary metabolites responsible for strong cytotoxic activities of *Onosma* species. For this purpose, firstly phytochemical and bioactivity screenings at genus level including 12 *Onosma* species were performed by HPLC fingerprinting and cytotoxicity studies. Cytotoxicity together with DNA Topoisomerases I and II inhibition assays guided our species selection and their further fractionation studies to isolate active constituents responsible for bioactivities.

1.1. Botanical Descriptions

1.1.1. Boraginaceae Family

Boraginaceae family distributed in the tropical, subtropical and temperate regions of the world, most intensively in the temperate areas of the Iranian-Turanian and Mediterranean regions, and also in the central parts of Central, North and South America.[1] The family represents about 34 genera including 325 species. The rate of endemism among native species is 42.2 % in Turkey.[2]

In the "*Flora of Turkey and The East Aegean Islands",* Boraginaceae family is defined as: "Annual, biennial or perennial herbs, rarely shrublets or trees. Leaves alternate, exstipulate, simple, often with marked setose indumentum. Ultimate branches of inflorescence cymose, cymes scorpioid or ± circinnate (cincinni), or inflorescence rarely thyrsoid. Calyx gamosepalous, 5-lobed (rarely 9-lobed or irregularly dentate), often accrescent after anthesis. Corolla 5-lobed, actinomorphic or rarely zygomorphic, usually with distinct tube and ± deeply lobed limb; throat often with 5 appendages or tufts or a zone of hairs, or smooth and glabrous. Stamens 5, epipetalous, alternating with corolla lobes. Ovary superior, 4-(rarely 2-) locular; style gynobasic, more rarely terminal, usually undivided, stigma entire or 2C-4)-lobed. Fruit usually of 4 nutlets, rarely fewer by abortion or fusion, or of 2 corky mericarps, or a drupe; nutlets berne on flat to pyramidal gynobase, attachment scar narrow to broad, without or with a sub-basal ring or stalk, with erect to subhorizontally incurved beak or unbeaked, keeled or not, often differentiated into disc and margin; margin sometimes prolonged into a spreading or incurved wing, or spiny-glochidiate; surface smooth or variously ornamented, glabrous, hairy or with glochidiate tubercles and/or spines. Syn: Ehretiaceae*."*.[3]

1.1.2. *Onosma* L.

The systematic status of *Onosma* L. is given below:

Kingdom : Plantae

Subkingdom : Tracheobionta

Division : Magnoliophyta

Class : Magnoliopsida

Subclass : Asteridae

Order : Lamiales

Family : Boraginaceae

Genus : *Onosma*

The *Onosma* genus, largest group in the family Boraginaceae, including over 230 species which are distributed in Asia, Eurasia, Mediterrian regions and Europe mostly Iran, Syria, Turkey, China, Pakistan, India and Sri Lanka etc. that have arid, steep rocky and sunny habitats.[4]

The genius represented by 102 species and 108 taxa in the flora of Turkey and almost 50% of the genus is endemic with 48 species; therefore, it can be seen that Anatolia is the gene center of *Onosma* species.[5,6]

Onosma is derived from the words 'osma' in Latin which taken its source from the Greek words 'onos' and 'osmé' (smell and perfume) by the Swedish botanist, Linnaeus, who formalized the modern botanical nomenclature the species is known as 'Golden Drop' in the UK, it is called as witch, pacifier, pseudo air and gold drop among people.[7–9]

Onosma genus is a diffucult group to identify taxonomically and most diagnostic features depend on hair characteristics.[10,11] Leaves and stems have great significance to describe the sectional categories of the genus member swhich are divided into three sections; *Protononosma*, *Podonosma* and *Onosma.* Both section *Protonosma and Podonosma* are composed of one species, while *Onosma* is originally described to have two subsections which are *Asterotricha* with basal leaves covered by stellate bristles or asterosetae and *Haplotricha* with basal leaves covered by simple setae only.[12]

In the "*Flora of Turkey and The East Aegean Islands", Onosma* genus is defined as: "Perennial, usually suffruticose, herbs or biennial herbs. Indumentum typically hispid, with patent to adpressed setae arising from glabrous or stellately setulose tubercles; tubercles rarely absent, setae rarely reduced to hairs. Flowers in terminal, or terminal and lateral, bracteate cymes; flowers usually nodding at anthesis. Calyx 5-sect to base, or rarely with a short tubular cup near base, lobesscarcely to greatly accrescent after flowering. Corolla tubular, cylindricalcampanulate to clavate, white, cream, yellow or orange at first, turning reddish, brownish or bluish after anthesis in some species; lobes usually very short, patent or ± deflexed. Annulus glabrous, rarely hairy. Filaments

flattened, adnate to corolla tube in lower part, free above; anthers ± sagittate, usually coherent at base, rarely free or laterally coherent, included or rarely exserted, usually with a sterile apical connective. Style filiform, usually exserted; stigma small, capitate to minutely bilobed. Nutlets 4 or fewer, ovoid to trigonous or bipyramidal, acute or with ± laterally compressed beak, usually smooth and glossy, rarely rugose or tuberculate. Syn: *Podonosma* Boiss., *Colsmannia* Lehm.".[3]

1.1.3. *Onosma mollis*

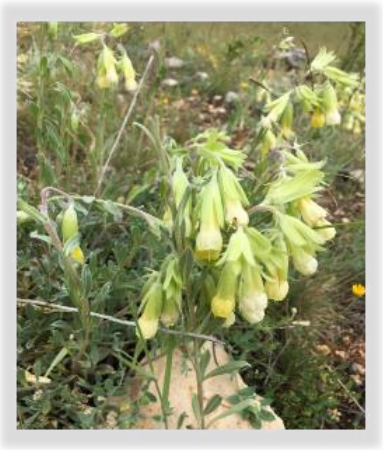


Figure 1.1. *Onosma mollis*

In the "*Flora of Turkey and The East Aegean Islands", Onosma mollis* is defined as: "Perennial. Plant greyish-white throughout with dense adpressed hairs (reduced setae) arising from stellate-hairy tubercles. Stems 15-35(-40) cm, ± unbranched. Leaves 40-100 x 3.5-9 mm, linear-spathulate to linear, obtuse to subacute; basal and lower cauline tapering towards base, upper sessile, broad at base. Inflorescence of 1-3 terminal and lateral cymes, scorpioid at first, becoming elongated and straight. Bracts linear-lanceolate, acute, shorter than calyx. Pedicels 1-3 mm. Calyx 12-14 mm in flower, to 22 mm in fruit, lobes narrowly triangular. Corolla lemon yellow to creamy white, 20-22 mm, cylindrical-campanulate, shortly pubescent. Anthers included, with sterile truncate tips, longer than filaments. Nutlets 5x3-5 mm, bipyramidate, with sharp ventral keel and acute apex, strawcoloured. Fl. 4-7. Fields, rocky places, steppe, 430-1800 m.".[3]
Type: [Turkey *C7]* in Mesopotamia, *Aucher* s.n. (G).

Inner Anatolia. **B6** Sivas: Gök Pinar S. of Gürün, 1800 m, *Sorger* 71-50-12! Maraş: 15 km N.E. of Elbistan towards Darende, 1240 m, *Hub.-Mor.* 12524. **B7** Sivas: Divriği, Dumluca Da., *T.Baytop* (lSTE 12886)! Malatya: S.E. of Malatya, S. of road to Elaziğ, *Alava* 6834! **C6** Gaziantep: Gaziantep to Nizip, nr Orul, 430-490 m, 29 iv 1865, *Hausskn.l* **C7** Urfa: nr Urfa, Birecik, *Hausskn.*

1.1.4. *Onosma taurica* var. t*aurica*

In the "*Flora of Turkey and The East Aegean Islands", Onosma mollis* is defined as:"Perennial, caespitose. Stems 12-25(-30) cm, usually simple, covered with quite long hairs usually arising from stellate-hairy tubercles, shortly hairy. Leaves adpressed-setose and shortly hairy, often whitish; basal 25-60 mm, gradually tapering into a rather long petiole; cauline linear-lanceolate to linear-oblong, obtuse, uppermost sessile. Inflorescence of 1-2 terminal cymes, straightened and elongated in fruit. Bracts usually shorter than calyx. Pedicels 0-1.5 mm. Calyx 12-13 mm, scarcely accrescent in fruit, lobes linear, adpressed- to subpatentsetose. Corolla white, cream or yellow, 22-25 mm, campanulate, gradually tapering towards base, glabrous. Anthers included, much shorter than filaments. Nutlets c. 2.5 mm, bipyramidate, dorsal keel indistinct, with short acute beak, brownish. Basal leaves (2-5-)3-7 mm wide, spathulate to linear-lanceolate, acute; plant 12-25(-30)cm var, tauricum var. tauricum**.** Syn: *O. velenovskyi* Davidov in Spis. Bâlg. Akad. Nauk. 12:104 (1915). Ic: Bot. Mag. 23: t. 889 (1805); Fl. RPR 7: t. 32 f. 3 (1960), rocky and sandy slopes, steppe, etc., 50-2400 m.".[3]

Type: Crimea, *Pallas* (Hb. B-Willd. 3367).
N. Turkey, W., S. & C. Anatolia; rare in E. Anatolia. **A2(E)** Istanbul: nr Kizilcaali, *A. & T.Baytoç* (lSTE 6647)! **A2(A)** Bursa: Mudanya, Arnavutköy, *Bornm.* 1899:5308! **A3** Bolu: nr Abant G., *H. & E.Walter* 4662. **A4** Kastamonu: Kastamonu, 900 m, *D.* 21692! **A7** Trabzon: 35 km S. of Trabzon, 610 m, *Apold et al.* 83! **B2** Manisa: 30 km from lnegöl to Buldan, *Hub.-Mor.* 5627. B3 Eskişehir: 18 km from Eskişehir to Kütahya, 880 m, *Hub.-Mor.* 12537. **B4** Kirşehir: 5 km E. of Sofular, Bozcaali Da., 1200 m, *Sorger* 64-20-5! **B5** Nevşehir; 8 km E. of Ürgüp, 1200 m, *Sorger* 64-32-66! **B6** Yozgat: Akdağmadeni to Büyük Nalbant Da., 1800 m, *Coode & Jones* 2087! **B9** Van: Çatak, *T.Baytop* (lSTE 4481)! **C2** Aydin: below Karacasu, 400-500 m, *D.* 41648! **C3** Burdur: 5 km S. of Burdur,

1100 m, *Sorger* 63-43-32! **C4** lçel: Büyük Eğri Da. to Mut, 1500 m, *Coode & Jones* 887! **C5** Adana: Bürücek nr Pozanti, 1250 m, *Demiriz* 1254. **C6** Hatay: Yayladağ, 600 m, *D.* 27188!

Figure 1.2. *Onosma taurica* var. *taurica*

1.2. Traditional Uses of *Onosma* species

Onosma L. species have been used traditionally for the treatment of various diseases such as rheumatism, kidney problems and heart attack in folk medicine. The underground parts are used for its diuretic, hemostatic and sedative activity. Apart from these, they have been used in hypertension treatment, fever and nervous system disorders in India[4], and inflammatory disorders such as pain, hemorrhoids, and tonsillitis in Turkey.[2]

O. hispidum is used to treat various cases such as fevers, pain relief and wounds. Dried roots are rubbed in outwardly tu cutaneous eruptions while the flowers are applied as cardiotonic and stimulant.[13]

O. argentatum Hub.-Mor. and *O. chlorotricum* roots extracted with oil and have been used for wound and burn treatment in folk medicine in Lorestan province.[4]

The root extract of *O. armeniacum* preparing with butter is used in treatment of wound, hemorrhoids, and gynecological problems in Turkey.[14]

The leaves of *O. fruticosa* Sibth are used for treatment of respiratory problems in the Turkish Republic of Northern Cyprus.[15]

The root of *O. echioides* was applied externally to skin eruptions while leaves are used as purgative for children. The usage of flowers is to treat rheumatism and papitations of the heart as a cardiac tonic and stimulant.[14]

The root of *O. paniculata* is used for treatment of different disorders with the inclusion of cancer in China.[4,13]

O. bracteosum, which is called as Gaozaban in India, is used as a tonic that supports to form the body's immune defense with regulation of urination.[4] Moreover, they have been used for asthma, bronchitis, demulcent treatments. Decoction of the aerial parts is using for the treatment of stomach, palpitation of heart and wound in folk medicine.[16]

1.3. Naphthoquinones

Naphthoquinones are one of the class of secondary metabolites widespread in many families which are Avicenniaceae[17], Bignoniaceae[18,19], Boraginaceae[20], Droseraceae[21], Ebenaceae[22], Juglandaceae[23], Nepenthaceae[24] and Plumbagnaceae.[25] In addition, microorganisms such as some actinomycetes, fungi, lichens. Naphthoquinones are oxygen-derivatives of naphthalene constitute on C_6-C_4 skeleton. They present monomeric structure in general but dimers and trimers are known.

Depending on forms, there are various biosynthesis pathway including acetate and malonate (plumbagin), shikimate/succinyl CoA combined pathway (lawsone) and shikimate/mevalonate pathway (alkannin/shikonin). Because of their colors changing from yellow to Brown, they are used for industrial purposes as natural colorant. Naphthoquinones have exhibited wide spectrum of bioactivities such as antimicrobial, antifungal and antiviral. In the light of these research, shikonin and alkannins are potential candidate to anticancer agents.[26–30]

1.3.1. Alkannin and Shikonin

Alkannin **(1)** and shikonin **(2)** are enantiomeric naphtoquinones that R and S enantiomers respectively. Shikonin had been isolated from the roots of *L. erythrorhizon* for the first time and alkannin had been discovered from *A. tinctorial*. More recently, these enantiomers were analyzed. They are biosynthesized by combination of shikimate and mevalonate pathways.[26,27]

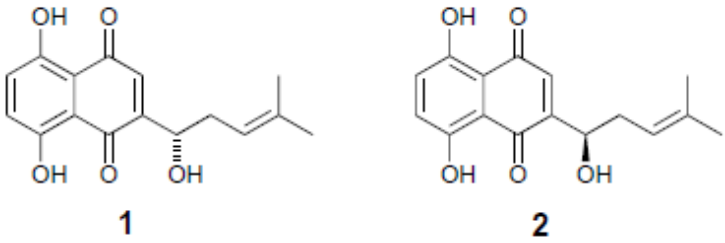
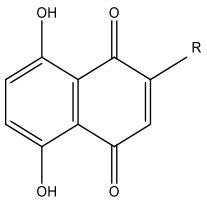
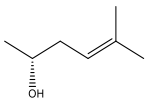
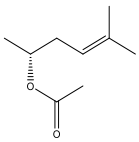
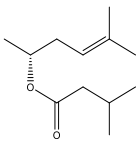
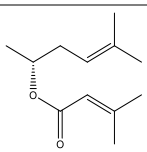


Figure 1.3. Structures of Alkannin **(1)** and Shikonin **(2)**

Alkannin and shikonin derivatives from natural products are illustrated with their occurrence in Table 1.1.

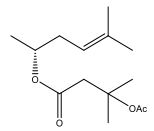
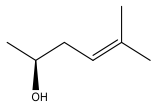
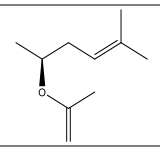
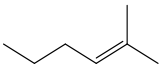
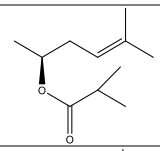
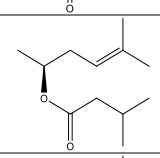
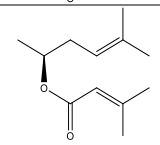
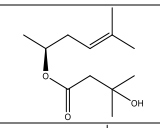
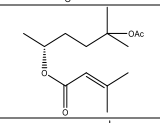
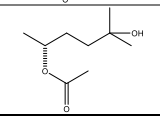
Table 1.1 Most of alkannin and shikonin derivatives from *Onosma* species



R	Name	Occurrence	References
	alkannin/ arnebin-4	*O. echioides, O. paniculata*	4,26
	acetylalkannin/ arnebin-3	*O. paniculata, O. arenaria*	4,26,31
	isovalerylalkannin	*O. heterophylla, O. paniculata*	4,26,32
	β, β - dimethylacrylalkannin/ arnebin-1	*O. heterophylla, O. hookeri, O. paniculata.*	4,26,32

(cont. on next page)

Table 1.1. (Cont.)

R	Name	Occurrence	References
	β-acetoxyisovalerylalkannin	*Onosma heterophylla, O. paniculata*	4,32
	shikonin	*O. caucasicum, O. conferitum, O. hookeri, O. livanovii, O. polyphyllum, O. sericium,O. setosum, O. visianii, O. zerizaminium*	4,26
	acetylshikonin	*O. confertum, O. hookeri, O. paniculatum, O. echioides, O. argentatum, O. leptanhtha*	4,26,33,34
	Deoxyshikonin/ deoxyalkannin/ arnebin-7	*O. argentatum, O. paniculatum, Onosma confertum, O. heterophylla*	4,26,34,35
	isobutyrylshikonin	*O. visianii*	36
	isovalerylshikonin	*O. argentatum, O. echioides, O. leptanhtha, O. visianii*	33,34,36,37
	β, β -dimethylacrylshikonin	*O. confertum, O. paniculatum, O. hookeri, O. zerizaminum, O. leptanhtha*	4,26,33
	β -hydroxyisovalerylshikonin	*O. visianii*	36
	acetylarnebin-2	*O. heterophylla*	2
	arnebin-6	*O. echioides*	37

1.3.2. Biosynthesis of Alkannin and Shikonin

A widely excepted pathway for the biosynthesis of alkannin and shikonin is shown in Figure 1.4. There are two main precursors which are 4-hydroxybenzoic acid (PHB,45) and geranyl pyrosphoshate (GPP,54). PHB geranyltransferase is used for maintenance of m-geranylPHB.[26]

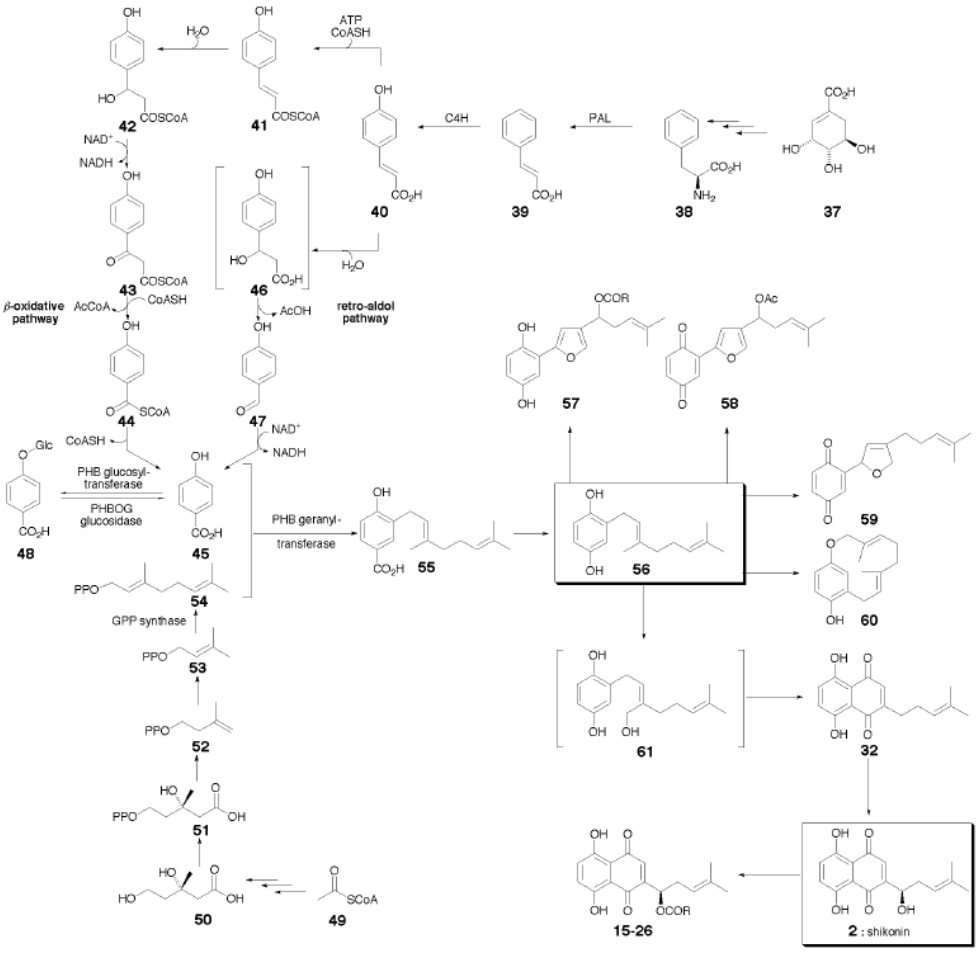


Figure 1.4. Biosynthesis of shikonin[26]

1.3.3. Phytochemistry Studies on *Onosma* Species

Hence *Onosma* species with high endemism ratio in Turkey, phytochemistry studies of these species were rare. Within the scope of phytochemical studies of *Onosma* genus naphtoquinones had been isolated.

Ahmad *et al*. studied on chloroform extract of leaves of *O. limitaneum* to isolate onosmone and bauerenone along with β-sitosterol glycoside. These compounds were reported for the first time from this plant.[38]

Ozgen *et al.* examined the *n*-hexane–dichloromethane root extract of *O. argentatum* that resulted in identification of four shikonin derivatives, *viz.* deoxyshikonin, acetylshikonin, 3-hydroxy-isovaleryl shikonin and 5,8-*O*-dimethylacetylshikonin.[39–41]

Alkannin and shikonin components with derivations of quinone which are deoxyalkannin or deoxyshikonin, arnebin-6 and 5, 8-dihydroxy-2-(4-methyl-6-oxo-5,6-

dihydro-2H-piran-2-yl)-[1,4] napthoquinone were isolated using high-performance liquid chromatography-mass spectrometry in the study of *O. echioides.*[42] Apart from this, Volatile components, from aerial parts such as leaves and flowers, were identified by gas chromatography.[43]

O. paniculata was subject of a phytochemical study. By preparative HPLC, three pure compounds (β-hydroxyisovalerylshikonin, acetylshikonin, dimethylacrylshikonin) together with a mixture of α-methylbutyrylshikonin–isovalerylshikonin were isolated and determined from the petroleum ether extract.[44]

Two naphthazarin derivatives from cyclohexane extract of *O. arenaria* and their *in vitro* cytotoxicities were reported. In this study, β-hydroxyisovalerylalkannin and acetylalkannin were isolated compounds, and their cytotoxic activities were found to be IC50 values of 0.55 μM and 2.30 μM on human cervix adenocarcinoma cells (HeLa).[31]

The cyclohexane root extracts of *O. leptanhtha* found to be the most active extracts in cytotoxicity and anti-inflammatory activity screenings, was investigated via bioactivity-guided fractionation to give β,β-dimethylacrylshikonin, isovalerylshikonin and acetylshikonin.[33]

O. visianii was extracted with *n*-hexane to isolate 11 secondary metabolites as isovalerylshikonin, isobutyrylshikonin, acetylshikonin, hydroxyisovalerylshikonin, shikonin-β,β-dimethylacrylate, propionylshikonin, 5,8-dimethoxy acetylshikonin, 1-(5,8-dimethoxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-en-1-yl2-methyl-butanoate, 5,8 dimethoxy isobutyrylshikonin, 5,8-O-dimethyldeoxyshikonin and (*E*)-2-(4-hydroxy-4-methylpent-2-en-1-yl)-5,8-dimethoxy-naphthalene-1,4-dione.[36]

Dong *et al.* identified four new naphthoquinones as Shikometabolin G, Naphthofuranin A, Naphthofuranin B, Naphthofuranin C, together with six known ones as deoxyshikonin, β,β-dimethylacrylshikonin, acetylshikonin, bothriodumin, shikonofuran E, from methanol extract of *O. paniculatum*.[45]

Onosmanones A and B, two novel quinonoid xanthenes with two geranyl groups, were isolated that the whole plants of *Onosma paniculatum* was extracted with 70 % Me_2CO/H_2O.[46]

1.3.4. Bioactivity studies of Naphthoquinones from *Onosma* Species

Different bioactivity studies, such as wound healing, antimicrobial, antithrombotic, and antifungal, of different *Onosma* species and metabolites which were

obtained by bioactivity-guided isolation studies were examined. Naphthoquinone and flavonoid derivatives considered these activities, and some phytochemical studies promotes the bioactivity of naphthoquinones. As a result, it is stated that alkannin, shikonin and their derivatives are potent pharmaceutical compounds due to the broad spectrum biological activities.[4,35]

Ozgen *et al.* studied on antimicrobial and antioxidant activity of *O. argentatum*. It is found that the *n*-hexane-dichloromethane extract of *O. argentatum* root showed high antioxidant activity (98%) at concentration of 0.1% and the IC_{50} was 0.0076% w/v. Moreover, the extract had demonstrated antimicrobial activity on *B. subtilis*, *E. coli* and *Staphyloccoccus aureus* with inhibition zone 28, 13 and 32 mm.[39] In another study, The root extract stimulate the growth of the human amnion fibroblast at 0.1g/mL and isolated 5,8-O-dimethylacetylshikonin at 0.05–5 g/mL whereas any activity had not showed for deoxyshikonin and acetylshikonin.[47]

The study of *O. gigantea* examined biological investigations of ethyl acetate, methanolic, and aqueous extracts for the antioxidant and cholinesterase inhibitor activity. the aqueous extract exhibited the highest antioxidant activity (134.31 μmol TEs/g adm), whereas the ethyl acetate extract had the less (2.97 μmol TEs/g adm). Moreover, aqueous extract did not showed cholinesterase inhibitor activity while the methanolic and aqueous extracts had the highest AChE and BChE inhibitory activities (31.57 and 6.87 μmol GALAEs/g adm, respectively).[48]

Moghaddam *et al.* reported antibacterial activity of *O. dichroanthum* various root extracts which are acetone, chloroform, methanol, ethanol and *n*-hexane-dichloromethane (1:1). Acetone extract was the most effective plant extracts and B. cereus with 17 mm inhibition zone and its lowest minimum inhibitory concentration (MIC) was 0.078 mg/ml against *Bacillus cereus*.[49]

Kretschmer *et al.* studied on bioactivity guided isolation studies of *O. paniculata* to obtain β-hydroxyisovalerylshikonin, acetylshikonin, dimethylacrylshikonin and α-methylbutyrylshikonin/isovalerylshikonin mixture. β-hydroxyisovalerylshikonin had demonstrated high cytotoxic activity on human CCRF-CEM leukemia, MDA-MB-231 and HCT 116 colon cancer cell lines with IC_{50} values of 0.6, 4.6 and 4.1 μM, respectively and acetylshikonin showed cytotoxicity on human U251 glioblastoma cell line being IC_{50} value of 15.9 μM. Moreover, α-methylbutyrylshikonin the highest on human SBcl2, WM35, WM9, and WM164 melanoma cell lines (IC_{50} values: 1.1, 2.3, 2.7 and 8.3 μM, respectively). Furthermore, α-methylbutyrylshikonin had led to increase the number of

cells has been shown the subG1 phase of cell cycle as well as to caspase-3/7 activation, indicating apoptotic cell death.[32,50]

Hemmati et al. studied on healing effect of *n*-hexane dichloromethane root extract (1: 1) of *Onosma bulbotrichum and* the second degree burn in rabbit model was investigated. In this study, the dose of 5% had the best effect for burn improvement; shown to have healing, and anti-inflammatory effects.[51]

Ahmet et al. focused on pharmaceutical activity of *O. griffithii* and its antifungal and parasitical activities were investigated. The crude methanolic extract had showed antifungal activity against *Aspergillus flavus* (55%) and *Fusarium solani* (40%) and had demonstrated parasiticidal activity (IC_{50}: 31.03 μg/ml) on *Leishmania major*.[52]

In the study of *O. arenaria* cyclohexane extract, β-hydroxy-isovalerylalkannin and acetylalkannin had isolated by bioactivity guided study. Acetylalkannin exhibited five-fold cytotoxic activity on HeLa cell lines (IC_{50}: 0.55 μM) compared to β-hydroxyisovalerylalkannin (IC_{50}: 2.30 μM). Acetylalkannin demonstrated the strongest activity both K562 (IC_{50}: 0.40 μM) and PBMC cells (-PHA, IC_{50}: 0.39 and +PHA, IC_{50}: 1.55 μM).[31]

The study of *O. leptantha* investigated that β, β -dimethylacrylshikonin, isovalerylshikonin and acetylshikonin had been isolated from cyclohexane extract and cytotoxic activities were screened versus a murine leukaemia and a human solid tumour cell line. β, β -dimethylacrylshikonin exhibited the highest activity against both cell lines (IC_{50}: 390 nM for L1210 cells, and approx. 2 mM for HT-1080) and acetylshikonin is active only in high concentrations.[33]

The study of methanol extract of *O. paniculatum* reported that ten naphthoquinones as Shikometabolin G, Naphthofuranin A, Naphthofuranin B, Naphthofuranin C, deoxyshikonin, β,β-dimethylacrylshikonin, acetylshikonin, bothriodumin and shikonofuran E. Whole compounds were investigated for inhibitory effects on NO production inmurine macrophage RAW264.7 to determine the potential property of anti-inflammation. and displayed good activity on the inhibition; IC_{50} values varied between 0.4 and 16.5 μM.[45]

1.3.5. Antitumor Activity of Alkannin/Shikonin Derivatives

Recent studies illustrated that derivatives of shikonin/alkannin have chemotherapeutic effects by blocking cancer cell growth, stimulating of apoptosis, having

an inhibitory activity of DNA topoisomerase, antimitogenic effect, reduction of carcinogenesis and angiogenesis.[28]

The mechanism providing cytotoxic activity of alkannin, shikonin and their derivatives had been investigated; there has been several theses to block cellular processes as shown in Figure 1.5.[26]

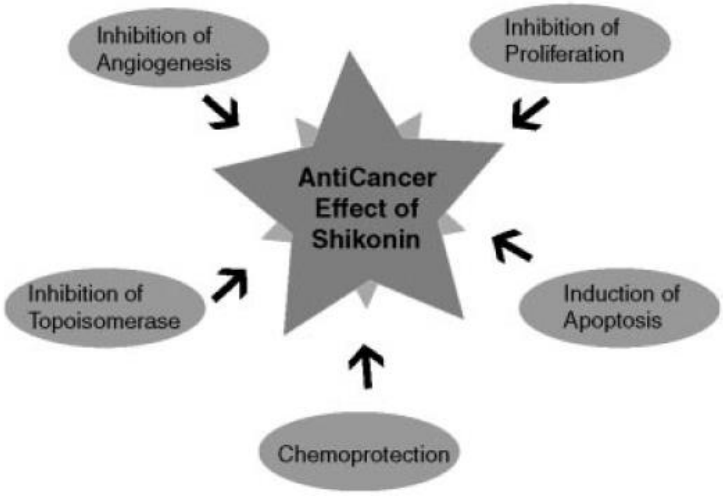


Figure 1.5. Potential targets of alkannin/shikonin[28]

1.3.5.1. Oxidative Stress

Oxidative stress, caused by the capacity to enter to the redox cycle, is considered to be responsible for cytotoxic activity. NADPH-cytochrome P-450 reductase can catalyse one electron reduction on quinonic compounds to generate semiquinone radical that start a redox cycle; it can autooxidize for regeneration of quinone and formation of superoxide radical anion in the existence of dioxygen. Formed free radicals and/or semiquinone with a chain mechanism can cause damage cells which could make contribution to cytotoxic activity.[26]

1.3.5.2. Bioreductive Alkylation

Bioreductive alkylation has been suggested to be responsible for attractiveness of cytotoxicity mechanisms for quinones. In the mechanism, generation of a highly effective

alkylating agent, is shown by the reduction of molecules with proper leaving groups. This electrophile can serve cellular nucleophiles as captive, which can lead to cell death or DNA glutathione. Since the mechanism can be nonspecific, this can cause the death of both healthy and tumor cells without discrimination.[26,53,54] The mechanism of bioreductive alkylation was proposed by Moose is shown in Figure 1.6.[55]

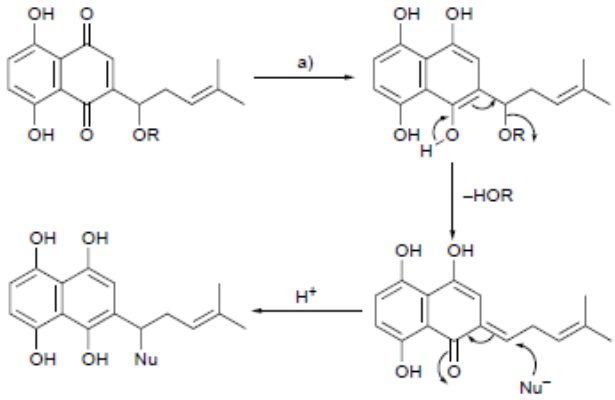


Figure 1.6. A possible bioreductive alkylation mechanism of alkannin and shikonin **(2)** (Moore) **a.** Reduction, **Nu.** nucleophile.

1.4. DNA Topoisomerases

In 1971, The DNA Topoisomerase enzyme was described for the first time by James Wang when working with supercoiled DNA of *E. coli* and that called as "omega protein" which is responsible for confers supercoiled DNA with nuclease and ligase activity.[56] Topoisomerases are classified are classified, mainly, as Type I and Type II which are divided into subgroups.[57]

DNA Topoisomerases break single or both chains of the DNA strand to reduce the stress generated in during DNA replication or transcription, and enable the DNA-interacting enzymes to function properly.[58,59] If stress on the DNA does not be removed by topoisomerases, this stress will form abnormal structures such as D loop (pairing of DNA double strand with complementary single stranded DNA segment), R loop (pairing of RNA with template DNA strand), guanosine quadruple structures and Z-DNA, resulting in the death of many chromosomal fractures.[59,60]

Topoisomerases which is responsible for topological changes on DNA by breakage of chains perform; these tasks with transesterification process that takes place

between protein and DNA. the hydroxyl group of the tyrosine amino acid, which is located in the centre, attacks the phosphate group of DNAs, resulting a transient phosphotyrosine binding while breaking phosphodiester bond. After that, a reverse transesterification reaction takes place between protein and DNA; phosphotyrosine binding is broken and ligation takes place (Figure 1.7).[61,62]

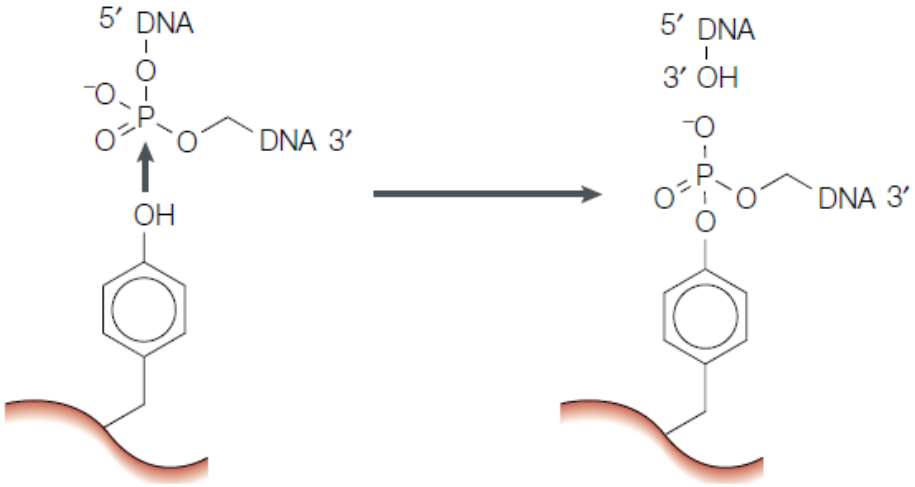


Figure 1.7. Reaction Mechanism of DNA Topoisomerases[61]

Topoisomerase enzymes act by breaking single strand (type I) or breaking two strands (type II). While Type I DNA Topoisomerases produce single strand breaks and do not require ATP energy, Type II DNA Topoisomerases are enzymes that require ATP and function by forming double strand breaks.[61]

1.4.1. DNA Topoisomerase I

Topoisomerase I are monomeric enzymes that solve problems by the helical nature of DNA.[63] Because of stored energy at torsional tension of DNA, Topoisomerase I do not need high energy cofactor for activity.[64]

According to the active site of topoisomerase binding on the broken DNA strand, Type I enzymes are classified by two classes. Consequently, the topoisomerases bound to the 5'P end of the broken DNA are Type IA, whereas those bound to the 3'OH end are Type IB and Type IC enzymes that differ from Type IB depending on sequence.[57,60]

When type IA links to DNA, double-stranding of DNA cannot easily rotate freely around each other. Instead of this, each reaction passes through the thread that is intact from the threads and the thread that is broken is reassembled. On the other hand, Topoisomerase IB enzymes relax how the 5 'OH end of the broken DNA around the intact chain (Figure 1.8).

Type IA DNA Topoisomerases can relax negative supercoiled DNA with mechanism of enzyme bridge. If it is broken in a double helix, DNA construct may also exhibit activity decatenation. They do not require ATP for activity without gyrase but their activity relies on Mg^{+2} and Zn^{+2} ions.[62,66] Type IB enzymes can relax both positive and negative supercoiled DNA and act by DNA rotation. The DNA rotation mechanism of type IB enzymes allows multiple strands to pass through each strand breakage and recombination cycle.[61,67] They do not require ATP and divalent cations for activity. Mechanically important difference is that the reactions catalyzed by the Type IA enzyme occur in single stranded regions whereas in reactions catalyzed by Type IB the space is created in the double stranded DNA segment.[61]

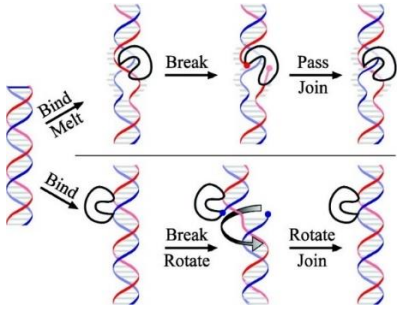


Figure 1.8. Mechanisms for type I topoisomerases **A.** Type IA mechanism **B.** Type IB mechanism[65]

Type IC had been isolated from only *Methanopyrus kandle* that relax both positive and negative supercoiled DNA like type IB enzyme. After sequence analysis and structural studies, it is discovered that Type IC belongs to Type IB class.[68,69]

1.4.2. DNA Topoisomerase II

Topoisomerase II act by complex mechanism including relaxation and decatenation, furthermore, they can play a role on both positive and negative relaxation.

These enzymes, which need ATP for their activities, provide to an elimination of super helical twist on DNA.[70] In this mechanism, G strand which is one of the DNA strand is entrapped and broken by the dimeric enzyme, the other segment (T strand) passes through the fracture formed. In the existence of Mg^{+2}, breakage of G strand is happened by binding tyrosine to DNA symmetrically with phosphotyrosine linkage between strands. The hydrolysis of ATP takes place in two steps in the reaction cycle for two times.[71,72]

Type II enzymes are divided into two subgroups which are type IIA and type IIB. Initially, all topoisomerases having ability of forming double chain fracture are accepted as Topoisomerase II; with the discovery of topoisomerase VII and VIII enzymes, class IIA and type IIB classifications are required.[73,74]

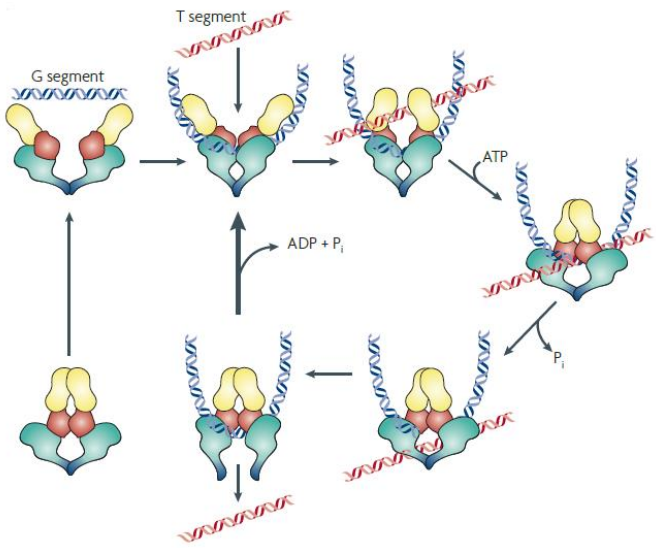


Figure 1.9. Action mechanism of Type II topoisomerase[71]

1.4.3. Classification of DNA Topoisomerases as Targeting Anticancer Drugs

Cancer is perhaps one of the most difficult human life-threatening diseases and in today's world, tremendous efforts are being made all over the scientific world to stop its dreadful effects. For that reason, increasing use of antiproliferative agents for therapeutic studies rather than signal pathways inhibitors and gene therapy which led to the recognition of DNA topoisomerase I and II as attractive targets for anticancer drug design and its development. Drugs targeting topoisomerase enzymes act by three steps which are

binding to DNA, the creation of fractures on DNA and relegation.[75] Compounds, which transform the enzyme to agents, that damage DNA by blocking the relegation, are called topoisomerase poisons.[76] These poisons are, also, known as Class I drugs that stabilizes the covalent complex of DNA-enzyme. Acridines, anthracyclines, actinomycins, ellipticines, alkaloids, isoflavones and quinolones belong to this class). Class II drugs are called as "Topoisomerase inhibitors" which inhibit enzyme catalytic activity without effecting covalent complex.[77]

Capable of binding to DNA via broad hydrophobic surfaces is called as intercalative properties; some of drugs must the DNA-binding ability to inhibit protein synthesis, RNA and DNA (Figure 1.10b-d and 1.10f). In contrast to some of drugs don't need intercalative properties to inhibit topoisomerases enzyme (Figure 1.10a and 1.10e).[78]

Table 1.2. DNA Topoisomerases inhibitors[77]

Class	Sub-class	Group	Example (s)	Target
Class I (Topoisomerase Poisons)	Intercalative	Acridines	m-AMSA	euk. topo II,
		Anthracyclines	Adriamycin[a], daunomycin	T4 topo II euk. topo II
		Actinomycins	Actinomycin D[a]	euk. topo II
		Ellipticines	2-methyl-9-OH- ellipticinium acetate	euk. topo II
		Miscellaneous	Intoplicine[a], mitoxan, bisantrene, cisplatin, aclacinomycin A[a], amirolide	euk. topo II
	Non-intercalative	Alkaloids	Camptothecin (CPT), irinotecan, topotecan, 9-amino-CPT	euk. topo I
		Epipodophyllotoxins	VP16, VM26	euk. topo II
		Isoflavodins	Genistein[a]	euk. topo II
		Quinolones	Nalidixic acid, oxolinic acid, norfloxacin, ciprofloxacin	gyrase, T4 topo II
		Miscellaneous	Azatoxin	euk. Topo II
Class II (Catalytic Inhibitors)		Coumarins	Novobiocin, coumermycin A_1, chlorobiocin	gyrase β, euk. topo II, rev. gyrase
		Fostriecin analogues	Fostriecin[a]	euk. topo II
		Miscellaneous	Merbarone, suramin, topostin[a,b]	euk. topo II

[a]Also shown to interfere with human topoisomerase I.
[b]No toxicity was reported on tumour cells.

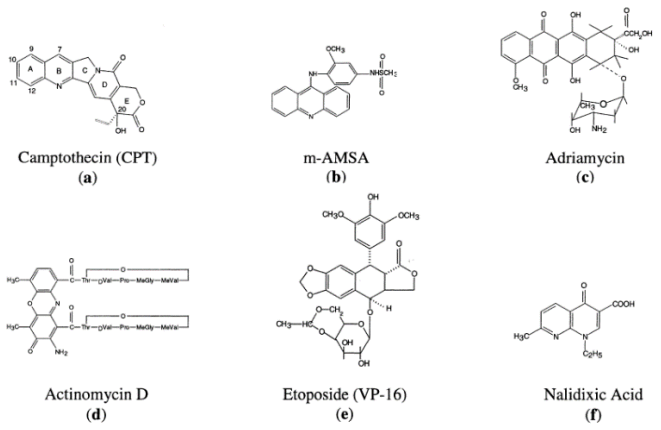


Figure 1.10. Chemical structures of chosen topoisomerase-targeting anticancer molecules[78]

1.4.3.1. Drugs targeting Type I Topoisomerases

Camptothecin known as antitumor agent was isolated from *Camptotheca acuminate* for the first time[79] and has been described as targeting the enzyme topoisomerase I.[80] It acts by the stabilization of covalent enzyme-DNA complex as poison. In previous studies, camptothecin did not interact directly to neither DNA nor type I enzyme; binding to TOP1-camptothecin-DNA ternary complex in a reversible manner that is apparently non-productive in catalysis. However, this situation is not fatal for the cell. that is why covalent complex must interact with the DNA replication tool.[77]

With discovery of the 6-unit lactone ring (ring E) which is responsible for inhibitory activity, different modifications were applied and various derivatives were produced. The main reason behind the production of various derivatives, was, to reduce the side effects with increasing water solubility and bioavailability of the molecule. Although its derivatives such as topotecan and irinotecan are used for treatment, there are limitations on the activity of camptothecin because the compounds lose their activity by opening lactone E ring having at physiological pH.[59,76,77]

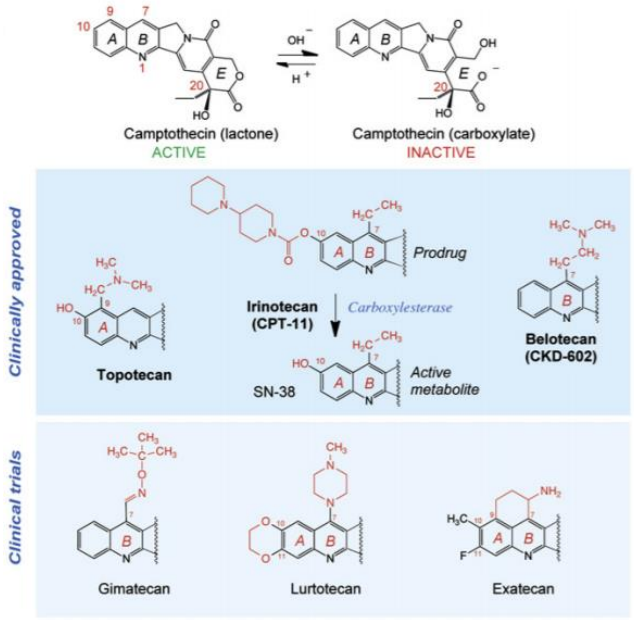


Figure 1.11. Camptothecin and its derivatives[59]

The instability of camptothecin and its derivatives originating from the E-ring has led to the discovery of different classes of compounds. In this context, indotecan and

indimitecan derivatives originating from the family of indenoisoquinoline have been discovered and clinical trials have been made on compounds founding that these compounds are more stable than camptothecin derivatives. Furthermore, TOP1-camptothecin-DNA ternary complexes are more permanent with fewer side effects and have multidrug resistance predecessor.[81]

Apart from the mechanism of action of camptothecin and its derivatives with intercalator properties, there are different compounds that bind to the minor groove of DNA and exhibit inhibitory properties. The poison effect of bis- and terbenzimidazoles is connected with binding strength to the DNA minor groove.[80]

1.4.3.2. Drugs Targeting Type II Topoisomerases

The activity of the enzyme topoisomerase II is based on a delicate balance in the cell. The preservation of this equilibrium provides to carry out appropriate chromosome segregation and other processes can be carried out in a healthy manner and the cell normally grows (Figure 1.12). DNA fragments generated by the enzyme are reversible and tolerable for cell.[78] However, the cell leads to death because of disruption of the balance through inhibitors or poisons which lead to recognition of inhibitory activity as attractive targets for anticancer drug design and its development.[70,82]

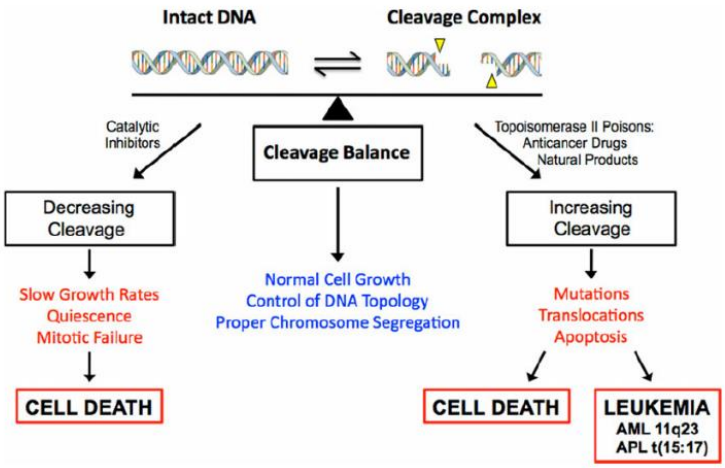


Figure 1.12. Equilibrium of topoisomerase II enzyme breakdown reaction in the cell[82]

According to alteration of topoisomerase II activity, compounds are classified by two categories which are catalytic inhibitors that reduce overall enzyme activity and

poisons that rise the rate of topo II-DNA cleavage complexes and transform into a cellular toxin initiating mutagenic and fatal consequences.[77]

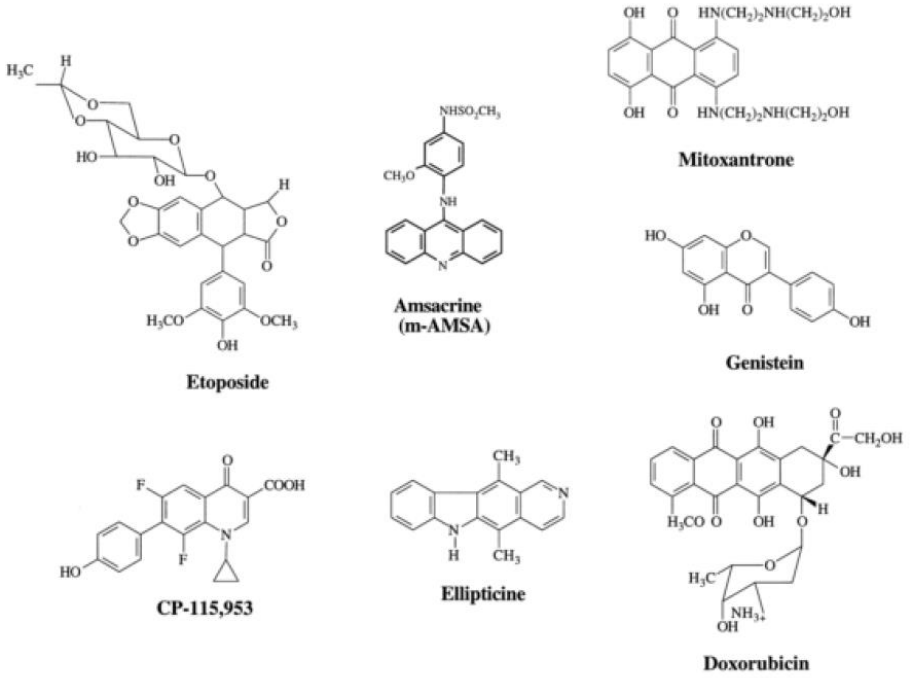


Figure 1.13. Topoisomerase II inhibitors[78]

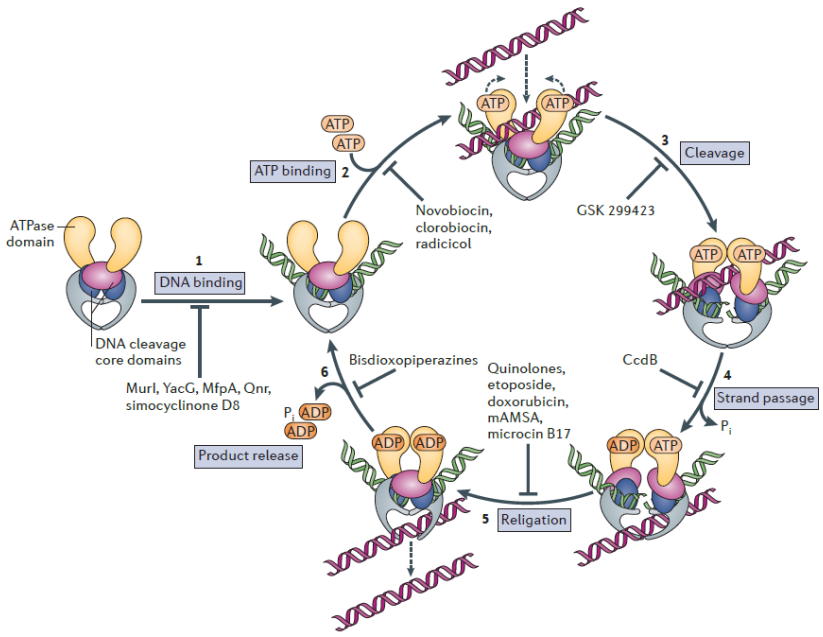


Figure 1.14. Topoisomerases II inhibition or poisoning points[83]

The topoisomerase II poisons are used as anticancer agents stabilizing covalent complex in three different ways: drug can interact with DNA to form complex, drug can bind to the enzyme-DNA double complex or drug may interact with enzyme to form a ternary covalent complex with the interaction of enzymes. However, in kinetic studies related to agents such as etoposide and elliptice, drugs form ternary covalent complex with interaction of enzyme.[78]

Some inhibitors of topoisomerase II enzyme without stabilizing the covalent complex change conformation of DNA with intercalation to lead to bind to DNA for enzyme and prevent covalent complex formation. The others such as merbone and bisdoxopiperazine affect the conformational change of the enzyme by inhibition of ATP hydrolysis (Figure 1.14).[59,83]

1.4.4. DNA Topoisomerases Studies of Naphthoquinone Derivatives

In 1998, according to Ray *et. al.*, diospirin, a bisnaphthoquinone with anticancer activity, showed complete inhibition of relaxation activity at a concentration of 15 μg/mL diospirin. As the dose increased, the inhibition increased.[85]

Plyta *et al.* reported that naphthoquinones due to the presence of phenolic hydroxyl group were potent inhibitors for Topoisomerase I. Synthetic and naturally occurring such as shikonin, alkannin and acetylalkannin isolated from *Alkanna tinctorial* were tested and minimum inhibition concentration (MIC) of each substance were observed (MIC: 50, 500 and 5 μM, respectively).[86]

In a study conducted by Fujii et al., it was reported that plumbagin and shikonin obtained from the plant induce mammalian topoisomerase II mediated breakage *in vitro.* Topo II-naphthoquinone mixture reduced DNA break at 65°C. This shows that the mechanism of topo II mediated DNA breakage induced by these naphthoquinone is related to the breakable complex formation. In addition, plumbagin is a weak intercalator at 50 μM, whereas shikonin does not intercalate into DNA even at 250 μM .[87]

EWS-FLI1 is a multiple mutant transcription factors that result in aberrant gene expression patterns which create the neoplastic phenotype. Chen *et al*. studied on screening of 5200 small molecules to disrupt the binding of EWS-FLI1 by comparing DNA-binding chemotherapeutics such as omycin D, cisplatin, doxorubicin, daunorubicin, and epirubicinand found that shikonin isolated from *Lithospermum erythrorhizon,* similarly disrupted protein-DNA interactions. Shikonin inhibited the

binding of EWSFLI1 with an IC_{50} of 600 nM. Shikonin only weakly blocked Topo I-mediated DNA relaxation at 1000 μM, whereas inhibited DNA relaxation by Topo II at 20 μM. The comparison of shikonin with doxorubicin and actinomycin D as known DNA intercalator suggested that shikonin is not a DNA intercalator.[84]

Ogawa et al. studied on inhibitory effect of shikonin on Topoisomerases II that relationships between topoisomerase inhibition and the transcriptional oscillation of *Bmal1* which drives gene expression with circadian rhythms. 125 μM concentrations of shikonin showed inhibition of Topoisomerase II activity and shikonin was incorporated into the nucleus and Top2 was located in the Bmal1 promoter, suggesting the relationship between Bmal1 transcription and Top2 inhibition.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Materials

For collection of different *Onosma* species from Southwest Anatolia, "*Flora of Turkey and The East Aegean Islands*" and other sources including herbarium records, related graduate theses, articles were searched to determine localities. Later, field studies were carried out for the collection of selected species in anthesis period that was mainly between April and July. Collected species were identified by 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 plant materials were firstly dried in shade, afterwards the roots and aerial parts were separated. All the plant materials were powdered and stored in non-light plastic bottles (Figure 2.1).

Table 2.1. Collected *Onosma* species and their locations

Name	Analysis Code	Herbarium Code	Location	Date
O. nana	G1-12	42709	Ballıkköy road-Ören-Fethiye Muğla, 1357 m 36°49.886'K 29°24.849'D	13.05.2017
O. oreodoxum	G2-01	42710	Manavgat Çayı road–Antalya, 189 m 37°11.048'K 31°12.237'D	19.05.2017
O. armenum	G2-07	42711	Başlar-Beydiğin, 1159 m 37°4.726'K 31°27.314'D	21.05.2017

(cont. on next page)

Table 2.1 (cont.)

Name	Analysis Code	Herbarium Code	Location	Date
O. frutescens	G2-11	42712	Eskiavdan Kızılkaya 796 m 37º16.769'K 30º26.390'D	21.05.2017
O. aucheranum	G2-12	42713	Yazır Yayla road, 984m 36º59.185'K 30º18.322'D	21.05.2017
O. mite	G2-13	42714	Termessos National Park, 395 m 37º1.004'K 30º31.306'D	21.05.2017
O. heterophyllum	G3-01	42715	Serinhisar-Yeşilova Salda Lake, Burdur road, 1158 m 37º31.459'K 29º39.200'D	28.05.2017
O. bracteosum	G3-03	42716	Vicinity of Burdur Gençlik Hiz. Ve Spor İl Müd. Çemdik Su Sporları ve Kamp Eğ. Mrkz. 37º41.498'K 30º11.540'D	28.05.2017
O. taurica* var. *taurica	G3-18	42717	Kütahya-Uşak highway, Alikahya Village 38.831862 'K 29.266381' D	31.05.2017
O. aksoyii	G4-09	42718	Sütçüler-Beyşehir, Ayvalıpınar Village 1146 m 37º41.614'K 31º01.340'D	16.06.2017
O. bornmuelleri	G4-15	42719	Beyşehir- Derebucak highway, 1080 m 37º32.146'K 31º29.999'D	17.06.2017
O. mollis	G4-18	42720	Gencek Neighborhood, Derebucak/Konya, 1258 m 37º25.688'K 31º31.756'D	17.06.2017

***Onosma* species selected for bioactivity-guided isolation studies

Figure 2.1. Powdered and bottled root materials of *Onosma* species

2.1.2. Used Chemicals

Acetonitrile: VWR Chemicals

Ethyl acetate: Sigma-Aldrich

Chloroform: Sigma-Aldrich

Methanol: Sigma-Aldrich

n-butanol: Sigma-Aldrich

n-hexane: Sigma-Aldrich

Petroleum Ether: VWR Chemicals

Dichloromethane: VWR Chemicals Sulfuric Acid: Merck

Formic acid: Sigma-Aldrich

Agarose: Basica LE

Camptothecin: Sigma-Aldrich

Dimethyl Sulfoxide: Sigma-Aldrich

EDTA: Sigma-Aldrich

Ethanol: Merck

Ethidium Bromide: Sigma-Aldrich

Glycerol: Sigma-Aldrich

HCl: Merck, Darmstadt

Sodium Dodecyl Sulfate (SDS): Sigma-Aldrich

2.1.3. Cell Lines and Medium Components for Cytotoxicity Studies

DU145 (metastatic prostate cancer), **HeLa** (human cervix carcinoma)**, MCF-7** (Human breast adenocarcinoma), **A-549** (human lung carcinoma) in DMEM with 10% FBS; **MRC-5** (human lung fibroblast), **HEP-G2** (Human hepatocellular carcinoma) in

EMEM with 10% FBS; **Capan-1** (human pancreatic ductal adenocarcinoma) in IMDM with 20% FBS and **HCC-1937** (human breast carcinoma cell) in RPMI with 10% FBS were cultured in 5% CO_2 at 37°C.

2.1.4. DNA Topoisomerases Studies

2.1.4.1. Solutions

TAE buffer solution: 10x TAE buffer solution were prepared with 400 mM Tris-acetate and 10 mM EDTA at pH value 8.0; diluted 1:10 for experiments.

Ethidium Bromide: 1.5 μg/mL of ethidium bromide solution was prepared from stock solution (10 mg/mL Etidium bromide).

Cleavage reaction buffer solution: 10 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 100 mM KCl, 1.1 mM EDTA, and 2.5% (v/v) glycerol.

2.1.4.2. Enzymes

Topoisomerase I (Human topoisomerase I) and II enzymes (Human topoisomerase II) (Inspiralis).

2.1.4.3. Substrates

Plasmid DNA substrate pBR322 (Inspiralis) and kinetoplast DNA (kDNA) (Inspiralis) were used for DNA Topoisomerase related experiments.

2.1.5. Instruments

Nuclear Magnetic Resonance Spectrometry: Varian AS400 (400 MHz)

HPLC: Thermo Scientific-Dionex Ultimate 3000

Optical rotation: Rudolph Research Analytical, AutoPol I

Mass Spectrometry: Agilent 1200/6530 Instrument – HRTOFMS

Microplate Reader: Versa MAX

Electrophoresis power supply: Thermo

UV Imaging system: Vilber Lourmat

Horizontal Electrophoresis Device: Thermo

2.2. Methods

2.2.1. Preliminary Studies

Preliminary extractions of the plant materials (Table 2.1) were carried out as mentioned in Methods 2.2.1.2. After that, HPLC analysis (Methods 2.2.1.2) and cytotoxicity studies (Methods 2.3) were performed. According to obtained results (Results 3.2 and 3.4), *Onosma* species with higher cytotoxicity (IC_{50}<8 µg/mL) and chemical diversity were chosen for further bioactivity-guided isolation studies.

2.2.1.1. Extraction

Soxhlet extractions in 150 ml of petroleum ether, dichloromethane and methanol in turn were performed with 10 g of dried and pulverized roots of *Onosma* species. Phytochemical profiles of the extracts were visualized by TLC experiments [Silica gel 60 F_{254}; *n*-hexane:Ethyl acetate:Formic acid (8:2:0.2)]. Precipitates were discarded after paper filtration process, and clear extraction solutions were concentrated by rotary evaporation at 40°C and stored in tightly capped vials in refrigerator until further use.

2.2.1.2. Preparing Samples for HPLC Analysis

1.5 g of powdered roots were extracted with 10 ml of petroleum ether using ultrasonic bath for 20 minutes and this process was repeated four times. The solvent was evaporated by rotary evaporator at 40°C. The final concentrations of samples were adjusted approximately as 1000 ppm with 750 µl $CHCl_3$ and 1250 µl MeOH solution (37.5% $CHCl_3$ - 62.5% MeOH). Before analysis, samples were filtered with 1.22 µm

PTFE. The same extraction procedures were performed with dichloromethane on the remaining plant pulp, and samples were prepared using abovementioned method.

Identification of HPLC analysis Conditions

Thermo Scientific-Dionex Ultimate 3000 system was used consisting of a quadruple pump, automatic sample injection section, column furnace and sequential diode detector (DAD) equipment.

Table 2.2. HPLC Analysis Condition

Time	1.025% TFA UPW	1.025% TFA ACN
1.00	40	60
10	27	73
13	25	75
20	20	80
25	0	100
30	0	100
31	40	60
35	40	60

All analyses were carried out with Synergi Max C18 column, and 1.025% TFA-ultra pure water and 1.025% TFA acetonitrile were used as solvent system. Analysis condition is given above.

2.2.2. Bioactivity-guided Extraction, Isolation and Purification Studies

Methods 2.2.2 were applied for isolation and purification studies, and methods for cytotoxicity studies were shown in Methods 2.3. Based on the results, *O. taurica* var. *taurica* and *O. mollis* were selected for further fractionation and purification studies.

2.2.2.1. Extraction

2.2.2.1.1. *Onosma taurica* var. *taurica*

O. taurica var. *taurica* which showed significant cytotoxic activity (IC_{50} < 8 μg/mL) and high chemical diversity, was taken to the further bioactivity-guided isolation. For this purpose, the powdered root of *O. taurica* var. *taurica* (350 g) was extracted using an equal volume (500 ml) of petroleum ether, dichloromethane and methanol, respectively, until red color deriving from naphthoquinones was not visible in the extractor unit of Soxhlet apparatus. The extracts were concentrated by rotary evaporation at 40°C (Petroleum ether = 4.76 g, dichloromethane = 1.46 g, methanol = 3.85 g). The petroleum ether and dichloromethane extracts showed great similarity in TLC chromatograms; therefore, these extracts were combined for further studies.

2.2.2.1.2. *Onosma mollis*

O. mollis was also selected for further bioactivity-guided isolation studies due to its noteworthy cytotoxic activity (IC_{50} < 8 μg/mL) and distinctive chemical profile. For this purpose, the powdered root of *O. mollis* (400 g) was extracted using an equal volume of petroleum ether, dichloromethane and methanol respectively in a 1000-ml volume system for each 100 g powdered root by the maceration method. After filtration to remove ruins, . The extracts were concentrated by rotary evaporation at 40°C and the final extracts were measured to give quantities (Petroleum ether = 5.048 g, dichloromethane = 2.46 g, methanol = 7.5 g). Since the cytotoxic activity of the petroleum ether extract illustrated significant cytotoxic activity (IC_{50} < 8 μg/mL), further isolation and purification procedures were carried out.

2.2.2.2. Isolation and Purification

Column chromatography with silica gel 60 (Merck 7734), Li Chroprep RP (C-18, Merck 9303) and Sephadex LH-20 as well as the preparative TLC on Silica gel 60 F254 (Merck, 1.05554) and reversed-phase Silica gel 60 RP-18 F254s (Merck, 1.05559) were utilized to obtain bioactive compounds. TLC was conducted on pre-coated silica gel 60

F254 aluminium sheets (Merck 5554) and RP-18 F254 (Merck) plates. Compounds were detected at 254 and 366 nm in the UV cabin; the spots were visualized by spraying 20% aq.H_2SO_4 onto the TLC plates followed by heating up to 110°C until the spots became visible.

During the chromatographic studies (CC, MPLC and TLC controls) the following solvent and/or solvent systems were used:

I. ACN:H_2O
II. ACN:BuOH:H_2O
III. MeOH
IV. $CHCl_3$:MeOH
V. Hex:EtOAc:FA
VI. Hex:EtOAc:MeOH:FA
VII. PE:$CHCl_3$:MeOH:FA

Compositions of the solvent systems were provided in detail on the isolation schemes.

2.2.2.2.1. *O. taurica* var. *taurica*

Isolation studies on the combined petroleum ether-dichloromethane extracts (6.22 g) started with medium pressure liquid chromatography using 85 g RP-C18 that was employed with ACN-H_2O to give 10 main fractions (see Figure 2.2). Then, cytotoxicity (Table 3.2) and DNA topoisomerase inhibition tests were performed to find bioactive fractions, and **ES-06**, **ES-07**, **ES-08** and **ES-09** fractions turned out to be the active ones. ES-09 was crystallized with 100% ACN to obtain **OT-1**. ES-06 was chromatographed over Sephadex LH-20 column chromatography with solvent systems containing MeOH-H_2O mixtures to give six main fractions. The subfraction ES-$06_{42\text{-}49}$ were subjected to a preparative TLC employing with 95% ACN to obtain **OT-2**. Consecutive Sephadex LH-20 columns were applied to purify the components of ES-07, and ES-$07_{16\text{-}19}$ subfraction was developed on preparative TLC using PE-$CHCl_3$-MeOH-FA (2:5:0.3:0.2) to give **OT-3**. The isolation steps were illustrated in detail in Figure 2.2. For structure elucidation of **OT-1**-**OT-3**, *see* results 3.4.2 section.

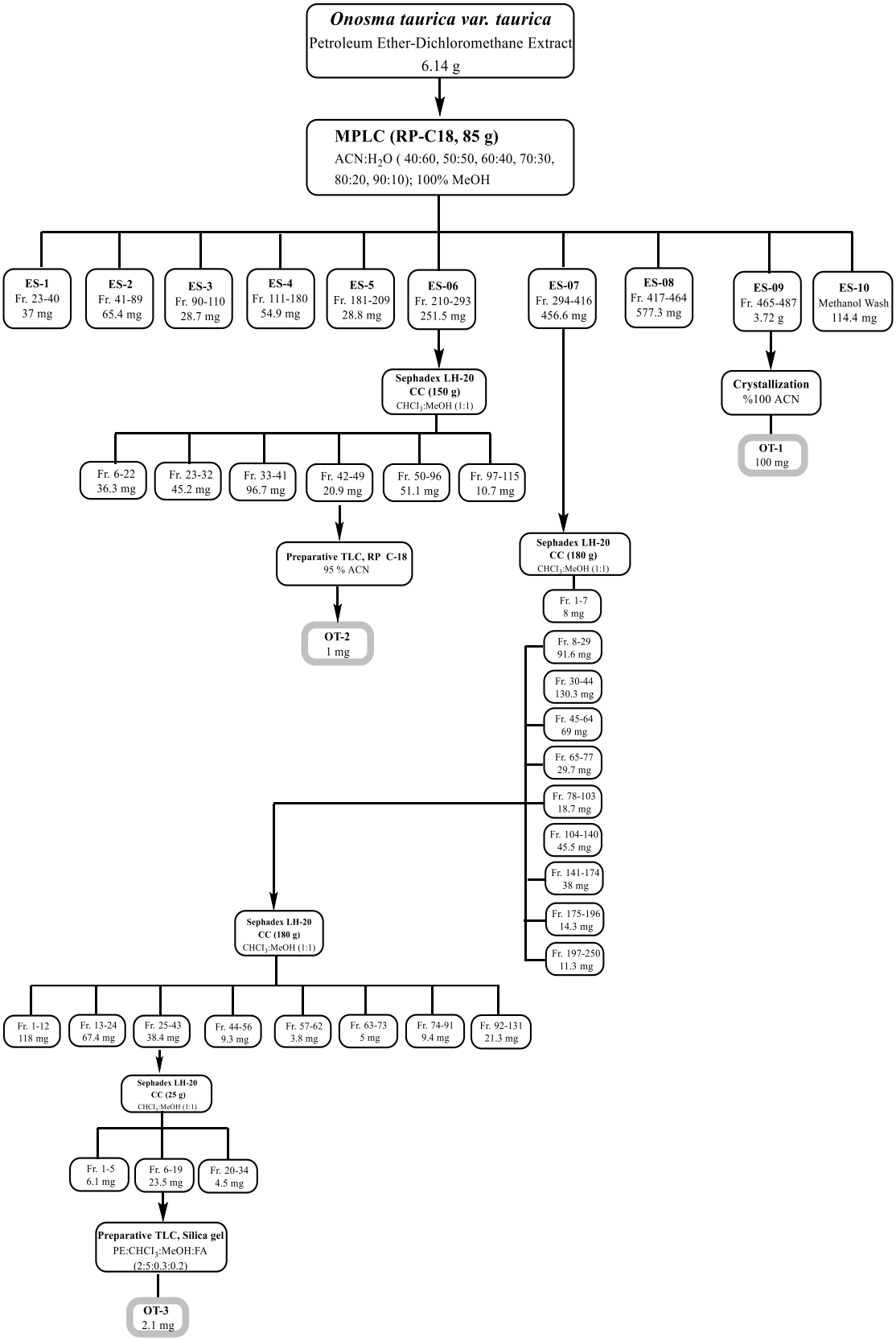


Figure 2.2. Isolation scheme of *O. taurica* var. *taurica*

2.2.2.2.2. *O. mollis*

Isolation studies on *O. mollis* commenced with the PE root extract due to its stronger cytotoxicity (IC_{50} < 8 μg/mL). Firstly, 6.22 g of PE extract was subjected to a silica gel (470 g) column and eluted with *n*-Hexane-EtOAc (95: 5 → 40:60). The fractions with similar profiles were pooled together, and 16 main fractions were obtained. Cytotoxicity (Table 3.3) and DNA topoisomerase inhibition tests showed **ES-23, ES-24, ES-25, ES-26, ES-27, ES-28, ES-29, ES-31, ES-32, ES-35** and **ES-36** as the most active fractions. Each active fraction was subjected to Sephadex LH-20 and the columns were employed with the solvent system $CHCI_3$-MeOH (1:1). Preparative TLC (silica gel, RP-C18) experiments were run as final purification step affording five pure compounds (**OM-1**-**OM-5**). Details of the isolation procedures are shown in Figures 2.3 and 2.4. For structure elucidation of the isolated molecules, *see* results section 3.4.1.

2.3. Cytotoxicity Studies

For this purpose, one of the colorimetric cell viability tests, MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was used. Degradation of the stabile tetrazolium salt MTT by the complex cellular mechanism yielding formazan, is directly related to the metabolic activity of the cells. The cytotoxic activity of the compounds and doxorubicin used as positive controls on the various cancer cells. Cells in the exponential growth phase were placed in 96-well plates to make 7000 cells / well for HeLa, MCF-7 and A549; 10000 cells / well for MRC-5; 8500 cells / well for Capan-1, HEP-G2 and HCC-1937. After 24 h of incubation and adding sample solutions in concentrations ranging from 8 to 32 μg/mL for main fractions and from 0.1 to 25 μM for pure compounds and, respectively, in each well, they were incubated. After 48 hours, the medium (100 μl) containing 10% MTT solution was added. MTT solution-containing 96-well cell culture dishes were incubated for 4 hours at 37°C in 5% CO_2 environment., and blue formazan crystals were dissolved in 150 μL of DMSO per well. Quantities of blue formazan product were measured at 570 nm using a microplate reader. For the cells, strong correlations between numbers of cells present and amounts of MTT formazan product were observed. The data were obtained from three independent assays, using three sets of wells for each assay. Cytotoxic effects of the compounds were determined according to percent cell viability.

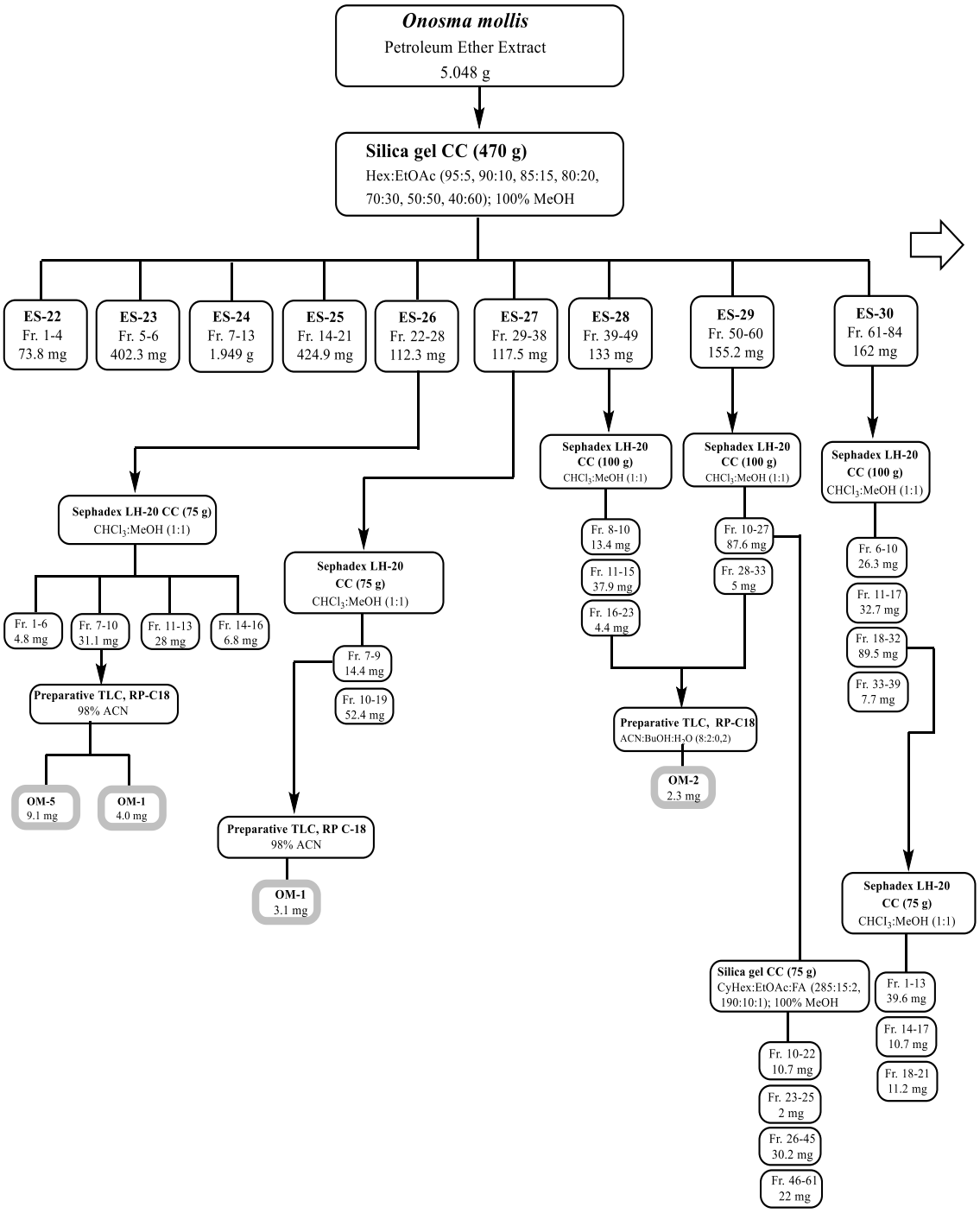


Figure 2.3. Isolation scheme of *O. mollis*

(cont. on next page)

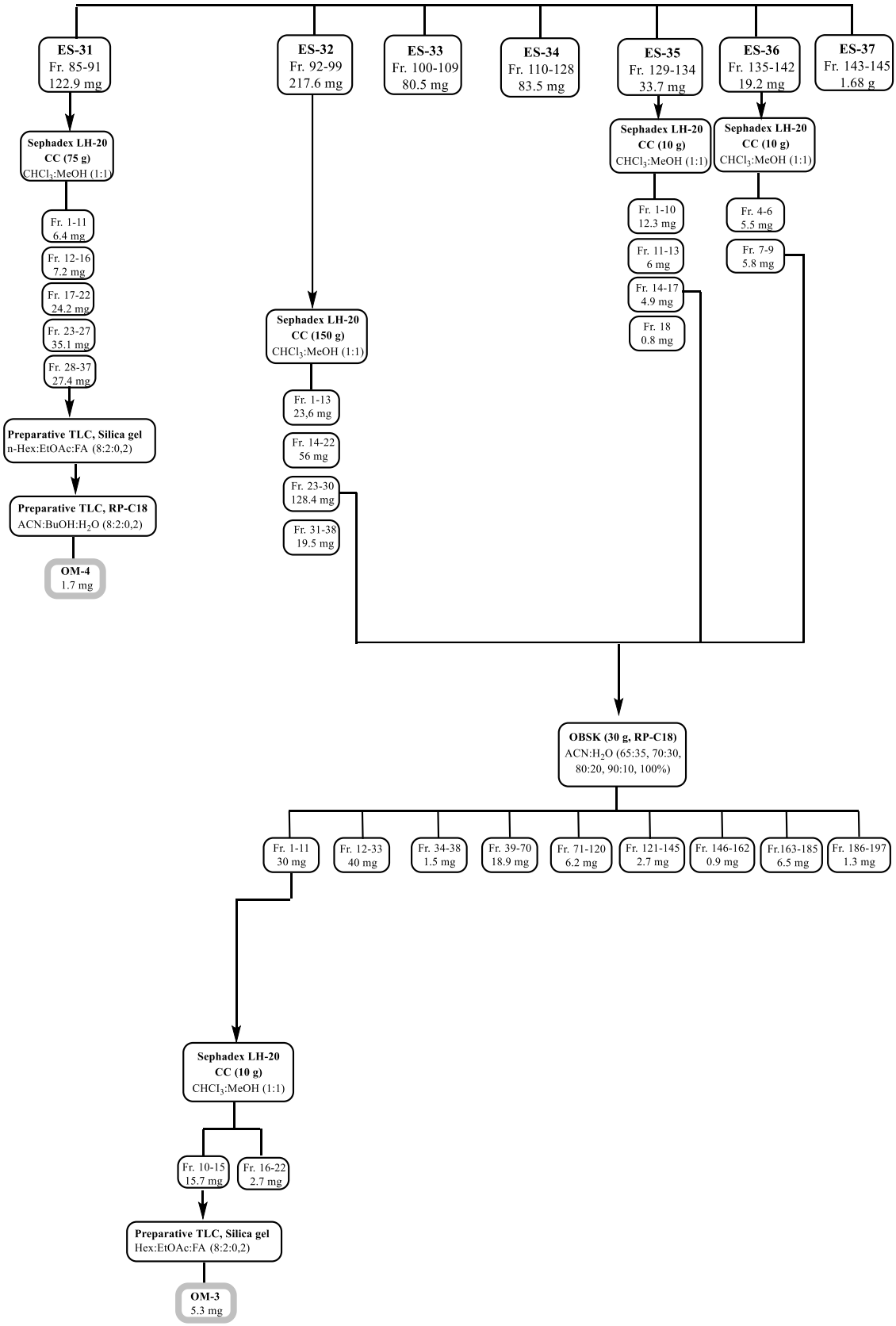


Figure 2.3. (cont.)

2.4. Inhibition of DNA Topoisomerases Studies

Activity tests for enzymes were performed to determine the activity of commercially obtained enzymes (unit / µl) as explained below.

2.4.1. DNA Topoisomerase I Activity

Plasmid supercoil relaxation assay was carried out to determine the effect of main fractions and pure compounds on hTopo I enzyme. The unit activity of topoisomerases I enzyme is described as the removal activity of supercoils from 500 ng of supercoiled plasmid substrate at 37°C for 30 min. In this purpose, substrate DNA (pBR322) and DNA topoisomerase I were subjected to incubation in the presence or absence of the test compounds at 37°C for 30 minutes. Briefly, relaxation assay was carried out using 500 ng of supercoiled (sc) pBR322 and one unit topoisomerase I in final volume of 20 µl reaction buffer solution (72 mm KCl, 5 mm MgCl, 2.5mm DTT, 5 mm spermidine and 0.1% BSA). Following the incubation at 37°C for 30 minutes, reaction products were on 1% agarose gels by electrophoresis (5 V/cm). Before photographed under UV light, gel was stained with ethidium bromide (Etd-Br). TAE buffer (40 mm Tris-acetate, pH 8.3, 2 mM EDTA) was used for preparation and running of agarose gel.

2.4.2. DNA Topoisomerase II Activity

The effects of the extracts on the hTopo II enzyme was analysed with decatenation reaction. This method is based on the separation of open circular DNA monomers (OC-DNA) from covalently closed circular DNA molecules (CCC-DNA). The activity of topoisomerase II enzyme is defined as the decatenation activity of 200 ng of kinetoplast DNA (kDNA) at 37°C for 30 min. Briefly, 200 ng of kDNA and one unit topoisomerase II were incubated in reaction buffer (50 mM Tris-Cl, pH 8.0, 120 mM KCl, 10 mM MgCl2, 0.5 mM ATP, 0.5 mM dithiothreitol) in 20 µl of reaction volume. The reactions in the presence and the absence of extracts were incubated at 37°C for 30 minutes; following this the reaction products were seperated on 1% agarose gels by electrophoresis (5 V/cm). Before photographed under UV light, gel was stained with ethidium bromide

(EtdBr). TAE buffer (40 mm Tris-acetate, pH 8.3, 2 mM EDTA) was used for preparation and running of agarose gel.

2.4.3. DNA Topoisomerase II Mediated-DNA Cleavage by Naphthoquinone Derivatives

One of the studies for determining the mechanism action of napthaquinone derivatives on topoisomerase II enzyme is analyzing of clevage complex formation. DNA strand breaks were analyzed in 20 ul reaction volumes containing 500 ng substrat DNA (pBR322) using 4–20 units of enzyme. Reactions were incubated with 2 µl of test substance at 37°C for 6 minutes; and terminated by the addition of 2 µl of 5% SDS, 2 µl of 250 mM EDTA. Proteinase K (0.8 mg/ml) was added and the reactions were incubated at 45°C for 30 minutes to digest the type II enzyme. The reaction products subjected to electrophoresis using 1% agarose gel containing 0.5 µg/ml Etd-Br (5 V/cm) and photographed under UV light. Known topoisomerase II poison Etoposide was used as positive control for DNA cleavage reaction.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Collection and Preliminary Processing of *Onosma* Species

Plant materials were collected from different locations of Southwest Anatolia between May and June 2018 (Table 2.1). Twelve different *Onosma* species were collected, and they were identified from their herbarium samples as *O. aucheranum, O. oreodoxum, O. bornmuelleri, O. nana, O. armenum, O. bracteosum, O. heterophylum, O. taurica* var. *taurica, O. mite, O. mollis, O. frutescens and O. aksoyii* by Ademi Fahri Pirhan. All plant materials were deposited at the Ege University Herbarium, Izmir, Turkey. The plant materials (the aerial and root parts) were dried in shade and then powdered by a grinder. The powdered root materials were extracted with petroleum ether, dichloromethane and methanol, respectively.

Table 3.1. Collected *Onosma* species and their locations

Name	Analysis Code	Herbarium Code	Location	Date
O. nana	G1-12	42709	Ballıkköy road-Ören-Fethiye Muğla, 1357 m 36º49.886'K 29º24.849'D	13.05.2017
O. oreodoxum	G2-01	42710	Manavgat Çayı road–Antalya, 189 m 37º11.048'K 31º12.237'D	19.05.2017
O. armenum	G2-07	42711	Başlar-Beydiğin, 1159 m 37º4.726'K 31º27.314'D	21.05.2017
O. frutescens	G2-11	42712	Eskiavdan Kızılkaya 796 m 37º16.769'K 30º26.390'D	21.05.2017
O. aucheranum	G2-12	42713	Yazır Yayla road, 984m 36º59.185'K 30º18.322'D	21.05.2017

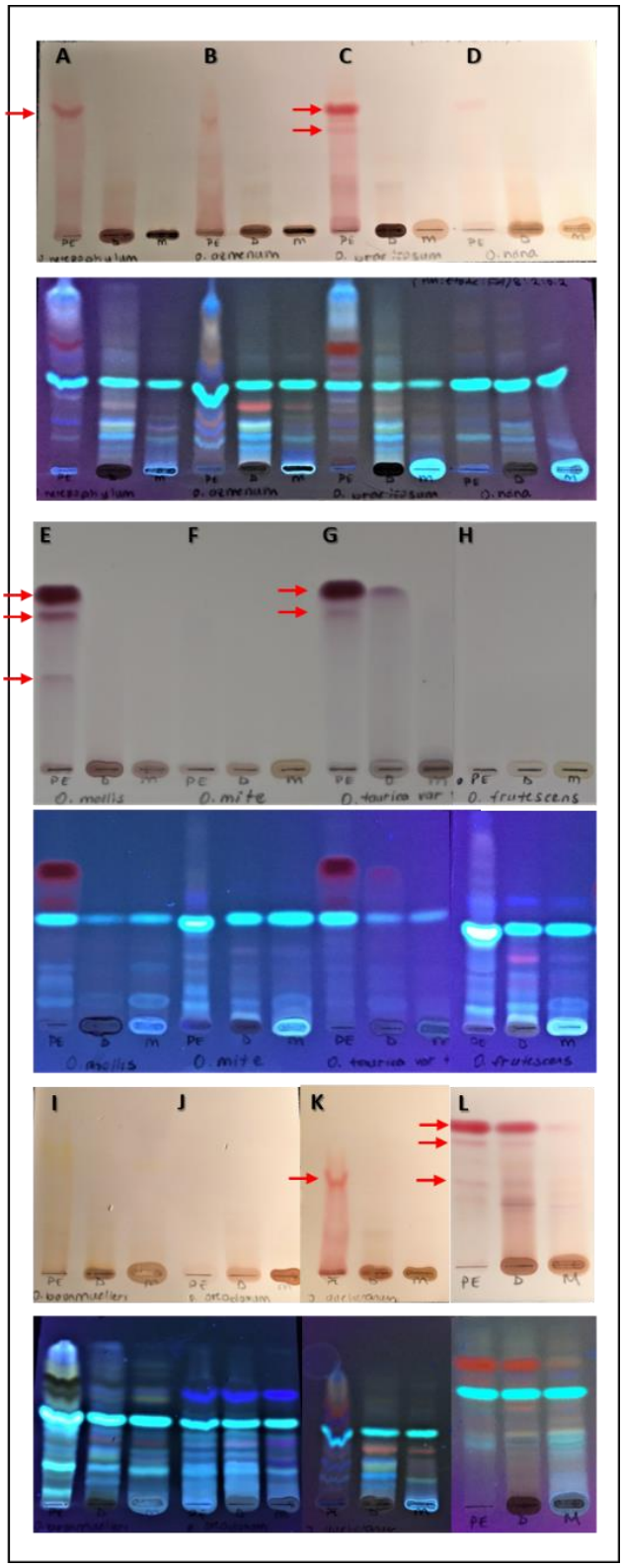
(Cont. on next page)

Table 3.1. (cont.)

Name	Analysis Code	Herbarium Code	Location	Date
O. mite	G2-13	42714	Termessos National Park, 395 m 37º1.004'K 30º31.306'D	21.05.2017
O. heterophyllum	G3-01	42715	Serinhisar-Yeşilova Salda Lake, Burdur road, 1158 m 37º31.459'K 29º39.200'D	28.05.2017
O. bracteosum	G3-03	42716	Vicinity of Burdur Gençlik Hiz. Ve Spor İl Müd. Çemdik Su Sporları ve Kamp Eğ. Mrkz. 37º41.498'K 30º11.540'D	28.05.2017
O. taurica* var. *taurica	G3-18	42717	Kütahya-Uşak highway, Alikahya Village 38.831862 'K 29.266381' D	31.05.2017
O. aksoyii	G4-09	42718	Sütçüler-Beyşehir, Ayvalıpınar Village 1146 m 37º41.614'K 31º01.340'D	16.06.2017
O. bornmuelleri	G4-15	42719	Beyşehir- Derebucak highway, 1080 m 37º32.146'K 31º29.999'D	17.06.2017
O. mollis	G4-18	42720	Gencek Neighborhood, Derebucak/Konya, 1258 m 37º25.688'K 31º31.756'D	17.06.2017

3.2. TLC Profiles of the Extracts

The root extracts of the *Onosma* species were monitored by TLC to realize their chemical diversity. The mobile phase was *n*-Hexane:Ethyl acetate:Formic acid (8:2:0.2) (Chromatogram 3.1). During evaluation of the TLC plates, the reddish/pinkish colored bands under visible and 365 nm UV lights was considered to be positive result for the presence of naphthoquinones. Based on the results, it was inferred that the petroleum ether and dichloromethane extracts were richer than the methanol extracts for their naphthoquinone contents as shown below.



Chromatogram 3.1. TLC chromatogram of different *Onosma* species of preliminary studies *(***A.** *O. heterophylum,* **B.** *O. armenum,* **C.** *O. bracteosum,* **D.** *O. nana,* **E.** *O. mollis,* **F.** *O. mite* **G.** *O. taurica var. taurica,* **H.** *O. frutescens,* **I.** *O. bornmuelleri,* **J.** *O. oreodoxum,* **K.** *O. aucheranum* **L.** *O. aksoyii)* (Silica gel, 8:2:0.2; *n*-Hex:EtOAc:FA)

3.3. HPLC Fingerprinting Analysis

A literature survey revealed that 520 nm was the wavelength of choice for the analysis of the target compounds (naphthoquinones).[35,88] Petroleum ether extracts of *O. nana* and *O. taurica* var. *taurica* were selected to check the suitability of this wavelength (Figure 3.1). The strongest absorbance values of the major component showing up at 17.5 min were taken into consideration. Our further studies revealed that the major compound in the extracts was β,β-dimethylacrylshikonin, and this compound was used as a reference in the HPLC fingerprinting studies.

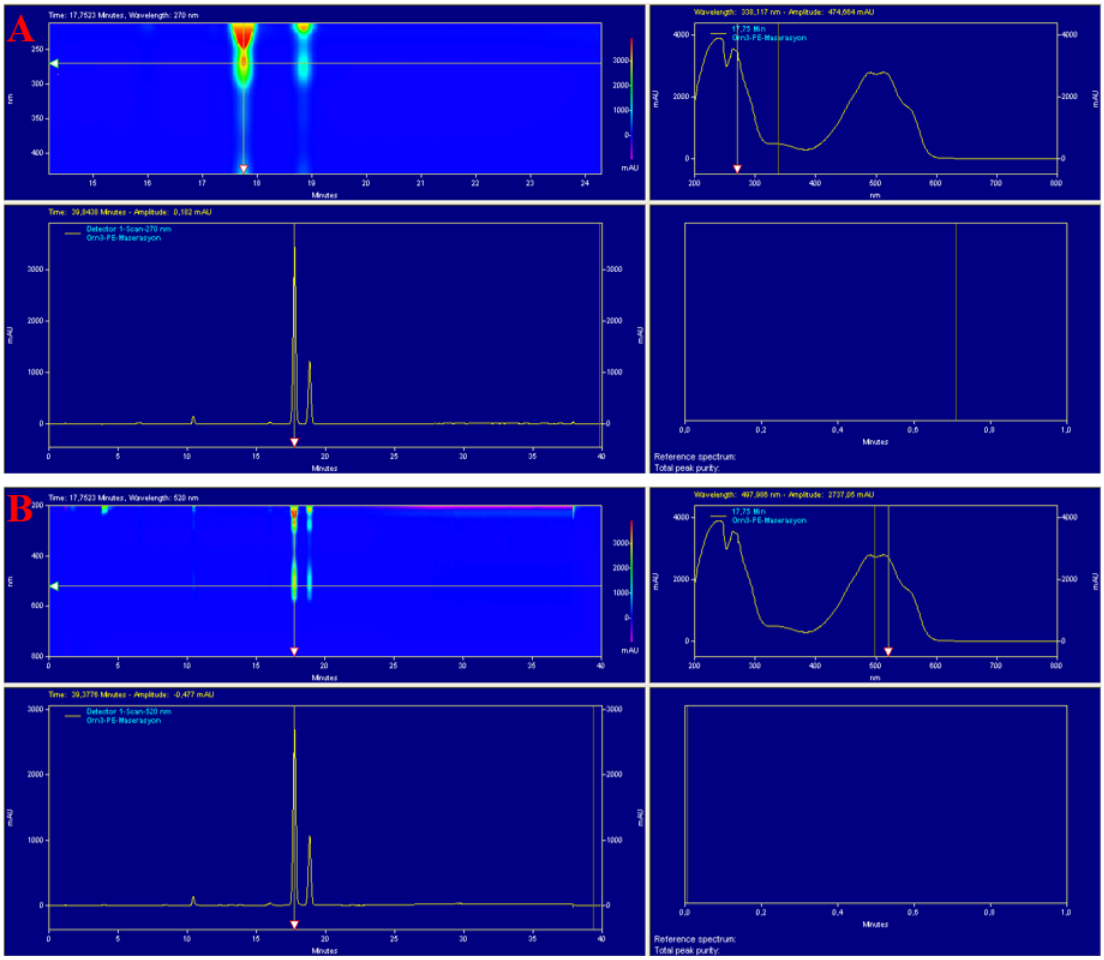
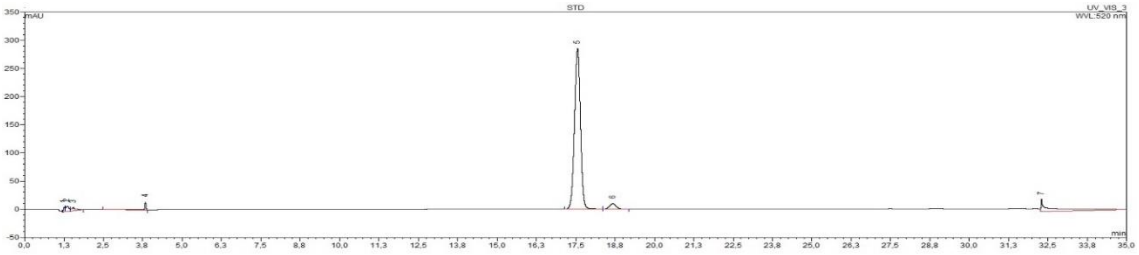


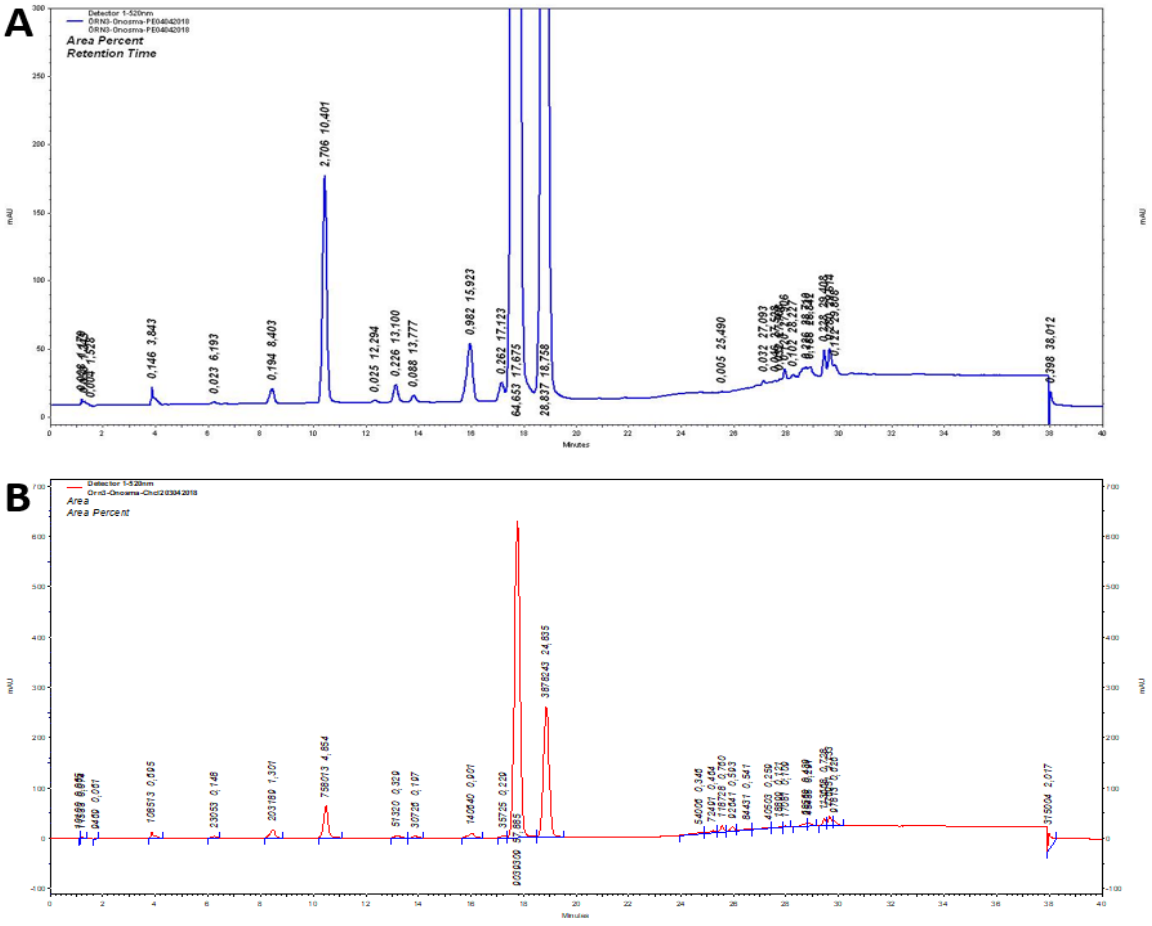
Figure 3.1. Scanning Results for *O. taurica* var. *Taurica*

As a result of the spectral scans, 270 and 520 nm were chosen for HPLC analyses. In addition to these, the peaks at 320 nm were also recorded in order to monitor the other non-polar compounds.

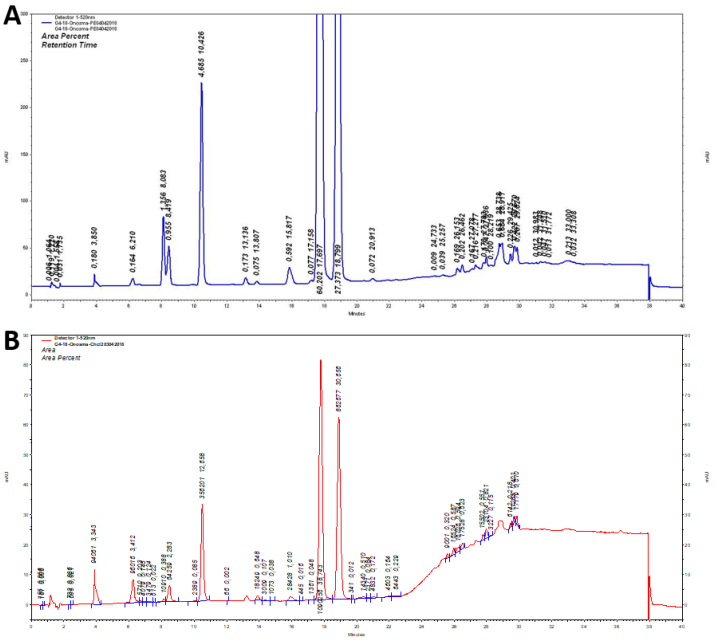
As a result of the analysis carried out, the purity of the standard compound was found to be 95%, and the retention time was 17.5 min.



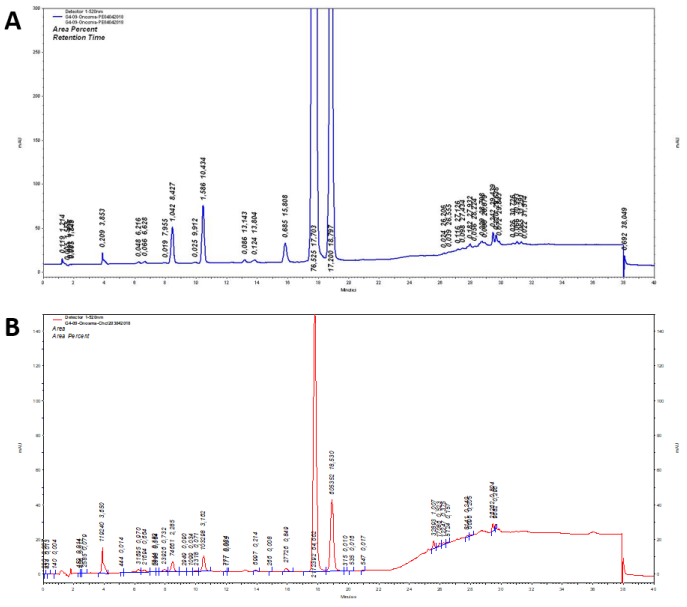
Chromatogram 3.1. HPLC chromatogram of β,β-dimethylacrylshikonin (reference compound)



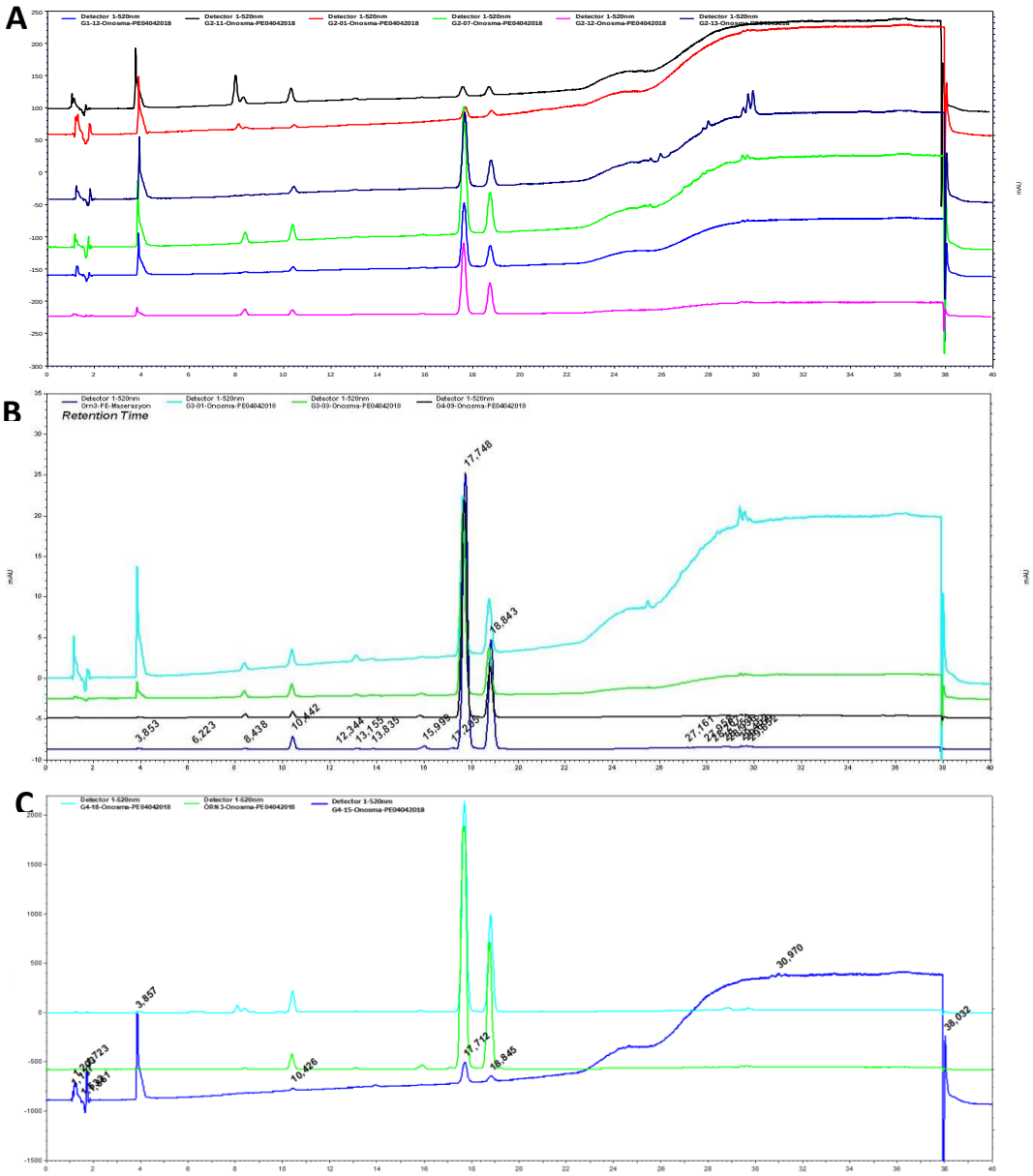
Chromatogram 3.2. HPLC chromatograms of *O. taurica* var. *taurica* extracts at 520 nm
A. Petroleum ether **B.** Dichloromethane



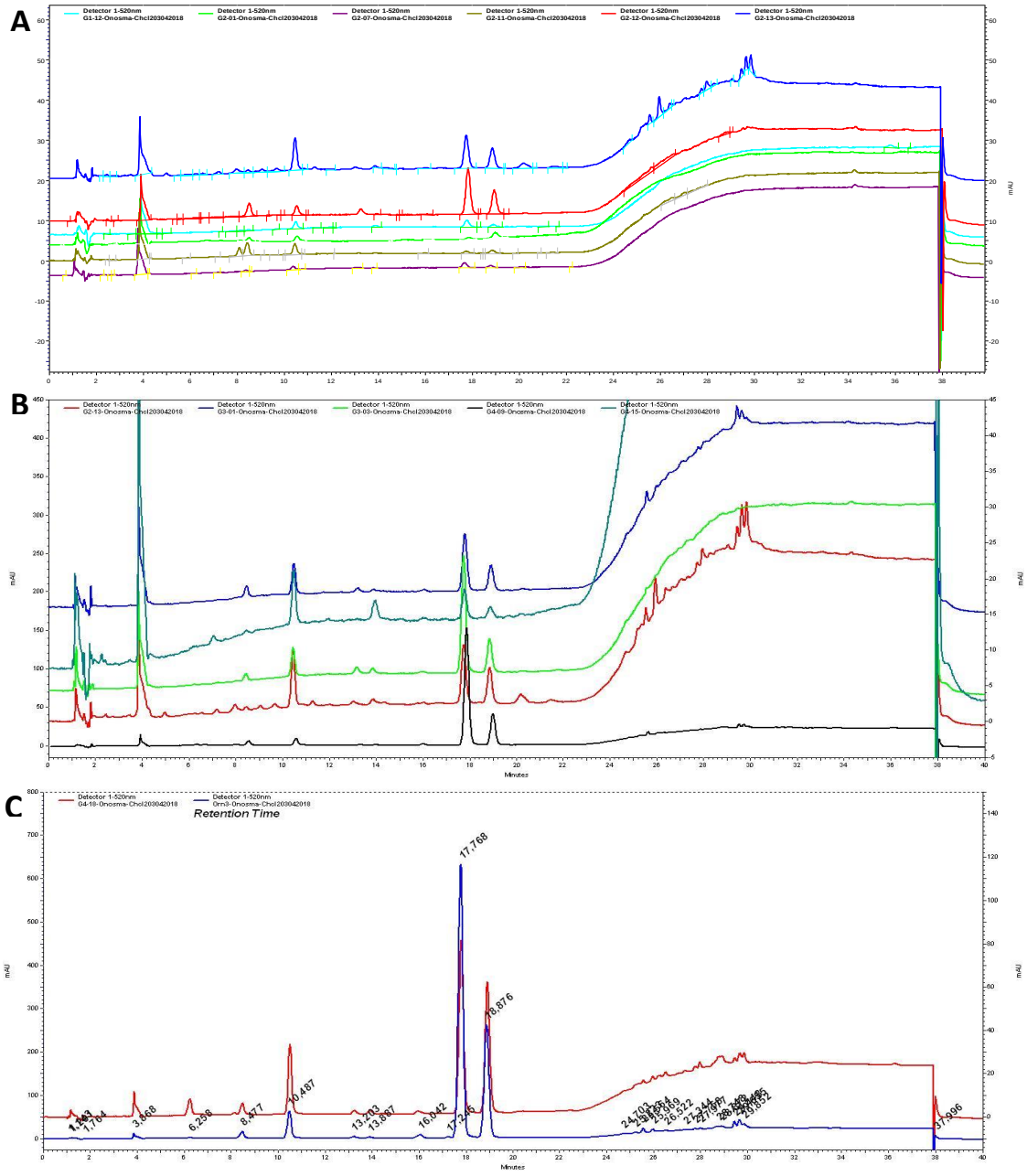
Chromatogram 3.3. HPLC chromatograms of *O. mollis* extracts at 520 nm **A.** Petroleum ether **B.** Dichloromethane



Chromatogram 3.4. HPLC chromatograms of *O. aksoyii* extracts at 520 nm **A.** Petroleum ether **B.** Dichloromethane



Chromatogram 3.5. HPLC chromatograms of the petroleum ether (PE) extracts from different *Onosma* species at 520 nm. **A.** *O. nana* (G1-12), *O. oreodoxum* (G2-01), *O. aucheranum* (G2-12), *O. frutescens* (G2-11), *O. mite* (G2-13) **B.** *O. heterophylum* (G3-01), *O. bracteosum* (G3-03), *O. aksoyii* (G4-09), *O. taurica* var. *taurica* (Örn 3) **C.** *O. mollis* (G4-18), *O. bornmuelleri* (G4-15), *O. taurica* var. *taurica* (Örn 3)



Chromatogram 3.6. HPLC chromatograms of the dichloromethane (D) extracts from different *Onosma* species at 520 nm. **A.** *O. nana* (G1-12), *O. armenum* (G2-07), *O. oreodoxum* (G2-01), *O. aucheranum* (G2-12), *O. frutescens* (G2-11), *O. mite* (G2-13) **B.** *O. mite* (G2-13), *O. heterophylum* (G3-01), *O. bracteosum* (G3-03), *O. aksoyii* (G4-09), *O. bornmuelleri (*G4-15) **C.** *O. mollis* (G4-18), *O. taurica* var. *taurica* (Örn 3)

Table 3.2. Area percentages of major components for PE and D extracts. (Minor compounds whose area percentage less than 0.8 % were not taken into consideration and chromatograms up to 25 minutes were considered for comparison.)

	Retention Time (min.)										
Extracts	**3.85**	**6.22**	**8.10**	**8.43**	**9.37**	**10.42**	**13.15**	**13.85**	**15.99**	***17.75**	**18.84**
***O. frutescens* (PE)**	32.78	-	4.47	28.45	-	13.91	-	-	-	9.94	9.56
***O. frutescens* (D)**	41.21	-	8.06	14.23	-	12.55	-	-	-	3.04	4.50
***O. aksoyii* (PE)**	-	-	-	1.40	-	-	-	-	-	55.54	30.14
***O. aksoyii* (D)**	12.23	-	-	-	-	-	-	-	-	5.54	2.26
***O. mollis* (PE)**	-	-	-	1.04	-	1.59	-	-	-	76.53	17.2
***O. mollis* (D)**	3.34	3.41	2.28	-	-	12.66	-	-	12.01	38.74	30.66
***O. mite* (PE)**	27.64	-	-	-	-	4.96	-	-	-	31.57	14.03
***O. mite* (D)**	18.64	-	2.32	1.44	1.59	13.30	-	-	-	16.82	10.83
***O. taurica* var. *taurica* (PE)**	-	-	-	-	-	0.937	-	-	-	59.96	10.52
O. taurica* var. *taurica (D)	-	-	-	1.30	-	4.85	-	-	0.90	57.89	24.84
***O. heterophylum* (PE)**	25.48	-	-	-	-	6.55	-	-	-	47.52	19.45
***O. heterophylum* (D)**	28.81	-	-	5.57	-	12.16	1.57	-	1.23	26.64	12.51
O. bracteosum (PE)	4.97	-	-	-	-	6.38	-	-	-	67.60	20.83
O. bracteosum (D)	24.15	-	-	2.25	-	8.92	2.03	0.82	0.89	44.62	13.28
O. armenum (PE)	15.94	-	-	-	-	6.94	-	-	-	39.95	15.47
O. armenum (D)	70	-	-	2.22	-	5.96	1.74	-	-	12.21	4.88
O. nana (PE)	-	19.61	-	-	-	2.88	-	-	-	56.49	18.45
O. nana (D)	65.1	-	-	-	-	13.25	-	-	-	15.58	5.
O. bornmuelleri (PE)	60.47	-	-	-	-	3.10	-	-	-	27.47	6.22
O. bornmuelleri (D)	60.65	1.70	-	-	-	13.15	-	4.61	-	9.01	3.50
O. oreodoxum (PE)	-	48.42	-	-	-	3.85	-	-	-	14.66	8.25
O. oreodoxum (D)	57.17	-	-		7.02	11.48	-	-	-	5.12	15.86
O. aucheranum (PE)	3.66	-	-	3.68	-	3.02	-	-	-	52.79	24.14
O. aucheranum (D)	19.86	-	-	7.44	-	5.29	-	3.92	-	31.34	17.11

* the retention time of β,β-dimethylacrylshikonin (*see* Chromatogram 3.1)

There were a number of studies in which fingerprinting of Boraginеceae family members and some *Onosma* species were investigated.

Hu et al. studied nine different species of the Boraginaceae family including six *Onosma* species to determine naphthoquinones by HPLC-DAD. For comparison of the contents, eight marker substances were used which were shikonin, acetylshikonin, deoxyshikonin, β-acetoxyisovalerylshikonin, isobutylshikonin, β,β-dimethyl-acrylshikonin, 2-methyl-*n*-butyrylshikonin, isovalerylshikonin. β-acetoxy-isovalerylshikonin was not present in *Onosma* species. The results revealed that *O. paniculatum* was found to contain the highest amount of naphthoquinones where β,β-dimethylacrylshikonin, acetylshikoin and 2-methyl-*n*-butyrylshikonin were the main components with the quantities of 4.9, 2.86 and 2.14 mg/g ratio, respectively. Moreover, *O. confertum* was found to contain the least number of naphthoquinones among all the nine samples (<0.08 mg/g). The study remarked that some *Onosma* species might be suitable for the development of an alternative naphthoquinone source.[88]

Vukic *et al.* examined the ethyl acetate extract of *O. visianii* by HPLC to reveal its naphthoquinone profile. On the basis of HPLC-PDA analysis with a mobile phase consisting of tetrahydrofuran, acetonitrile and water, seven shikonin derivatives including acetylshikonin, isobutyrylshikonin and α-methylbutyrylshikonin as major compounds were determined within 25 minutes.[89]

Another study dealing with cytotoxicity of *O. paniculatum* had also examined the chemical profile of the petroleum extract to propose the compounds responsible for bioactivity. The HPLC fingerprinting performed on RP C-18 column eluted with acetonitrile and water (0–35 min; 65–74% Water) displayed 3 major compounds with retention times of 10.8, 22.7 and 24.3 min. Rinner *et al.* tentatively suggested acetylshikonin, dimethylacrylshikonin and epoxyshikonin as main compounds.[35]

By going through the available literature, an appropriate HPLC method was developed [RP C-18 column, mobile phase consisting of acetonitrile and water (0–35 min; 60–100% Water)], and the collected *Onosma* species were analyzed. As mentioned above, the major constituent (retention time = 17.5 min.) was β,β-dimethylacrylshikonin in all petroleum ether extracts. The analyses showed that naphthoquinone derivatives were present in greater amounts in the PE extracts as compared to the D extracts, which were richer for the polar compounds. As a result of peak area comparisons, it was found that β,β-dimethylacrylshikonin was the major component in the PE extracts of *O. frutescens, O. oreodoxum* and *O. bornmuelleri* displaying less variability in quinones

compared to the other species. Eleven naphthoquinone derivatives were observed in the PE extract of *O. taurica* var. *taurica*. In the extracts of *O. aksoyii* and *O. mollis*, nine naphthoquinone derivatives were detected, and more polar nature of the compounds was inferred based on the retention times.

When the evaluation of area percentages according to retention time, β,β-dimethylacrylshikonin (retention time (RT) = 17.5 min.) was a major component in PE extracts of *O. taurica* var. *taurica* (59.96), *O. aksoyii* (55.54) and *O. mollis* (76.53). Moreover, three unknown main peaks with retention times of 3.85, 10.42 and 18.84 min, were observed. The highest percentage of an unknown compound at 3.85 min was D extract of *O. armenum* (70); it was followed by the D extracts of *O. nana* (65.1), O. *bornmuelleri* (60.65) and *O. oreodoxum* (57.17). A quinone with the retention time of 10.42 min. was the highest ratio in PE extracts of *O. frutescens* with 13.91 %. Additionally, *O. mite* (13. 30), *O. nana* (13.25) and *O. bornmuelleri* (13.15) exhibited higher ratios for the same compound. Therefore, non-polar compounds were richer in the PE extracts than D extracts. The other unknown compounds at 18.84 min. were more abundant in the D extracts of *O. mollis* (30.66), *O. taurica* var. *taurica* (24.84), and *O. aksoyii* (30.14).

Consequently, *O. taurica* var. t*aurica*, *O. mollis* and *O. aksoyii* were found to be richer sources of naphthoquinones.

3.4. Structure Identification of Pure Compounds Isolated from *O. taurica* var. *taurica* and *O. mollis*

3.4.1. Structure Identification of OM-2

In the HR-ESI-MS spectrum (negative mode) of **OM-2**, a major ion peak was observed at *m/z* 329.010285 $[M-H]^-$ indicating the molecular formula as $C_{18}H_{18}O_6$ (calc. 329.1025 for $C_{18}H_{17}O_6$).

By comparison of ^1H NMR spectrum of **OM-2** with that of β,β-dimethylacrylshikonin (**OM-5**), great similarity for both compounds was realized, except the acyl group extending from C-11 position. In the ^1H and ^{13}C NMR spectra of **OM-2**, additional resonances compared to those of shikonin were observed, which were

consistent with the presence of an acetyl group (^{1}H NMR: δ 2.20; ^{13}C NMR: 169.9 and 25.9). As a result, the structure of **OM-2** was identified as acetylshikonin.[50]

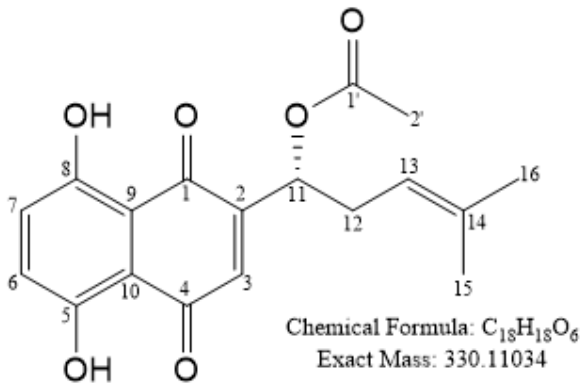
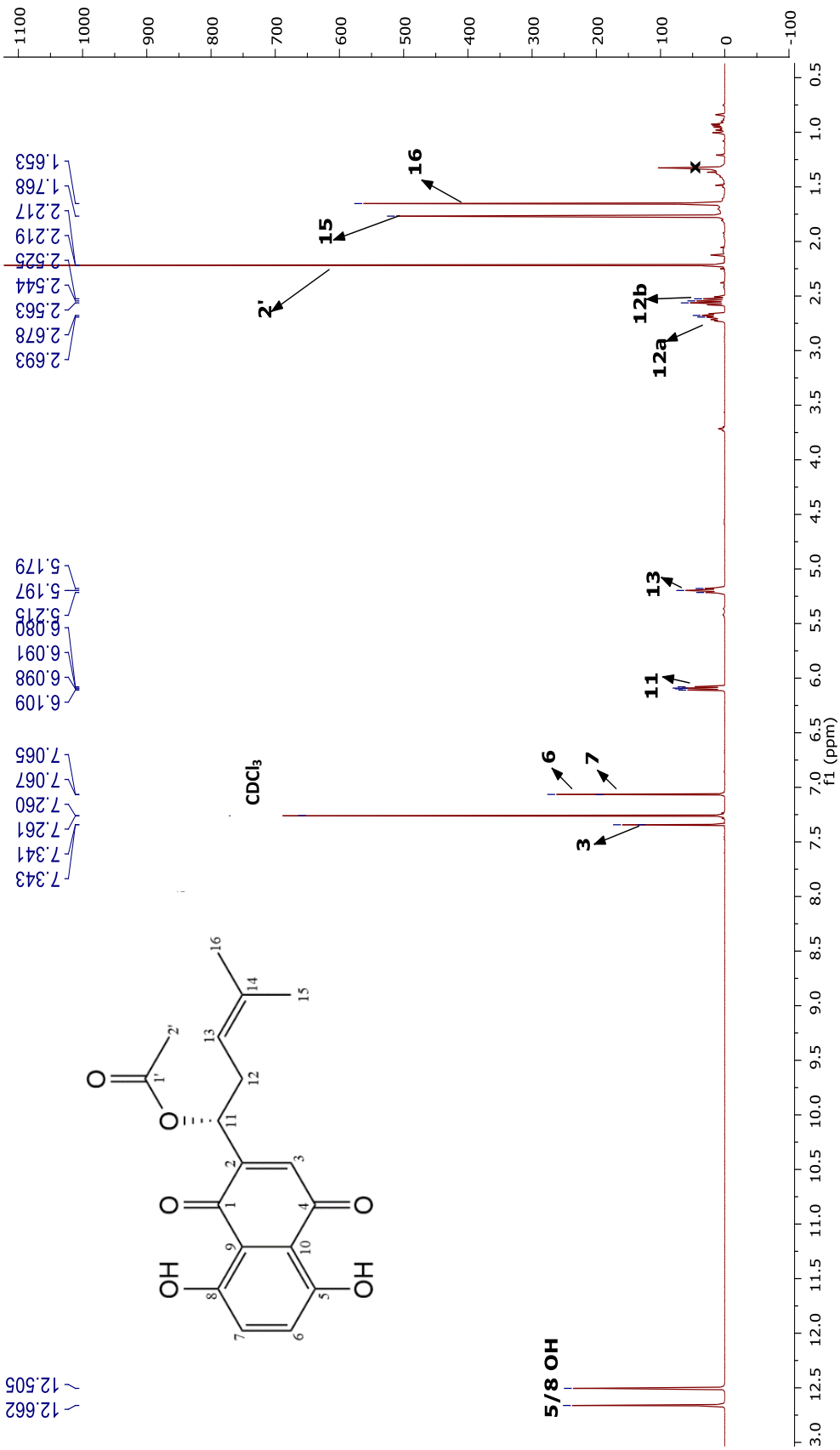


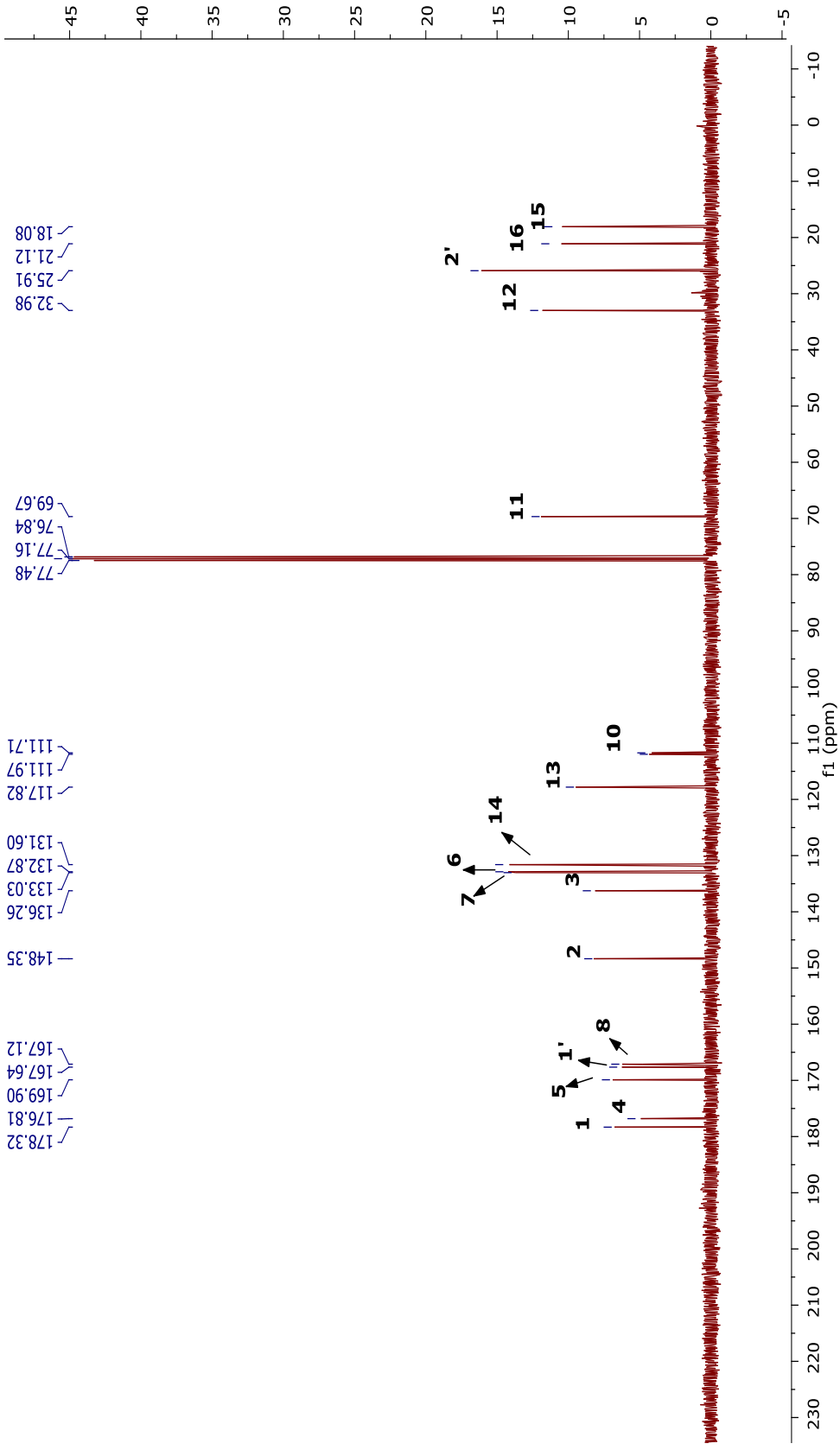
Figure 3.2. Chemical structure of acetylshikonin, [(1R)-1-(5,8-dihydroxy-1,4-dioxonaphthalen-2-yl)-4-methylpent-3-enyl] acetate

Table 3.3. ^{1}H and ^{13}C NMR Data of **OM-2** (400 MHz, δ ppm, in $CDCl_3$)

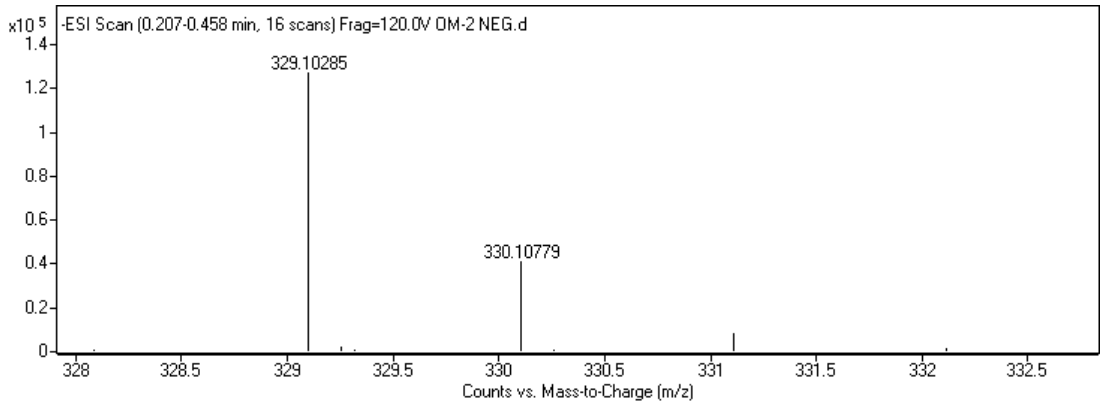
H/C	**Mult.**	**δ_H (ppm)**	***J* (Hz)**	**δ_C (ppm)**
1	C			178.3
2	C			148.4
3	CH	7.07	*d* (1.9)	136.3
4	C			176.8
5	C			167.1
6	CH	7.34	*s*	132.9
7	CH_2	7.34	*s*	133
8	C			167.6
9	C			112
10	C			111.7
11	CH	6.09	*m*	69.7
12	CH_2	2.78	*m*	33
		2.54	*m*	
14	C			131.6
15	CH	1.65	*s*	18.1
16	C	1.77	*s*	21.1
1'	C			169.9
2'	CH3	2.2		25.9



Spectrum 3.1. ^{1}H-NMR Spectrum of **OM-2**



Spectrum 3.2. ^{13}C-NMR Spectrum of **OM-2**



Specrum3.3. HR-ESI-MS of **OM-2** (Negative mode)

3.4.2. Structure Identification of OM-3

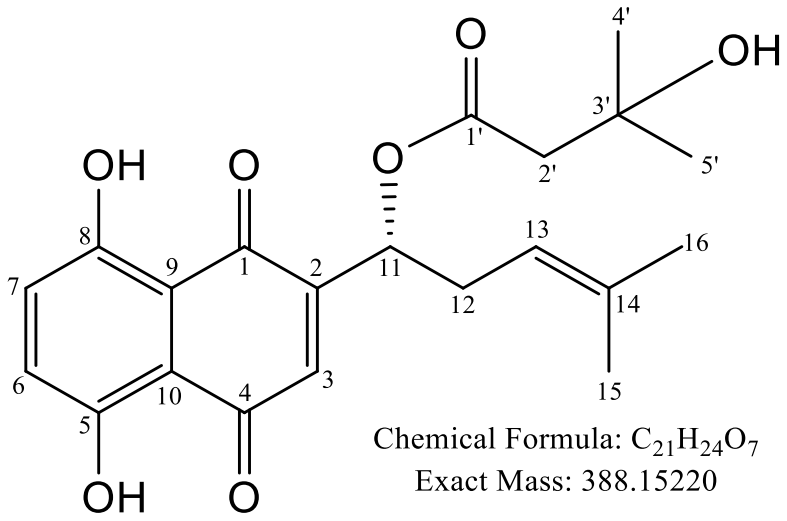


Figure 3.3. Chemical structure of β-hydroxyisovalerylshikonin, [(1R)-1-(5,8-dihydroxy-1,4-dioxonaphthalen-2-yl)-4-methylpent-3-enyl] 3-hydroxy-3-methylbutanoate

OM-3 was isolated as red solid that behaved more polar than **OT-1** (**OM-5**) on the thin-layer chromatogram (Mobile phase: *n*-Hexane:EtOAc:FA).

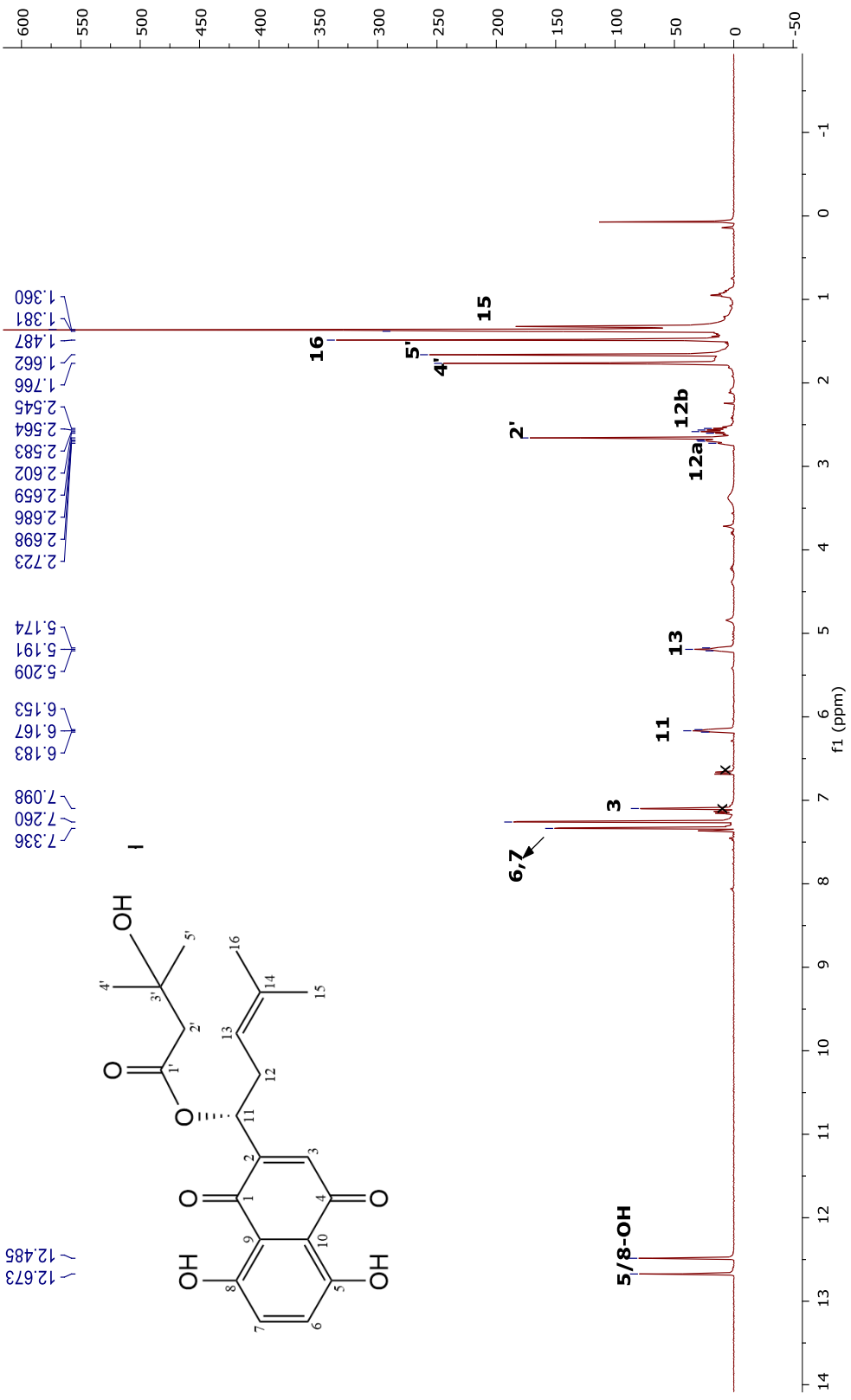
In the HR-ESI-MS spectrum (negative mode) of **OM-3**, a major ion peak was observed at *m/z* 387.1440 $[M-H]^-$ indicating the molecular formula of $C_{21}H_{24}O_7$ (calc. 387.1444 for $C_{21}H_{23}O_7$).

Detailed inspection of the ^1H NMR spectrum of **OM-3** revealed that it was another C-11(O)-acylated naphthoquinone derivative. A comparison of the mass data of **OM-3**

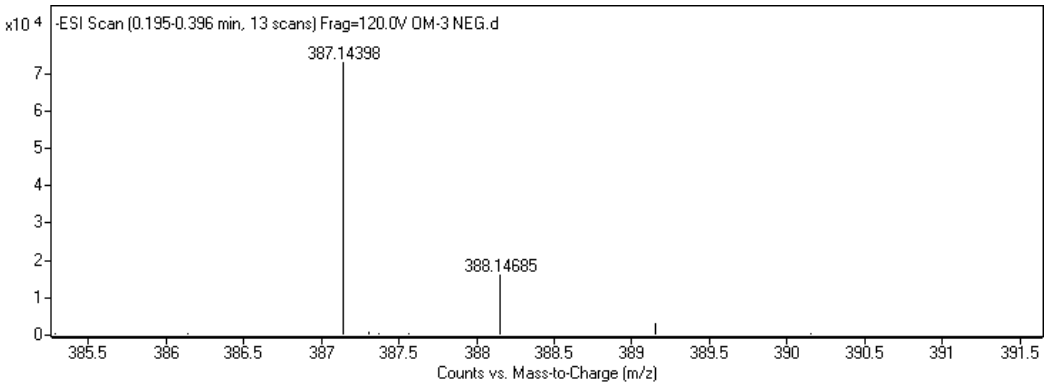
and **OM-5** was significant for hydration of the acyl moiety in **OM-5**. In the ^{1}H NMR spectrum of **OM-3**, the absence of the double bond proton (H-2' of **OM-5**) and comparison of the data with those of previously published analogs, clarified the structure as β-hydroxyisovalerylshikonin.[90]

Table 3.4. ^{1}H NMR Data of **OM-3** (400 MHz, δ ppm, in $CDCl_3$)

H/C	**Mult.**	**δ_H (ppm)**	***J* (Hz)**
1	C		
2	C		
3	CH	7.10	*d* (1.4, 1)
4	C		
5	C		
6	CH	7.33	*s*
7	CH	7.33	*s*
8	C		
9	C		
10	C		
11	CH	6.18	*m*
12	CH_2	2.67	*d* (10.6, 7)
		2.56	*m*
13	CH	5.20	*d* (7.1, 1)
14	C		
15	CH	1.38	*s*
16	C	1.48	*s*
1'	C		
2'	CH_2	2.65	*s*
3'	C		
4'	CH	1.76	*s*
5'	*CH*	1.66	



Spectrum 3.4. ^{1}H-NMR Spectrum of **OM-3**



Specrum3.5. HR-ESI-MS of **OM-3** (Negative mode)

3.4.3. Structure Identification of OM-4

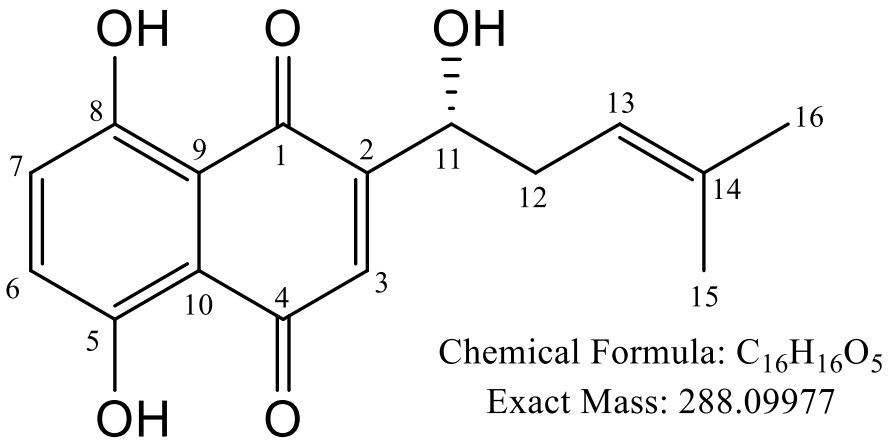


Figure 3.4 Chemical structure of shikonin, (R)-5,8-Dihydroxy-2-[1-hydroxy-4-methylpent-3-en-1-yl]naphthalene-1,4-dione

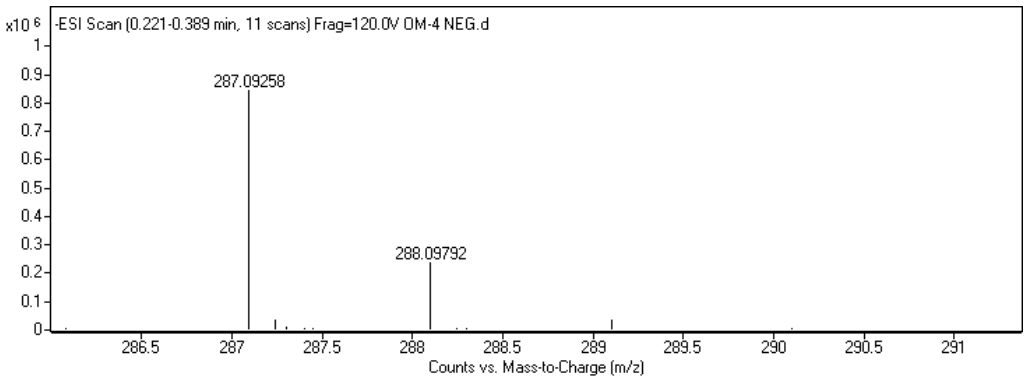
OM-4 was obtained as a reddish solid. Its TLC profile as a reddish band (under visible light) implied a naphthoquinone-type molecule. In the HR-ESI-MS spectrum (negative mode) of **OM-4**, a major ion peak was observed at *m/z* 287.0926 $[M-H]^-$ indicating the molecular formula as $C_{16}H_{16}O_5$ (calc. 287.0920 for $C_{16}H_{15}O_5$).

In the low field region of the ^1H NMR spectrum, resonances deriving from the exchangeable protons [δ 12.51 (*s*) and 12.61 (*s*)] together with the signals at δ 7.20 (s, 2H), 7.21 and 7.17 (H-3, H-6 and H-7, respectively) suggested that **OM-4** was a mono-substituted naphthoquinone as **OM-5**. The signals at δ 4.91 (1H, *bs*), 5.21 (1H, *t*, *J*=7.2 Hz),

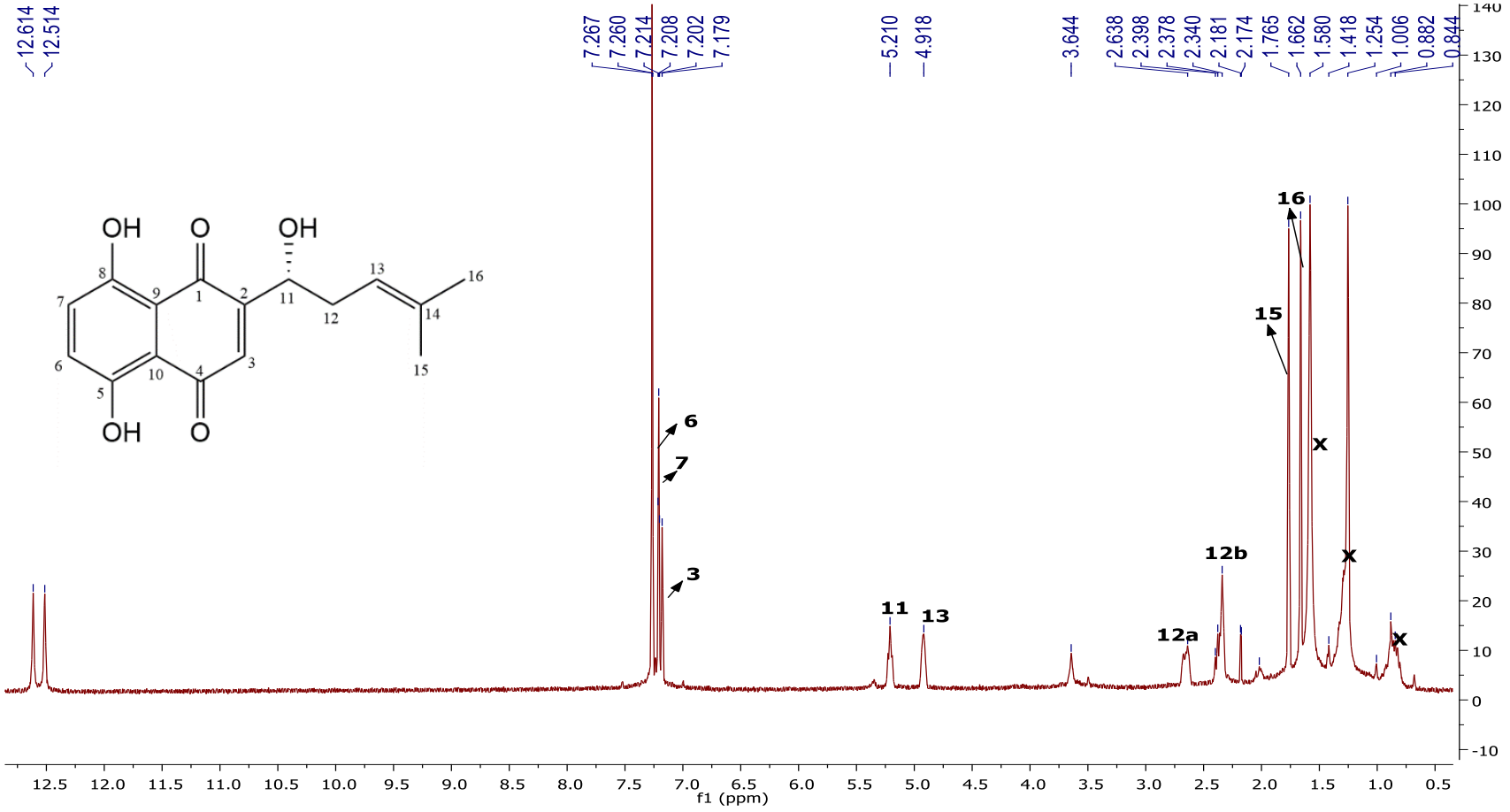
1.76 (3H, *s*) and 2.18 (3H, *s*) were evident for characteristic 4-methylpent-3-en-1-ol acyclic moiety. The ^{1}H NMR data of **OM-4** was identical with those of alkannin or shikonin (5,8-dihydroxy-2-[1-hydroxy-4-methylpent-3-enyl] naphthalene-1,4-dione), enantiomers of each other.[91] The positive optical rotation value $[\alpha]_{D}^{25}$: 40; *c* 0.1, MeOH) proved that **OM-4** was shikonin.

Table 3.5. ^{1}H NMR Data of **OM-4** (400 MHz, δ ppm, in $CDCl_3$)

H/C	**Mult.**	**δ_H (ppm)**	***J* (Hz)**
1	C		
2	C		
3	CH	7.17	*d (1.1)*
4	C		
5	C		
6	CH	7.20	*s*
7	CH	7.21	*s*
8	C		
9	C		
10	C		
11	CH	4.91	*s*
12	CH_2	2.63	*m*
		2.35	*m*
13	CH	5.21	*m*
14	C		
15	CH	1.76	*s*
16	C	2.18	*s*



Specrum 3.6. HR-ESI-MS of **OM-4** (Negative mode)



Spectrum 3.7. ^{1}H-NMR Spectrum of **OM-4**

3.4.4. Structure Identification of OM-5

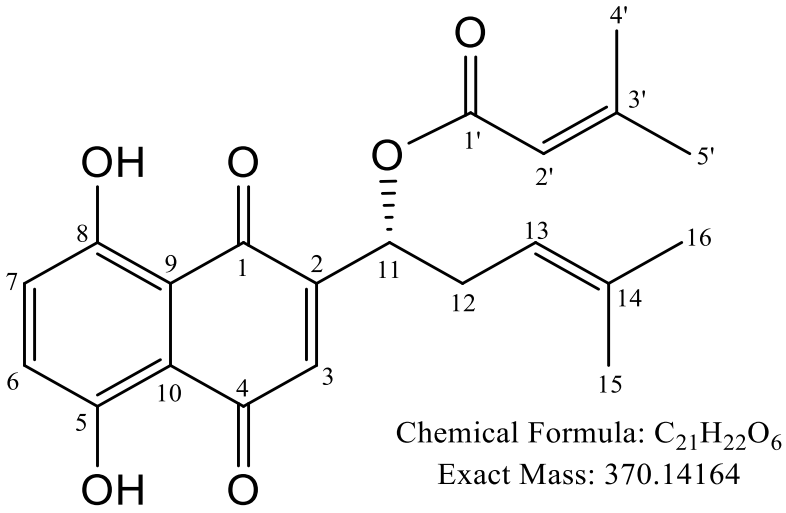


Figure 3.5. Chemical structure of β,β-dimethylacrylshikonin, [1-(5,8-dihydroxy-1,4-dioxonaphthalen-2-yl)-4-methylpent-3-enyl]3-methylbut-2-enoate.

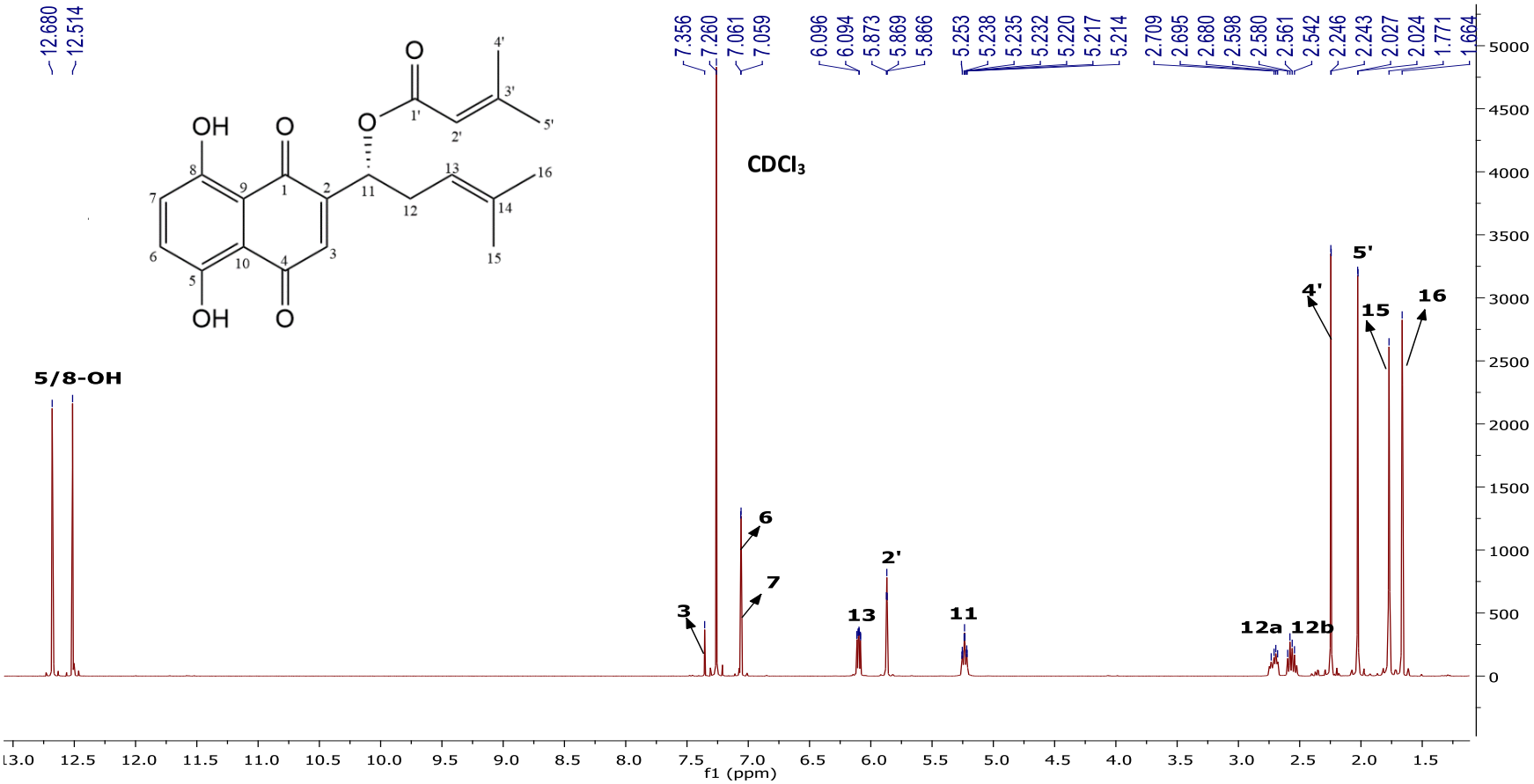
OM-5 was obtained as a reddish crystal, and observed as pinkish band in the TLC chromatogram (under visible light) implying its naphthoquinone-type nature. In the LC-MS spectrum of **OM-5**, a major ion peak was observed at *m/z* 415.50 $[M+2Na-H]^+$ indicating the molecular formula as $C_{21}H_{22}O_6$ (calc. 415.52 for $C_{21}H_{21}O_6Na_2$).

In the low field region of the ^{1}H NMR spectrum, the resonances at δ 12.68 (*s*) and 12.51 (*s*) belonging to the exchangeable protons of two aromatic hydroxy groups, and the signals at δ 7.06 (*d*, *J*=1.1 Hz; H-3) and 7.36 (s, 2H; H-6 and H-7) suggested that **OM-5** was a mono-substituted naphthoquinone derivative. The signals at δ 6.01 (*ddd*, *J*=7.2, 4.4, 0.8 Hz) and 5.24 (*m*) was evident for characteristic oxymethine (H-11) and tri-substituted double bond (H-13) protons, respectively, for the acyclic moiety. Besides, the methyl signals at δ 1.77 (*s*) and 1.66 (*s*) were attributed to H_3-15 and H_3-16, respectively. Together with the characteristic naphthoquinone resonances in the ^{13}C NMR spectrum, five additional resonances consistent with the presence of a dimethylacryl moiety were observed (δ 166.3, 115, 158.8, 25.6 and 17.8).[90] Due to the low-field shift of H-11 (δ 5.24, m) in the ^{1}H NMR spectrum, linkage position of dimethylacryl group was deduced to be C-11(O).

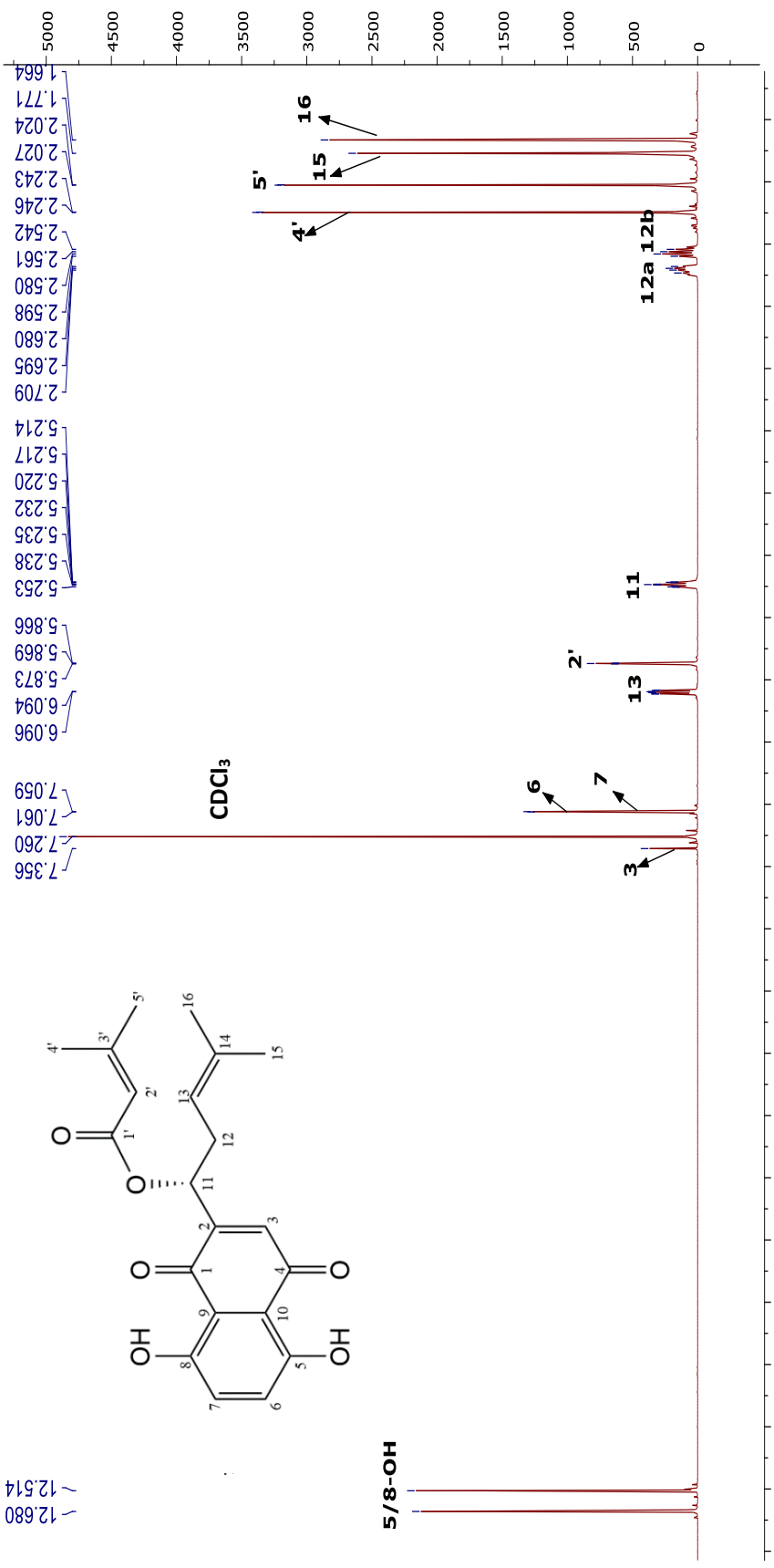
The ^{1}H and ^{13}C NMR data of **OM-5** was identical with those of *β,β*-dimethylacrylal-kannin (shikonin) [1-(5,8-dihydroxy-1,4-dioxonaphthalen-2-yl)-4-methylpent-3-enyl]3-methylbut-2-enoate.[90] The positive optical rotation value of **OM-5** ($[\alpha]_D^{25}$: +120; *c* 0.1, MeOH) showed that it was an shikonin derivative with C-11(R) absolute configuration. Consequently, the structure of **OM-5** was determined to be *β,β*-dimethylacrylshikonin.

Table 3.6. ^{1}H and ^{13}C Data of **OM-5** (400 MHz, δ ppm, in $CDCl_3$)

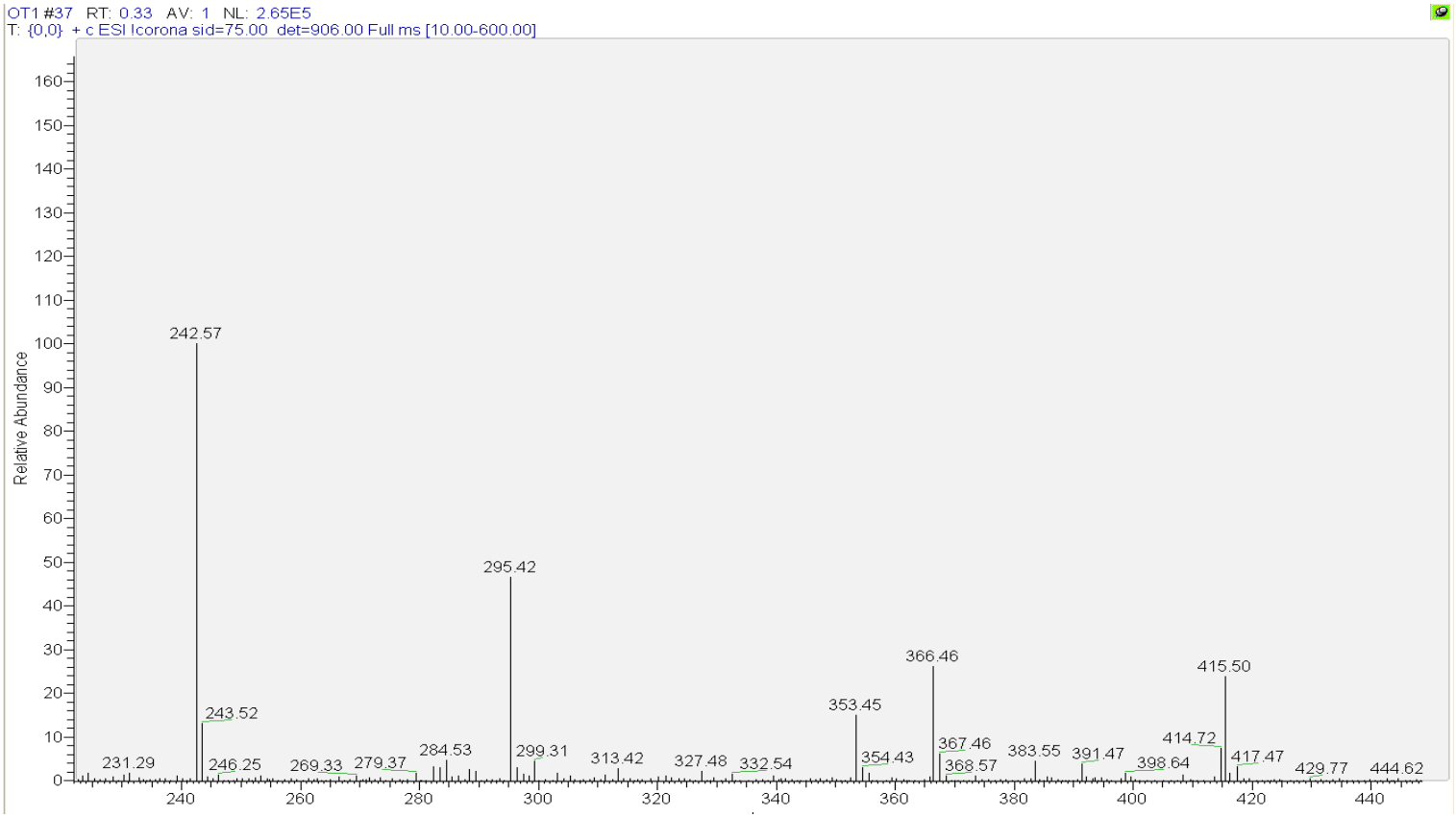
H/C	**Mult.**	**δ_H (ppm)**	***J* (Hz)**	**δ_C (ppm)**
1	C			178.8
2	C			148.8
3	CH	7.06	*d* (1.1)	135.5
4	C			177.3
5	C			166.9
6	CH	7.36	*s*	132.3
7	CH	7.36	*s*	132.2
8	C			165.4
9	C			111.6
10	C			111.4
11	CH	5.24	*m*	117
12	CH_2	2.71	*m*	32.7
		2.56	*dt* (14.8, 7.4)	
13	CH	6.10	*ddd* (7.2, 4.4, 1.8)	68.4
14	C			131.4
15	CH	1.77	*s*	27.4
16	C	1.66	*s*	21.2
1'	C			166.3
2'	CH	5.87	*m*	115
3'	C			158.8
4'	CH	2.03	*d* (1.2)	25.6
5'	CH	2.24	*d* (1.1)	17.8



Spectrum 3.8. [1]H-NMR Spectrum of **OM-5**



Spectrum 3.9. ^{13}C-NMR Spectrum of **OM-5**



Spectrum 3.10. LC-MS spectrum of **OM-5** (positive mode)

3.4.5. Structure Identification of OT-1

When NMR spectra of **OT-1**was inspected, it was found to be identical with **OM-5**. Thus, structure of **OT-1** was identified as β,β-dimethylacrylshikonin.

3.4.6. Structure Identification of OT-2

As a result of spectral characterization studies, the structure of **OT-2**, isolated from *O. mollis,* was determined to be same as **OM-4** (shikonin).

3.4.7. Structure Identification of OT-3

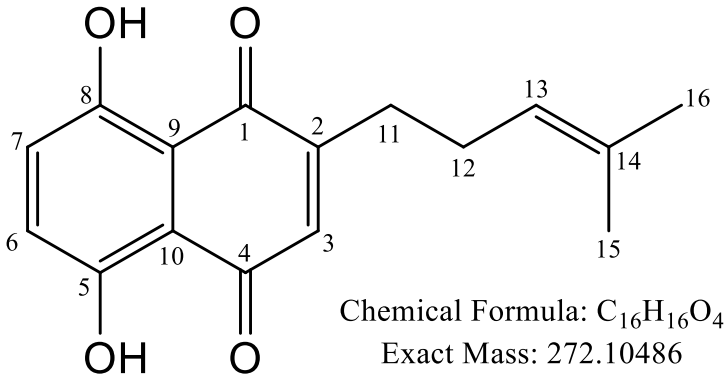


Figure 3.6. Chemical structure of 11-deoxyshikonin, 5,8-dihydroxy-2-(4-methylpent-3-enyl) naphthalene-1,4-dione

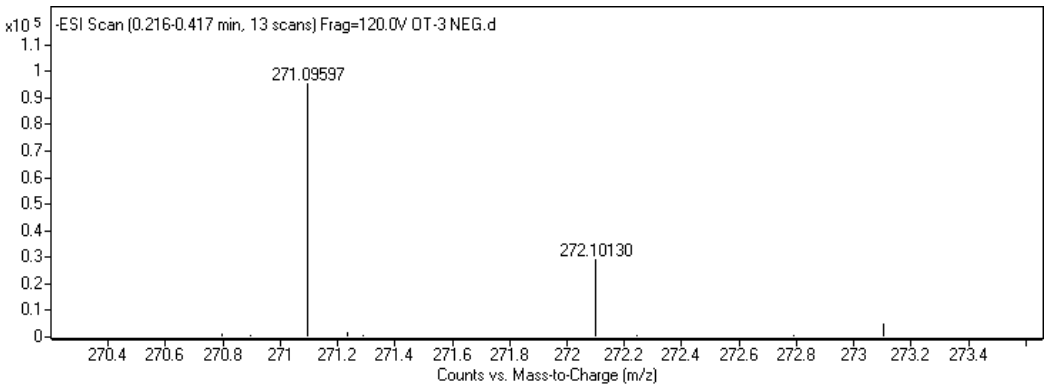
OT-3 was isolated as a red solid, and its thin-layer chromatogram illustrated a typical naphthoquinone profile. In the HR-ESI-MS spectrum (negative mode) of **OT-3**, a major ion peak was observed at *m/z* 271.0960 $[M-H]^-$ indicating the molecular formula as $C_{16}H_{16}O_4$ (calc. 271.0971 for $C_{16}H_{15}O_4$).

In the low field region of the ^1H NMR spectrum, resonances at δ 12.47 (s) and 12.63 (s) belonging to the exchangeable protons of two aromatic hydroxy group and the signals at δ 7.20 (s, 2H; H-6 and H-7) suggested another naphthoquinone framework as in **OT-1** and **OT-2**. The methyl signals at δ 1.70 (s) and 1.60 (s) were readily assigned for the methyl groups H_3-15 and H_3-16, respectively. Furthermore, the characteristic hydroxymethine proton of C-11 was absent in the ^1H NMR spectrum. Comparison of the

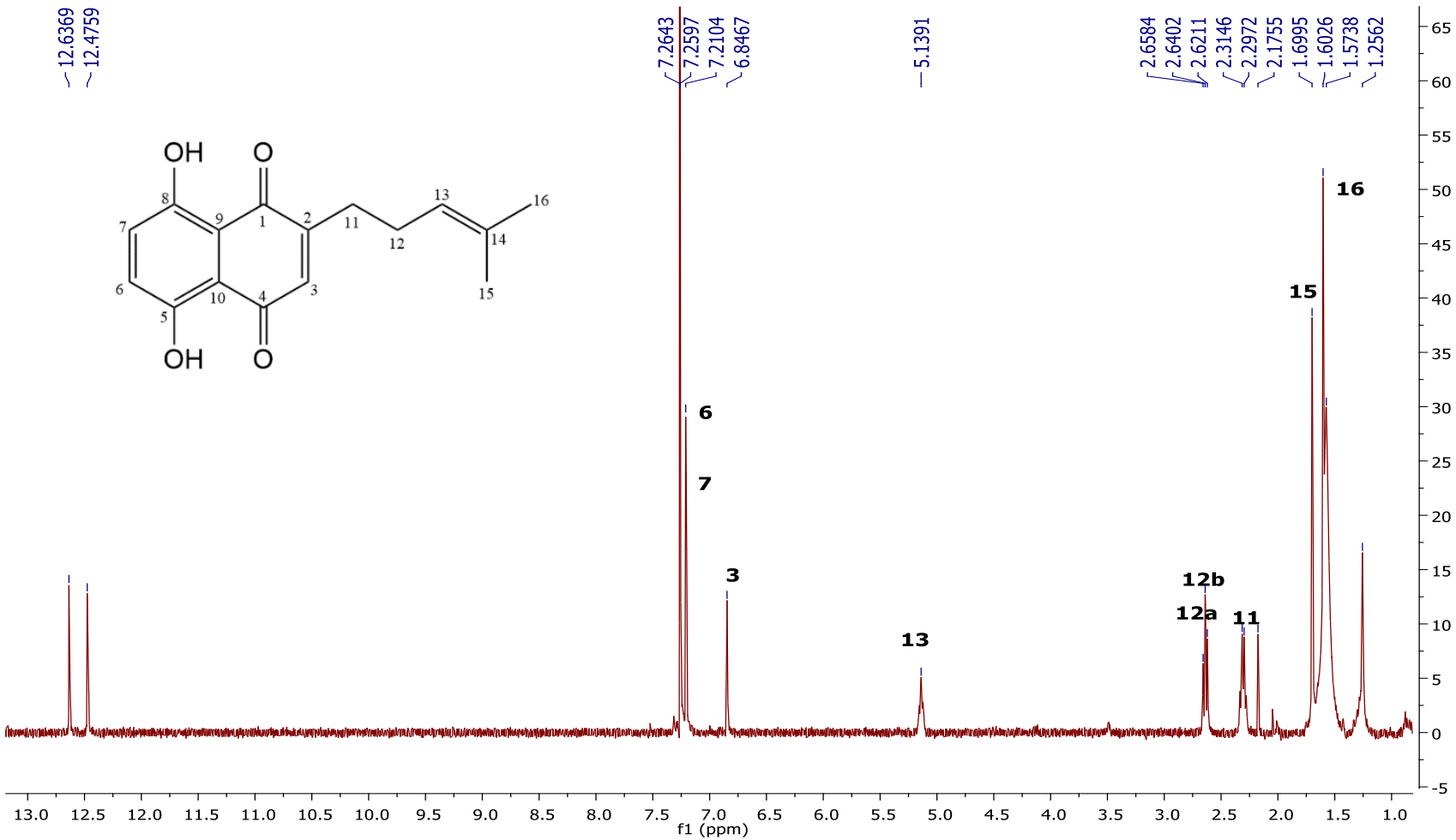
^{1}H NMR data of **OT-3** with those of previously published naphthoquinone derivatives allowed us to establish the structure as 5,8-dihydroxy-2-(4-methylpent-3-enyl) naphthalene-1,4-dione (11-deoxyshikonin).[50]

Table 3.7. ^{1}H Data of **OT-3** (400 MHz, δ ppm, in $CDCl_3$)

H/C	**Mult.**	**δ_H (ppm)**	***J* (Hz)**
1	C		
2	C		
3	CH	7.17	*d (1.1)*
4	C		
5	C		
6	CH	7.21	*s*
7	CH	7.21	*s*
8	C		
9	C		
10	C		
11	CH	4.91	*s*
12	CH_2	2.63	*m*
		2.35	*m*
13	CH	5.21	*m*
14	C		
15	CH	1.70	*s*
16	CH	1.60	*s*



Spectrum 3.11. HR-ESI-MS spectrum **OT-3** (negative mode)



Spectrum 3.12. ^{1}H-NMR Spectrum of **OT-3**

The structure elucidation studies let us establish five naphthoquinone-type compounds, viz. shikonin**,** β,β-dimethylacrylshikonin, deoxyshikonin, acetylshikonin and β-hydroxyisovalerylshikonin from O. taurica var. taurica and O. mollis.

A number of phytochemical studies focusing on isolation and characterization of naphthoquinone derivatives from *Onosma* species were reported.

β, β-dimethylacrylshikonin was reported several times from different *Onosma* species, including *O. leptanhtha*[33], *O. visianii*[36], *O. paniculata*[32], *O. confertum*, *O. hookeri* and *O. zerizaminum*.[4]

β-hydroxyisovalerylshikonin was previously isolated from *O. paniculata*[32], *O. leptanhtha*[33] and *O. visianii*.[36]

Acetylshikonin, another common naphthoquinone, was isolated from *O. paniculata*[32], *O. leptanhtha*[33], *O. visianii*[36] and *O. argentatum*[34], *O. confertum, O. hookeri* and *O. echioides.*[4]

Hu et al. studied naphthoquinone derivatives of Boraginaceous plants by high-performance liquid chromatography. Deoxyshikonin was found in the methanol extracts of *O. waltonii, O. hookeri, O. hookeri* var. *longiflorum, O. paniculatum, O. exsertum* and *O. confertum.*[88] Other studies was also reported deoxyshikonin in different extracts of *O. heterophylla*[92], *O. argentatum*[34] *and O. echioides.*[93]

Shikonin was previously isolated from *O. caucasicum, O. conferitum, O. hookeri, O. livanovii, O. polyphyllum, O. tauricum, O. sericium, O. setosum, O. visianii* and *O. zerizaminium.*[4,26]

From phytochemistry point of view, *Onosma* species, in this study, have not been subject of detailed isolation studies. Although structures of the obtained molecules were previously described, herein, they were reported for the first time from the title plants.

Based on the HPLC chromatograms, the number of molecules observed in the main extracts was higher than the isolated molecules. As the amounts of root materials were not in high quantity, we were not able to isolate the minor constituents. As well, during isolation studies, it was realized that the naphthoquinones in the root extracts of *O. taurica* var. *taurica* were decomposed by heat and day-light exposure resulting inseparable crude fractions and/or too minute artifacts.

3.5. Cytotoxicity Studies

3.5.1. Cytotoxic Activity of *Onosma* Species

Preliminary cytotoxicity tests were performed by screening the extracts of different *Onosma* species. Based on the results, the species to be undertaken bioactivity-guided isolation studies were selected.

Table 3.8. The results of cytotoxicity assay of the root extracts (**P.** Petroleum ether, **D.** Dichloromethane, **M.** Methanol)

IC_{50} VALUES (µg/mL) @ 48 h								
Extracts	**Du145**	**Capan-1**	**HCC-1937**	**MCF-7**	**HeLa**	**HEP-G2**	**A-549**	**MRC-5**
***O. frutescens* (PE)**	>32	>32	>32	>32	>32	>32	>32	>32
***O. frutescens* (D)**	>12	≈12	>12	≈12	≈12	11.12	≈12	11.9
***O. frutescens* (M)**	>32	>32	>32	>32	>32	>32	>32	>32
***O. aksoyii* (PE)**	<8	<8	<8	<8	<8	<8	<8	<8
***O. aksoyii* (D)**	>32	≈32	21.3	15.6	23	21.66	19	26.4
***O. aksoyii* (M)**	>32	>32	>32	>32	>32	>32	>32	>32
***O. mollis* (PE)**	<8	<8	<8	<8	<8	<8	<8	<8
***O. mollis* (D)**	>32	>32	>32	>32	>32	>32	>16	>32
***O. mollis* (M)**	>32	>32	>32	>32	>32	>32	>32	>32
***O. mite* (PE)**	22.1	30	17.6	25.3	22.6	23.5	>32	>32
***O. mite* (D)**	>32	>32	<8	>32	≈32	>32	26.15	>32
***O. mite* (M)**	>32	>32	>32	>32	>32	>32	>32	>32
***O. taurica* var. *tarica* (PE)**	<8	<8	<8	<8	<8	<8	<8	<8
***O. taurica* var. *tarica* (D)**	<8	7.95	<8	<8	<8	7.09	8.64	<8
***O. taurica* var. *tarica* (M)**	>32	>32	16,5	>32	≈32	>32	>32	>32
***O. heterophylum* (PE)**	>32	>32	>32	>32	>32	>32	>32	>32
***O. heterophylum* (D)**	>32	>32	>32	>32	>32	>32	>32	>32
***O. heterophylum* (M)**	>32	>32	>32	>32	>32	>32	>32	>32
O. bracteosum (PE)	>32	>32	>32	>32	>32	>32	>32	>32
O. bracteosum (D)	>32	>32	>32	>32	>32	>32	>32	>32
O. bracteosum (M)	>32	>32	>32	>32	>32	>32	>32	>32

(cont. on next page)

Table 3.8. (cont.)

IC_{50} VALUES (µg/mL) @ 48 h								
Extracts	**Du145**	**Capan-1**	**HCC-1937**	**MCF-7**	**HeLa**	**HEP-G2**	**A-549**	**MRC-5**
O. armenum (PE)	>32	>32	>32	>32	>32	>32	>32	>32
O. armenum (D)	>32	>32	>32	>32	>32	>32	>32	>32
O. armenum (M)	>32	>32	>32	>32	>32	>32	>32	>32
O. nana (PE)	>32	>32	>32	>32	>32	>32	>32	>32
O. nana (D)	>32	>32	>32	>32	>32	>32	>32	>32
O. nana (M)	>32	>32	>32	>32	>32	>32	>32	>32
O. bornmuelleri (PE)	>32	>32	>32	>32	>32	>32	>32	>32
O. bornmuelleri (D)	>32	>32	>32	>32	>32	>32	>32	>32
O. bornmuelleri (M)	>32	>32	>32	>32	>32	>32	>32	>32
O. oreodoxum (PE)	>32	>32	>32	>32	>32	>32	>32	>32
O. oreodoxum (D)	>32	>32	>32	>32	>32	>32	>32	>32
O. oreodoxum (M)	>32	>32	>32	>32	>32	>32	>32	>32
O. aucheranum (PE)	>32	>32	>32	>32	>32	>32	>32	>32
O. aucheranum (D)	>32	>32	>32	>32	>32	>32	>32	>32
O. aucheranum (M)	>32	>32	>32	>32	>32	>32	>32	>32
Doxorubicin	0.62	2.13	>2.72	0.53	0.44	0.38	0.53	0.58

Only a limited number of cytotoxicity screening on *Onosma* species were reported, which were taken into consideration for our cytotoxic activity tests.

The study of *O. arenaria* cyclohexane extract showed cytotoxic activity on HeLa (Human cervix carsinoma) and K562 (Human erythtoleukemic cell line) cells with IC_{50} values of 0.39 and 0.83 µM, respectively.[33]

Vukic *et al.* investigated cytotoxic activity of five different extracts of the *O. visianii* roots including petroleum ether, methanol, acetone, ethylacetate and chloroform. Acetone, chloroform and ethyl acetate extracts, in which acetylshikonin, isobutyrylshikonin and α-methylbutyrylshikonin were main components, showed strong cytotoxic activity versus HCT-116 and MDA-MB-231 cancer cell lines. Chloroform extract had the strongest activity on HCT-116 with IC_{50} value of 17.57 µg/mL at 48 hours. The ethyl acetate, chloroform and acetone extracts were the most potent *O. visianii* extracts versus MDA-MB-231 cells after 72 h, with IC_{50} values of 22.13, 13.16 and 8.11 µg/mL, respectively. The petroleum ether and methanol extracts were the least effective versus MDA-MB-231 and HCT-116 cells.[89]

It was reported that *n*-hexane-dichloromethane extract of *O. argentatum* root had cytotoxic activity at 10 µg/mL dose on human amnion fibroblast (HAFs).[47]

The overview of available literature revealed that there has been no study towards screening of *O. taurica* var. *taurica* and *O. mollis* extracts for cytotoxic activity.

The petroleum ether and dichloromethane extracts of *Onosma* species, rich in naphthoquinones, were more active than the methanol extracts in a dose range of 8 to 32 μg/mL. Specifically, the petroleum ether and dichloromethane extracts of ***O. taurica* var. *taurica*** along with the petroleum ether extract of ***O. mollis*** and ***O. aksoyii*** showed significant and stronger cytotoxic activity compared to the other extracts (IC_{50} < 8 μg/mL. Activity of the dichloromethane extract of ***O. mite*** exhibited selectivity towards human breast carcinoma cell line (HCC-1937) (IC_{50} < 8.0 μg/mL) than other cell lines.

Consequently, petroleum ether extract of *O. mollis* and *O. aksoyii* and petroleum ether-dichloromethane extracts of *O. taurica* var. *taurica* were found to have promising cytotoxicity in the current study, warranting further investigation to purify the specific compounds responsible for the activity.

3.5.2. Cytotoxic Activity of the Main Fractions from *O. taurica* var. *taurica*

The petroleum ether-dichloromethane extract of *O. taurica* var. *taurica* (6.22 g) was subjected to medium pressure liquid chromatography (MPLC) using reversed-phase material (RP-18) to give ten main fractions (from ES-01 to ES-10). These fractions were tested on seven human cancer cell lines, namely DU145, Capan-1, HCC-1937, MCF-7, HeLa, HEP-G2, A-549, and a normal cell line (MRC-5) to select active ones (Table 3.8).

When the activity of these fractions was evaluated based on their column elution order, it was realized that the active ones were more non-polar compounds. Fractions with stronger cytotoxicity (IC_{50} values less than 8 μg/mL), *viz.* **ES-06**, **ES-07**, **ES-08** and **ES-09** were selected for further bioactivity guided isolation (Table 3.9; Figure 2.2).

Table 3.9. Cytotoxicity results of the main fractions obtained from the root extracts of *O. taurica* var. *taurica*

IC_{50} VALUES (μg/mL) @ 48 h								
Fraction	**Du145**	**Capan-1**	**HCC-1937**	**MCF-7**	**HeLa**	**HEP-G2**	**A-549**	**MRC-5**
ES-01	>32	>32	>32	>32	>32	>32	>32	>32
ES-02	>32	>32	>32	>32	>32	>32	>32	>32

(cont. on next page)

Table 3.9. (Cont.)

IC_{50} VALUES (µg/mL) @ 48 h								
Fraction	**Du145**	**Capan-1**	**HCC-1937**	**MCF-7**	**HeLa**	**HEP-G2**	**A-549**	**MRC-5**
ES-03	>16	>16	>16	>16	>16	>16	>16	>16
ES-04	>32	>32	>32	>32	>32	>32	>32	>32
ES-05	26.45	22.5	21.6	>32	≈32	31.1	>32	11.32
ES-06	<8	<8	<8	11.1	9.06	9.01	9.38	<8
ES-07	<8	<8	<8	<8	<8	<8	14.75	<8
***ES-08**	<4	<4	<4	<4	<4	<4	4.73	<4
***ES-09**	5.12	<4	<4	5.6	6.85	6.1	7.29	<4
ES-10	>16	>12	>16	>16	>16	>16	>16	>16
Doxorubicin	0.62	2.13	>2.72	0.53	0.44	0.38	0.53	0.58

***Some extracts have been applied in a lower dose because of the solubility problem in the desired dose range.

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

The petroleum ether root extract of *O. mollis* (5.048 g) was chromatographed over silica gel column chromatography to yield sixteen main fractions (from ES-22 to ES-37); they were tested versus seven human cancer cell lines, namely DU145, Capan-1, HCC-1937, MCF-7, HeLa, HEP-G2, A-549 and a normal cell line, MRC-5 to identify active fractions (Table 3.10). When the cytotoxic activity of 16 main fractions obtained from silica column chromatography was examined, the active compounds were also realized to be separated according to their polarities. The results showed that the fractions with non-polar character and/or red and/or pink TLC bands under visible light due to the presence of naphthoquinones had higher activities. **ES-23**, **ES-24**, **ES-25**, **ES-26**, **ES-27**, **ES-28**, **ES-29**, **ES-31**, **ES-32**, **ES-35** and **ES-36** with IC_{50} values less than <8 mg/mL were chosen for further bioactivity-guided isolation studies.

Table 3.10. Cytotoxicity results of the main fractions obtained from the root extracts of *O. mollis*

IC_{50} VALUES (µg/ml) @ 48 h								
Fractions	**Du145**	**Capan-1**	**MRC-5**	**HCC-1937**	**MCF-7**	**HeLa**	**HEP-G2**	**A-549**
ES-22	8.3	8.6	7.4	9.1	>16	>16	10,2	>16
ES-23	<8	<8	<8	<8	<8	<8	<8	<8

(Cont. on next page)

Table 3.10. (Cont.)

Fractions	Du145	Capan-1	MRC-5	HCC-1937	MCF-7	HeLa	HEP-G2	A-549
ES-24	<8	<8	<8	<8	<8	<8	<8	<8
***ES-25**	<4	<4	<4	<4	≈4	5	<4	4.16
ES-26	<8	<8	<8	<8	<8	<8	<8	<8
***ES-27**	<4	<4	<4	<4	≈4	4.46	<4	≈4
***ES-28**	<4	<4	<4	<4	≈4	9.72	<4	6.6
***ES-29**	<4	<4	<4	<4	<4	8.45	<4	4.76
ES-30	<8	<8	<8	≈8	12.4	>16	11.8	>16
***ES-31**	4.2	5.6	4.1	6.7	13.56	≈16	7.8	≈16
***ES-32**	5	6.1	5.4	7.2	≈16	>16	8.9	>16
ES-33	13.39	16.7	12.8	18.4	18.26	25.2	19.7	≈32
ES-34	17.74	15.4	13.6	19.7	30	>32	20,3	>32
***ES-35**	<4	<4	<4	<4	5.45	11.7	<4	11.6
ES-36	11.74	12.6	11.1	14.8	15	>16	14.3	>16
ES-37	>16	>16	>16	>16	>16	>16	>16	>16
Doxorubicin	0.62	2.13	>2.72	0.53	0.44	0.38	0.53	0.58

*****Some extracts have been applied in a lower dose because of the solubility problem in the desired dose range.

3.5.4. Cytotoxic Activity of Pure Compounds from *O. taurica* var. *taurica* and *O. mollis*

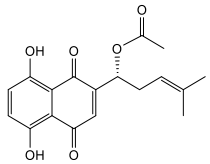
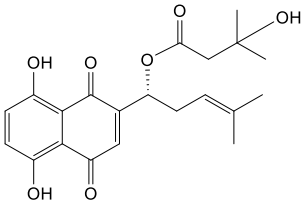
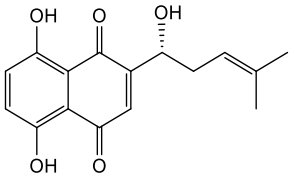
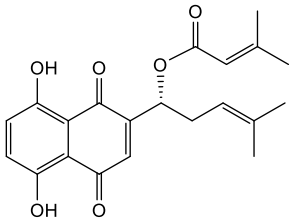
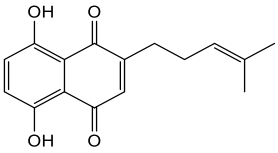
Further bioactivity guided fractionations led us to isolate five compounds from *O. mollis* and *O. taurica* var. *taurica*. The isolates were identified as shikonin**,** β,β-dimethylacrylshikonin, deoxyshikonin, acetylshikonin, β-hydroxy-isovalerylshikonin on the basis of their spectral data. Their cytotoxic activities were investigated on three human cancer cell lines, namely DU145, HCC-1937, HeLa, and a normal cell line, MRC-5, using MTT cell proliferation reagent. The IC_{50} values were unexpectedly broad ranging from 1.83 to 25 μg/mL.

Table 3.11. Cytotoxicity results of the pure compounds

Compounds		IC_{50} Values (μM) @ 48 h			
		HeLa	**HCC-1937**	**DU145**	**MRC-5**
OM-1	Unidentified	>25	>25	>25	>25

(cont. on next page)

Table 3.11. (cont.)

Compounds		IC_{50} Values (μM) @ 48 h			
		HeLa	HCC-1937	DU145	MRC-5
OM-2	 Acetylshikonin	21.95±1.6	2.26±1.7	3.07±0.261	2.51±1.44
OM-3	 β-Hydroxyisovalerylshikonin	>25	>25	>25	>25
OM-4	 Shikonin	>25	5.27±0,568	3.12±0.405	5.51±1.91
OM-5	 β,β-Dimethylacrylshikonin	16.51±1.2	1.84±1.18	1.91±0.437	1.83±1.30
OT-3	 Deoxyshikonin	>25	>25	>25	>25
	Doxorubicin	0.44	>2.72	0.62	0.58

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

Kretschmer *et al.* performed a bioactivity guided isolation study on *O. paniculata* to obtain β-hydroxyisovalerylshikonin, acetylshikonin, dimethylacrylshikonin and α-methylbutyrylshikonin/isovalerylshikonin mixture. β-Hydroxyisovalerylshikonin demonstrated higher cytotoxic activity on human CCRF-CEM leukemia, MDA-MB-231

and HCT 116 colon cancer cell lines with IC_{50} values of 0.6, 4.6 and 4.1 μM, respectively. Acetylshikonin exhibited cytotoxicity towards human U251 glioblastoma cell line with an IC_{50} value of 15.9 μM. Moreover, α-methylbutyrylshikonin was found to be the most active compound versus human SBcl2, WM35, WM9 and WM164 melanoma cell lines (IC_{50} values: 1.1, 2.3, 2.7 and 8.3 μM, respectively). Furthermore, α-methylbutyrylshikonin increased the number of cells in the subG1 phase of the cell cycle as well it resulted in caspase-3/7 activation, indicating apoptotic cell death.[32]

In the study of *O. leptantha*, β,β-dimethylacrylshikonin, isovalerylshikonin and acetylshikonin were purified from cyclohexane extract and their cytotoxic activities were examined versus a murine leukemia (L1210) and a human solid tumor cell line (HT-1080). β,β-dimethylacrylshikonin exhibited the highest activity on both cell lines (IC_{50}: 390 nM for L1210 cells, and approx. 2 μM for HT-1080), and acetylshikonin was found to be active only at higher concentrations.[33]

Yingkun *et al.* studied anti-proliferative effects and apoptotic properties of shikonin on human HepG2 cells. The growth of HepG2 cells was inhibited in a dose-dependent manner with an IC_{50} value of 4.30 μg/mL. The cell cycle studies revealed that HepG2 cell cycle progression was blocked at the S phase.[94]

Wu *et al.* examined the effects of shikonin isolated from *L. erythrorhizon*, which revealed that the caspase-3 activation and blockage of DNA synthesis were the reasons for HeLa cell death. Shikonin, inhibited HeLa tumor cell growth with an IC_{50} value of 18.9 μM, and significantly blocked the transition from G1 to S phase in the cell cycle at 10 μM. Increment of caspase-3 activity was observed within 12 h after 20 μM of shikonin treatment.[95]

Another study on apoptotic cell death, it was shown that 8-24 μM of shikonin treatment in human bladder cancer cells (T24) resulted in cell damage. Moreover, shikonin (16 μM) was found to induce G0/G1 arrest and apoptosis on T24 cancer cells.[96]

Acetylshikonin demonstrated cytotoxic activity on HepG2 cells with an IC_{50} value of 2 μM. As well 2.5 μM of acetylshikonin increased caspase3 and PARP1 cleavages, and the bax expression while the bcl2 expression level decreased in HepG2 cells. Thus it was inferred that caspase3 dependent mechanism along with regulation of the mitochondrial pro-apoptotic bax and anti-apoptotic bcl2 protein expression were important features for the action of acetylshikonin. Also, treatment of HepG2 cells with acetylshikonin at 2.5 μM dose resulted in an increase in p53 expression level, a typical apoptosis regulator in cancer cells.[97]

When the literature for cytotoxicity of naphthoquinones were reviewed, it was seen that our results were quite similar with the previous data obtained. The isolated compounds (**OM-1** – **OM-5** and **OT-3**) showed prominent cytotoxicity with IC_{50} values ranging from 1.83 to 25 μM.

When the magnitude of cytotoxic activity was evaluated, acetylshikonin (**OM-2**), and β,β-dimethylacrylshikonin (**OM-5**) were noticeable with greater activity towards HCC-1937 (IC_{50} values of 2.26 and 1.91, respectively). β,β-dimethylacrylshikonin was found to be the most active metabolite versus DU145 (IC_{50} values of 3.12 μM). Deoxyshikonin (**OT-3**) and β-hydroxyisovalerylshikonin (**OM-3**) showed lower cytotoxic activities versus HCC-1937, HeLa, DU145 and MRC- 5 cell lines with IC_{50} values higher than 25 μM. Shikonin (**OM-4**), acetylshikonin and β,β-dimethylacrylshikonin displayed cytotoxicity versus MRC-5, normal cell line with IC_{50} values of 5.51, 2.51 and 1.82, respectively. Therefore, it can be stated that the compounds have no selectivity for cancer cell lines. Among the cancer cells, DU145 and HCC-1937 turned out to be the most susceptible cell lines inhibited by naphthoquinones.

Predicting relationships between structure and activity, previous studies were taken into consideration. The results reveal that acylation of C-11(*O*) is important for higher cytotoxic activity because of the enhancing effect of acylation on the electrophilicity of the quinone moiety.[53,98] For example, in our screenings, acetylshikonin (**OM-2**) with the smallest ester group (IC_{50}: 2.26 μM versus HCC-1937) was found approximately 2.5-fold more cytotoxic than shikonin (IC_{50} : 5.27 μM).

Moreover, one of the least active naphthoquinones was deoxyshikonin (IC_{50}>25 μM) versus all the cell lines suggesting importance of C-11(OH) group. Indeed, its hydroxylated metabolite shikonin exhibited more prominent toxicity at 5.27 μM concentration on HCC-1937 cell line.

Additionally, chemistry of the acyl side chain but not the size was noteworthy in the case of β-hydroxyisovalerylshikonin (**OM-3**) and β,β-dimethylacrylshikonin (**OM-5**). The presence of α,β-unsaturated carbonyl functionality as in **OM-5** increased the cytotoxicity greatly compared to the β-hydroxy carbonyl residue as in **OM-3** (IC_{50} values 1.84 μM versus 25 μM on HCC-1937).

Cytotoxicity together with DNA Topoisomerases I and II inhibition assays guided our species selection and their further fractionation studied to isolate active constituents responsible for bioactivities.

3.6. Inhibitory Activity of DNA Topoisomerases

3.6.1. Inhibitory Activity of the Main Fractions

3.6.1.1. Inhibitory Activity of the Main Fractions from *O. taurica* var. *taurica*

The relaxation reactions were initiated with 1 mg/mL concentration of the extracts and a sample agarose gel image of the hTopo I relaxation assay is given in Figure 3.7.

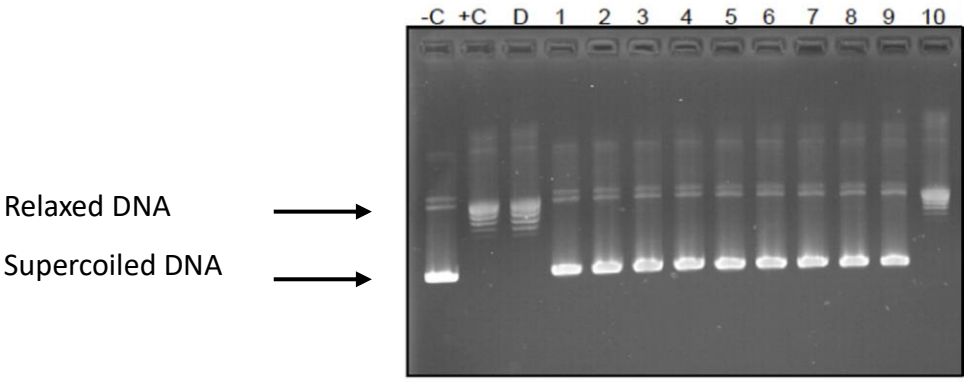


Figure 3.7. Inhibitory effects of *O. taurica* var. *taurica* main fractions at final concentration of 0.1 mg/mL on hTopo I activity. The final volumes of the reactions were 20 µL; 500 ng of pBR322 substrate incubated with 1 unit of hTopo I enzyme at 37°C for 30 min in the presence and absence of the test compounds. The reaction products were separated using agarose gel electrophoresis and monitored under UV after Etd-Br staining. Experiments were carried out using a 1:10 dilution of the stocks at concentrations of 1 mg/mL of the test compounds (1-10 encoded applications), **-C**. negative control, **+C**. positive control (enzyme only) and **D**. DMSO at a concentration of 2.5%.

The main fractions of *O. taurica* var. *taurica*, except the sample ES-10, showed inhibitory effect on hTopo I relaxation activity at 0.1 mg/mL concentration (Figure 1, 10 encoded application). To determine whether their effects on hTopo I enzyme were dose-dependent, the relaxation activity was repeated with dilutions at different concentrations, analysed on 1% agarose gel, and photographed under UV light (Figure 3.8).

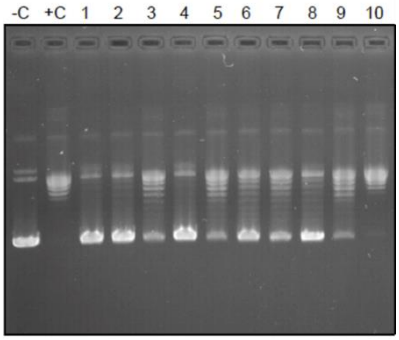


Figure 3.8. Dose dependent effects of *O. taurica* var. *taurica* main fractions at final concentration of 0.01 mg /mL on hTopo I activity. The reactions were carried out under the conditions detailed above at concentrations of 0.1 mg /mL of the test substances. Experiments were carried out using a 1:10 dilution of the stocks (1-10 encoded applications) **-C**. negative control, **+C**. positive control (enzyme only). DMSO control was not included because DMSO concentration in the samples was negligible in this dilution rate.

hTopo I relaxation analyses have shown that a significant proportion of the main fractions (samples ES-1, 2, 4, 6, 8) were effective on hTopo I activity up to working concentration of 0.01 mg/mL (Figure 3.8).

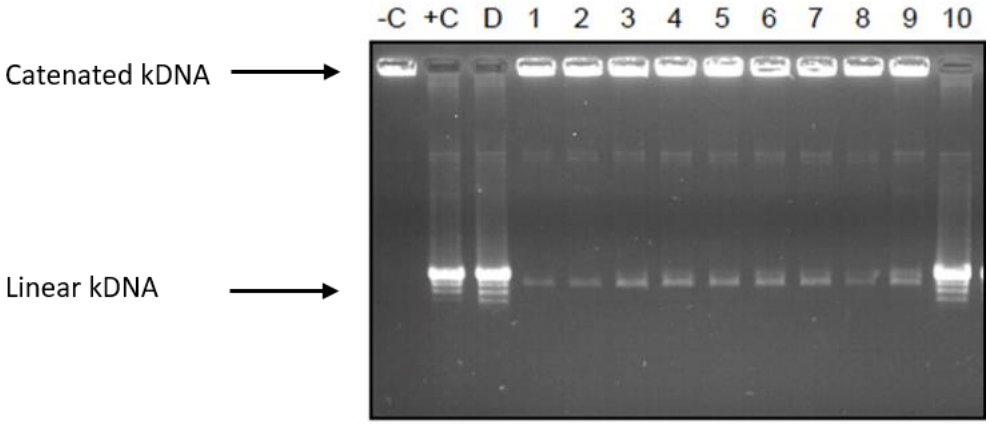


Figure 3.9. Inhibitory effects of *O. taurica* var. *taurica* main fractions at final concentration of 0.1 mg/mL on hTopo II activity. The final volumes of the reactions were 20 µL; 200 ng of kDNA substrate incubated with 1 unit of hTopo II enzyme at 37°C for 30 min in the presence and absence of the test compounds. The reaction products were separated using agarose gel electrophoresis and monitored under UV after Etd-Br staining. Experiments were carried out using a 1:10 dilution of the stocks at concentrations of 1 mg/mL of the test substances (1-10 encoded applications) **-C**. negative control, **+C**. positive control (enzyme only) and **D**. DMSO at a concentration of 2.5%.

Decatenation assay was carried out to determine the inhibitory effect of the main fractions of *O. taurica* var. *taurica* on hTopo II activity at a concentration of 0.1 mg/mL and reactions were visualized on agarose gel (Figure 3.9).

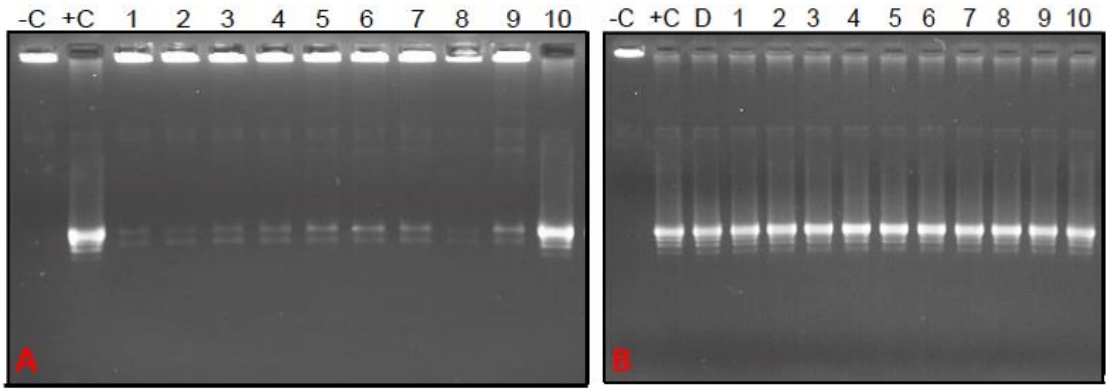


Figure 3.10. Inhibitory effects of *O. taurica* var. *taurica* main fractions at final concentration of 0.05 and 0.01 mg/mL (A and B, respectively) on hTopo II. Experiments were carried out using a 1:10 dilution of the stocks (1-10 encoded applications) at concentrations of 0.5 and 0.1 mg/mL (Figure 3.10A and B respectively) **-C**. negative control, **+C**. positive control (enzyme only) and **D**. DMSO at a concentration of 2.5%.

The main fractions isolated from *O. taurica* var. *taurica,* except the sample ES-10, showed inhibitory effect on hTopo II decatenation activity at 0.1 mg/mL concentration (Figure 3.9, 10 encoded application). To determine whether their effects on hTopo II enzyme were dose-dependent, the analyses were repeated with dilutions of 0.05 and 0.01 mg/mL final concentrations of the test compounds (Figure 3.10A and B).

hTopo II decatenation reactions showed that main fractions lost their inhibitory effect on the enzyme at concentration of 0.01 mg/mL.

3.6.1.2 Inhibitory Effect of the Main fractions from *O. mollis*

The main fractions of *O. mollis,* except the samples ES-22 and ES-26, showed inhibitory effect on hTopo I relaxation activity at 0.01 mg/mL concentration (Figure 3.11).

Main fractions of *O. mollis* showed inhibitory effect except the samples ES-22 and ES-26 on hTopo I relaxation activity at 0.01 mg/mL concentration.

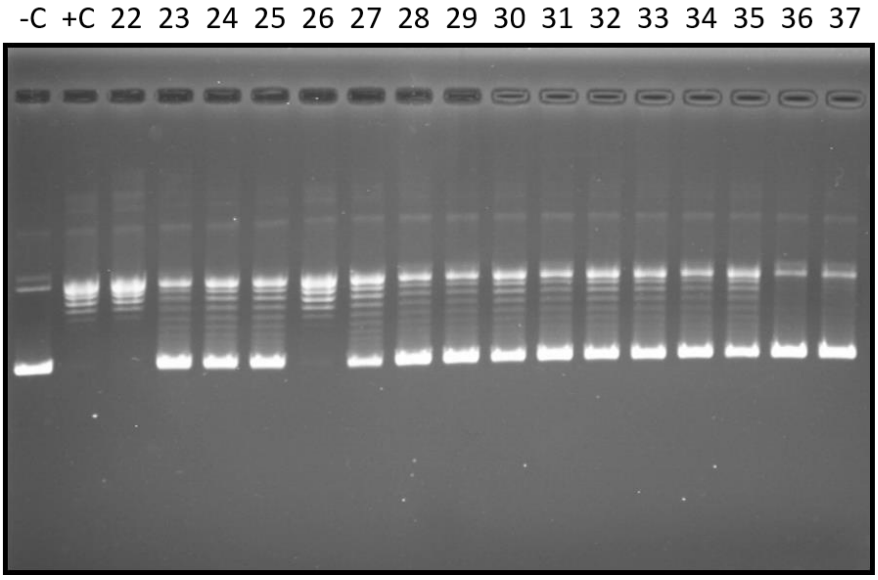


Figure 3.11. Inhibitory effects of *O. mollis* main fractions at final concentration of 0.01 mg/mL on hTopoI. The protocol of reaction has implemented as described above. Experiments were carried out using a 1:10 dilution of the stocks at concentrations of 0.1 mg/mL of the test compounds (22-37 encoded applications) **-C**. negative control, **+C**. positive control (enzyme only)

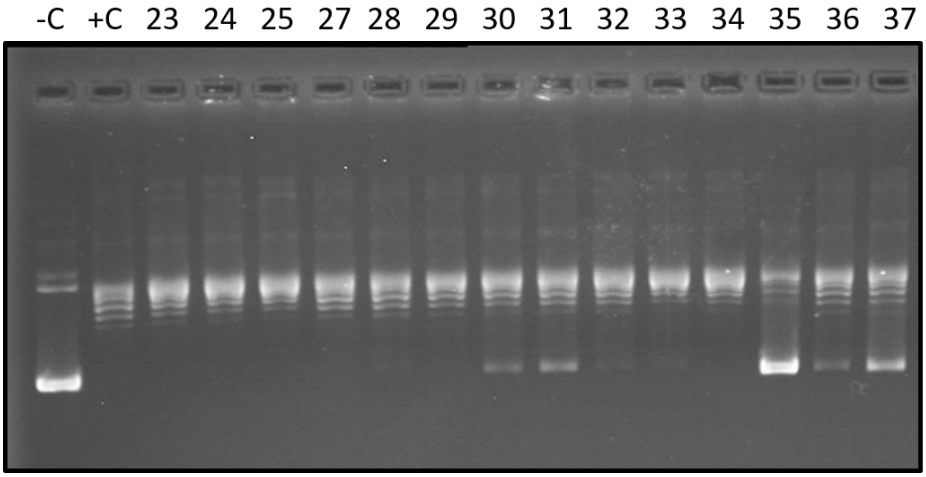


Figure 3.12. Inhibitory effects of *O. mollis* main fractions at final concentration of 0.001 mg/mL on hTopo I. The protocol of reaction has implemented as described above. Experiments were carried out using a 1:10 dilution of the stocks at concentration of 0.01 mg/mL of the test compounds (23-37 encoded applications) **-C**. negative control, **+C**. positive control (enzyme only).

hTopo I relaxation reactions illustrated that the main fractions, except samples ES-30, 31, 35, 36 and 37, lost their effect on the enzyme (Figure 3.12) at working concentration of 0.001 mg/mL; furthermore, ES-30, 31 and 36 showed less inhibitory effect on hTopo I enzyme than ES-35 and 37.

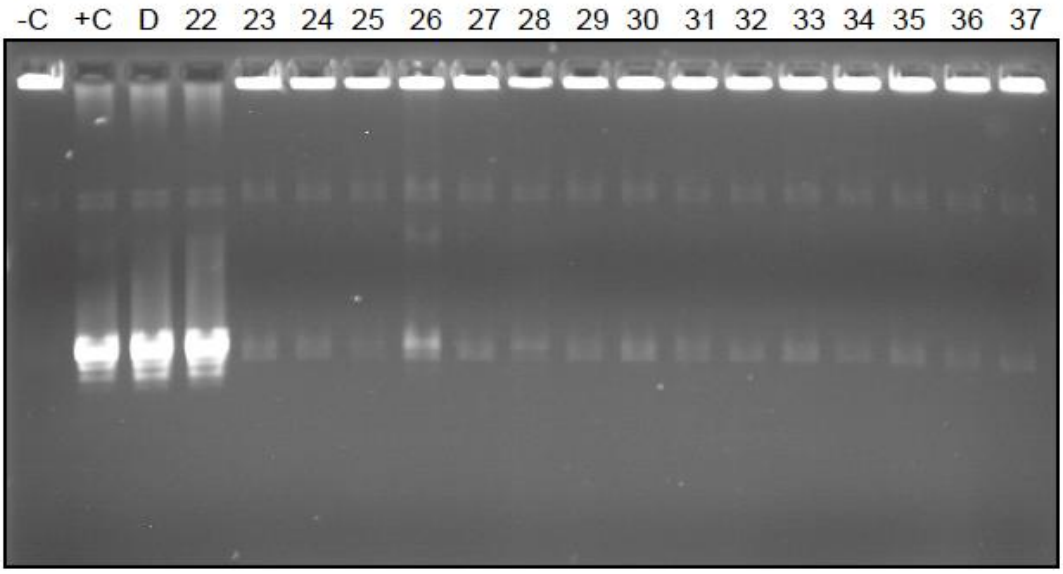


Figure 3.13. Inhibitory effects of *O.mollis* main fractions at final concentration of 0.1 mg/mL on hTopoII. Experiments were carried out using a 1:10 dilution of the stocks at concentrations of 1 mg/mL of the test substances (22-37 encoded applications) **-C**. negative control, **+C**. positive control (enzyme only) and **D**. DMSO at a concentration of 2.5%.

According to hTopo II decatenation reactions, ES-26 (Figure 3.13, 26 encoded application) did not exhibit inhibitory effect on the hTopo II enzyme while ES-22 had less effect than the other samples at concentration of 0.1 mg/mL. The analyzes were repeated with dilutions at concentrations of 0.01, 0.005, and 0.001 mg/mL (Figures 3.14A, B and C, respectively) to determine whether their effects on hTopo II enzyme were dose-dependent.

hTopo II decatenation reactions have shown that main fractions, except ES-25 and ES-26, had inhibitory effects on the enzyme (Figure 3.14A; 25 and 26 encoded applications, respectively) at a working concentration of 0.01 mg/mL while ES-24 and ES-37 (Figure 3.14A; 24 and 37 encoded applications, respectively) were less effective than the other samples.

hTopo II decatenation assays showed that only the sample ES-28 (Figure 3.14B, application 28 code) influenced the activity of the enzyme as compared to the other samples at 0.005 mg/mL.

hTopo II decatenation reactions showed that *O. mollis* fractions lost their effects on the enzyme at a working concentration of 0.001 mg/mL.

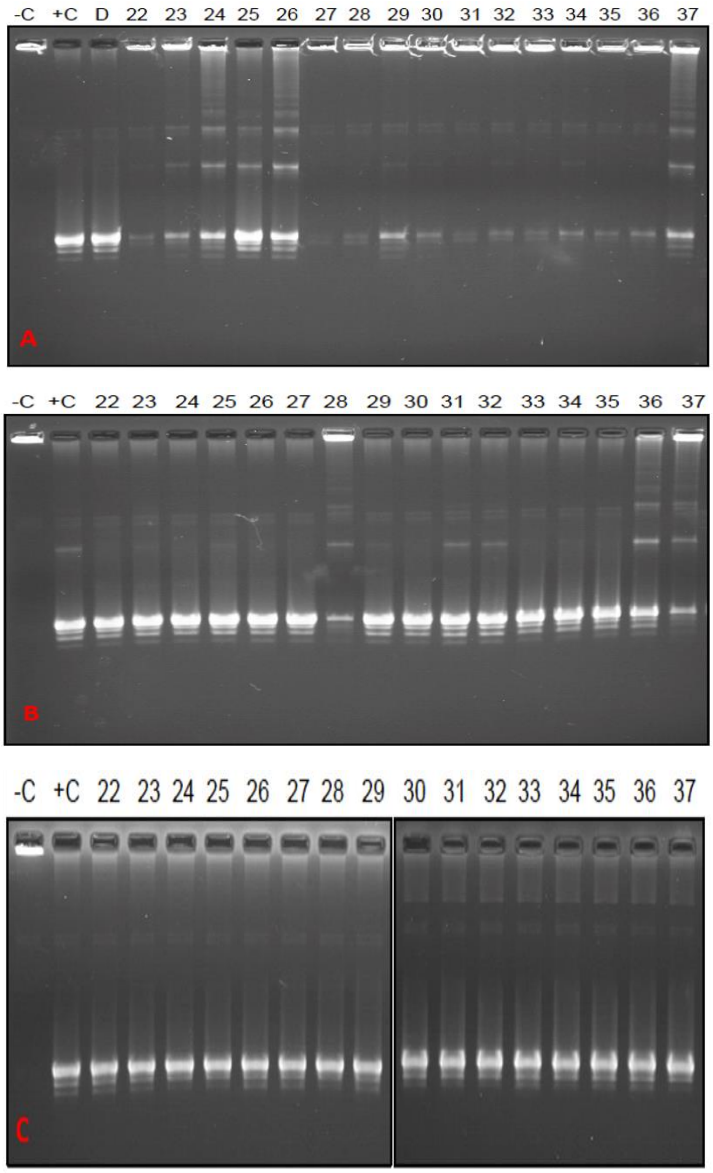


Figure 3.14. Inhibitory effects of *O. mollis* main fractions at final concentration of 0.01, 0.005 and 0.001 mg/mL on hTopo II. The reactions were carried out under the same conditions detailed above at stock concentrations of 0.1 mg/mL (A), 0.05 mg/mL (B) and 0.01 mg/mL (C) of the test substances. Experiments were carried out using a 1:10 dilution of the stocks (22-37 encoded applications) **-C.** negative control, **+C**. positive control (enzyme only) and **D**. DMSO at a concentration of 2.5%.

As a result, activity screening tests of the main fractions were completed consistently. The next part of study includes the tests for pure compounds of the main fractions that are effective at low concentrations and determination of the mode of action of effective samples.

3.6.2. Inhibitory Activity of Pure Compounds

3.6.2.1. Inhibitory Activity of Pure Compounds from *O. taurica* var. *taurica*

The structure determination studies revealed that the pure compounds isolated from *O. taurica* var. *taurica* **OT1** and **OT-2** were same with **OM5** and **OM-4**, respectively. The inhibitory effect of **OT-1** and **OT-2** encoded compounds on topoisomerases I and II were given in 3.6.2.2**.**

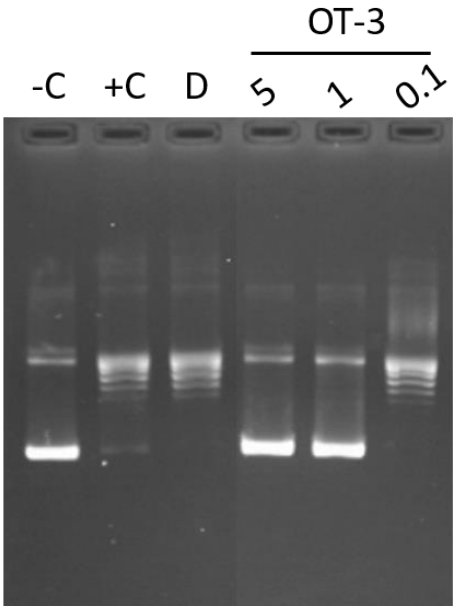


Figure 3.15. Dose dependent effect of OT-3 on hTopo I -**C**. negative control, **+C**. positive control (enzyme only), **D**. DMSO at a concentration of 2.5% and **OT-3.** Deoxyshikonin with 0.5 mg/mL (1.8 mM), 0.1 mg/mL (0.36 mM) and 0.01 mg/mL (0.036 mM), respectively.

Dose-dependent analysis of inhibitory effect of deoxyshikonin on hTopo I enzyme were done with dilutions at different concentrations and it lost its inhibitory effect on hTopo I relaxation activity at 0.01 mg/mL concentration (Figure 3.15).

Deoxyshikonin isolated from *O. taurica* var. *taurica* lost its inhibitory effect on hTopo II between 0.1 mg/mL (0.36 mM) and 0.5 mg/mL (1.8 mM) concentration (Figure 3.16).

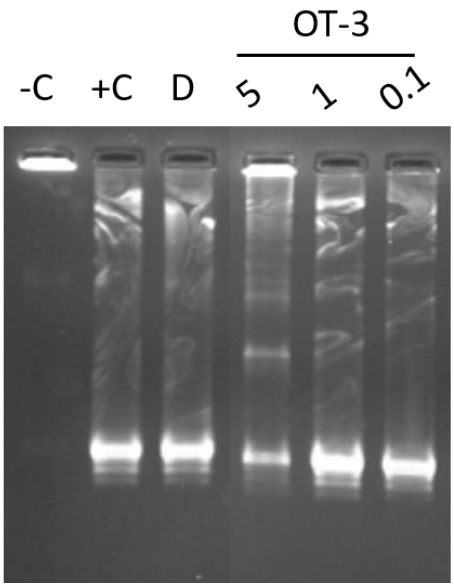


Figure 3.16. Dose-dependent inhibitory activity of OT-3 on hTopo II**. -C**. negative control, **+C**. positive control (enzyme only), **D**. DMSO at a concentration of 2.5% and **OT-3.** Deoxyshikonin with 0.5 mg/mL (1.8 Mm), 0.1 mg/mL (0.36 Mm) and 0.01 mg/mL (0.036 Mm), respectively.

3.6.2.2. Inhibitory Activity of Pure Compounds from *O. mollis*

Inhibitory effect of napthoquinone derivatives obtained from *O. mollis* on human topoisomerase I and II enzymes were analyzed.

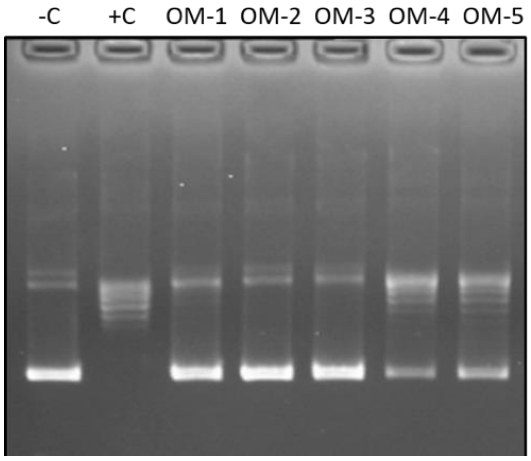


Figure 3.17. Inhibitory effect of the pure compounds at final concentration of 0.1 mg/mL on hTopo I. **-C**. negative control, **+C**. positive control (enzyme only), **OM-1**. Unidentified Compound **OM-2**. Acetylshikonin **OM-3**. β-hydroxy-isovalerylshikonin **OM-4**. Shikonin **OM-5**. β,β-dimethylacrylshikonin.

The pure compounds from *O. mollis* showed inhibitory effect on hTopo I relaxation activity at 1 mg/mL concentration (Figure 3.17). To determine whether their effects on hTopo I enzyme were dose-dependent, the analyses were repeated with dilutions at different concentrations and were carried out.

hTopo I relaxation analyses showed that **OM-1**, **OM-2** and **OM-4** lost their effect on the enzyme at a working concentration of 0.01 mg/mL.

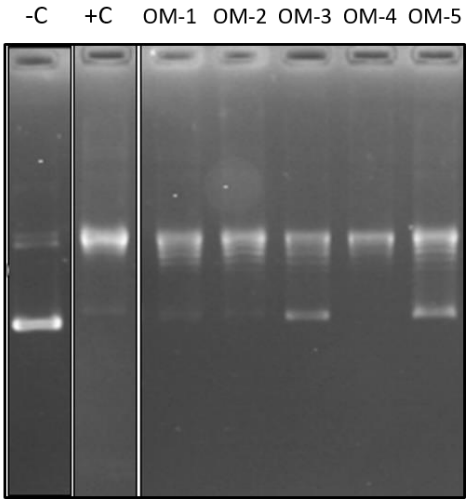


Figure 3.18. Inhibitory effect of the pure compounds at final concentration of 0.01 mg/mL on hTopo I. **-C**. negative control, **+C**. positive control (enzyme only), **OM-1**. Unidentified Compound **OM-2**. Acetylshikonin **OM-3**. β-hydroxy-isovalerylshikonin **OM-4**. Shikonin

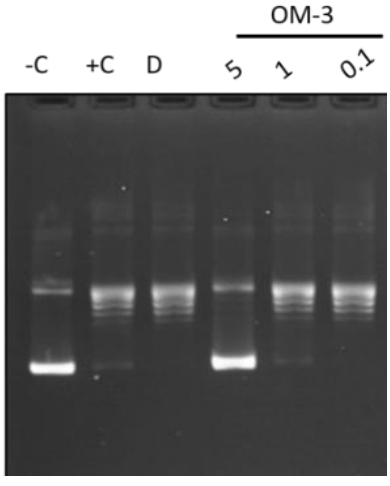


Figure 3.19. A dose-dependent effect of **OM-3** on hTopo I. **C**. negative control, **+C**. positive control (enzyme only), **D**. DMSO at a concentration of 2.5%, **OM-3**. β-hydroxyisovalerylshikonin with 0.5 mg/mL (1.25 mM), 0.1 mg/mL (0.25 mM) and 0.01 mg/mL (0.025 mM), respectively.

β-hydroxyisovalerylshikonin was selected as a promising test compound for its efficacy on topoisomerase I enzyme; therefore, it was tested at different concentrations to determine whether the inhibitory effect on the enzyme was dose-dependent (Figure 3.19).

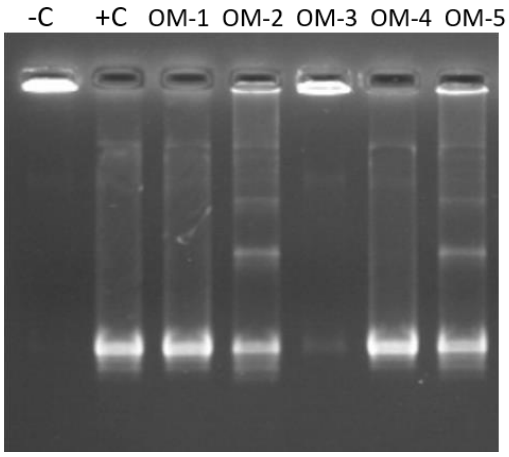


Figure 3.20. Inhibitory effect of the pure compounds at final concentration of 0.1 mg/mL on hTopo II. **-C**. negative control, **+C.** positive control (enzyme only), **OM-1**. Unidentified Compound **OM-2**. Acetylshikonin **OM-3**. β-hydroxy-isovalerylshikonin **OM-4**. Shikonin **OM-5**. β,β-dimethylacrylshikonin

hTopo II decatenation reactions showed that pure compounds except **OM-3** did not have inhibitory effect on the enzyme at a working concentration of 0.1 mg/mL.

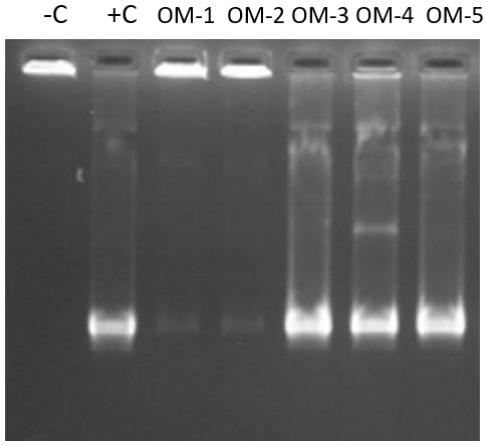


Figure 3.21. Inhibitory effect of the pure compounds on hTopo II **-C**. negative control, **+C**. positive control (enzyme only), **OM-1**. Unidentified Compound (0.5 mg/mL) **OM-2**. Acetylshikonin (0.5 mg/mL) **OM-3**. β-hydroxyisovalerylshi-konin (0.01 mg/mL) **OM-4**. Shikonin (0.5 mg/mL) **OM-5**. β,β-dime-thylacrylshikonin (0.5 mg/mL).

Decanation reaction showed that **OM-1** and acetylshikonin (**OM-4)** had inhibitory effects at 0.5 mg/mL concentration on topoisomerase II enzyme. Moreover, β-hydroxyisovalerylshikonin (**OM-3**) lost its inhibitory effect on topoisomerases II enzyme at 0.01 mg/mL concentration.

3.6.3. DNA Topoisomerase II Mediated-DNA Cleavage by Naphthoquinone Derivatives

One of the action mechanisms of naphthoquinones on topoisomerase II enzyme is explained as cleavage complex formation. The enzyme was fixed on the DNA by SDS addition, followed by removal of protein by addition of proteinaseK, and cleavages were stabilized and screened by gel separation (Figure 3.22). Etoposide was used as reference compound and linear DNA band was observed in the reactions with the addition of etoposide. However, the absence of linear DNA bands in the reactions, in which the naphthoquinone derivatives were tested, indicated that the test compounds had no activity to form cleavage on DNA.

The fact that many anticancer agents act as topoisomerase inhibitors, DNA topoisomerase I and II have become attractive targets in drug development. Fuji *et al.* and Ahn *et al.* reported that naphthoquinones containing phenolic residues were identified as Topoisomerase inhibitors.[87,99]

Topoisomerase I and II inhibitory activities of the main extracts and fractions together with the isolated molecules were investigated in addition to the cytotoxicity studies.

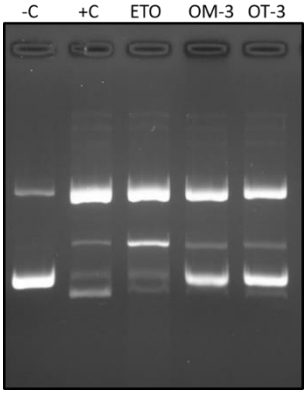


Figure 3.22. The effect of naphthoquinone derivatives on DNA Topoisomerase II Mediated-DNA Cleavage. **-C**. negative control, **+C**. positive control (enzyme only), **ETO.** Etoposide (25 mM), **OM-3**. β-hydroxyisovalerylshikonin (12.5 mM) and **OT-3** Deoxyshikonin (18 mM).

In this study, the extracts and main fractions of *O. taurica* var. *taurica* lost their inhibitory effect on hTopoI and hTopoII enzymes at 0.01 mg/mL dose while the pure compounds lost at higher than 0.01 mg/mL. Similar results were seen for *O. mollis*; the fractions were more active than the pure compounds (0.001 versus 0.1 mg/mL, respectively) Evans stated that the isolated compounds might not show the same clinical response as the extracts. Often, total the therapeutic activity may be more or less different than the individual therapeutic activities because of synergism or antagonism.[100]

Plyta *et al.* reported that naphthoquinones due to the presence of phenolic hydroxyl group were potent inhibitors for Topoisomerase I. Shikonin, alkannin and acetylalkannin isolated from *Alkanna tinctoria* were tested and minimum inhibitory concentrations (MIC) were found to be 50, 500 and 5 μM, respectively.[86]

EWS-FLI1 is a multiple mutant transcription factor that result in aberrant gene expression patterns which leads to a neoplastic phenotype. Chen *et al*. screened 5200 small molecules to disrupt the binding of EWS-FLI1 by comparing with DNA-binding chemotherapeutics such as omycin D, cisplatin, doxorubicin, daunorubicin, and epirubicinand. Shikonin isolated from *Lithospermum erythrorhizon*, interrupted protein-DNA interactions like chemotherapeutic agents. Shikonin inhibited binding of EWSFLI1 with an IC_{50} of 600 nM. Moreover, shikonin weakly blocked Topo I-mediated DNA relaxation at 1000 μM, whereas inhibited DNA relaxation by Topo II at 20 μM. The comparison of shikonin with DNA intercalators doxorubicin and actinomycin D suggested that shikonin was not a DNA intercalator.[84]

Ogawa et al. reported the inhibitory effect of shikonin on Topoisomerases II and also investigated the relationships between topoisomerase inhibition and the transcriptional oscillation of *Bmal1* which drives gene expression with circadian rhythms. Shikonin at 125 μM concentration displayed Topoisomerase II inhibitory activity. It was incorporated into the nucleus and Topo2, and also was located in the Bmal1 promoter. It was indicated that there was a relationship between Bmal1 transcription and Topo2 inhibition.[100]

In the light of inhibition studies in the literature, the inhibitory effects of the isolated compounds were examined.

β-hydroxyisovalerylshikonin showed the strongest inhibitory effect at the concentrations of 0.025 mM (0.01 mg/mL) on Topoisomerase I. Moreover, decanation reactions demonstrated that β-hydroxyisovalerylshikonin lost its inhibitory effect on Topoisomerase II with the lowest concentration of 0.025 mM (0.01 mg/mL).

Topoisomerase II Mediated-DNA Cleavage analysis was carried out using etoposide as a reference[101], known to act as poison and increasing the formation of fractures. The naphthoquinone derivatives did not have activity such as etoposide that stabilized the covalent complex. Therefore, based on the results, we hypothesize that the test compounds inhibit the catalytic activity compared to reference compound, rather than stabilizing the covalent complex because of non-forming cleavage.

CHAPTER 4

CONCLUSION

Onosma species, distributed mainly in the Mediterranean region and Central Asia, contains about 230 species.[5] Phytochemical investigations performed on *Onosma* genus led to the identification of various naphthoquinone derivatives, which showed a broad spectrum of biological activities including wound healing, anti-inflammatory, antitumor, antimicrobial and antithrombotic.[26]

In this study, 12 *Onosma* species collected from Southwest Anatolia were specifically studied for their naphthoquinone contents. Cytotoxic activities of the root extracts were investigated using MTT assay on human cancer and normal cell lines as well as enzyme inhibitory properties towards DNA Topoisomerase I and II.

First part of the study focused on the chemical profiling of the PE, D and M extracts of the root materials by TLC and HPLC followed by cytotoxicity tests, data of which directed us to select the active plant(s)/extract(s) for bioactivity-guided isolation studies.

HPLC fingerprinting revealed that β,β-dimethylacrylshikonin was the chief constituent amongst the naphthoquinone-type secondary metabolites. The detailed inspection of the chromatograms also showed that naphthoquinone derivatives were present in greater amounts in the PE extracts as compared to the D extracts.

As expected, the cytotoxicity results demonstrated superior activity for non-polar petroleum ether and dichloromethane extracts, which were rich in naphthoquinones. Particularly, with IC_{50} values less than 8 μg/mL, PE and D extracts of *O. taurica* var. *taurica* and *O. aksoyii* along with the PE extract of *O. mollis* showed significant and stronger cytotoxic activity compared to the other extracts. Thus, in the current study, *O. mollis* and *O. taurica* var. *taurica* were taken to the bioassay guided fractionation studies to purify the compounds responsible for bioactivity.

During bioactivity guided purification studies, different chromatographic methods were utilized to obtain the compounds. As a result, five compounds were isolated, and their structures were identified as shikonin (**OM-4**), β,β-dimethylacrylshikonin (**OM-5**), deoxyshikonin (**OT-3**), acetylshikonin (**OM-2**), β-hydroxyisovalerylshikonin (**OM-3**) by spectral methods (NMR and MS). The compounds

were previously isolated from different *Onosma* species shown in Table 1.1. As there has been no phytochemical study performed, the isolated compounds with previously known structures, are reported for the first time from *O. taurica* var. *taurica* and *O. mollis*.

When cytotoxic activities of the pure compounds were evaluated, acetylshikonin and β,β-dimethylacrylshikonin were found to be more active versus HCC-1937 cell line with IC_{50} values of 2.26 and 1.84, respectively, whereas shikonin and acetyl shikonin had higher toxicity on DU145 cells (IC_{50} values: 3.12 and 3.07 μM, respectively). Amongst the cancer cell lines, HeLa was found to be more resistant to the test compounds (IC_{50} values from 16.5 to >25 μM). Moreover, the positive control doxorubicin was almost 3 to 5 times more potent than the active naphthoquinones.

The bioactivity results also suggested no selectivity towards cancer cell lines because of the toxicity observed for the normal cell line MRC-5. Generally narrow therapeutic window means that the chance of the bioactive compound to become a drug candidate is little. In the case of anticancer drug development, therapeutic value of the candidate needs to be evaluated thoroughly, as most of the chemotherapy agents are also quite toxic to normal tissues in the body. Indeed, in this thesis, similar cytotoxicity findings with the positive control doxorubicin, clinically used for the treatment of different cancers (leukaemia, Ewing sarcoma, Hodgkin/non-Hodgkin lymphomas, neuroblastoma, ovary and breast etc.), suggest that such direct statement(s)/conclusion(s) need to be put forward carefully.

It should also be taken into consideration that MRC-5 is a sensitive cell line, and utilization of normal cervix, prostate and breast cancer cell lines in further screenings might provide more appropriate data to evaluate therapeutic potential of the isolated compounds. Additionally, Xia *et al.* and Kontogiannopoulos *et al.* managed to decrease toxicity and improve therapeutic index of the naphthoquinones by using shikonin-loaded liposomes.[102,103] Thus, drug delivery strategies should also be investigated as an alternative approach in further studies.

From structure-activity relationship perspective, shikonin was approximately 5-fold more cytotoxic than deoxyshikonin on HCC-1937 implying the importance of hydroxy group at C-11 position. Additionally, the presence of acyl group extending from C-11(*O*) and its chemistry noticed to be important for cytotoxic activity on HCC-1937 and MRC-5 cell lines; however, similar conclusion was not evident versus the other cell lines (HeLa and DU 145). Furthermore, it was undeniable that the presence of β-hydroxy carbonyl residue in the acyl side chain (i.e. β-hydroxyisovalerylshikonin) diminished the

cytotoxic activity greatly, which underlined importance of non-polar side chain. It is hypothesized that the hydroxy group at C-3' position forms hydrogen bond(s) with the other hydrogen donor/acceptor centers, viz. C-1', C-5 and C-8. Indeed, when the minimized energy conformers were calculated by Chem3D software, the most stable conformer(s) exhibited hydrogen bonding for C-3'(OH) between either with C-1 carbonyl group or C-8(OH) (Figure 4.1).

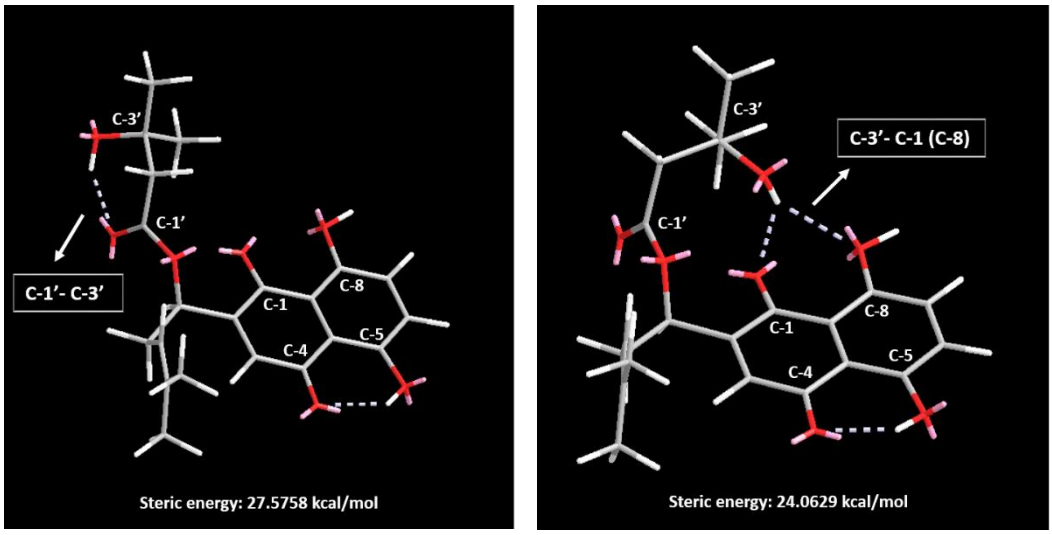


Figure 4.1. The conformers of β-hydroxyisovalerylshikonin

Naphthoquinones are potent inhibitors of topoisomerases by intercalation of DNA subsequently inducing DNA cleavage and apoptosis.[104] Furthermore, Chen *et al*. report that naphthoquinones with phenolic nature have more inhibitory effects on topoisomerases.[84] Thus inhibitory effects of the *Onosma* root extracts, fractions and purified compounds were investigated using *in vitro* plasmid relaxation and decatenation assays to determine minimum inhibitory concentrations.

β-hydroxyisovalerylshikonin was found to be the strongest inhibitory compound at 25 μM (0.01 mg/mL) concentration on Topoisomerase I. Moreover, decatenation reactions demonstrated that β-hydroxyisovalerylshikonin lost its inhibitory effect on Topoisomerase II with the lowest concentration at 25 μM (0.01 mg/mL). As deoxyshikonin and β-hydroxyisovalerylshikonin were considerably potent compounds, they were taken into further analyses for mediated-DNA cleavage. The selected compounds were not active as the positive control etoposide in stabilization of the covalent complex. Therefore, we speculate that the naphthoquinone derivatives isolated from *Onosma*

species exert their effects via inhibition of catalytic activity rather than stabilizing the covalent complex because of non-forming cleavage.

One of the proposed mechanisms for the cytotoxicity of naphthoquinones is oxidative stress due to semiquinone radical formation resulting damage of macromolecules inside the cell.[105] Another suggested mechanism is bioreductive alkylation. In this mechanism, naphthoquinones undergo nucleophilic attack; generation of a highly effective alkylating agent causes cell death and/or DNA damage.

As the less- or non- cytotoxic compounds are more effective in regards to topoisomerase inhibition, one might postulate that a decrease in bio-alkylation property of naphthoquinones enable them to pass the cytoplasm intact to reach nucleus, and subsequently to act on topoisomerases.

Consequently, based on the results obtained, isolation/semi-synthesis of new naphthoquinone derivatives and investigation of their bioactivities are warranted to establish structure-activity relationships thoroughly, as well further molecular mechanism studies are needed to prove the speculations that are put forward above.

REFERENCES

(1) Ihsan A . Al-Shehbaz. The Genera Of Boraginaceae In The Southeastern United States. *J. Arnold Arbor. . Suppl. Ser.* **2018**, *1* (May), 1–169.

(2) Yıldırımlı Ş. The Chorology of the Turkish Species of Boraginaceae Family. *Herb J. Systatics Bot.* **2000**, *7* (2), 257–272.

(3) Davis, P. *Flora of Turkey and The East Aegean Islands*, Vol. 9.; 1985.

(4) Kumar, N.; Kumar, R.; Kishore, K. Onosma L.: A Review of Phytochemistry and Ethnopharmacology. *Pharmacogn. Rev.* **2013**, *7* (14), 140.

(5) Binzet, R. A New Species of *Onosma* L. (Boraginaceae) from Anatolia. *Turk. J. Botany* **2016**, *40* (2), 194–200.

(6) Binzet, R.; Orcan, N. Anatomical and Palynological Investigations on Endemic *Onosma Mersinana* Riedl, Binzet & Orcan. *Pakistan J. Bot.* **2009**, *41* (2), 503–510.

(7) Öztürk, M., Özçelik, H. Useful Plants of East Anatolia. *SISKAV* **1991**, 196.

(8) Baytop, T. *Türkçe Bitki Adları Sözlüğü*; 1994.

(9) T, S. W. The Gender of the Generic Name Onosma (Boraginaceae). *Int. Assoc. Plant Taxon. (IAPT) Stable* **1993**, *42* (3), 679–681.

(10) Teppner, H., Tuzlacı, E. *Onosma* Propontica Azanavour (Boraginaceae-Lithospermae). *Stapfia* **1994**, *34*, 77–83.

(11) Peruzzi, L.; Passalacqua, N. G. Taxonomy of the *Onosma Echioides* (L .) L . Complex (Boraginaceae) Based on Morphometric Analysis. **2008**, No. May, 763–774.

(12) Riedl, H. *Onosma* L. In *Flora of Turkey and the East Aegean Island*; Edinburg University Press, 1978; Vol. 6, pp 326–378.

(13) Ghahremaninejad F, Joharchi M, V. E. New Plant Records for Khorassan Province, Iran. *Ann Narurhist Mus Wien B* **2005**, *106*, 255-93.

(14) Kandemir, A. The Rediscovery of Some Taxa Thought to Have Been Extinct in Turkey. *Turk. J. Botany* **2009**, *33* (2), 113–122.

(15) D., V. *An Illustrated Flora of North Cyprus.*; Koeningstein: Koeltz Scilentific Books., 1994.

(16) Binzet, R.; Akçin, Ö. E. The Anatomical Properties of Two *Onosma* L . (Boraginaceae) Species from Turkey. **2012**, *6* (17), 3288–3294.

(17) Itoigawa, M.; Ito, C.; Tan, H. T. W.; Okuda, M.; Tokuda, H.; Nishino, H.; Furukawa, H. Cancer Chemopreventive Activity of Naphthoquinones and Their Analogs from Avicennia Plants. *Cancer Lett.* **2001**, *174* (2), 135–139.

(18) Itokawa, H.; Matsumoto, K.; Morita, H.; Takeya, K. Cytotoxic Naphthoquinones From Mansoa-Alliacea. *Phytochemistry* **1992**, *31* (3), 1061–1062.

(19) De Moura, K. C. G.; Emery, F. S.; Neves-Pinto, C.; Pinto, M. D. C. F. R.; Dantas, A. P.; Salomão, K.; De Castro, S. L.; Pinto, A. V. Trypanocidal Activity of Isolated Naphthoquinones from Tabebuia and Some Heterocyclic Derivatives: A Review from an Interdisciplinary Study. *J. Braz. Chem. Soc.* **2001**, *12* (3), 325–338.

(20) Brigham, L.; Michaels, P.; Flores, H. Cell-Specific Production and Antimicrobial Activity of Naphthoquinones in Roots of *Lithospermum Erythrorhizon*. *Plant Physiol.* **1999**, *119* (2), 417–428.

(21) Culham, A.; Gornall, R. J. The Taxonomic Significance of Naphthoquinones in the Droseraceae. *Biochem. Syst. Ecol.* **1994**, *22* (5), 507–515.

(22) Zakaria, M. Bin; Jeffreys, J. A. D.; Waterman, P. G.; Zhong, S. M. Naphthoquinones and Triterpenes from Some Asian Diospyros Species. *Phytochemistry* **1984**, *23* (7), 1481–1484.

(23) Binder, R. G.; Benson, M. E.; Flath, R. A. Eight 1,4-Naphthoquinones from Juglans. *Phytochemistry* **1989**, *28* (10), 2799–2801.

(24) Likhitwitayawuid, K.; Kaewamatawong, R.; Ruangrungsi, N.; Krungkrai, J. Antimalarial Naphthoquinones from *Nepenthes Thorelii*. *Planta Med.* **1998**, *64* (3), 237–241.

(25) Dinda, B.; Das, S. K.; Hajra, A. K.; Bhattacharya, A.; De, K.; Chel, G.; Achari, B. Chemical Constituents of Plumbago Indica Roots and Reactions of Plumbagin: Part II. *Indian J. Chem. - Sect. B Org. Med. Chem.* **1999**, *38* (5), 577–582.

(26) Papageorgiou, V. P.; Assimopoulou, A. N.; Couladouros, E. A.; Hepworth, D.; Nicolaou, K. C. The Chemistry and Biology of Alkannin, Shikonin, and Related Naphthazarin Natural Products. *Angew. Chemie - Int. Ed.* **1999**, *38* (3), 270–300.

(27) Babula, P.; Adam, V.; Havel, L.; Kizek, R. Noteworthy Secondary Metabolites Naphthoquinones – Their Occurrence, Pharmacological Properties and Analysis. *Curr. Pharm. Anal.* **2009**, *5* (1), 47–68.

(28) Chen, X.; Yang, L.; Oppenheim, J. J.; Zack Howard, O. M. Cellular Pharmacology Studies of Shikonin Derivatives. *Phyther. Res.* **2002**, *16* (3), 199–209.

(29) Lu, Q.; Liu, W. J.; Ding, J.; Cai, J. C.; Duan, W. H. Shikonin Derivatives: Synthesis and Inhibition of Human Telomerase. *Bioorg. Med. Chem. Lett.* **2002**, *12* (10), 1375–1378.

(30) Bogurcu, N.; Sevimli-Gur, C.; Ozmen, B.; Bedir, E.; Korkmaz, K. S. ALCAPs Induce Mitochondrial Apoptosis and Activate DNA Damage Response by Generating ROS and Inhibiting Topoisomerase I Enzyme Activity in K562 Leukemia Cell Line. *Biochem. Biophys. Res. Commun.* **2011**, *409* (4), 738–744.

(31) Kundaković, T.; Stanojković, T.; Juranić, Z.; Kovačević, N. Cytotoxicity in Vitro of Naphthazarin Derivatives from *Onosma Arenaria*. *Phyther. Res.* **2006**, *20* (7), 602–604.

(32) Kretschmer, N.; Rinner, B.; Deutsch, A. J. A.; Lohberger, B.; Knausz, H.; Kunert, O.; Blunder, M.; Boechzelt, H.; Schaider, H.; Bauer, R. Naphthoquinones from *Onosma Paniculata* Induce Cell-Cycle Arrest and Apoptosis in Melanoma Cells. *J. Nat. Prod.* **2012**, *75* (5), 865–869.

(33) Kundakovic, T.; Fokialakis, N.; Dobric, S.; Pratsinis, H.; Kletsas, D.; Kovacevic, N.; Chinou, I. Evaluation of the Anti-Inflammatory and Cytotoxic Activities of Naphthazarine Derivatives from *Onosma Leptantha*. *Phytomedicine* **2006**, *13* (4), 290–294.

(34) Özgen, U.; Coşkun, M.; Kazaz, C.; Seçen, H. Naphthoquinones from the Roots of *Onosma Argentatum* Hub.-Mor. (Boraginaceae). *Turkish J. Chem.* **2004**, *28* (4), 451–454.

(35) Rinner, B.; Kretschmer, N.; Knausz, H.; Mayer, A.; Boechzelt, H.; Hao, X. J.; Heubl, G.; Efferth, T.; Schaider, H.; Bauer, R. A Petrol Ether Extract of the Roots of *Onosma Paniculatum* Induces Cell Death in a Caspase Dependent Manner. *J. Ethnopharmacol.* **2010**, *129* (2), 182–188.

(36) Sut, S.; Pavela, R.; Kolarcik, V.; Cappellacci, L.; Petrelli, R.; Maggi, F.; Dall'Acqua, S.; Benelli, G. Identification of *Onosma Visianii* Roots Extract and Purified Shikonin Derivatives as Potential Acaricidal Agents against Tetranychus Urticae. *Molecules* **2017**, *22* (6).

(37) Nikita, G.; Vivek, P.; Chhaya, G. Wound-Healing Activity of an Oligomer of Alkannin/Shikonin, Isolated from Root Bark of *Onosma Echioides*. *Nat. Prod. Res.* **2015**, *29* (16), 1584–1588.

(38) Ahmad, U.; Kousar, F.; Khan, A.; Zubair, M.; Iqbal, S. A New Ketone and a Known Anticancer Triterpenoid from the Leaves of *Onosma Limitaneum* Introduction. **2005**, *88*, 309–311.

(39) Özgen, U.; Houghton, P. J.; Ogundipe, Y.; Coşkun, M. Antioxidant and Antimicrobial Activities of *Onosma Argentatum* and *Rubia Peregrina*. *Fitoterapia* **2003**, *74* (7–8), 682–685.

(40) Larin AP, A. N. On the Efficacy of *Onosma* in Experimental Hypertension. *Farmakol Toksikol* **1967**, *30*, 694-696.

(41) Pavol, M.; Mártonfiová, L.; Kolarčik, V.; Pavol, M.; Mártonfiová, L.; Kolar, V. Karyotypes and Genome Size of *Onosma* Species from Northern Limits of the Genus in Carpathians Karyotypes and Genome Size of Onosma Species from Northern Limits of the Genus in Carpathians. **2014**, *7114*.

(42) Bains S, Kaur K, K. S. Colourfastness Properties of Dyeing Wool with Goldendrop *(Onosma Echioides)* Dye Using Combination of Mordants. *Colourage* **2005**, *51*, 51–55.

(43) Maggi, F.; Tirillini, B. Analysis of the Volatile Components of *Onosma Echioides* (L .) L . Var . Coiumnae Lacaita Growing in Central Italy. **2009**, *21* (September 2007), 441–448.

(44) Attar, F.; Joharchi, M. R. *Onosma Khorassanica*, a New Species from Northeast of Iran. *Rostaniha* **2006**, *7*, 111–114.

(45) Dong, M.; Liu, D.; Li, Y. H.; Chen, X. Q.; Luo, K.; Zhang, Y. M.; Li, R. T. Naphthoquinones from *Onosma Paniculatum* with Potential Anti-Inflammatory Activity. *Planta Med.* **2017**, *83* (7), 631–635.

(46) Quan, L.; Dai, W.; Li, F.; Li, Y.; Chen, X.; Li, R. Onosmanones A and B , Two Novel Quinonoid Xanthenes from *Onosma Paniculatum*. *Nat. Prod. Res.* **2018**,

6419, 1–6.

(47) Ozgen, U.; Ikbal, M.; Hacimuftuoglu, A.; Houghton, P. J.; Gocer, F.; Dogan, H.; Coskun, M. Fibroblast Growth Stimulation by Extracts and Compounds of *Onosma Argentatum* Roots. **2006**, *104*, 100–103.

(48) Sarikurkcu, C.; Kirkan, B.; Ozer, M. S.; Ceylan, O.; Atilgan, N.; Cengiz, M.; Tepe, B. Chemical Characterization and Biological Activity of *Onosma Gigantea* Extracts. *Ind. Crops Prod.* **2018**, *115* (January), 323–329.

(49) Moghaddam, P. Z.; Mazandarani, M.; Zolfaghari, M. R.; Badeleh, M. T.; Ghaemi, E. A. Antibacterial and Antioxidant Activities of Root Extract of *Onosma Dichroanthum* Boiss . in North of Iran. **2012**, *6* (8), 1776–1781.

(50) Damianakos, H.; Kretschmer, N.; Syklowska-Baranek, K.; Pietrosiuk, A.; Bauer, R.; Chinou, I. Antimicrobial and Cytotoxic Isohexenylnaphthazarins from A*rnebia Euchroma* (Royle) Jonst. (Boraginaceae) Callus and Cell Suspension Culture. *Molecules* **2012**, *17* (12), 14310–14322.

(51) Hemmati, A.; Namjuyan, F.; Yousefi, S.; Housmand, G. The Healing Effect of N-Hexan- Dichloromethane Extract Root *Onosma Bulbotrichum* in Second Degree Burns. **2018**, *7* (1), 1–9.

(52) Ahmad, B.; Ali, N.; Bashir, S.; Choudhary, M. I.; Azam, S.; Khan, I. Parasiticidal , Antifungal and Antibacterial Activities of *Onosma Griffithii* Vatke. *African J. Biotechnol.* **2009**, *8* (19), 5084–5087.

(53) Wang, R.; Zhang, X.; Song, H.; Zhou, S.; Li, S. Synthesis and Evaluation of Novel Alkannin and Shikonin Oxime Derivatives as Potent Antitumor Agents. *Bioorganic Med. Chem. Lett.* **2014**, *24* (17), 4304–4307.

(54) Huang, G.; Zhao, H. R.; Meng, Q. Q.; Zhang, Q. J.; Dong, J. Y.; Zhu, B. quan; Li, S. S. Synthesis and Biological Evaluation of Sulfur-Containing Shikonin Oxime Derivatives as Potential Antineoplastic Agents. *Eur. J. Med. Chem.* **2018**, *143*, 166–181.

(55) Moore, H. W.; Moore, H. W. Bioactivation as a Model for Drug Design Bioreductive Alkylation Published by : American Association for the Advancement of Science Stable URL : Http://Www.Jstor.Org/Stable/1745040 Digitize , Preserve and Extend Access to Science Bioactivation as a Model. **1977**, *197* (4303), 527–532.

(56) Wang, J. C. Interaction between DNA and an Escherichia Coli Protein ω. *J. Mol. Biol.* **1971**, *55* (3), 523–533.

(57) Forterre, P.; Gribaldo, S.; Gadelle, D.; Serre, M. C. Origin and Evolution of DNA Topoisomerases. *Biochimie* **2007**, *89* (4), 427–446.

(58) Nitiss, J. L. Investigating the Biological Functions of DNA Topoisomerases in Eukaryotic Cells. *Biochim. Biophys. Acta - Gene Struct. Expr.* **1998**, *1400* (1–3), 63–81.

(59) Pommier, Y.; Leo, E.; Zhang, H.; Marchand, C. DNA Topoisomerases and Their Poisoning by Anticancer and Antibacterial Drugs. *Chem. Biol.* **2010**, *17* (5), 421–433.

(60) Bjornsti, M.-A.; Osheroff, N. DNA Topoisomerase Protocols Volume I: DNA Topology and Enzymes. *Methods Mol. Biol.* **1999**, *94*.

(61) Wang, J. C. Cellular Roles of DNA Topoisomerases: A Molecular Perspective. *Nature Reviews Molecular Cell Biology*. 2002, pp 430–440.

(62) Schoeffler, A. J.; Berger, J. M. DNA Topoisomerases: Harnessing and Constraining Energy to Govern Chromosome Topology. *Q. Rev. Biophys.* **2008**, *41* (1), 41–101.

(63) Goodsell, D. S. The Molecular Perspective: DNA Topoisomerases. **2002**, 381–382.

(64) Baker, N. M.; Rajan, R.; Mondragń, A. Structural Studies of Type I Topoisomerases. *Nucleic Acids Res.* **2009**, *37* (3), 693–701.

(65) Dekker, N. H.; Rybenkov, V. V; Duguet, M.; Crisona, N. J.; Cozzarelli, N. R.; Bensimon, D.; Croquette, V. The Mechanism of Type IA Topoisomerases. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99* (19), 12126–12131.

(66) Chen, S. H.; Chan, N.-L.; Hsieh, T. New Mechanistic and Functional Insights into DNA Topoisomerases. *Annu. Rev. Biochem.* **2013**, *82* (1), 139–170.

(67) Forterre, P. Phylogenomics of DNA Topoisomerases : Their Origin and Putative Roles in the Emergence of Modern Organisms. **2018**, *37* (3), 679–692.

(68) Belova, G. I.; Prasad, R.; Kozyavkin, S. a; Lake, J. a; Wilson, S. H.; Slesarev, a I. A Type IB Topoisomerase with DNA Repair Activities. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98* (11), 6015–6020.

(69) Taneja, B.; Patel, A.; Slesarev, A.; Mondragón, A. Structure of the N-Terminal Fragment of Topoisomerase V Reveals a New Family of Topoisomerases. *EMBO J.* **2006**, *25* (2), 398–408.

(70) McClendon, A. K.; Osheroff, N. DNA Topoisomerase II, Genotoxicity, and Cancer. *Mutat. Res. - Fundam. Mol. Mech. Mutagen.* **2007**, *623* (1–2), 83–97.

(71) Nitiss, J. L. Repertoire of Biological Functions. **2009**, *9* (MAy).

(72) Wendorff, T. J.; Schmidt, B. H.; Heslop, P.; Austin, C. A.; Berger, J. M. The Structure of DNA-Bound Human Topoisomerase II Alpha: Conformational Mechanisms for Coordinating Inter-Subunit Interactions with DNA Cleavage. *J. Mol. Biol.* **2012**, *424* (3–4), 109–124.

(73) Gadelle, D.; Krupovic, M.; Raymann, K.; Mayer, C.; Forterre, P. DNA Topoisomerase VIII: A Novel Subfamily of Type IIB Topoisomerases Encoded by Free or Integrated Plasmids in Archaea and Bacteria. *Nucleic Acids Res.* **2014**, *42* (13), 8578–8591.

(74) Nichols, M. D.; DeAngelis, K.; Keck, J. L.; Berger, J. M. Structure and Function of an Archaeal Topoisomerase VI Subunit with Homology to the Meiotic Recombination Factor Spo11. *EMBO J.* **1999**, *18* (21), 6177–6188.

(75) Liu, L. F. DNA Topoisomerase Poisons as Antitumor Drugs. *Annu. Rev. Biochem.* **1989**, *58* (1), 351–375.

(76) Denny, W. a. Emerging DNA Topoisomerase Inhibitors as Anticancer Drugs.

Expert Opin. Emerg. Drugs **2004**, *9* (1), 105–133.

(77) Topcu, Z. DNA Topoisomerases as Targets for Anticancer Drugs [Review]. *J. Clin. Pharm. Ther.* **2001**, *26* (6), 405–416.

(78) Burden, D. A.; Oshero, N. Burden, Osheroff - 1998 - Mechanism of Action of Eukaryotic Topoisomerase II and Drugs Targeted to the Enzyme. **1998**, *1400*.

(79) Wall, M. E.; Wani, M. C.; Cook, C. E.; Palmer, K. H.; McPhail, A. T.; Sim, G. A. Plant Antitumor Agents. I. The Isolation and Structure of Camptothecin, a Novel Alkaloidal Leukemia and Tumor Inhibitor from Camptotheca Acuminata. *J. Am. Chem. Soc.* **1966**, *88* (16), 3888–3890.

(80) Li, T.; Liu, L. F. Tumor Cell Death Induced By Topoisomerase-Targeting Drugs. *Annu. Rev. Pharmacol. Toxicol.* **2001**, 53–77.

(81) Pommier, Y. Drugging Topoisomerases: Lessons and Challenges. *ACS Chem. Biol.* **2013**, *8* (1), 82–95.

(82) Pendleton, M.; Jr, R. H. L.; Felix, C. A.; Grimwade, D.; Osheroff, N. Topoisomerase II and Leukemia. **2014**, *1310*, 98–110.

(83) Vos, S. M.; Tretter, E. M.; Schmidt, B. H.; Berger, J. M. All Tangled up: How Cells Direct, Manage and Exploit Topoisomerase Function. *Nat. Rev. Mol. Cell Biol.* **2011**, *12* (12), 827–841.

(84) Chen, C.; Shanmugasundaram, K.; Rigby, A. C.; Kung, A. L. Shikonin, a Natural Product from the Root of Lithospermum Erythrorhizon, Is a Cytotoxic DNA-Binding Agent. *Eur. J. Pharm. Sci.* **2013**, *49* (1), 18–26.

(85) Ray, S.; Hazra, B.; Mittra, B.; Das, a; Majumder, H. K. Diospyrin, a Bisnaphthoquinone: A Novel Inhibitor of Type I DNA Topoisomerase of Leishmania Donovani. *Mol. Pharmacol.* **1998**, *54* (6), 994–999.

(86) Plyta, Z. F.; Li, T.; Papageorgiou, V. P.; Mellidis, A. S.; Assimopoulou, A. N.; Pitsinos, E. N.; Couladouros, E. A. Inhibition of Topoisomerase I by Naphthoquinone Derivatives. *Bioorganic Med. Chem. Lett.* **1998**, *8* (23), 3385–3390.

(87) Fujii, N.; Yamashita, Y.; Arima, Y.; Nagashima, M.; Nakano, H. Induction of Topoisomerase II-Mediated DNA Cleavage by the Plant Naphthoquinones Plumbagin and Shikonin. *Antimicrob. Agents Chemother.* **1992**, *36* (12), 2589–2594.

(88) Hu, Y.; Jiang, Z.; Leung, K. S. Y.; Zhao, Z. Simultaneous Determination of Naphthoquinone Derivatives in Boraginaceous Herbs by High-Performance Liquid Chromatography. *Anal. Chim. Acta* **2006**, *577* (1), 26–31.

(89) Vukic, M. D.; Vukovic, N. L.; Obradovic, A. D.; Popovic, S. L.; Zaric, M. M.; Djurdjevic, P. M.; Markovic, S. D.; Baskic, D. D. Naphthoquinone Rich Onosma Visianii Clem (Boraginaceae) Root Extracts Induce Apoptosis and Cell Cycle Arrest in HCT-116 and MDA-MB-231 Cancer Cell Lines. *Nat. Prod. Res.* **2017**, *6419*, 1–5.

(90) Zhou, W.; Jiang, H. D. G. La; Peng, Y.; Li, S. S. Comparative Study on Enantiomeric Excess of Main Akannin/Shikonin Derivatives Isolated from the

Roots of Three Endemic Boraginaceae Plants in China. *Biomed. Chromatogr.* **2011**, *25* (10), 1067–1075.

(91) Spyros, A.; Assimopoulou, A. N.; Papageorgiou, V. P. Structure Determination of Oligomeric Alkannin and Shikonin Derivatives. *Biomed. Chromatogr.* **2005**, *19* (7), 498–505.

(92) Mellidis, A. S.; Papageorgiou, V. P. Naphthazarins from *Onosma Heterophylla*. *J. Nat. Prod.* **1987**, *50* (4), 618–619.

(93) Sagratini, G.; Cristalli, G.; Giardinà, D.; Gioventù, G.; Maggi, F.; Ricclutelli, M.; Vittori, S. Alkannin/Shikonin Mixture from Roots of *Onosma Echioide*s(L.) L: Extraction Method Study and Quantification. *J. Sep. Sci.* **2008**, *31* (6–7), 945–952.

(94) Yingkun, N.; Lvsong, Z.; Huimin, Y. Shikonin Inhibits the Proliferation and Induces the Apoptosis of Human HepG2 Cells. **2010**, *1146*, 1138–1146.

(95) Wu, Z.; Wu, L. J.; Li, L. H.; Tashiro, S. I.; Onodera, S.; Ikejima, T. Shikonin Regulates HeLa Cell Death via Caspase-3 Activation and Blockage of DNA Synthesis. *J. Asian Nat. Prod. Res.* **2004**, *6* (3), 155–166.

(96) Yeh, C. C.; Kuo, H. M.; Li, T. M.; Lin, J. P.; Yu, F. S.; Lu, H. F.; Chung, J. G.; Yang, J. S. Shikonin-Induced Apoptosis Involves Caspase-3 Activity in a Human Bladder Cancer Cell Line (T24). *In Vivo (Brooklyn).* **2007**, *21* (6), 1011–1020.

(97) Park, S. H.; Phuc, N. M.; Lee, J.; Wu, Z.; Kim, J.; Kim, H.; Kim, N. doo; Lee, T.; Song, K. S.; Liu, K. H. Identification of Acetylshikonin as the Novel CYP2J2 Inhibitor with Anti-Cancer Activity in HepG2 Cells. *Phytomedicine* **2017**, *24*, 134–140.

(98) Kyong-Up, B.; Yong, S. G.; Yong, K.; Dai-Eun, S.; Byung-Zun, A. 2-Substituted Naphthazarins; Synthesis and Antitumor Activity. *Arch. Pharm. (Weinheim).* **1997**, *330* (12), 377–382.

(99) Ahn, B.; Baik, K.; Kweon, G.; Lim, K.; Hwang, B. Acylshikonin Analogues : Synthesis and Inhibition of DNA Topoisomerase-I. **1995**, 1044–1047.

(100) Ogawa, Y.; Kawano, Y.; Yamazaki, Y.; Onishi, Y. Shikonin Shortens the Circadian Period: Possible Involvement of Top2 Inhibition. *Biochem. Biophys. Res. Commun.* **2014**, *443* (1), 339–343.

(101) Hande, K. R. Etoposide: Four Decades of Development of a Topoisomerase II Inhibitor. *European Journal of Cancer*. 1998, pp 1514–1521.

(102) Kontogiannopoulos, K. N.; Assimopoulou, A. N.; Hatziantoniou, S.; Karatasos, K.; Demetzos, C.; Papageorgiou, V. P. Chimeric Advanced Drug Delivery Nano Systems (Chi-ADDnSs) for Shikonin Combining Dendritic and Liposomal Technology. *Int. J. Pharm.* **2012**, *422* (1–2), 381–389.

(103) Xia, H.; Tang, C.; Gui, H.; Wang, X.; Qi, J.; Wang, X.; Yang, Y. Preparation , Cellular Uptake and Angiogenic Suppression of Shikonin-Containing Liposomes in Vitro and in Vivo. **2013**, 207–215.

(104) Wang, W.; Dai, M.; Zhu, C.; Zhang, J.; Lin, L.; Ding, J.; Duan, W. Synthesis and Biological Activity of Novel Shikonin Analogues. *Bioorganic Med. Chem. Lett.* **2009**, *19* (3), 735–737.

(105) Yang, J. T.; Li, Z. L.; Wu, J. Y.; Lu, F. J.; Chen, C. H. An Oxidative Stress Mechanism of Shikonin in Human Glioma Cells. *PLoS One* **2014**, *9* (4).