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ÖZGE GÜZEL

SEDA DUMAN

SİNEM YILMAZ

ÇİĞDEM KARAKOYUN

DEMET KUL

See next page for additional authors

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Authors
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Screening of cytotoxicity and DNA topoisomerase IIa inhibitory activity of Turkish *Onosma* species

Özge GÜZEL[1], Seda DUMAN[1], Sinem YILMAZ[2,3], Çiğdem KARAKOYUN[4], Demet KUL[1], Ademi Fahri PİRHAN[5], Erdal BEDİR[6,*]

[1]Graduate School, İzmir Institute of Technology, İzmir, Turkey
[2]Department of Bioengineering, Graduate School of Natural and Applied Sciences, Ege University, İzmir, Turkey
[3]Department of Bioengineering, Faculty of Engineering, University of Alanya Aladdin Keykubat, Antalya, Turkey
[4]Department of Pharmacognosy, Faculty of Pharmacy, Ege University, İzmir, Turkey
[5]Department of Biology, Faculty of Science, Ege University, İzmir, Turkey
[6]Department of Bioengineering, İzmir Institute of Technology, İzmir, Turkey

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Abstract: *Onosma* L., the largest genus of Boraginaceae, is represented by 105 species in Turkey with an endemism rate of 52%. Phytochemical studies indicate that Boraginaceae plants mainly comprise naphthoquinones with a wide range of biological activities including anticancer, antiinflammatory, wound healing, and antioxidant effects. However, few taxa of the genus *Onosma* have been investigated in detail for their bioactivities. Considering the high rate of endemism and an inadequate number of bioactivity screening studies in literature, we aimed to evaluate the cytotoxic effects and topoisomerase inhibitory activities of some *Onosma* species growing in southwestern Turkey. Here, we describe a comprehensive cytotoxic activity screening study on petroleum ether, dichloromethane, and methanol extracts of the roots of 20 identified and one unidentified *Onosma* taxa. The MTT cell viability assay has been performed to investigate the cytotoxicity of the extracts against seven cancer cell lines (MCF-7, HeLa, Hep G2, A549, Capan-1, HCC-1937, and DU-145) and a noncancerous cell line (MRC-5), while doxorubicin was served as a positive standard. The petroleum ether extracts of *O. aksoyii* Aytaç&Türkmen, *O. isaurica* Boiss. and Heldr., *O. taurica* Pallas ex Willd. var. *taurica* and *O. alborosea* Fisch. & C.A. Mey subsp. *alborosea* var. *alborosea* were determined as the most active ones based on their IC_{50} values. DNA topoisomerase IIα inhibition assay was conducted on the petroleum ether and dichloromethane extracts of these four active species, and almost all tested extracts demonstrated strong inhibition on the enzyme at a concentration of 0.1 mg/mL. Our cytotoxicity screening results were consistent with the findings of the topoisomerase IIα inhibition test. This study advocates the significant role of *Onosma* species in the field of anticancer drug discovery.

Key words: *Onosma*, endemic, cytotoxic, bioactivity, screening, topoisomerase.

1. Introduction

Cancer is one of the most common types of health problems, ranking as the second leading cause of mortality, worldwide. According to current reports published by World Health Organization (WHO), cancer was estimated to account for 9.6 million deaths in 2018 (WHO, 2019).

Enormous efforts have been devoted to the discovery of anticancer agents. But despite the great development in science and technology, anticancer drug discovery still remains a challenging endeavor. The two major impediments in cancer research are a narrow therapeutic index of anticancer drugs and drug resistance developed by cancer cells. Therefore, it is very important to find out novel drug molecules with high efficiency and low toxicity apart from overcoming drug resistance (McAllister et al., 2004; Mandelblatt et al., 2013).

Natural product research has been one of the most important approaches in anticancer drug discovery either having the potential directly to be a drug candidate or acting as drug lead molecules for drug design strategies for years (Lee, 1999; Mukherjee et al., 2001). During the past decades, discoveries of highly potent cytotoxic natural products encouraged scientists to deepen phytochemical research on plants, microorganisms, fungi, and marine invertebrates. Vinca alkaloids (vincristin, vinblastin), taxanes (taxol, taxotere), podophyllotoxins (etoposide, teniposide), and quinones (doxorubicine, daunorubicin, epirubicin) are the main FDA-approved anticancer natural

* Correspondence: erdalbedir@iyte.edu.tr

products being frequently prescribed for various types of cancer (Bachur et al., 1978; Safarzadeh et al., 2014).

The genus *Onosma* L., one of the richest genera within Boraginaceae family, is represented by 105 species (110 taxa) in Turkey, with an endemism rate of 52% (Binzet et al., 2018; Akcin and Binzet, 2019). Even if *Onosma* species contain secondary metabolites related to a great number of chemical skeletons including quinones, terpenes, alkaloids, coumarins, flavonoids etc. the members are popularly known for their content of 1,4-naphthoquinone derivatives such as alkannins and shikonins (Kumar et al., 2013).

Alkannin (S) and shikonin (R), alkyl substituted 1,4-naphthoquinone derivatives, are enantiomers mainly present in the roots of Boraginaceae plants being responsible for a wide range of bioactivities, including wound healing, antimicrobial, antiinflammatory, antioxidant, antithrombotic, and particularly anticancer effects (Damianakos et al., 2012; Kretschmer et al., 2012; Dong et al., 2017; Hemmati et al., 2018).

The earliest studies on cytotoxic activities of shikonins were recorded in the 1970s, right after the official declaration of anticancer effects of alkannin and shikonin against Walker carcinoma 256 and sarcoma 180 by the National Cancer Institute (Sankawa et al., 1977). However, the traditional use of shikonin-containing plants for cancer treatment dates back to the 12th century AD and has a very strong history especially in traditional Chinese medicine (TCM) (Papageorgiou et al., 1999).

Zicao is one of the naphthoquinone-rich herbal remedies suggested by TCM for cancer treatment and officially contains three Boraginaceae plants; *Arnebia euchroma* (Royle) Johnst., *A. guttata* Bunge, and *Lithospermum erythrorhizon* Sieb.et Zucc. Principal components of Zicao are reported to be shikonin/alkannin derivatives, especially estimated as acetylshikonin, dimethylacrylshikonin, and epoxyshikonin (Rinner et al., 2010).

Providing a large amount of shikonin derivatives, *Onosma* species (*Onosma paniculatum* Bur. et Franch., *O. exsertum* Hemsl., *O. confertum* W. W. Smith, *O. hookerii* Clarke var. *longiflorum* Duthie, *O. hookerii* Clarke, and *O. waltonii* Duthie) have also been used as interchangeable sources for Zicao by native people in southwestern China (Hu et al., 2006). These historical and current pieces of evidence indicate that strong cytotoxic effects of shikonins are ultimately responsible for both traditional and modern usage of *Onosma* roots in cancer therapy (Kumar et al., 2013).

Cellular mechanistic studies reveal that shikonin and its derivatives exert their anticancer activities via multiple mechanisms including the activation of classic caspase-3 mediated apoptosis pathway, producing reactive oxygen species (ROS), and inhibiting the human DNA topoisomerases (topos) (Chen et al., 2002).

Since topos have emerged as promising targets for cancer therapy, interest in these enzymes exponentially increased over the last two decades. Topos are crucial enzymes modulating the topological state of DNA and have essential roles in metabolic processes including DNA replication, repairmen, and transcription during the cell cycle (Wang, 1985). These enzymes are categorised into two groups, Type I and Type II, depending on breaking and resealing one or both strands of DNA during their catalytic cycle, respectively.

Currently prescribed popular antitumor agents, camptothecin, doxorubicin, and etoposide are representative inhibitors of topo I and II. Doxorubicin (hydroxydaunorubicin) is a quinone-containing anthracycline antibiotic and an FDA-approved anticancer drug which strongly inhibits topo II (Ross, 1985).

Although all topoisomerases have crucial roles during mitosis, only the level of topo IIα proteins rapidly increases at the proliferative stage (Ohashi et al., 1999; Mu et al., 2000). In other words, the concentration of topo IIα is always higher in cancer cells than in normal cells. That is the reason why topo IIα was stated as a primary cellular target for cancer therapeutics (Gurbani et al., 2012). This enzyme executes its effect by controlling the decatenation checkpoint and maintaining the genomic stability (DNA cleavage) during the cell cycle (Bower et al., 2010). Consequently, topo IIα inhibitory activity tests are usually carried out via investigation of either or both of these two cellular mechanisms.

The present study was designed to assess the cytotoxic effects and DNA topoisomerase IIα inhibitory activities of some *Onosma* species collected from southwestern Turkey. For this purpose, petroleum ether, methanol, and dichloromethane extracts of the roots of 21 *Onosma* members were screened against seven cancer cell lines (MCF-7, HeLa, Hep G2, A549, Capan-1, HCC-1937, and DU-145) and a noncancerous cell line (MRC-5) using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cell viability assay.

2. Materials and methods

The anatomical structures and morphological characteristics of collected plant materials were double checked for accurate identification. Species names, local names, herbarium codes, collection dates, localities and altitudes are provided in Table 1. The plant species and taxa were identified by Prof. Dr. Ademi Fahri Pirhan (Ege University). Voucher specimens were deposited in Herbarium of Science Faculty, Ege University, İzmir, Turkey. Air-dried and fine-powdered root materials were stored inside light-proof plastic bottles at room temperature.

Table 1. Details of collected *Onosma* taxa.

Taxa	Local name	Localities
Onosma bracteosa Hausskn. & Bornm.*	Küpeliemcek	Turkey, C2 Muğla: Kavaklıdere District, Kuyualan Village, 37°28.103'K 28° 25.585D, 961 m, 13.05.2017, (EGE 2357)
Onosma bornmuelleri Hausskn. & Bornm.*	Küpeliemcek	Turkey, C2 Muğla: Kavaklıdere District, Kuyualan Village, 37° 28.297'K 28° 26.981'D, 965 m, 13.05.2017, (EGE 2358).
Onosma bornmuelleri Hausskn. & Bornm.*	Küpeliemcek	Turkey, C2 Muğla: Kavaklıdere District, Kuyualan Village, 37° 28.269'K 28° 26.573'D, 1003 m, 13.05.2017 (EGE 2359)
Onosma bornmuelleri Hausskn. & Bornm.*	Küpeliemcek	Turkey, C2 Muğla: Kavaklıdere District, Kuyualan Village, 37° 28.269'K 28° 26.573'D, 1011 m, 13.05.2017, (EGE 2360)
Onosma heterophylla Griseb.	Deliemzik	Turkey, C2 Muğla, Kavaklıdere-Nebiler District, 37° 27.38'K 28° 25.29'D, 1080 m, 13.07.2017, (EGE 2361)
Onosma heterophylla Griseb.	Deliemzik	Turkey, C2 Muğla: Yılanlı Mountain, Muğla District, 5th km, 37° 13.929'K 28° 23.652'D, 959 m, 13.07.2017, (EGE 2362)
Onosma frutescens Lam.	Sarkıemcek	Turkey, C2 Muğla: Masa Mountain, 37° 13.149'K 28° 23.162'D, 881 m, 13.07.2017, (EGE 2363)
Onosma frutescens Lam.	Sarkıemcek	Turkey, C2 Muğla: Masa Mountain, 37° 13.328'K 28° 22.116'D, 747 m, 13.07.2017, (EGE 2364)
Onosma taurica Pallas ex Willd var. *taurica*#	Emzikotu	Turkey, C2 Muğla: Köyceğiz-Fethiye Road Göcek Tunnel, 36° 45.518'K 28° 53.251'D, 246 m, 13.07.2017, (EGE 2366)
Onosma frutescens Lam.	Sarkıemcek	Turkey, C2 Muğla: Göcek Tunnel – Fethiye 36° 45.93'K 28° 53.76'D, 315 m, 13.07.2017, (EGE 2367)
Onosma frutescens Lam.	Sarkıemcek	Turkey, C2 Muğla: Ballık Village Road of Ören, Fethiye, 36° 46.925'K 29° 23.691'D, 544 m, 13.07.2017, (EGE 2368)
Onosma nana DC.*#	Tavşangözü	Turkey, C2 Muğla: Ballık Village Road of Ören, Fethiye, 36° 49.886'K 29° 24.849'D, 1357 m, 13.07.2017, (EGE 2369)
Not identified		Turkey, C2 Muğla. Boncuk Mountain, Ören, Altınyayla, 36° 52.144'K 29° 24.740'D, 1700 m, 14.05.2017
Onosma taurica Pallas ex Willd var. *taurica*	Emzikotu	Turkey, C2 Denizli: Altınyayla-Gökhisar Road, 37° 06,781'K 29° 31.263'D, 989 m, 14.05.2017, (EGE 2374)
Onosma bornmuelleri Hausskn. & Bornm.*	Küpeliemcek	Turkey, C2 Denizli: Altınyayla-Gökhisar Road, 36° 04.452'K 29° 31.840'D, 1018 m, (EGE 2375)
Onosma oreodoxa Boiss. & Heldr.	Darışincarı	Turkey, C3 Antalya: Çay Neighborhood, Manavgat Road /Antalya 37° 11.048'K 31° 12.237'D, 189 m, 19.05.2017, (EGE 2416)
Onosma oreodoxa Boiss. & Heldr.#	Darışincarı	Turkey, C3 Antalya: Manavgat Burmahan Village 37° 9.791'K 31° 12.331'D, 255 m, 19.05.2017, (EGE 2417)
Onosma oreodoxa Boiss. & Heldr.	Darışincarı	Turkey, C3 Antalya: Manavgat Burmahan Village 37° 9.500'K 31° 11.801'D, 194 m, 19.05.2017, (EGE 2418)
Not identified		Turkey, C3 Antalya: Manavgat, Taşağıl, Beydiğin Plateau 37° 12.054'K 31° 24.741'D, 1070 m, 20.05.2017 (EGE 2419)
Onosma taurica Pallas ex Willd var. *taurica*	Emzikotu	Turkey, C3 Antalya: Manavgat, Toka Road 37° 12.291'K 31° 24.699'D, 1084 m, 20.05.2017 (EGE 2420)
Onosma armena DC.*#	Hevajo	Turkey, C3 Antalya: Başlar-Beydiğin Tunnel 37° 5.372'K 31° 27.102'D, 1029 m, 20.07.2017 (EGE 2421)

Table 1. (Continued).

Onosma armena DC.*	Hevajo	Turkey, C3 Antalya: Başlar-Beydiğin Tunnel 1-1.5 km (towards Beydiğin) 37° 4.726'K 31° 27.314'D, 1159 m, 20.05.2017, (EGE 2422)
Onosma oreodoxa Boiss. & Heldr.	Darışincarı	Turkey, C3 Antalya: Akseki Highway Gençler District 36° 47.236'K 31° 43.236'D, 241 m, 20.05.2017 (EGE 2423)
Onosma oreodoxa Boiss. & Heldr.	Darışincarı	Turkey, C3 Antalya: Ekşili Village 37° 9.684'K 30° 41.689'D, 286 m, 21.05.2017 (EGE 2424)
Onosma frutescens Lam.	Sarkıemcek	Turkey, C3 Antalya: Eskiavdan Kızılkaya Highway, Rocky Hill 37° 16.532'K 30° 25.678'D, 856 m, 21.05.2017 (EGE 2425)
Onosma frutescens Lam.	Sarkıemcek	Turkey, C3 Antalya: Eskiavdan Kızılkaya District 37° 16.769'K 30° 26.390'D, 796 m, 21.07.2017 (EGE 2426)
Onosma aucheriana DC.#	Emcek	Turkey, C2 Antalya: Yazır Plateau Highway, 36° 59.185'K 30° 18.322'D, 984 m, 21.05.2017 (EGE 2427)
Onosma mitis Boiss. & Heldr.#	Çamşincarı	Turkey, C3 Antalya: Termessos National Park, 37° 1.004'K 30° 30.306'D, 395 m, 21.05.2017, (EGE 2428/42714)
Onosma frutescens Lam.	Sarkıemcek	Turkey, C3 Antalya: Termessos National Park Summit, 37° 0.528'K 30° 29.434'D, 590-650 m, 21.05.2017 (EGE 2429)
Onosma heterophylla Griseb.#	Deliemzik	Turkey, C2 Burdur: Serinhisar-Yeşilova Salda Lake, Burdur Highway, 37° 31.459'K 29° 39.200'D, 1158 m, 28.05.2017 (EGE 42715)
Onosma bracteosa Hausskn. & Bornm.*#	Küpeliemcek	Turkey, C3 Burdur: Hacilar Village (towards Burdur) 37° 36.539'K 30° 8.137'D, 889 m, 28.05.2017 (EGE 42716)
Onosma bracteosa Hausskn. & Bornm.*	Küpeliemcek	Turkey, C3 Burdur: Vicinity of Burdur 37° 41.498'K 30° 11.540'D, 862 m, 28.05.2017 (EGE 42716)
Onosma bracteosa Hausskn. & Bornm.*	Küpeliemcek	Turkey, C3 Isparta: Yakaören (Entry) 37° 46.6'K 30° 28.53'D, 1207 m, 28.05.2017, (EGE 42716)
Onosma taurica Pallas ex Willd var. *taurica*	Emzikotu	Turkey, C3 Isparta: Yakaören (Entry) 37° 46.6'K 30° 28.53'D, 1207 m, 28.05.2017
Onosma oreodoxa Boiss. & Heldr.	Darışincarı	Turkey, C3 Isparta: Eğirdir- Gelendost Highway 5th km 37° 54.017'K 30° 54.212'D, 927 m, 29.05.2017
Onosma bracteosa Hausskn. & Bornm.*	Küpeliemcek	Turkey, B3 Isparta: Yeşilköy- Hacılar District (Yeşilköy) 37° 0.891'K 30° 58.250'D, 1023 m, 29.05.2017
Not identified		Turkey, B3 Isparta: Yeşilköy-Mountain Side Road 37° 58.107'K 30° 0.481'D, 1427 m, 29.05.2017
Onosma oreodoxa Boiss. & Heldr.	Darışincarı	Turkey, C3 Isparta: Aksu-Eğirdir Highway 37° 79.970' K 30° 93.260' D, 1072 m, 29.05.2017
Onosma bracteosa Hausskn. & Bornm.*	Küpeliemcek	Turkey, C3 Isparta: Aksu-Dedegöl Mountain District, 37° 43.730'K 30° 11.665'D, 1172 m, 29.05.2017
Onosma bracteosa Hausskn. & Bornm.*	Küpeliemcek	Turkey, C3 Isparta: Dedegöl-Melikler Plateau 37° 41.940'K 31° 17.478'D, 1650-1707 m, 30.05.2017
Onosma bornmuelleri Hausskn. & Bornm.*	Amasyaşincarı	Turkey, C3 Isparta: Dedegöl Mountain (towards Beyşehir) 37° 41.136'K 31° 22.664'D, 1204 m, 30.05.2017
Onosma bornmuelleri Hausskn. & Bornm.*	Küpeliemcek	Turkey, C3 Konya: Yeşildağ-Kurucaova Highway, 37° 40.196'K 31° 26.481'D, 1234 m, 30.05.2017
Onosma bracteosa Hausskn. & Bornm.*	Küpeliemcek	Turkey, C3 Isparta: 37° 44.008'K 31° 15.542'D, 1563 m, 30.05.2017
Onosma oreodoxa Boiss. & Heldr.	Darışincarı	Turkey, C3 Isparta: Melikler-Aksu Lowland 37° 44.321'K 31° 10.783'D, 1187 m, 30.05.2017
Onosma taurica Pallas ex Willd var. *taurica*	Emzikotu	Turkey, C3 Burdur: Afyon-Burdur Highway, 37° 55.509'K 30° 15.870'D, 1128 m, 30.05.2017 (EGE 42717)
Onosma taurica Pallas ex Willd var. *taurica*	Emzikotu	Turkey, B2 Uşak: Kütahya-Uşak Highway, Alikahya Village 38° 83.1862 'K 29° 26.6381' D, 1156 m, 30.05.2017 (EGE 42717)

Table 1. (Continued).

Onosma bornmuelleri Hausskn. & Bornm.*	Amasyaşincarı	Turkey, B3 Afyon: Dinar-Karacahalı District, (D-320 Highway) 38º 2.132'K 31º 4.237'D, 1013 m, 30.05.2017
Onosma bornmuelleri Hausskn. & Bornm.*	Amasyaşincarı	Turkey, A2 Bilecik: (around the lake) 37º 59.709'K 32º 15.970'D, 580 m, 30.05.2017
Onosma oreodoxa Boiss. & Heldr.	Darışincarı	Turkey, C3 Antalya: Antalya-Isparta Highway, Sakızlık Village, 37º 37.009'K 30º 44.229'D, 510 m, 30.05.2017
Onosma oreodoxa Boiss. & Heldr.	Darışincarı	Turkey, C3 Isparta: Aksu – Sütçüler Highway, Sağrak Village 37º 34.390'K 30º 58.638'D, 1156 m, 15.06.2017
Onosma oreodoxa Boiss. & Heldr.	Darışincarı	Turkey, C3 Isparta: Sütçüler, Tota Mountain, 37º 34.931'K 31º 58.956'D, 1191 m, 15.06.2017
Not identified		Turkey, C3 Isparta: Tota Mountain (towards Sütçüler), 37º 33.341'K 31º 03.890'D, 1507 m, 16.06.2017
Not identified		Turkey, C3 Isparta: Sütçüler/Belen, 37º 29.595'K 31º 0.590'D, 1231 m, 16.06.2017
Onosma oreodoxa Boiss. & Heldr.	Darışincarı	Turkey, C3 Isparta: Sütçüler 37º 29.591'K 30º 58.969'D, 1149 m, 16.06.2017
Onosma oreodoxa Boiss. & Heldr.	Darışincarı	Turkey, Isparta: Sütçüler, Yazılıkanyon National Park, 30º 29.187'K 30º 55.974'D, 851 m, 16.06.2017
Onosma aksoyii Aytaç & Turkmen *#	Beyşincarı	Turkey, C3 Isparta: Sütçüler-Beyşehir, Ayvalıpınar Village, 37º 41.614'K 31º 01.340'D, 1146 m, 16.06.2017
Onosma oreodoxa Boiss. & Heldr.	Darışincarı	Turkey, C3 Isparta: Melikler Plateau Road 3rd km, 37º 43.820'K 31º 15.898'D,1594 m, 16.06.2017
Onosma armena DC.*	Hevajo	Turkey, C3 Isparta: Derebucak – Beyşehir District, Yenişarbademli Highway, 37º 22.227'K 31º 28.077'D, 1619 m, 16.06.2017
Onosma bracteosa Hausskn. & Bornm.*	Küpeliemcek	Turkey, C3 Isparta: Derebucak – Beyşehir District, Yenişarbademli Highway, 37º 21.951'K 31º 28.060'D, 1610 m, 16.06.2017
Onosma mollis DC.	Divanköşk	Turkey, C3 Isparta: Sütçüler-Beyşehir District, Yenişarbademli Highway, 37º 22.267'K 31º 29.430'D, 1386 m, 16.06.2017
Onosma bornmuelleri Hausskn. & Bornm.*	Amasyaşincarı	Turkey, C3 Isparta: Beyşehir- Derebucak District, Akseki Turnout, 37º 34.741'K 31º 34.332'D, 1175 m, 17.06.2017
Onosma bornmuelleri Hausskn. & Bornm.*#	Amasyaşincarı	Turkey, C3 Konya: Beyşehir- Derebucak Highway, 37º 32.146'K 31º 29.999'D, 1080 m, 17.06.2017 (EGE 42719)
Onosma bornmuelleri Hausskn. & Bornm.*	Amasyaşincarı	Turkey, C3 Konya: Huğlu Lowland, 37º 34.741'K 31º 29.821'D, 1227 m, 17.06.2017
Onosma bornmuelleri Hausskn. & Bornm.*	Amasyaşincarı	Turkey, C3 Konya: Huğlu Lowland, Gecek Stream, 37º 25.693'K 31º 30.753'D, 1249 m, 17.06.2017
Onosma mollis DC.#	Divanköşk	Turkey, C3 Konya: Gencek Neighborhood, Derebucak, 37º 25.688'K 31º 31.756'D, 1258 m, 17.06.2017 (EGE 42720)
Not identified		Turkey, C3 Antalya: İbradi Highway (8th km) 3º 09.216'K 31º 32.202'D, 1297 m, 17.06.2017
Onosma aucheriana DC.	Emcek	Turkey, C3 Antalya: Toka Plateau, 37º 13.653'K 31º 23.540'D, 1275 m, 17.06.2017
Onosma bornmuelleri Hausskn. & Bornm.*	Amasyaşincarı	Turkey, C3 Antalya: İbradi- Korkuteli Highway 37º 59.865'K 31º 18.052'D, 860 m, 17.06.2017
Onosma frutescens Lam.	Sarkıemcek	Turkey, C3 Antalya: İbradi- Korkuteli Highway 37º 59.191'K 31º 18.333'D, 967 m, 17.06.2017
Onosma strigosissima Boiss.*#	Yalışincarı	Turkey, C3 Antalya: Kemer-Alacasu Koyu Mountain 36º 32.086'K 30º 33.464'D, 109 m, 24.08.2018 (EGE 3661)
Onosma nydeggeri Hub.-Mor.*#	Allışincar	Turkey, C3 Antalya: Kumluca/Altınyaka Street, near Kıranköşk Mosque 36º 33.825'K 30º 20.843'D, 994 m, 27.04.2018 (EGE 3662)

Table 1. (Continued).

Onosma frutescens Lam.	Sarkıemcek	Turkey, C2 Antalya: Fethiye-Korkuteli Highway 36º 43.235'K 29º 26.586'D, 888 m, 24.08.2018 (EGE 3663)
Onosma frutescens Lam.	Sarkıemcek	Turkey, C2 Muğla: Fethiye-Korkuteli Highway, near Urluca Fountain, 36º 49.426'K 29º 33.258'D, 1106 m, 28.04.2018 (EGE 3664)
Onosma frutescens Lam.#	Sarkıemcek	Turkey, C3 Antalya: Seki Turnout, 36º 49.568'K 29º 33.343'D, 1086 m, 28.04.2018 (EGE 3665)
Onosma taurica Pallas ex Willd var. *taurica*	Emzikotu	Turkey, C2 Burdur: Fethiye-Korkuteli Highway, Dirmil Turnout, 36º 53.963'K 29º 41.020'D, 1245 m, 28.04.2018 (EGE 3666)
Onosma isaurica Boiss. & Heldr.*	Külemcek	Turkey, C2 Antalya: Antalya-Fethiye Highway(D350), 36º 56.347'K29º 45.195'D, 1332 m, 28.04.2018 (EGE 3667)
Not identified#		Turkey, C2 Antalya: Antalya-Denizli Highway (E87), Antalya-Burdur Provincial Border, 37º 2.946'K 9º 51.854'D, 1399 m, 28.04.2018 (EGE 3668)
Not identified		Turkey, C2 Denizli: 37º 1.973'K 28º 53.686'D, 1412 m, 28.04.2018 (EGE 3669)
Onosma alborosea Fisch. & C. A. Mey. subsp. *sanguinolenta* (Vatke) Bornm.#	Morşincar	Turkey, C2 Muğla: 36º 45.93'K 28º 53.76'D, 1412 m, 28.04.2018 (EGE 3670)
Onosma alborosea Fisch. & C. A. Mey. subsp. *sanguinolenta* (Vatke) Bornm.	Morşincar	Turkey, C3 Antalya: Kızılcadağ, near Umut Fountain/ Çığlık Plateau 37º 2.080'K 29º 59.941'D, 1576 m, 28.04.2018
Onosma inexspectata Teppner.*#	Moremzik	Turkey, C6 Adana: Osmaniye/Hasanbeyli Road, 37º 6.888'K 36º 35.789'D, 1005 m, 05.06.2020 (EGE 3671)
Onosma gigantea Lam.#	Kocaemcek	Turkey, C4 Mersin: Cemilli Village /Fındıkpınarı District, 36º 47.814'K 34º 27.441'D, 412 m, 05.06.2020 (EGE 3672)
Onosma alborosea Fisch. & C. A. Mey. subsp. *alborosea* var. *alborosea* #	Kayaemceği	Turkey, C4 Mersin: Gülnar-Ermenek Highway/Bereket Street, 36º 21.318'K 33º 13.687'D, 1127 m, 06.06.2020 (EGE 3673)
Onosma roussaei DC.#	Yamaçemceği	Turkey, C4 Mersin: Gülnar-Ermenek Highway 24.th km, 36º 24.577'K 33º 10.337'D, 1389 m, 06.06.2020 (EGE 3675)
Onosma isaurica Boiss. & Heldr.*#	Külemcek	Turkey, C4 Konya: Hadim-Bozkır Highway, 5th km towards Bozkır, 36º 58.765'K 32º 23.291'D, 1840 m, 06.06.2020 (EGE 3676)
Onosma alborosea Fisch. & C. A. Mey. subsp. *alborosea* var. *alborosea*	Kayaemceği	Turkey, C4 Mersin: Gülnar-Ermenek District, 36º 21.211'K 33º 13.650'D, 1206 m, 19.07.2020 (EGE 3677)
Onosma mitis Boiss. & Heldr.	Çamşincarı	Turkey, C3 Antalya: Termessos National Park, 37º 1.004'K 30º 30.306'D, 395 m, 20.07.2020 (EGE 3678)

* endemic, # selected for bioactivity screening studies.

2.1. Preparation of plant extracts

Air-dried and powdered roots (10 g each) were sequentially macerated with 100 mL of petroleum ether (ChemLab), methanol (Fisher Optima), and dichloromethane (JT Baker, Inc.) using an ultrasonic bath (Ultrasonic LC 30, Singen, Germany) for 30 min. Organic solvents were evaporated in vacuo (Heidolph Laborata 4001, Germany) to obtain crude extracts.

All solvents used in the present study were of analytical grade.

2.2. Cytotoxic activity test

2.2.1. Cell culture and drug treatment

Adenocarcinoma human alveolar basal epithelial cells (A549), human endometrial carcinoma (HeLa), human pancreatic ductal adenocarcinoma (Capan-1), human hepatocellular carcinoma (Hep G2), human breast adenocarcinoma (MCF-7), human breast carcinoma (HCC-1937), human lung fibroblasts (MRC-5), and human prostate carcinoma (DU-145) cell lines were obtained from American Type Culture Collection and maintained as exponentially growing monolayers by culturing according to the supplier's instructions. For cytotoxic activity analysis, each cell line was exposed to organic extracts at final concentrations of 4, 8, 16, and 32 µg/mL, for 48 h.

2.2.2. MTT cell viability assay

The cytotoxicity screening assay was performed by a modification of the MTT method against seven cancer cell lines and a noncancerous cell line (Mosmann, 1983; Marks et al., 1992). This method is based on the conversion of a pale-yellow tetrazolium salt (MTT), into blue, colorimetrically measurable formazan products when incubated with living cells. A noncancerous cell line MRC-5 was treated with samples so as to evaluate the selectivity of cytotoxic samples. Doxorubicin, a wide-spectrum anticancer antibiotic, was served as a positive control. The cells were seeded at supplemented sterile culture media (RPMI-1640 and DMEM (Invitrogen, USA) containing 10% fetal bovine serum (5% CO_2 in air, Gibco, USA) in 96-well plates (Sigma-Aldrich) at 37 °C for 24 h. After incubation, cells were treated with gradient concentrations (range 8 to 32 µg/mL) of the samples for 48 h. The culture liquid was removed and MTT dye in serum-free medium (100 µL/well) was added to the wells (5% CO_2 atmosphere) and left for another incubation at 37 °C for 4 h. MTT dye solution was drained, and the residue was dissolved in dimethyl sulfoxide (DMSO; 150 µL per each). The absorbance of occurred formazan products was measured at 570-690 nm via a microplate reader (Varioskan™). Absorbance readings were processed using GraphPad Prism 5 (San Diego, CA, US). Results were expressed as IC_{50} values (concentration that is required for 50% inhibition).

2.3. Topoisomerase IIα inhibition assay

Human topoisomerase IIα was purchased from Inspiralis (Norwich, UK). Enzymes were stored at -80 °C. Negatively supercoiled pBR322 DNA (Inspiralis, Norwich, UK) was commercially purchased and the purity of DNA products was checked on 1.2% (w/v) agarose gel electrophoresis (Basica LE). EDTA, DMSO, and EtBr were purchased from Sigma-Aldrich. All chemicals and reagents were of analytical grade.

One unit of Topo IIα was defined as the amount of enzyme required to relax 200 ng of PBr322 in 30 min at 37 °C under the standard test conditions. All reactions were carried out according to manufacturer's specifications in a final volume of 20 µL containing 50 mM Tris-HCl, (pH: 8.0), 120 mM KCI, 10 mM $MgCI_2$, 5 mM DTT, 5 mM spermidine, 2 µL of 0.1% ATP, 12 µL dH_2O, 0.2 µg supercoiled pBR322 and test solutions of extracts [1:10 dilution of 1 mg/mL in DMSO: dH_2O, (1:7)]. Solvent control (**S**), negative control (**-C**) (no enzyme), and positive control (**+C**) (1 U of topo IIα) were prepared in a similar manner but excluded plant extracts. Enzyme stock solutions were diluted in Topo IIα buffer. Reactions were initiated by the addition of 1 U of topo IIα. After incubation of the reaction mixture at 37 °C for 30 min, the reaction was stopped by adding 5 µL of loading buffer (0.0025% bromophenol blue, 25% glycerol, and 5% sarcosyl). To visualize the relaxed species, DNA loading dye (Thermo Fisher, R0611) was added and the reaction products were separated by 1.2% agarose gel electrophoresis (5V/cm). Finally, gel was stained with ethidium bromide (0.5 µg/mL) and photographed under UV light.

3. Results and discussion

3.1. MTT cytotoxicity screening

Within the scope of this study, 87 *Onosma* specimens belonging to 20 *Onosma* taxa were collected from southwestern Anatolia. Together with an unidentified species, 21 specimens were selected for MTT cytotoxic activity screening studies (Table 1). The petroleum ether, dichloromethane, and methanol extracts of 21 *Onosma* roots were tested against seven cancer cell lines (A549, Capan-1, DU-145, HCC-1937, HeLa, Hep G2, MCF-7) and a noncancerous human cell line (MRC-5) to evaluate their anticancer potential and determine the selectivity (Table 2). MTT assay was performed testing four different concentrations of the root extracts (4, 8, 16, 32 µg/mL) and serving doxorubicin as a positive control.

Within the context of this study, the sample with an IC_{50} of less than 8 µg/mL was considered to represent potent cytotoxicity. Thus, our findings in MTT assay indicated that the petroleum ether root extracts of *O. aksoyii* (endemic), *O. isaurica* (endemic), *O. mollis* DC., *O. alborosea* var. *alborosea*, and *O. taurica* var. *taurica*

Table 2:. Cytotoxic activities of some *Onosma* species.

	IC_{50} Values (µg/mL)								
***Onosma* species**	**Organic extracts**	**MRC-5**	**MCF-7**	**HeLa**	**Hep G2**	**A549**	**Capan-1**	**HCC-1937**	**DU-145**
	P	<8	<8	<8	<8	<8	<8	<8	<8
Onosma aksoyii Aytaç & Turkmen	D	<8	<8	<8	15	14	<8	<8	<8
	M	>32	>32	>32	>32	>32	>32	>32	>32
	P	>32	>32	>32	>32	>32	>32	>32	>32
Onosma strigosissima Boiss.	D	18.8	19.05	≈32	>32	>32	17	18.9	16.6
	M	>32	>32	>32	>32	>32	>32	>32	>32
	P	16.6	≈32	>32	>32	>32	>32	>32	>32
Onosma nydeggeri Hub.-Mor.	D	>32	>32	>32	>32	>32	>32	>32	>32
	M	>16	>16	>16	>16	>16	>16	>16	>16
	P	>8	>8	>8	>8	>8	>8	>8	>8
Onosma frutescens Lam.	D	>8	>8	>8	>8	>8	>8	>8	>8
	M	>16	>16	>16	>16	>16	>16	>16	>16
	P	4.46	<4	≥16	>16	>16	5.4	6.8	4.8
Onosma taurica Pallas ex Willd var. *taurica*	D	<8	<8	28.75	31.75	>8	<8	<8	<8
	M	>16	>16	>16	>16	>16	>16	>16	>16
	P	≈16	>16	>16	>16	≈16	≈16	≈16	≈16
Not identified	D	>16	>16	>16	>16	>16	>16	>16	>16
	M	>32	>32	>32	>32	>32	>32	>32	>32
	P	7.28	15.9	>16	>16	>16	8.4	8.82	13.3
Onosma alborosea Fisch. & C. A. Mey. subsp. *sanguinolenta* (Vatke) Bornm.	D	>32	>32	>32	>32	>32	>32	>32	>32
	M	>16	>16	>16	>16	>16	>16	>16	>16
	P	>16	>16	>16	>16	>16	>16	>16	>16
Onosma roussaei DC.#	D	>16	>16	>16	>16	>16	≈16	>16	>16
	M	>32	>32	>32	>32	>32	>32	>32	>32
	P	<4	<4	6.82	12.35	<4	<4	<4	10
Onosma isaurica Boiss. & Heldr.	D	>32	>32	>32	>32	>32	>32	>32	>32
	M	>32	>32	>32	>32	>32	>32	>32	>32
	P	>16	>16	>16	>16	>16	≈16	≈16	≈16
Onosma inexpectata Teppner.*#	D	>16	>16	>16	>16	>16	>16	>16	>16
	M	>16	>16	>16	>16	>16	>16	>16	>16
	P	>16	>16	>16	>16	>16	>16	>16	>16

Table 2. (Continued).

Onosma gigantea Lam.#	D	>16	>16	>16	>16	>16	>16	>16	>16
	M	>32	>32	>32	>32	>32	>32	>32	>32
	P	<4	5	5.63	≈16	8.88	<4	<4	<4
Onosma alboroseum Fisch. & C. A. Mey. subsp. *alborosea* var. *alborosea*	D	>16	>16	>16	>16	>16	>16	>16	>16
	M	>32	>32	>32	>32	>32	>32	>32	>32
	P	>16	>16	>16	>16	≈16	≈16	≈16	15
Onosma mitis Boiss. & Heldr.	D	>16	>16	>16	>16	>16	>16	>16	>16
	M	>32	>32	>32	>32	>32	>32	>32	>32
	P	<8	<8	<8	<8	<8	<8	<8	<8
Onosma mollis DC.	D	>32	>32	>32	>32	>32	>32	>16	>32
	M	>32	>32	>32	>32	>32	>32	>32	>32
	P	>32	>32	>32	>32	>32	>32	>32	>32
Onosma heterophylla Griseb.	D	>32	>32	>32	>32	>32	>32	>32	>32
	M	>32	>32	>32	>32	>32	>32	>32	>32
	P	>32	>32	>32	>32	>32	>32	>32	>32
Onosma bracteosa Hausskn. & Bornm.	D	>32	>32	>32	>32	>32	>32	>32	>32
	M	>32	>32	>32	>32	>32	>32	>32	>32
	P	>32	>32	>32	>32	>32	>32	>32	>32
Onosma armena DC.	D	>32	>32	>32	>32	>32	>32	>32	>32
	M	>32	>32	>32	>32	>32	>32	>32	>32
	P	>32	>32	>32	>32	>32	>32	>32	>32
Onosma nana DC.*	D	>32	>32	>32	>32	>32	>32	>32	>32
	M	>32	>32	>32	>32	>32	>32	>32	>32
	P	>32	>32	>32	>32	>32	>32	>32	>32
Onosma bornmuelleri Hausskn. & Bornm.*	D	>32	>32	>32	>32	>32	>32	>32	>32
	M	>32	>32	>32	>32	>32	>32	>32	>32
	P	>32	>32	>32	>32	>32	>32	>32	>32
Onosma oreodoxa Boiss. & Heldr.	D	>32	>32	>32	>32	>32	>32	>32	>32
	M	>32	>32	>32	>32	>32	>32	>32	>32
	P	>32	>32	>32	>32	>32	>32	>32	>32
Onosma aucheriana DC.	D	>32	>32	>32	>32	>32	>32	>32	>32
	M	>32	>32	>32	>32	>32	>32	>32	>32
Doxorubicin		>2.72	0.47	0.803	0.79	0.41	0.81	0.53	0.71

P: Petroleum ether, D: Dichloromethane, M: Methanol extracts of *Onosma* root.

were significantly cytotoxic (IC_{50} < 8 µg/mL). *O. isaurica* and *O. alborosea* var. *alborosea* were strikingly effective against MCF-7, Capan-1, and HCC-1937 with IC_{50} values of less than 4 µg/mL, which corresponds to minimum test concentration for this study. Besides, the petroleum ether and dichloromethane extracts of *O. frutescens* Lam. also displayed moderate cytotoxicity against all tested cancer cell lines (IC_{50} range of 8-16 µg/mL).

Evaluating the cancer cells individually; MCF-7, Capan-1, and HCC-1937 were relatively more sensitive to cytotoxic root extracts, while Hep G2 was reasonably resistant.

Naphthoquinone type compounds are known for their lipophilic characteristics which make them very soluble in nonpolar solvents (Kumar et al., 2013). Thus, it was not surprising to observe that petroleum ether extracts have displayed higher cytotoxic activity compared to relatively polar dichloromethane and methanol extracts since *Onosma* roots mainly contain nonpolar naphthoquinone pigments.

On the other hand, these results are not adequate to prove that polar extracts are deprived of bioactive components. Since polar extracts include compounds with various polarities, the estimation of behaviors of each compound is quite difficult. The presence of several groups of secondary metabolites together in one extract may lead to synergistic, additive, or antagonistic effects (Fandiño et al., 2020). Hence, deep phytochemical investigations should be carried out also on the extracts with high IC_{50} values.

Previously some *Onosma* species were subjected to cytotoxic activity tests and promising results were reported. Kundavic et al. reported cytotoxic activity of cyclohexane extract of *O. arenaria* Waldst. & Kit. on HeLa (Human cervix carcinoma) and K562 (Human erythtoleukemic cell line) cells with IC_{50} values of 0.39 and 0.83 µM, respectively (Kundaković et al., 2006).

Vukic et al. (2018) investigated cytotoxic activities of root extracts of *O. visianii* Clem. According to their findings, acetone, chloroform, and ethyl acetate extracts, rich in acetylshikonin, isobutyrylshikonin, and α-methylbutyrylshikonin, had significant cytotoxic effects on HCT-116 and MDA-MB-231 cancer cell lines with IC_{50} values of 8.11, 13.16, 22.13 µg/mL, respectively (Vukic et al., 2018).

Mašković et al. (2015) examined aqueous extracts of *Onosma aucheriana* DC. collected from Serbia, for their cytotoxic activities using human rhabdomyosarcoma (RD), human cervix carcinoma (Hep2c), and murine fibroblast (L2OB) cell lines. Inorganic root extract inhibited the survival of cell lines with IC_{50} ranging from 25.54 to 40.34 µg/mL (Mašković et al., 2015).

Sharma et al. (2004) investigated the protective effects of *O. echioides* L. on skin carcinogenesis, tumor promoter induced markers, and oxidative stress in mice. Pretreatment of plant extract, restored the levels of reduced glutathione and cellular protective enzymes at 5 mg and 10 mg/kg b.wt doses (Sharma et al., 2004).

Root extracts of *O. taurica* var. *taurica*, *O. aucheriana* and *O. isaurica* collected from central Anatolia were evaluated for antiinflammatory and antinociceptive activities, in vivo (Tosun et al., 2008). Also, *O. isaurica* and *O. bracteosa* Hausskn. & Bornm. were assessed for their antioxidant and α-amylase and tyrosinase enzyme inhibition activities (Saravanakumar et al., 2019).

Recently, our team has investigated root extracts of 12 *Onosma* species collected from southwestern Anatolia in terms of their cytotoxic activity and stated that *O. aksoyii, O. taurica* var. *taurica*, and *O. mollis* were the most effective ones with IC_{50} values less than 8 µg/mL. Based on these promising results, bioactivity guided isolation studies were performed on the root extracts of *O. taurica* var. *taurica* and *O. mollis* resulting in the isolation of five known naphthoquinone derivatives (Güzel, 2018). Similarly, another bioactivity guided isolation study on *Onosma aksoyii* is in progress (Kul et al., 2019).

Nonetheless, bioactivity screening studies and phytochemical investigations on the genus *Onosma* still remain insufficient.

Therefore, this study is served as a national guide for researchers, suggesting strategic foresight for various phytochemical investigations such as natural product isolation studies and qualitative/quantitative profiling assays, on the genus *Onosma*. The present findings will not only encourage research teams to evaluate this highly endemic genus, but also help selecting promising species for further isolation studies. Moreover, we provide supportive information for crucial roles and usage of some *Onosma* species in traditional cancer therapy.

This is the first comprehensive bioactivity screening research on Turkish *Onosma,* and these findings obviously will draw researchers' attention to endemic *Onosma* species mainly growing in the flora of southwestern Turkey.

3.2. Topoisomerase IIα inhibition assay

O. aksoyii, *O. isaurica*, *O. taurica* var. *taurica* and *O. alborosea* subsp. *alborosea* var. *alborosea* were selected for ATP dependent topoisomerases II mediated supercoil relaxation assays based on the results of the MTT cytotoxic activity test. The petroleum ether and dichloromethane root extracts of selected *Onosma* taxa (except for dichloromethane root extract of *Onosma aksoyii*) strongly inhibited Topo IIα at 0.1 mg/mL doses (Figure).

These findings suggest that interfering with Topo IIα mediated relaxation might contribute to the mode of action of cytotoxicity of *Onosma* extracts. The petroleum ether

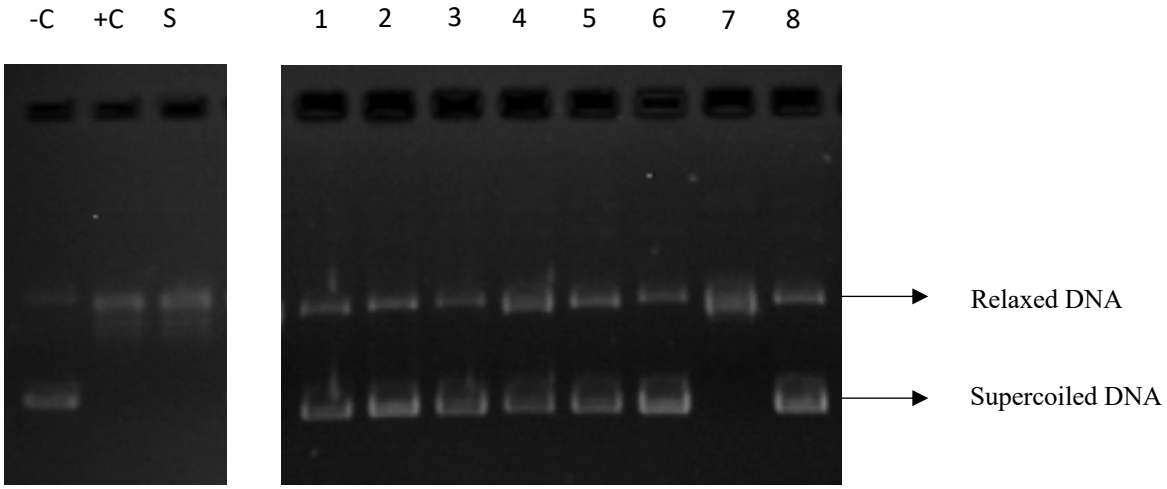

Figure. Topoisomerase IIα inhibition effects of *Onosma* root extracts.

extracts of *O. aksoyii*, *O. isaurica*, *O. taurica* var. *taurica* and *O. alborosea* subsp. *alborosea* var. *alborosea* and the dichloromethane extracts of *O. isaurica*, *O. taurica* var. *taurica* and *O. alborosea* subsp. *alborosea* var. *alborosea* were also determined as potent Topos IIα-interfering agents.

Previously, topo IIα mediated DNA cleavage activity assays were performed on a group of naphthoquinone derivatives including shikonin, plumbagin, lawson, and lapachol. Promising inhibitory activities were reported for those pigments at a dose range of 0.5 and 12.5 μM (Fujii et al., 1992). Additionally, topo I and topo II inhibitory effects of naphthoquinones have been reported by several research groups (Plyta, 1998; Ogawa et al., 2014).

Synthetic 6-(1-hydroxyiminoalkyl) derivatives of 1,4 naphthoquinones were also evaluated for their effects on topo I and the optimal synthetic group was suggested as propylated derivatives with IC_{50} range of 36-27 μM (Song et al., 2000).

More recently, our team has reported isolation studies guided by topo I and II inhibitory activity on the root extracts of *O. taurica* var. *taurica* and *O. mollis* collected from Turkey (Güzel, 2018). Shikonin, acetylshikonin, β-hydroxyisovalerylshikonin, deoxyshikonin were isolated and subjected to Topo I and II inhibition tests. All compounds were found to be effective at concentrations of 0.1 mg/mL on Topo I.

Although naphthoquinone derivatives were evaluated for their topoisomerases inhibitory activities and found highly effective (Fujii et al., 1992), bioactive potentials of *Onosma* species, which are great sources of naphthoquinones, have not been deeply investigated yet.

Since research on topoisomerase inhibitory effects of *Onosma* species are partially less, the present study will contribute to the literature with prominent results of the promising species; *O. aksoyii*, *O. isaurica*, *O. taurica* var. *taurica* and *O. alborosea* subsp. *alborosea* var. *alborosea*.

Cytotoxic agents may affect the cells via several mechanisms which involve, intercalating into DNA, generating reactive oxygen species, inducing apoptosis, and inhibiting specific enzymes such as RNA polymerases, cytochrome c oxidases, or topoisomerases. Since the cytotoxic agents may act through complex mechanisms, it was not favorable to bring up any direct correlation between our topoisomerase inhibition results and MTT findings. Still all the inhibition values of the petroleum ether extracts of tested *Onosma* species were consistent with MTT cell viability results.

MTT cell viability assays and Topo IIα inhibition tests, together suggest that the mentioned *Onosma* species strongly deserve further investigations for anticancer drug discovery.

Bioactivity screening results not only promote research on the therapeutic potential of the genus *Onosma*, but also increase the economic value of these highly endemic plants in Turkey.

One of the major factors underlying both the endemism and unique chemical diversity of *Onosma* species is their unusual serpentinic habitat. The abiotic stress and specific mineral composition of soils in high altitudes and serpentine floras, make plants prone to chemical and morphologic differentiation (Özdeniz et al., 2017). The locations where specimens have been collected for this study were mostly serpentine rocky grasslands and highlands (see Table 1). Therefore, that is possible to claim that *Onosma* species investigated within this study are likely to have rich contents with chemically unique secondary metabolites.

Taking these findings together, the present bioactivity screening study gives points to the important role of Turkish *Onosma* species in natural product chemistry and anticancer drug discovery. Further investigations are required for *Onosma* species growing in Turkey.

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