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Evaluation of Cytotoxic Effect of *Onosma armeniacum* Extract on Various Cancer Cells

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ABSTRACT

The aim of this study was to investigate the antioxidant properties of the root extract of *Onosma armeniacum* and its cytotoxic effect against human liver (HepG2), lung (A549) and colon (WiDr) cancer cell lines compared with a normal fibroblast cell line. Antioxidant properties of the extract was determined using the colorimetric methods. The cytotoxic effect of the extract were investigated using MTT assay. The total phenolic content and reducing power values of extract were 26.1±0.4 mg gallic acid equivalent and 52.6±1.1 mg trolox equivalent per g sample, respectively. The extract exhibited selective cytotoxic effect in all studied cancer cell lines, especially HepG2 (3.6-fold). The results of this study reveal that *O. armeniacum*, which has been found to have acceptable antioxidant and cytotoxic effects, should be examined with more comprehensive studies.

Research Article

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Onosma armeniacum Ekstraktının Çeşitli Kanser Hücrelerindeki Sitotoksik Etkisinin Değerlendirilmesi

ÖZET

Bu çalışmanın amacı *O. armeniacum* kök ekstraktının antioksidan özellikleri ile normal bir fibroblast hücre hattına kıyasla insan karaciğer (HepG2), akciğer (A549) ve kolon (WiDr) kanser hücre hatlarındaki sitotoksik etkisini araştırmaktır. Ekstraktın antioksidan özellikleri kolorimetrik yöntemler kullanılarak belirlenmiştir. Ekstraktın sitotoksik etkisi MTT yöntemi kullanılarak araştırılmıştır. Ekstraktın toplam fenolik içerik ve indirgeyici güç değerleri, g örnek başına sırasıyla 26.1±0.4 mg gallik asit eşdeğeri ve 52.6±1.1 mg troloks eşdeğeri olarak hesaplanmıştır. Ekstrakt, çalışılan tüm kanser hücre serilerinde, özellikle HepG2 (3.6 kat), seçici sitotoksik etki göstermiştir. Çalışmanın sonuçları, kabul edilebilir antioksidan ve sitotoksik etkilere sahip olduğu saptanan *O. armeniacum*'un daha kapsamlı çalışmalarla incelenmesi gerektiğini ortaya koymaktadır.

Araştırma Makalesi

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INTRODUCTION

Cancer, a non-communicable disease, is one of the largest causes of death, and is expected to increase by 75% over the next two decades to reach nearly 25 million cases (Wellington, 2015). Developing country populations are inexplicably affected by an increasing number of cancer cases (Pereyra et al., 2019). Africa, Asia, Central and South America account for over 60% of total cases worldwide and about 70% of cancer deaths in the world (Wellington, 2015). The loss of the cell cycle control and avoiding apoptotic death of the cells are two of the most important factors in transforming a normal cell into a malignant state (Wellington, 2015; Erdogan et al., 2020). Surgery, radiotherapy and chemotherapy are the three main approaches to cancer treatment (Pereyra et al., 2019). Although chemotherapy is a frequently used method in the treatment of cancer, some disadvantages reduce the success rate of chemotherapy (Boulos et al., 2019). The most important disadvantages of chemotherapy

are developing of drug resistance against chemotherapeutics in cancer cells and formation of harmful effect in normal cells in time (Pereyra et al., 2019). Both reasons prevent the administration of drug doses high enough to kill of all cancer cells in the body (Boulos et al., 2019). Therefore, there is an urgent need for new generation drugs with improved pharmacological properties for cancer treatment. Since 60% of the current anticancer drugs are derived from natural products, the search for new drug candidates from natural products is logical and promising (Albaqami et al., 2018; Boulos et al., 2019).

Mankind has been looking for ways to deal with diseases since ancient times, and this seeking still continues (Calhan and Gundogan, 2019). People have used the traditional herbal remedies to treat many diseases for centuries (Stanković et al., 2020). Today, the use of traditional medicines is increasing daily in developed and developing countries. According to the World Health Organization, more than half of the world population uses traditional treatment methods to relieve their ailments (Singh, 2015). The use of traditional medicine and medicinal plants has therefore attracted considerable interest in the scientific arena in recent years, and the plants are still considered to be the main source for the largest natural antioxidant and anticancer components (Moghaddam et al., 2012). Natural products are drug raw materials that need to be further tested due to their anticancer properties (Pereyra et al., 2019). However, many plants have been used in traditional therapy for centuries but have not been scientifically studied (Asadi-Samani et al., 2019). Turkey has a wide variety of plant species diversity due to its geographical location and climate (Calhan and Gundogan, 2019). The genus *Onosma* L. belongs to Boraginaceae family and contains more that 200 species which mostly spreads of China, Turkey, Iran and Pakistan. In particular, Anatolia is a central geography for 95 *Onosma* species and 48 of these species are endemic (Kumar et al., 2013). One of these species is also *Onosma armeniacum* Klokov which is especially grown in Eastern Anatolia Region in Turkey (Ozgen et al., 2004). Traditionally, *Onosma* species are used to treat rheumatism, bladder pain, kidney irritation, hypertension, tonsillitis, hemorrhoids, bronchitis, dyspnea, gynecological problems, asthma, cancer and infectious diseases (Mazandarani et al., 2011; Kumar et al., 2013; Engel et al., 2016; Stanković et al., 2020). The main secondary metabolites of this genus are alkaloids, naphthoquinones, phenolics, phytosterols, terpenoids and fatty acids (Mazandarani et al., 2011; Engel et al., 2016). Wound-healing, antioxidant, anti-inflammatory, antimicrobial and anticancer activities of this genus have been demonstrated in previous studies and these activities are attributed to the above mentioned compounds (Moghaddam et al., 2012; Kumar et al., 2013; Engel et al., 2016). In addition, some *Onosma* species have been used in the cosmetic industry in recent years (Sivaci et al., 2015).

Researching the cytotoxic effect of Boraginaceae species has become very popular in recent years and the number of scientific works in this field is increasing daily. Poma *et al.* (2018) reported that *Glandora rosmarinifolia* extract exhibits antiproliferative effect on human liver and breast cancer cells through inducing oxidative stress, while Aïssaoui *et al.* (2019) demonstrated that *Heliotropium bacciferum* extract has selective cytotoxic effect on human colon cancer (HCT-116 and DLD1) cells compared with human normal fibroblast cells. Recently, Erdogan *et al.* (2020) demonstrated that *Paracaryum bingoelianum* extract has moderate cytotoxic effect on human breast (MCF-7) and colon (HT-29) cancer cells through inducing apoptosis. However, to the best of knowledge, the cytotoxic effect of *Onosma armeniacum* is not explored yet. The aim of this study was to investigate the antioxidant properties of *Onosma armeniacum* extract and its cytotoxic effect against human liver (HepG2), lung (A549) and colon (WiDr) cancer cell lines compared with a normal fibroblast (BJ) cell line.

MATERIALS and METHODS

Chemicals

All the chemicals and solvents used in extract preparation, antioxidant activity analysis and cytotoxicity experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the chemicals and solutions used in cell culture studies were purchased from Lonza (Verviers, Belgium) and Biological Industries (Kibbutz Beit Haemek, Israel).

Plant Material

The roots of *Onosma armeniacum* were collected from Erzurum province of Turkey. The plant samples were authenticated by Professor Ufuk Ozgen (Karadeniz Technical University, Faculty of Pharmacy, Department of Pharmacognosy). A voucher specimen was deposited in the Herbarium of the Faculty of Pharmacy of Ankara University (AEF-23796).

Preparation of Extract

The root samples were left to dry at room temperature for 15 days. The root parts of the dried plant were carefully powdered using a mechanical grinder (Retsch ZM 200, Haan, Germany). For preparing stock extract, 1 g of the powder material was weighed and mixed with 20 mL dimethyl sulfoxide (DMSO) and then mixture was continuously stirred at 45ºC for 24 h. At the end of the period, the mixture was filtered through filter paper (Whatman Grade 1) and 0.2 µM filter and stored at -20ºC by aliquoting (Mazandarani et al., 2011; Demir et al., 2018a).

Total Phenolic Content (TPC) Analysis

The total phenolic content of the extract was determined with Folin-Ciocalteu reagent using the method of developing by Slinkard and Singleton (1977). Gallic acid was used as a standard and the result was expressed as mg gallic acid equivalent per g powder of dry plant (mg GAE g sample^{-1}) (Aliyazicioglu et al., 2017).

Total Flavonoid Content (TFC) Analysis

The total flavonoid content of the extract was determined with aluminium chloride method (Moreno et al., 2000). Quercetin was used as a standard and the result was expressed as mg quercetin equivalent per g powder of dry plant (mg QE g sample^{-1}) (Demir et al., 2018b).

Reducing Power Analysis

The ferric reducing power of extract was determined using the method of developing by Oyaizu (1986). Trolox was used as a standard and the result was expressed as mg trolox equivalent per g powder of dry plant (mg TE g sample^{-1}) (Ozkan et al., 2017).

Cell Culture

A549, WiDr, HepG2 and BJ cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). All cells were cultivated in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin solution and with a 5% CO_2 supply at 37°C (Demir et al., 2019a).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl-Tetrazolium Bromide (MTT) Assay

The cytotoxic effect of extract and cisplatin (was used as a positive control) was determined using colorimetric MTT assay (Mosmann, 1983) with slight modifications (Turan et al., 2018). Cancer cells were seeded in 96-well plates at 5000 cells per well, while BJ cells were seeded at 2500 cells per well and overnight incubated at 37ºC. After incubation, the medium was discarded and the cells were exposed with different concentrations of extracts (2.5-250 µg mL^{-1}) (Engel et al., 2016) and cisplatin (0.09-12 µg mL^{-1}) (Demir et al., 2019b) for 72 h. DMSO concentration to which cells were exposed was kept below 0.1% (v/v), and only cells treated with 0.1% (v/v) DMSO formed the negative control group (Engel et al., 2016). At the end of the period, the media were removed and fresh medium containing 0.25 mg mL^{-1} MTT dye was added to each well. After two hours of incubation, the media were removed, and the formed formazan crystals were dissolved in DMSO. The absorbance value in wells were read using a microplate reader reader (Versamax Molecular Devices, CA, USA) at 570 nm. Cell viability (%) was calculated used with following formula (Turan et al., 2017):

$$\text{Cell viability (\%)} = (OD_{\text{treatment group}} / OD_{\text{control group}}) \times 100$$

The dose-response curves were drawn using %logarithmic concentrations against cell viability and the IC_{50} value of extract and cisplatin were calculated for each cell line (Demir et al., 2020). The IC_{50} values calculated for extract and cisplatin in all cell lines were used to determine the selectivity index (SI) value with the following formula (Turan et al., 2019):

$$SI = \text{BJ cells } IC_{50} / \text{Cancer cells } IC_{50}$$

Statistical Analysis

All experiments were performed at least four times. The distribution of the data was examined with the Kolmogorov-Smirnov test. Data showing normal distribution were expressed as arithmetic mean±standard deviation. Statistical analyzes between the groups were revealed by ANOVA and post-hoc Tukey tests. $p<0.01$ was regarded as significant.

RESULTS and DISCUSSION

The situation that endogenous and exogenous redox reactions do not balance by the antioxidant system is called “oxidative stress” (Turkmen et al., 2016). It is known that increased oxidative stress causes cell damage and cell death and oxidative stress is among the most important causes of fatal diseases, such as cancer, diabetes, neurodegenerative and cardiovascular diseases (Yalcin et al., 2016). Antioxidant molecules prevent diseases by preventing the formation of free radicals, scavenging the free radicals or repairing the harmful effects of free radicals (Misir et al., 2018). There is no doubt that plants are a good source for biologically active phytochemicals and to date more than 4000 phytochemicals have been identified in plants, including phenolic acid, tannine, coumarin, anthraquinone, flavonoid, phenolic diterpene and anthocyanin subgroups (Mazandarani et al., 2011). Phenolics are known to have various biological activities, such as antioxidant, anti-inflammatory, antimicrobial and anticancer (Mazandarani et al., 2011; Yalcin et al., 2016). The determination of phenolic content and antioxidant activity in plant materials is seen as a starting point for investigating potential health benefits (Stanković et al., 2020). The antioxidant potential of the extract were therefore determined using the TPC, TFC and reducing power methods and the results were shown in Table 1.

In other studies examining the antioxidant properties of different *Onosma* species, Ebrahimzadeh *et al.* (2010) reported that TPC and TFC values of *O. demawendicum* extract are 47.2±2.2 mg GAE g sample^{1}, and 13.7±0.8 mg QE g sample^{-1}, respectively.

Table 1. Antioxidant properties of *O. armeniacum* extract (n=6)
Çizelge 1. O. armeniacum ekstraktının antioksidan özellikleri (n=6)

Antioxidant Parameters	
Total Phenolic Content (mg GAE g sample^{-1})	26.1±0.4
Total Flavonoid Content (mg QE g sample^{-1})	13.7±0.9
Reducing Power (mg TE g sample^{-1})	52.6±1.1

While Mazandarani *et al.* (2011) reported that TPC and TFC values of different *O. dichroanthum* extracts are range from 4.5 to 125.6 mg GAE g sample^{-1}, and 9.8 to 41 mg QE g sample^{-1}, respectively. Gharib and Godarzee (2016) demonstrated that TPC and TFC values of different *Onosma* species (*O. bulbotrichum*, *O. microcarpum* and *O. sericeum*) are range from 3.4 to 19.3 mg GAE g sample^{-1}, and 2.5 to 14.5 mg QE g sample^{-1}, respectively, while Khaledi *et al.* (2018) reported that TPC and TFC values of *O. sericeum* extract are 54.5 mg GAE g sample^{-1}, and 13.8 mg QE g sample^{-1}, respectively. Calhan and Gundogan (2019) reported that TPC and TFC values of different *O. sericeum* extracts are range from 58.4 to 93.3 mg GAE g sample^{-1}, and 21.8 to 60.3 mg QE g sample^{-1}, respectively, while Stanković *et al.* (2020) reported that TPC and TFC values of different *Onosma* species (*O. sericea* and *O. stenoloba*) are range from 32.5 to 69.8 mg GAE g sample^{-1}, and 8.4 to 52.6 mg rutin equivalent g sample^{-1}. This results were consistent with the findings of many research groups investigating the antioxidant properties of different *Onosma* extracts. The minor differences between this results and the results of other studies may be due to the plant species, the extraction method and the solvent type.

Cancer is one of the most dangerous and rapidly spreading diseases today, and colon, lung and liver cancer are among the most common types of cancer (Erdogan et al., 2020). Herbal products with the ability to inhibit cell proliferation and induce apoptosis are widely researched for the development of anticancer drugs (Albaqami et al., 2018). Although there are some reports about the cytotoxic effect of various Boraginaceae species (Poma et al., 2018; Aïssaoui et al., 2019; Erdogan et al., 2020), there is no study about the cytotoxic effect of *Onosma armeniacum*. Therefore, this study aimed to determine the cytotoxic effect of *O. armeniacum* extract on various cell lines, including A549, HepG2 and WiDr, representing common cancers. The cytotoxic effect of the extract on these cell lines was determined using MTT assay and the growth curves of the cells were shown in Figure 1.

When all cells are evaluated together, statistically significant cytotoxic effect of extract was emerged starting at a concentration of 5 µg mL^{-1} and extract exhibited cytotoxic effect in all studied cancer cells in a dose-dependent manner.

Cisplatin was used as a positive control in cytotoxicity experiments and the growth inhibitor effect of cisplatin on all studied cell lines was shown in Figure 2.

When all cells are evaluated together, statistically significant cytotoxic effect of cisplatin was emerged starting at a concentration of 0.09 µg mL^{-1} and cisplatin exhibited cytotoxic effect in all studied cancer cells in a dose-dependent manner. In order to make the results more understandable, the IC_{50} values (µg mL^{-1}) of extract and cisplatin in all studied cell lines were calculated and presented in Table 2.

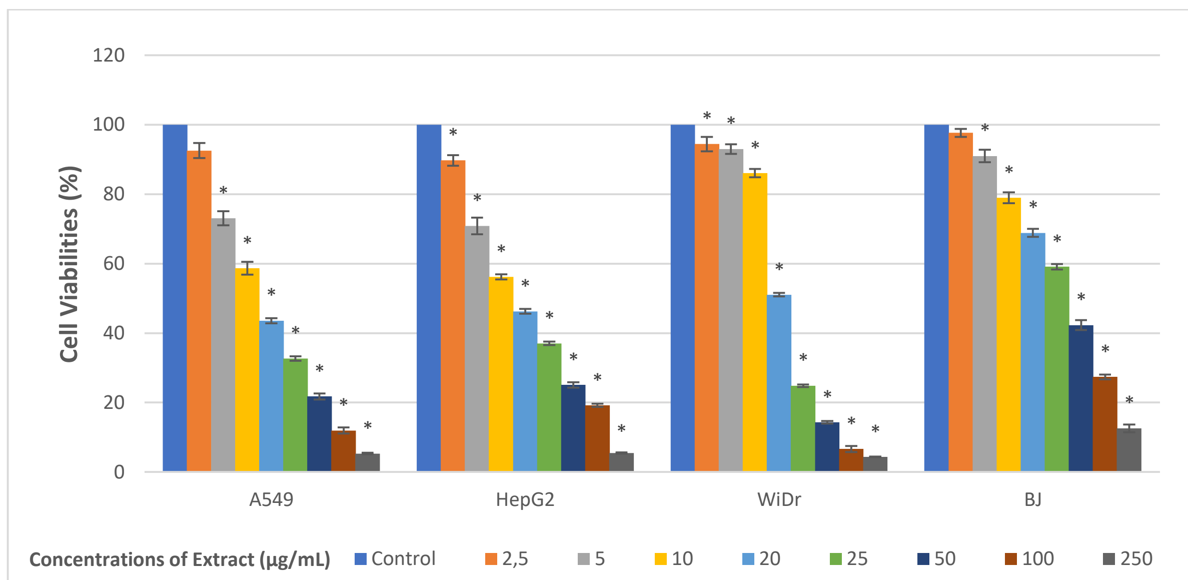

Figure 1. The cytotoxic of *O. armeniacum* extract on cancer and BJ cell lines.
*Denotes statically significant differences in comparison with control (p<0.01).
Şekil 1. O. armeniacum ekstraktının kanser ve BJ hücre hatları üzerindeki sitotoksik etkisi.
**Kontrol ile kıyaslandığında istatistiksel olarak anlamlı farkı belirtir (p<0.01).*

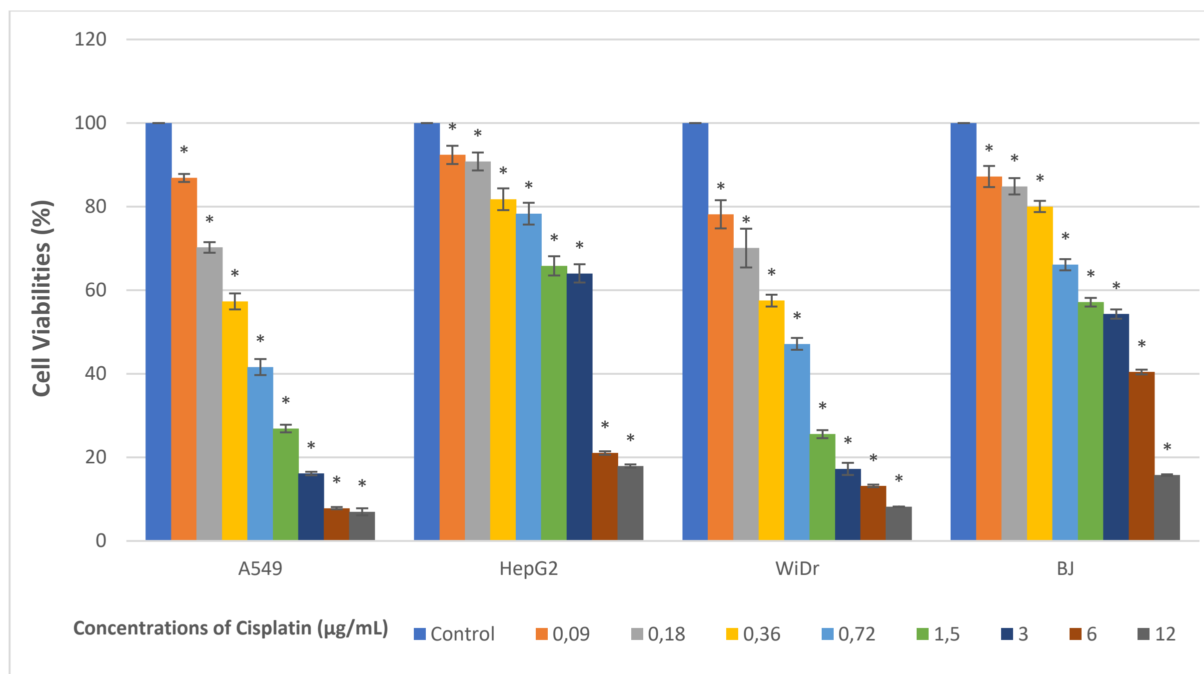

Figure 2. The cytotoxic of *cisplatin* on cancer and BJ cell lines.
*Denotes statically significant differences in comparison with control (p<0.01).
Şekil 2. Cisplatinin kanser ve BJ hücre hatları üzerindeki sitotoksik etkisi.
**Kontrol ile kıyaslandığında istatistiksel olarak anlamlı farkı belirtir (p<0.01).*

Table 2. IC_{50} values (µg mL^{-1}) calculated for *O. armeniacum* extract and cisplatin (n=4)
Çizelge 2. O. armeniacum ekstraktı ve cisplatin için hesaplanan IC_{50} (µg mL^{-1}) değerleri (n=4)

	Extract	Cisplatin
A549	17.5±0.5	0.73±0.02
HepG2	12.8±0.5	4.11±0.01
WiDr	18.3±0.3	0.78±0.03
BJ	45.9±1.0	5.15±0.09

When Table 2 is examined, it is seen that the IC_{50} values for extract ranged from 12.8 to 45.9 µg mL^{-1}. According to the US National Cancer Institute, the extract with an IC_{50} value lower than 30 µg mL^{-1} against tumor cell lines in *in vitro* conditions are considered promising for anticancer drug development (de Oliveira et al., 2016). From this point of view, it is seen that the cytotoxic effect of the extract on all three cell lines meets this condition. In previous studies showing the cytotoxic effect of different *Onosma* species, Engel *et al.* (2016) reported that *Onosma hispida* extract exhibits moderate cytotoxic effect on two bone (MG-63, Saos-2) and two breast (BT-20, MCF-7) cancer cell lines, while Vukic *et al.* (2018) reported that *Onosma visianii* extract exhibits antiproliferative effect on human colon (HCT-116) and breast (MDA-MB-231) cell lines through inducing apoptosis and cell cycle arrest at G_2/M phase. Albaqami *et al.* (2018) reported that *Onosma bracteatum* extract exhibits cytotoxic effect on human prostate (PC-3), lung (A549) and breast (BT549) cancer cells in a dose-dependent manner through inducing caspase-3 activity, while Asadi-Samani *et al.* (2019) reported that *Onosma sericeum* extract exhibits pretty low cytotoxic effect (IC_{50}>300 µg mL^{-1}) on human breast cancer (MCF-7 and MDA-MB-231) cells. Recently, Calhan and Gundogan (2019) reported that *Onosma sericeum* extract exhibits cytotoxic effect on human breast cancer (MCF-7) cells in a dose-dependent manner.

Selectivity is one of the most important criteria for a compound to be evaluated as a chemotherapeutic (Aliyazicioglu et al., 2019). For this reason, one normal cell line (BJ) was used along with three cancer cells in the study. The SI of extract and cisplatin for all studied cancer cells were calculated using the formula described in the "Materials and Method Section" of the IC_{50} values obtained for each cell and results were presented in Table 3.

Table 3. SI values of *O. armeniacum* extract and cisplatin
Çizelge 3. O. armeniacum ekstraktı ve cisplatin için hesaplanan SI değerleri

	Extract	Cisplatin
A549	2.6	7.0
HepG2	3.6	1.3
WiDr	2.5	6.6

The results showed that the extract exhibited a highly selective cytotoxic effect, especially in the HepG2 cell lines. It is emphasized that the SI value for a compound or extract whose cytotoxic activity has been investigated should be at least 2 (de Oliveira et al.,

2016). From this point of view, the SI value of the extract in all three cell series satisfied this condition.

Onosma species are reported to be rich in phenolics (benzoic acid, caffeic acid, chlorogenic acid, coumaric acid, ferulic acid, protocatechuic acid, rosmarinic acid, apigenin, catechin, kaempferol, quercetin and luteolin) and naphthoquinones (shikonin derivatives) (Ozgen et al., 2004; Kumar et al., 2013; Stanković et al., 2020). Various studies reported that phenolic and naphthoquinone derivatives exhibit antiproliferative effect on various types of cancer, such as leukemia, melanoma, glioma, prostate, liver, breast, bladder, gastric, thyroid, cervix, colon and lung. The antiproliferative activities of these compounds are explained by several cellular mechanisms, such as their ability of modulating apoptosis, necroptosis, reactive oxygen species (ROS) formation, glycolysis, inflammation, angiogenesis, metastasis, autophagy, ferroxitosis, cell cycle arrest, mitochondrial function, endoplasmic reticulum stress, cytoskeleton formation, and PI3K/AKT/mTOR and RAS/RAF/MEK/ERK signaling pathways (Zhou et al., 2016; Abotaleb et al., 2019; Boulos et al., 2019; Wang et al., 2019). Therefore, it could be considered that the selective cytotoxic activity of the extract may be due to the synergistic effect of its phenolic and naphthoquinone content.

CONCLUSION

This is the first study to determine the antioxidant potential and selective cytotoxic effect of *Onosma armeniacum* in the literature. It is believed that it would be beneficial to identify the active compounds of the plant and the cytotoxic mechanisms of these compounds, as well as to support studies with *in vivo* experiments.

Researchers Contribution Rate Declaration Summary

The authors declare that they have contributed equally to the article.

Conflicts of Interest Statement

None of the authors had any financial or personal relationships with other individuals or organizations that might inappropriately influence their work during the submission process.

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