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Onosma heterophylla'nın Antioksidan, Yara İyileştirici ve Sitotoksik Özellikleri

Laçine AKSOY[1*], Ömer HAZMAN[1], Ahmet BÜYÜKBEN[2], Şevval CANDAN[1], Emine COŞKUN[1], Hanife Sena ARSLAN[1], Feyza EFİLOĞLU[1]

Öne Çıkanlar:
- Akciğer kanser hücresi
- MTT
- Proliferasyon etkisi

Anahtar Kelimeler:
- A549
- Antikanserojen
- Hücre çiziği
- L929
- *Onosma heterophylla*

ÖZET:

Çalışmada, *Onosma heterophylla*'nın toprak üstü kısımları kullanılarak hazırlanan sulu ekstraktların antioksidan, antikarsinojenik ve yara iyileştirici aktivitelerinin belirlenmesi amaçlanmıştır. Sulu ekstraktlar infüzyon ve maserasyon yöntemleri kullanılarak hazırlanmıştır. Ekstraktların antioksidan aktivitesini belirlemek için toplam fenolik madde içeriği, total antioksidan ve oksidan kapasitesi ile oksidatif stres indeks düzeyleri belirlenmiştir. Sitotoksik aktiviteyi belirlemek için L929 hücreleri ve A549 hücreleri kullanılmıştır. L929 hücreleri yara modelinde kullanılmıştır. *O. heterophylla*'nın aynı familyadaki türlere benzer antioksidan özelliklere sahip olduğu görülmektedir. Bu türle ilişkili en önemli biyolojik aktivite antikarsinojenik ve yara iyileştirici etkidir. Uygulanan hiçbir dozun sağlıklı L929 hücrelerinde hücre canlılığını azaltıcı bir etkisi yoktur. 500-1000 µg/mL konsantrasyonunda sağlıklı hücrelere zarar vermeden kanserli hücrelerin canlılığını azaltırken, 125-250 µg/mL konsantrasyonunda sağlıklı hücrelerin canlılığını artırmış kanserli hücrelerin canlılığını azaltmıştır, bu da çok etkili antikarsinojenik etkiye sahip fitokimyasallar içeren bir bitki olduğunu göstermektedir. *O. heterophylla* özütünün yara üzerinde %83.72±2.53 oranında kapanma oranına sahip olduğu gözlenmiştir. Gelecekteki çalışmalarda bitkinin biyolojik aktivitelerinden sorumlu bileşen, özellikle antikarsinojenik ve yara iyileştirici etkileri analiz edilebilir.

Antioxidant, Wound Healing and Cytotoxic Properties of *Onosma heterophylla*

Highlights:
- Lung cancer cell
- MTT
- Proliferation effect

Keywords:
- A549
- Anticarcenogenic
- Cell scratch
- L929
- *Onosma heterophylla*

ABSTRACT:

The study was aimed to determine the antioxidant, anticarcinogenic and wound healing activities of aqueous extracts prepared using *Onosma heterophylla* aerial parts. Aqueous extracts were prepared using infusion and maceration methods. Total phenolic substance content, total antioxidant and oxidant capacity with oxidative stress index levels were determined for to state the antioxidant activity. L929 cells and A549 cells were used to determine cytotoxic activity. L929 were used in the wound model. It is seen that the *O. heterophylla* has antioxidative properties like the species in the same family. The most important biological activity associated with this species is the anticarcinogenic and wound healing effect. No dose applied has a decreasing effect on cell viability on healthy L929 cells. While it reduces the viability of cancerous cells without harming healthy cells at a concentration of 500-1000 µg/mL, it increases the viability of healthy cells and reduces the viability of cancerous cells at a concentration of 125-250 µg/mL, indicating that it is a plant containing phytochemicals with a very effective anticarcinogenic effect. It was observed that *O. heterophylla* extract had a closure rate of 83.72±2.53% on the wound. In future studies, the component responsible for the biological activities of the plant, especially its anticarcinogenic and wound healing effects, can be analyzed.

[1] Laçine AKSOY (**Orcid ID:** 0000-0001-8086-5079), Ömer HAZMAN (**Orcid ID:** 0000-0002-2702-6847), Şevval CANDAN (**Orcid ID:** 0009-0009-9772-2758), Emine COŞKUN (**Orcid ID:** 0009-0007-9507-6444), Hanife Sena ARSLAN (**Orcid ID:** 0009-0006-2285-5562), Feyza EFİLOĞLU (**Orcid ID:** 0009-0004-7443-6357), Afyon Kocatepe University, Faculty of Science and Arts, Department of Chemistry, 03200, Afyonkarahisar, Türkiye
[2] Ahmet BÜYÜKBEN (**Orcid ID:** 0000-0002-5639-7217), Afyon Kocatepe University, Çay Vocational School, Program of Chemistry Technology, 03200, Afyonkarahisar, Türkiye
***Sorumlu Yazar/*Corresponding Author**: Laçine AKSOY, e-mail: lacinetur@aku.edu.tr

INTRODUCTION

Plants face environmental challenges. Various abiotic factors cause the production of reactive oxygen species. This condition is known as oxidative stress. Plants have a wide antioxidant defense system oxidative damage. Secondary metabolites (polyphenols, flavonoids, terpenoids) formed under different environmental stresses. Antioxidants found in plants play an important role in the acclimatization of plants and their adaptation to environmental challenges. They are also beneficial to human health (Liu et al., 2015; Maury et al., 2020; Khare et al., 2020).

Boraginaceae is a family containing approximately 2000 species. Many plants used in medicine and cosmetics are from this family. It is used therapeutically with its content of many biologically active compounds, including flavonoids, terpenoids and phenols. They exhibit antimicrobial, contraceptive, antitumor, cardiotonic, and antiplatelet activities due to the phytochemicals found in their structures (Taravati et al., 2014; Dresler et al., 2017). *Onosma* L. (Boraginaceae) is a genus whose systematics is quite difficult to study (Aytaç and Türkmen, 2011). *O. heterophylla* is a perennial plant and is a semi-shrub species. The plant has a long branched, sharp stem. It has several stems, 15-40 cm, short hairy, awned, simple or branched. Base and lower stem leaves are 25-150 x 3-5 mm, both surfaces are hairy, but the hairs on the upper surface are longer. It is linear-rectangular in shape. Crown color is pale yellow. It is 20-30 mm and tapers from top to base and has a hairy appearance. It grows in a temperate ecosystem. It grows naturally in meadows, rocky lands, clearings of mixed oak forests, river crossings and pastures. The flowering period occurs in June and July. It is also known as Deli Emzik (Davis, 1978; Mellidis et al., 1993).

A wound is defined as the disruption of the cellular, anatomical and functional continuity of a living tissue. To restore the structure of injured tissue, a complex process must occur that involves migration, proliferation, and a complex signaling network (Masson-Meyers et al., 2020).

Cancer is one of the leading causes of morbidity and mortality. Because cancer cells lose many of the regulatory functions found in normal cells, they continue to divide when normal cells do not. Various toxicities such as cardiotoxicity, myelotoxicity, and renal toxicity may occur as a result of chemotherapy treatments (Manil et al., 1995; Desai et al., 2008).

Plants have tremendous potential to provide new medicines. They are considered to be a repository of natural chemicals that may be effective against cancer, and oxidative stress for this reason. In this study, the antioxidative, anticarcinogenic effects and wounds healing effects of the aqueous extract of the *O. heterophylla*, about which there are very limited studies, were investigated.

MATERIALS AND METHODS

Plant Material and Extraction

O. heterophylla plant was collected from Sinanpaşa district of Afyonkarahisar (38° 44′ 40″ North, 30° 14′ 34″ East) in July 2023. It was identified by Dr. Mustafa Kargıoğlu (Herbarium code: AKU10824) The aboveground part of the plant species was used in the studies. The aerial parts of the species were pulverized using a blender (Waring 32BL80, Connecticut, USA). The extraction process was carried out for this plant species, which was homogenized by pulverization. Maceration and infusion processes were applied for the extraction of the plant species. 10 grams of the aerial parts, which were ground into powder, were weighed. 100 mL of 60 °C deionized water was added. The prepared solutions were incubated in an ultrasonic bath. Extracts taken from the dark environment were filtered. The solvent was removed using the rotary evaporator and the extract was obtained. Studies were started with the prepared plant extracts.

Cell Culture Studies

Cell culture studies were started to evaluate the cytotoxicity activities of extracts prepared using the aerial parts of the *O. heterophylla* plant species. A549 lung cancer cell line and L929 mouse fibroblast cell line were used. Before starting cell culture studies, the required medium was prepared for processes such as production, thawing, freezing and propagation of cells. All necessary substances were added to the nutrient solutions in certain amounts in order for the cells in the laboratory environment to continue their vital activities. An incubator (Nüve EC 160) was used, providing 5% CO_2 environmental conditions at 37 °C. All A549 and L929 cells were prevented from dissolving and transferred into falcons containing medium. and centrifuged. The cell pellet was suspended undissolved in medium. After the procedure, the cells were placed in the incubator for growth and maintenance. Observations were made daily by taking images of the cells in the incubator with an invert microscope (VWR ECN: 630-2738). At this stage, the media were changed every two days until the cells were confluent. After the passaging process was completed, when a sufficient amount of confluent was reached, the cells were counted.

3-4,5-dimethyl-thiazolyl-2,5-diphenyltetrazolium bromide (MTT) analysis is a test used to evaluate cell metabolic activity by determining the rate of cell viability and toxic effects on living cells. Cells (A549 and L929) were planted in 96-well plates for MTT analysis, with 10^4 cells per well. One day later, different concentrations of plant extracts were added to the prepared concentrations and the aqueous mixtures were added to the first set wells in three repetitions. MTT solution was added to wells. Color changes were observed at the end of the waiting process. As a result, purple colored formosan crystals were dissolved. Measurements were made using a microplate reader (Biotek, ELx800) to measure the absorbance of the well at 540 nm. The control groups, to which no treatment was applied, were considered 100% alive, and the effect of the doses on cell viability was calculated with the formula below (Hazman et al., 2021).

Cell Viability (%) = $[(100*\text{Absorbance}_{\text{sample}}) / (\text{Absorbance}_{\text{control}})]$

Determination of Total Phenolic Substance Amount

Total phenolic acid measurements were made using the Folin-Ciocalteu method. Since caffeic acid is a phenolic antioxidant, the total phenolic substance amount of caffeic acid was determined to compare the phenolic substance amounts in the extracts. First, a standard curve was drawn for gallic acid. Standard solutions of gallic acid at different concentrations were prepared. Folin-Ciocalteu reagent was added to the prepared caffeic acid, standards and plant extracts. After 5 minutes, Na_2CO_3 was added. The absorbance against water was measured at 763 nm on a UV spectrophotometer (Nuve NF 400). With the straight equation obtained from the gallic acid standard curve, the total amount of phenolic acid in 1 mg of plant extracts was found as gallic acid equivalent (µg GAE/mg extract) (Singleton and Rossi, 1965).

Total Antioxidant, Oxidant Capacity and Oxidative Stress Index

The materials we used for TAC and TOC levels were measured with commercial kits (Rell Assay, Gaziantep, Turkey) that work with the spectrophotometric method. Aqueous solutions of the plant extracts at a concentration of 1 mg/ml were prepared. Ethyl alcohol was used as a blank solution. The total amount of antioxidants in the samples was determined to be equal to trolox. TAC levels of all samples were determined with the line equation (Erel, 2004). Standard hydrogen peroxide (H_2O_2) was used for TOC levels. Reagents were added to the aqueous solutions of the extract at a concentration of 1 mg/ml in accordance with the protocol, and TOC levels of each sample were determined with optical densities determined by spectrophotometric (540 nm) measurments (Erel, 2005). The oxidative stress

index (OSI) calculated by dividing TOC levels by TAC levels (OSI=TOC/TAC) (Hazman et al., 2022).

Wound Healing Effect

The wound healing effect of the plant was determined by in vitro cell scratch wound healing test. The mouse fibroblast cells (L929) were used in the study. The wound healing model was created using a 24-well tissue culture dish. 10^5 number L929 cells were planted in each well in 800 µl of medium. The cells were cultured until they became confluent. A wound model was created by making linear scratches with a pipette tip. The images obtained at the end of 48 hours were delimited by the edges of the cells participating in the formation of the wound model using imageJ software. Based on the pixels, the amount of cell-free area (wound) in between was calculated as a percentage. The wound closure rates in the zero-hour acellular area ($Area_{t0}$) and the forty-eighth hour acellular area ($Area_{t48}$) were calculated with the following formula (Kaptaner İğci and Aytaç, 2020).

Cover ratio = $[(Area_{t0}-Area_{t48})/Area_{t0}] \times 100$

Statistical Analysis

SPSS20 was used to evaluate the data obtained. As a result of the evaluation, Duncan post test and ANOVA (one-way analysis of variance) were performed on the data obtained for each parameter with "mean±standard deviation". Differences with statistical significance ($p<0.05$) between study groups were indicated using (a, b, c..) or (*) symbols.

RESULTS AND DISCUSSION

Plants are an important part of nature's arsenal of medicinal substances and are almost completely free of the side effects often caused by synthetic drugs and chemicals. Various compounds from plants are evaluated for their phytotherapeutic properties. However, it is accepted that the medicinal effects of plants result from the synergistic and/or antagonistic interaction of compounds present in the whole plant (Vaou et al., 2022). Although there are studies examining the biological characteristics of different species of the Onosma genus, no studies have been found on the *O. heterophylla* plant species. The antioxidant, cytotoxic, anticarcinogenic levels and wound healing effects of aqueous *O. heterophylla* extracts were examined in the presented study.

Total Phenolic Substance Content, Total Antioxidant and Oxidant Capacity, Oxidative Stress Index

Phenolic compounds have a structure consisting of an aromatic ring containing hydroxyl. There are approximately 8000 phenolic substances naturally. Plant phenolics act as primary antioxidants or hydrogen atom donors, reducing agents, free radical and oxygen scavengers. Phenolic compounds found in plants have redox properties and these properties enable them to act as antioxidants. The Folin-Ciocalteu is a more preferred method for the determination of total phenolics (Hatami et al., 2014)

In this study, when the extract was reacted with Folin-Ciocalteau reagent in alkaline environment, a blue colored solution was obtained due to the presence of the complex. As a result of the analyses, the total phenolic substance amount of caffeic acid, which is known to be a phenolic antioxidant substance, was determined. Figure 1A shows the amount of caffeic acid and the total phenolic substance of the extract. While the total phenolic substance amount of caffeic acid was 1054.1±22.2 µg GAE/mg, the total phenolic substance amount of the extract was found to be 134.11±11.02 µg GAE/mg extract. In a study conducted in the literature on *Onosma armeniacum,* which belongs to the same genus, the total phenolic content of this species was determined as 26.1±0.4

µg GAE/mg. It is seen that *O. heterophylla* has more total phenolic content than *O. armeniacum* (Demir et al., 2021).

In TAC analyses, a fixed radical is generally used. TAC analyzes are widely used on biological samples. The most commonly used direct assay is the Trolox equivalent antioxidant capacity test. TAC analysis, also known as TEAC, is based on the inhibition of absorbance by antioxidants of the ABTS•+ radical cation, which has a characteristic absorption spectrum. TEAC analysis measures a compound's ability to reduce ABTS+. The biggest advantage of the ABTS test compared to other analyzes is that ABTS easily dissolves in both organic and aqueous solvents, therefore it can be applied to both hydrophilic and lipophilic antioxidants (Silvestrini et al., 2023). In the presented study, the total antioxidant capacity of *O. heterophylla* was determined as 7.26±0.86 mmol Trolox Eqiv./L. In a study conducted in the literature, the chemical compositions and antioxidant and enzyme inhibitor activities of *O. sintenisii* and *O. mutabile* were investigated. *O. mutabile* extract was found to exhibit higher activity in all antioxidant activity tests. The ABTS radical scavenging activity of *O. sintenisii* was determined to be approximately 80 mg Trolox Equiv./g extract, while the radical scavenging activity of *O. mutabile* was determined to be approximately 125 mg Trolox Equiv./g extract (Ozer et al., 2022).

Direct measurement of oxidative stress levels due to free radicals is extremely difficult due to their very short half-lives. The three most commonly used colorimetric methods to measure oxidative stress are the TMB-peroxidase analysis, the free oxygen radical test, also called the FORT test, and the oxidation of ferrous ion to ferric ion and its analysis with xylenol orange (FOX1). In addition, FOX2 analysis applied by updating FOX1 is also a very common spectrophotometric method. The FOX2 test, which was also applied in the presented study, is based on the oxidation of ferrous iron to ferric iron by various sample peroxide types can be measured (Buico et al., 2009). The TOC value of *O. heterophylla* was measured as 21.37±1.53 µmol H_2O_2 Eqiv./L.

Oxidative Stress Index (OSI) is an indicator of the degree of oxidative stress. The oxidative stress index of *O. heterophylla* is 2.95±0.17 Arbitrary units. Figure 1B shows the total antioxidant and oxidant capacity (TOC), oxidative index values of the plant extract.

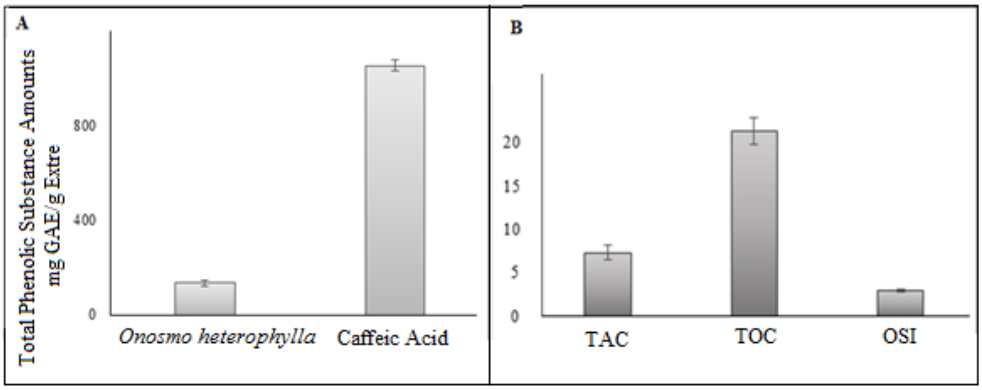


Figure 1. A. *Onosma heterophylla* total phenolic substance amount of plant extract and caffeic acid, B: *Onosma heterophylla* TAC (Total Antioxidant Capacity), Total Oxidant Capacity (TOC), and OSI (Oxidative stress index). TAC; mmol Trolox Eqiv./L, TOC; µmol H_2O_2Eqiv./L, OSI; Arbitrary Unit

Cytotoxicity Levels and Anticarcinogenic Activity of *Onosma heterophylla* Aqueous Extracts

Medicinal plants can be used directly as medicine and contain powerful bioactive compounds. These herbs have been found to have healing wounds, treating skin diseases, urethral infection, rheumatism and cancer activities. There are approximately 250,000 plant species in the plant kingdom, and only 10% of them have been investigated for the treatment of many diseases (Iqbal et al., 2017; Patel et al., 2010).

In the analysis of the cytotoxicity levels and anticarcinogenic activity of the aqueous extracts of the *O. heterophylla* plant on the A549 lung cancer cell line, it was observed that the aqueous extracts prepared at high concentrations reduced the A549 cell viability. It is desirable that the viability of cancerous cells decreases at high concentrations (Fig.2.). Because for anticarcinogenic activity, it is desired that cancer cells decrease, not increase. Based on this, it can be said that the anticarcinogenic activity of *O. heterophylla* in high doses on the A549 lung cancer cell line is high. Considering the low doses (15.625 µg/mL, 31.25 µg/mL and 62.5 µg/mL), it was seen that the anticarcinogenic activity was close to the control group. In other words, it is understood that the aqueous extracts of the plant at low concentrations do not have much effect on the A549 lung cancer cell line. Briefly, it was observed that application of *O. heterophylla* aqueous extract to A549 cells at concentrations of 125 µg/mL and higher reduced cell viability to less than 20% compared to the control group. It appears that application of the aqueous extract to A549 cells at concentrations of 62.5 µg/mL and lower has no effect on cell viability.

When the cytotoxicity of L929 cells, which is preferred as a healthy cell line, was examined, it was seen that it increased cell viability at low doses. It appears that high doses do not have negative effects on cell viability. In this case, it appears that it only reduces the viability of cancerous cells without damaging healthy cells, especially at a concentration of 500-1000 µg/mL. It is also noteworthy that it increases healthy cell viability and reduces cancerous cell viability at a concentration of 125-250 µg/mL. Besides all these, no dose has a decreasing effect on cell viability on healthy L929 cells. As for L929 cells, it is observed that the aerial parts of the *O. heterophylla* plant at different doses increase proliferation at 250 µg/mL and lower concentrations. In addition, it appears that it has no effect on cell viability at high concentrations. The cytotoxicity levels of aqueous extracts of *O. heterophylla* species in A549 and L929 cells are presented in Figure 2.

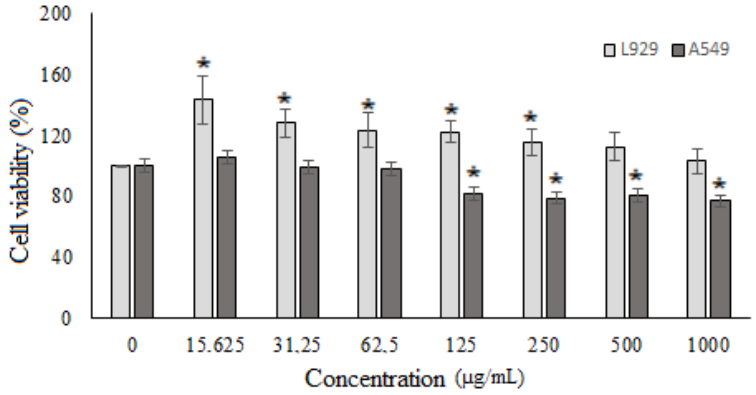


Figure 2. *Onosma heterophylla* extract cytotoxic effects on L929 cells and anticarcinogenic effects on A549 cells

Engel et al. concluded that *Onosma hispida* extract showed highly effective cytotoxicity on Saos-2, BT-20, MG-63, and MCF-7 cells. In another study, it was stated that *Onosma bracteatum* has a cytotoxic effect in a concentration-dependent manner against), PC-3 (human prostate), A549 (lung cancer) and BT549 (breast) cells by regulating caspase-3 activity (Engel et al., 2016; Albaqami et al., 2018).

Effects of *Onosma heterophylla* on the created wound model

Wound healing is an important process of restoring the integrity and function of tissue after injury or trauma. Hemostasis in wound healing occurs in four stages: inflammation, proliferation and tissue restructuring. There are different factors that affect the speed of wound healing, and a lot of research is being done in this field to find new drugs that accelerate wound healing. The scratch wound

healing assay is an in vitro assay that is a reliable, inexpensive and faster method and mimics the migration of cells during in vivo healing of wounds (Ameena et al., 2023)

In the presented study, a scratch wound model was created in mouse fibroblast cells (L929) cells. The amounts of cell-free areas (wound) at the beginning of the study and at the 48th hour of the study and the wound closure rates in each are given in Table 1.

Table 1. Cell-free area amounts and wound closure rates between the 0th and 48th hours of the study

	Amount of cell-free area (wound %)		**In the woud area close rate (%)**
	0. Hour	**48. Hour**	
Control	46.12±3.41	14.09±2.37	69.50±3.68
Onosma heterophylla	44.07±3.15	7.22±1.6[a]	83.72±2.53[b]

The data were given as±mean standard deviation (n≥3). a refers to data with statistically lower ($p<0.05$) wound rate than the control group. b refers to data with statistically higher ($p < 0.05$) wound rate than the control group.

It was determined that wounds occurred in both groups at the beginning of the study, and a decrease in the cell-free area was observed at the 48th hour of the study. The cell-free area in the group where *O. heterophylla* extract was applied was statistically ($p < 0.05$) lower than the control. When wound closure rates were compared as a percentage, it was seen that the closure rate in the group where *O. heterophylla* extract was applied was statistically higher than the control ($p < 0.05$). Microscope images of control and *O.heterophylla* extract-applied L929 cells created in the wound model at 0, 24 and 48 hours are presented in Figure 3.

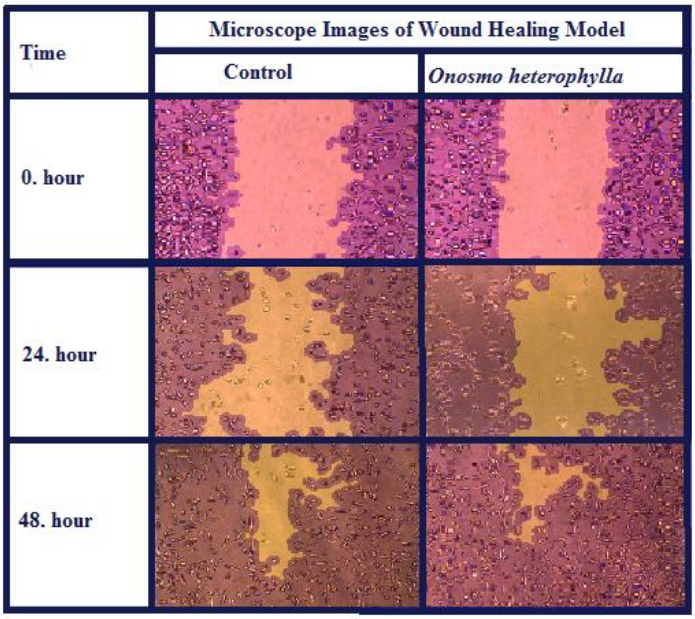


Figure 3. *Onosma heterophylla*'s wound healing activity in mouse fibroblast cells (L929)

Onosma species are used to treat kidney irritation, rheumatism, bladder pain, as well as wounds and burns. While the flowers of the *O. hispidum* plant are used for heart conditions, its roots are also used for application to treat bites, wounds, and skin conditions (Ahmad et al., 2010). A study was conducted to investigate the wound healing potential of extracts prepared from *O. dichroantha* Boiss roots using three different solvents (cyclohexane, ethyl acetate and methanol). Cyclohexane extract was found to show wound healing properties in all four mechanisms. LC-MS/MS studies revealed the

presence of naphthoquinone compounds in the cyclohexane extract. These results support the traditional use of *O. dichroantha* roots in the treatment of wounds and ulcers (Safavi et al., 2019)

CONCLUSION

As a result, in this study where the antioxidative, anticarcinogenic/cytotoxic, and wound healing effects of the *O. heterophylla* species were examined, it was seen that the species came to the fore with its anticarcinogenic and wound healing effects. While no dose of the plant extract applied had a decreasing effect on cell viability on L929, it was observed that some doses only reduced the viability of cancerous cells without damaging healthy cells. In addition, it increases healthy cell viability and reduces cancerous cell viability at a concentration of 125-250 µg/mL, indicating that it is a very effective anticarcinogenic species. It has been observed that *O. heterophylla* accelerates wound healing after creating a wound model and has a high wound closure rate. Studies can be conducted on the mechanisms of the anticarcinogenic and wound healing effects of the species. Apart from this, there may be components responsible for the biological activities of the plant.

Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

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

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