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Onobrychis argyrea subsp. *argyrea* Ekstrelerinin Antioksidan, Antimikrobiyal ve Antiproliferatif Aktivitelerinin Belirlenmesi

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Öne Çıkanlar:
- Tüm ekstraktlar endemik bir bitkiden hazırlandı
- Üç farklı biyolojik aktivite çalışması gerçekleştirildi
- Bazı ekstraktlar kanser hücreleri üzerinde etkili oldu

Anahtar Kelimeler:
- Onobrychis argyrea subsp. argyrea
- antioksidan
- antiproliferatif
- antimikrobial

ÖZET:
Fabaceae familyasına ait bitkiler, hayvan beslenmesinde ve insan sağlığının korunmasında (antidiyabetik, antikanserojen, antioksidan, antienflamatuar ve kardiyovasküler) büyük öneme sahiptir. Bu familyanın önemli cinslerinden biri olan *Onobrychis* yüksek fenolik içeriğe sahiptir ve güçlü bir antioksidan, antikanserojen ve antimikrobiyal ajan olarak bilinir. Bu çalışmada, *Onobrychis argyrea* subsp. *argyrea* (O. argyrea) türünün kloroform (OAC), etanol (OAE) ve su (OAW) ekstreleri hazırlanmış ve bu ekstrelerin antiproliferatif, antimikrobiyal ve antioksidan aktiviteleri incelenmiştir. Ekstraktların antiproliferatif etkileri farklı kanser hücre dizileri üzerinde XTT kolorimetrik yöntemi ile değerlendirilmiştir. Bulgulara göre, OAE ekstresi tüm hücre hatlarına karşı en yüksek etkinliği göstermiştir. MCF-7 %83.71, A549 %92.14 ve HT-29 %72.24 inhibe edildi. Tüm ekstrelerin *Staphylococcus aureus, Escherichia coli* ve *Streptococcus pneumoniae'ye* karşı antimikrobiyal aktivitesi, disk difüzyon tekniği kullanılarak belirlendi. Son olarak antioksidan gücü DPPH ve FRAP yöntemleri kullanılarak belirlendi. DPPH yönteminde, OAE eksrtesi en yüksek radikal süpürme aktivitesi gösterdi (IC_{50}: 34.12±0.2). OAE (257.95±1.40) ve OAW (282.14±0.83) ekstreleri, FRAP yönteminde benzer sonuçlarda yüksek aktivite göstermiştir. Toplam fenolik madde içeriği OAE için 226.15±1.89 mg GAE/g ve OAW için 146.52±0.71 mg GAE/g olarak belirlendi. Bu sonuçlar, çalışılan bitkinin önemli bir antioksidan kaynağı olduğunu göstermektedir. Bütün bu sonuçlardan bitkinin çeşitli biyolojik aktivitelere ve zengin ikincil metabolitlere sahip olduğu anlaşılmaktadır.

Determination of Antioxidant, Antimicrobial, and Antiproliferative Activities of *Onobrychis argyrea* subsp. *argyrea* Extracts

Highlights:
- All extracts were prepared from an endemic plant
- •Three different biological activity studies were performed
- Some extracts are effective on cancer cells

Keywords:
- *Onobrychis argyrea* subsp. argyrea
- antioxidant
- antiproliferative
- antimicrobial

ABSTRACT:
Plants belonging to the Fabaceae family have great importance in animal nutrition and the protection of human health (antidiabetic, anticarcinogenic, antioxidant, anti-inflammatory, and cardiovascular). *Onobrychis*, known as a potent antioxidant, anticarcinogenic and antimicrobial agent, its high phenolic substance is one of the important genera of this family. In this study, chloroform (OAC), ethanol (OAE), and water (OAW) extract of *Onobrychis argyrea* subsp. argyrea (*O. argyrea*) specie was prepared; and the antiproliferative, antimicrobial, and antioxidant activities were investigated. The antiproliferative effects of the extracts were evaluated on different cancer cell lines by XTT colourimetric method. OAE demonstrated the most excellent efficacy against all cell lines, according to the findings. MCF-7 83.71%, A549 92.14%, and HT-29 72.24% were all inhibited. The antimicrobial activity of OAE and OAW extracts against *Staphylococcus aureus, Escherichia coli*, and *Streptococcus pneumoniae* was determined using the disc-diffusion technique. Finally, antioxidant power was determined using DPPH and FRAP methods. In the DPPH research, OAE and OAW extracts showed the highest levels of inhibition (IC_{50}: 34.12±0.2 and 21.58±0.12, respectively). OAE (257.95±1.40) and OAW (282.14±0.83) extracts showed high activity with similar results in the FRAP method. Total phenolic content was determined as 226.15±1.89 mg GAE/g for OAE and 146.52±0.71 mg GAE/g for OAW, respectively. These results show that the species is an essential source of antioxidants. These studies are the first data for *Onobrychis argyrea* subsp. *argyre*a. All these results show that the plant has various biological activities and rich secondary metabolites.

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INTRODUCTION

From ancient times to the present, plants have been frequently employed in traditional medicine to treat disease. Approximately 60% of these species are medicinal herbs. Medicinal plants are important because they may produce therapeutic or health-promoting secondary metabolites in a variety of tissues. Secondary metabolism phytometabolites are a medically significant resource in disease treatment because they exhibit a wide range of polypharmacological actions (Hao et al., 2018).

Plants belonging to the Fabaceae family have great importance both in animal nutrition and in the protection of human health (antidiabetic, anticarcinogenic, antioxidant, anti-inflammatory, and cardiovascular) (Baytop, 1988; Foo et al., 2000). It is used in traditional medicine bleeding, cuts, and wounds in various countries (Özbek et al., 2019). There are 42 endemic species in our country. Among these species, *Onobrychis argaea, Onobrychis elata, Onobrychis argyrea*, and *Onobrychis tournefortii* have taken their place in the list of medicinal and aromatic plants in Turkey (Açıkgöz, 1998).

Onobrychis species, afzelin, arbutin, quercetin, rutin, and tannins (Karakoca et al.,2015) are potent antioxidants, anticancer and antimicrobial agents with rich phenolic content (Usta et al.,2014; Karamian et al.,2016). Quercetin and kaemferol from these flavonols in *Onobrychis* species have been reported to have antidiarrheal, antiulcer, and anti-inflammatory effects as well as in vitro biological effects such as inhibiting cellular proliferation, regulating enzymatic activity, and reducing free radicals (Bülbül, 2017; Marais et al., 2000).

O. argyrea, known as the 'Gümüş Korunga', is from the Fabaceae family. The literature review reveals that chemical studies on the specie are not sufficient, and biological activity tests have not been investigated sufficiently yet. Therefore, in our study, *O. argyrea* specie aimed to determine the total phenolic content of and to determine the in vitro antioxidant, antiproliferative and antimicrobial activities and to bring them to the literature.

MATERIALS AND METHODS

Collection of Plant Material

Plant samples were collected from the Erzincan Binali Yıldırım University campus in July 2022. The species was identified by Prof. Dr Ali Kandemir, and it was deposited in Erzincan Binali Yıldırım University Herbarium with the collector number Altın S.3.

Preparation of plant extract

The aerial parts of the collected plant were dried at room temperature and in the shade, then crushed with liquid nitrogen and turned into powder. 10 g plant material was extracted with chloroform, ethanol, and water at 25 °C for 45 minutes x2 in an ultrasonic bath. After the solvents were removed by evaporation, the extracts were stored at +4 ^{o}C.

Free radical scavenging activity (2,2-Diphenyl-1- picrylhydrazyl, DPPH)

The free radical (DPPH•) scavenging activities of the extracts were performed by making some modifications to the method reported by (Blois, 1958). Stock solutions (1 mg/mL) of extracts and standards were prepared for the test. Samples at concentrations of 20, 40, 80, 120, 160, 240, 320, and 400 µg/mL were prepared, and their final volume was adjusted to 3 mL using methanol. Then, 1 mL of DPPH· solution (0.26 mM, in methanol) was added, and the mixture was vortexed and incubated for 30 minutes in the dark at room temperature. After the incubation was completed, the reaction mixture was measured at 517 nm, the absorbance values were converted to % activity, and the IC_{50} (µg/mL) for each extract was calculated, and the results were given in comparison with Trolox.

Iron reduction power activity (FRAP)

The reducing power activity test was performed by performing with minor modifications to the method reported by (Oyaizu M.,1986). Stock solutions (1 mg/mL) of extracts and standards were prepared for the test. 100 µL of the prepared stock solutions were added to the test tubes, and the volume was made up to 1.25 mL with phosphate buffer (0.2 M, pH 6.6). 1.25 mL of potassium ferric cyanide [$K_3Fe(CN)_6$] (1%) was added to this mixture, and the mixture was incubated at 50 ˚C for 20 minutes. After incubation, 1.25 mL of 10% trichloroacetic acid (TCA) and 0.25 mL of 0.1% $FeCl_3$ solution were added to the reaction medium, respectively, and the absorbance was measured at 700 nm.

Determination of total phenolic compound quantity

The total phenolic content of the extracts was determined spectrophotometrically with the Folin-Ciocalteu reagent. In the study, 100 µL of the stock solutions of the samples at 1 mg/mL concentrations were taken into test tubes, and 4.5 mL of distilled water was added, followed by 100 µL of Folin-Ciocalteu reagent, and left for 10 minutes at room conditions. Then, 300 µL of 2% Na_2CO_3 solution was added, and the mixture was vortexed and incubated for 120 minutes at room conditions. After incubation, the absorbance of the mixture was measured at 760 nm. A calibration curve was created using different concentrations of gallic acid as a standard, and the phenolic contents of the extracts were given as mg gallic acid equivalent phenolic substance/g extract (Slinkard and Singleton, 1977).

Determination of total flavonoid content

The total flavonoid content of the extracts obtained from plants was determined by the aluminium chloride colourimetric method (Chang et al., 2002). For the test, the extracts and standards were taken from the stock solutions (1 mg/mL) prepared in methanol into 100 µL test tubes and the final volume was completed to 4.8 mL with methanol. Then, 100 µL of 1 M NH_4CH_3COO solution and 100 µL of 10% $AlCl_3$ solution were added to the mixture, and the final mixture obtained by vortexing was incubated for 45 minutes at room conditions. After incubation, the absorbance of the mixture was measured at 415 nm in a spectrophotometer. A calibration curve was created with different concentrations of quercetin as a standard, and the flavonoid content of the extracts was given as mg quercetin equivalent/g extract.

Antimicrobial Activity

Preparation of bacterial isolates

Onobrychis argyrea subsp. *argyrea* the aerial parts of (OA) plant, water (OAW), ethanol (OAE), and chloroform (OAC) extracts were tested on seven standard bacterial isolates (Gram (-) of these bacteria are *Pseudomonas aeruginosa* American Type Culture Collection (ATCC) 27853, *Klebsiella pneumonia* ATCC 700603, colistin-resistant *Escherichia coli* ATCC 19846, *Escherichia coli* ATCC 25922, Gram (+) *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* 25922 *Streptococcus pneumonia* is ATCC 45616) and one fungus(*Candida albicans*). Bacterial isolates were supplied from Erzincan Mengücek Gazi Training and Research Hospital Medical Microbiology Laboratory.

Inoculum preparation

Standard strains kept at -80^{0}C were inoculated on 5% sheep blood medium (Biomerieux, France) and incubated at 37°C for 16 to 18 hours. At the end of the period, a pure passage was made from the colonies grown in the sheep blood medium into a new 5% sheep blood medium, and the same process

was repeated for the second time. 0.5 MacFarland (1.5x108 CFU/ml) turbid solutions of bacterial isolates grown in the medium were adjusted in 0.9% saline and DensiCHEK Plus (Biomerieux, France) densitometer device.

Determination of antimicrobial activity disk diffusion method

The Kirby-Bauer disk diffusion method was used to determine the antimicrobial effect of plant extracts, taking into account the recommendations of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) (Bauer et al., 1966).

The prepared bacterial solutions were spread on Mueller Hinton agar medium (Biomerieux, France) with the help of a cotton swab and allowed to dry for 15 minutes. Mueller Hinton Fastidious (MH-F) agar supplemented with 5% defibrinated horse blood and 20mg/l β-NAD was used for *Streptococcus pneumoniae* isolate. Then, 20 µL of the extracts prepared as 200 mg/ml were added to 6 mm diameter standard blank discs (Bioanalyse Turkey) and allowed to dry for 15 minutes. After the extract-impregnated discs were dried, they were placed on the surface of the medium with the help of sterile pliers and the plates were incubated for 16-18 hours at 37 ^{0}C. At the end of the period, the diameter of the zone where bacteria did not grow around the discs was measured with a ruler and noted. The same procedures were repeated three times for each concentration. Vancomycin 5 µg/mL and Erythromycin 15 µg/ml for Gram (+) bacteria and Amikacin 30 µg/mL disc for Gram (-) bacteria were used as the positive control. Pure water was used as the negative control.

Cell Culture Studies

Cell lines for human breast adenocarcinoma MCF-7, human lung cancer A549 and colorectal adenocarcinoma HT-29 were commercially purchased from ATCC (American Type Culture Collection, USA). MCF-7 cells were cultured with EMEM media, while HT-29 cells were cultured with McCoy's 5A medium. All basal media were supplemented with fetal bovine serum (10%), Na-Pyruvate (10%), and antibiotics (Pen-Strep) before experimental procedures. Studies were carried out in a Class II biosafety cabinet. Incubation of cultures was carried out at 37°C in a 5% CO_2 incubator.

Cytotoxicity assay

Cell growth inhibitory capacities of *O. argyrea* extracts were evaluated against MCF-7, A549, and HT-29 cell lines by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H tetrazolium-5-carboxanilide (XTT) assay (Batool et al., 2022). Inhibition percentages of the extracts at a concentration of 250 µg/mL were calculated.

RESULTS AND DISCUSSION

Antioxidant Activity

Total phenolic and flavonoid amounts of *O. argyrea* chloroform, ethanol, and water extracts were determined and DPPH and FRAP methods were used for the determination of antioxidant capacity.

O. argyrea the total phenolic content of the extracts prepared from was determined using the Folin-Ciocalteu reagent (FCR). Phenolic content was found to be 30.60±0.13, 226.15±1.89, and 146.52±0.71 mg of GAE/g extract for chloroform, ethanol, and water, respectively. Total phenolic contents of chloroform, ethanol, and water extracts are presented in Table 1.

The $AlCl_3$ method was used to determine the total flavonoid content. The reducing power or potential is one of the most significant markers of antioxidant capacity. This reducing power is an

essential mechanism of antioxidant substances in phenolic structure. Total flavonoid contents of chloroform, ethanol, and water extracts are presented in Table 1.

Free radical scavenging activities of *O. argyrea* extracts were determined using DPPH radicals. DPPH, a stable radical, is the most commonly used radical in the determination of antioxidant capacity. Reacting with antioxidants, DPPH gains electrons and bleaching occurs therefore a decrease in absorbance occurs. If the added substance makes the DPPH solution lighten, the absorbance value is measured as low, and a low absorbance means that the activity is high. Trolox was used as standard. *O. argyrea* total phenolic, flavonoid contents and total antioxidant capacities of chloroform, ethanol, and water extracts are presented in Table 1.

Table 1. Total phenolic, flavonoid contents and total antioxidant capacities of *O. argyrea*

Extract/Positive Control	DPPH• scavenging IC_{50} (μg/mL)	Total phenolics mg GAE/g Extract	Total flavonoids mgQE/g Extract	Reducing power mgTE/g Extract
OAC	60.85±0.4	30.60±0.13	15.71±1.03	16.93±0.20
OAE	34.12±0.2	226.15±1.89	108.10±0.7	257.95±1.40
OAW	21.58±0.12	146.52±0.71	71.42±1.03	282.14±0.83
Trolox	11.95±0.15			

OAC; *O. argyrea* chloroform extract, OAE; *O. argyrea* ethanol extract, OAA; *O. argyrea* water extract.

There are no antioxidant studies on *O. argyrea* species. This study is the first antioxidant study of *O. argyrea*. When the results were examined, the ethanol extract was found to be the richest in terms of phenolic content with 226.15 mgGAE/g phenolic compound content. After the ethanol extract, the amount of phenolic content is followed by water (146.52 mgGAE/g) and chloroform extract (30.60 mgGAE/g).

Karadağ (2018), *O. argyrea* subsp. *isaurica* in the study investigating the antioxidant activity of phenolic content was observed the highest in the methanol extract among the extracts. According to their study, they determined methanol extract (78.09 mgGAE/g), ethyl acetate (76.83 mgGAE/g), and water extract (76.06 mgGAE/g).

When the total flavonoid content is considered, the richest extract in terms of flavonoid content is methanol extract with 41.57 mgRE/g content. This is followed by ethyl acetate (32.56 mgRE/g) and water extract (22.70 mgRE/g). They studied *O. isaurica* when extracts were evaluated regarding DPPH radical scavenging activity; the strongest effect was detected in the methanol extract (126.51 mgTE/g). This is followed by water (116.89 mgTE/g) and ethyl acetate (81.81 mgTE/g) extracts, respectively. In the FRAP test results, the highest result was found in methanol extract with 200.70 mgTE/g. This is followed by the water extract 187.45 mgTE/g and the ethyl acetate extract 179.19 mgTE/g. The results showed that the species *O. argyrea* has a substantially higher antioxidant content than the type *O. isaurica*.

In vitro antimicrobial effect results

In the literature, no study has been found on the antimicrobial activity of the *O.argyrea* plant, but there are studies conducted with other species. In the study by Karakoca et al. (2015), the antimicrobial activities of methanol, ethanol, and water extracts of the flower and root parts of the *O.armena* plant were tested by the disc diffusion method. They found that the ethanol extracts obtained from the flower part were highly effective against *S.aureus* (mean±SD: 21.81±0.65), but the water extract was ineffective against all tested microorganisms. Again, the ethanol extract of the root parts was found to be effective against *S.aureus* (18.18±0.23), *E.coli*, and *P.aeruginosa*, respectively, and the water extract was found to be only antifungal.

Aliahmadi et al. (2015), in their study in Iran, extracted the total water-soluble proteins of *O. sativa* Lam plant seeds and tested them by agar coating method on three different bacteria (*S.aureus*, *Enterococcus faecium*, and *E.coli*). As a result, they determined that the total water-soluble proteins of *Onobrychis sativa* Lam plant seeds showed strong antibacterial activity against *E. faecium* and *S. aureus* strains, respectively.

In our study, each of the OAE and OAS extracts was found to be effective against three microorganisms by the literature. Bacteria that both were effective were *S. pneumoniae* (mean±SD: 15.0±0.0), *S.aureus* (mean±SD: 8.7±0.57), and *E.faecalis* (mean±SD: 7.7±0.57), in order of effectiveness. OAE was found to be more effective than OAW against these bacteria. The inhibition results of the standards and extracts are shown in Table 2.

Table 2. Antimicrobial activities of *O. argyrea* extracts (mean ± SD, n = 3)

Extract and const. (mg/ml)	**Zone Diameter of Standard Strains (mm ± SD)**						
	S. aureus	***E. faecalis***	***S. pneumoniae***	***E.coli***	***E.colim COL-R***	***K. Pneumoniae***	***P.aeruginosa***
OAC (200)	-	-	-	-	-	-	-
OAE (200)	8.7±0.57	7.7±0.57	15.0±0.0	-	-	-	-
OAW (200)	7.7±0.57	7.0±0.0	7.7±0.57	-	-	-	-
E (15 µg)	**21.3±0.57**	nt	nt	**20.7±0.57**			
VA (5 µg)	nt	**14.3±0.57**	**25.3±0.57**	nt	nt	nt	nt

OAC: *O. argyrea* chloroform extract OAE: *O. argyrea* ethanol extract, OAW: *O. argyrea* water extract, E: Erythromycin, VA: Vancomycin, -: no inhibition, nt: Not tested

In vitro anticancer effect results

XTT colourimetric method is widely used to evaluate the antiproliferative effects of natural or synthetic compounds. Based on this, the various extracts including OAC, OAE, and OAW from O. argyrea subsp. argyrea were tested on breast cancer MCF-7 cells, lung cancer A549 cells, and colon cancer HT-29 cells for their cell growth inhibitory activities. For this, each cell line was treated with these extracts at a dose of 250 µg/mL and incubated for 24 hours. The 250 µg/mL dose-dependent cell growth inhibitory results are shown in Table 3.

Table 3. *O. argyrea* % inhibitory values of extracts on cancer cell lines (250 µg/mL)

***O. argyrea* extracts**	**MCF-7**	**A549**	**HT-29**
Chloroform (OAC)	75.28	85.00	67.20
Ethanol (OAE)	83.71	92.14	72.24
Water (OAW)	78.09	88.32	70.02

OA extracts showed significant inhibition on all cell lines at 250 µg/mL concentrations. Among all the extracts, the highest inhibition on the studied cancer lines was determined in the OAE extract. In particular, OAE extract exhibited almost 100% inhibition on A549 lung cancer cells. In addition, its inhibition of 83% for the MCF-7 breast cancer cell line is also highly significant. Although the effect of HT-29 on colon cancer is lower than other cell lines, the highest activity among all extracts belongs to the OAE extract with a value of 72%.

In the study by Bulbul (2017), aqueous extracts of four wild sainfoin species *Onobrychis argyrea*, *Onobrychis galegifolia*, *Onobrychis tournefortii*, and *Onobrychis albiflora* were used. Different doses of these extracts were tested on the human colon cancer cell line (HCT-116) and the

human embryonic stem cell line (HEK-293). The inhibitory concentration of the extracts obtained from the plants, which caused a decrease in the proliferation of both cell lines at 24 and 48 hours, was determined by MTT analysis. The results showed that *O. albiflora* extract has antiproliferative, apoptotic, and necrotic effects on HCT-116.

The genus *Onobrychis*, belonging to the Fabaceae family, contains many anticarcinogenic compounds. For example, afzelin (Naeem et al., 2022), quercetin (Hashemzaei et al., 2017), inositol (Yu et al., 2017), cinnamic acid (Zhu et al., 2016), vitexin (Scarpa et al., 2018), and Ebenfuran III (Roumeliotis et al., 2013). It has also been supported by studies that these molecules, which are in the content of the Onobrychis genus and are important from an oncological point of view, have significant antiproliferative effects, primarily on MCF-7, PC-3, and HT-29 cell lines

CONCLUSION

In our study, chloroform, ethanol, and water extracts were prepared by using the aerial parts of *Onobrychis argyrea* subsp. *argyrea* species. By determining the total phenolic and flavonoid content of the species, DPPH and FRAP methods were used for antioxidant studies. The extract with the highest total phenolic and flavonoid content was the ethanol extract, followed by the water extract. In antioxidant studies, the highest activity was calculated in water and ethanol extracts, respectively. Chloroform extract showed moderate activity. Antimicrobial studies were determined by the Kirby-Bauer disk diffusion method. The findings revealed that ethanol and water extract significantly inhibited *S. aureus*, *E. faecalis*, and *S. pneumoniae* bacteria while chloroform extract had no antibacterial impact on any bacteria. Antiproliferative studies were investigated by the XTT method on MCF-7, HT-29, and A549 cell lines. Percentages of inhibition were determined for each extract at a concentration of 250 µg/mL. All extracts showed good and remarkable activity on all three cell lines.

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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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