A Comparative Study on the \textit{in vitro} Antioxidant and Antimicrobial Potentials of Three Endemic \textit{Ononis} L. Species from Turkey

Türkiye'den Üç Endemik \textit{Ononis} L. Türünün \textit{in vitro} Antioksidan ve Antimikrobiyel Potansiyelleri Üzerine Karşılaştırmalı Bir Çalışma

**ABSTRACT**

Objectives: In this study, the antioxidant capacity, antimicrobial activity and phenolic contents of aerial parts and roots extracts of three endemic \textit{Ononis} L. (Leguminosae) species \textit{O. sessilifolia} Bornm., \textit{O. basiadnata} Hub. & Mor., \textit{O. macrosperma} Hub. & Mor.) were investigated for the first time.

Materials and Methods: The phenolic contents of the extracts [water and ethanol (EtOH)] and fractions [dichloromethane, EtOAc and n-butanol] were determined using Folin-Ciocalteu's phenol reagent. Also, their antioxidant capacities were studied using qualitative DPPH \(^{-1}\) (1-diphenyl-2-picrylhydrazyl radical) and TBA assays. The antimicrobial activity of these extracts and fractions compared with standard antibiotics were studied using disc diffusion assays against various Gram-positive and Gram-negative bacteria and fungi.

Results: The total phenolic contents of the water extracts were found to range between 14.78-80.33 mg/g, and the EtOH extracts ranged from 67.19-145.33 mg/g. EtOAc fractions of the three species were rich in terms of total phenolic contents when compared with other extracts (242.56-620.89 mg/g). The most significant results in the TBA assays were obtained in EtOH extracts of \textit{O. macrosperma} (IC\(_{50}=0.13\pm0.17 \mu\text{g/mL}\)), \textit{O. sessilifolia} (IC\(_{50}=1.41\pm0.58 \mu\text{g/mL}\)) and root (IC\(_{50}=1.96\pm0.39 \mu\text{g/mL}\)).

Conclusion: EtOAc fractions rich in phenolic content were also found to be the most effective in antioxidant activity assays. Although all water extracts had no antimicrobial activity, EtOH extracts and n-butanol fractions showed generally moderate activity against bacteria. Some EtOAc fractions except for \textit{O. sessilifolia} showed less activity against \textit{Escherichia coli}, \textit{Staphylococcus aureus}, MRSA and \textit{Candida albicans}.

Key words: \textit{Ononis sessilifolia}, \textit{Ononis basiadnata}, \textit{Ononis macrosperma}, antioxidant capacity, antimicrobial activity, endemic

**ÖZ**

Amaç: Bu çalışmada üç endemik \textit{Ononis} L. (Leguminosae) türünün (\textit{O. sessilifolia} Bornm., \textit{O. basiadnata} Hub. & Mor., \textit{O. macrosperma} Hub. & Mor.) antimikrobiyal aktivite, antioksidan kapasite ve fenolik içerikleri ilk kez araştırılmıştır.

Gereç ve Yöntemler: Ekstrerinin [su ve etanol (EtOH)] ve fraksiyonlarının [diklorometan, etil asttat (EtOAc) ve n-butanol] fenol içerikleri Folin-Ciocalteu’nün fenol reaktifi kullanılarak belirlendi. Ayrıca, antioksidan kapasiteleri kalitatif DPPH \(^{-1}\) (1-diphenyl-2-picrylhydrazyl radical) ve TBA reaktifi ile karşılaştırıldı. Eksere ve fraksiyonların antimikrobiyel aktiviteleri disk difüzyon tekniği kullanılarak standart antibiotiklere kıyaslanarak çeşitli Gram pozitif, Gram negatif ve mantara karşı değerlendirildi.

Bulgular: Su ekstrerinin toplam fenol içerikleri 14.78-80.33 mg/g aralığında değişirken, EtOH ekstrerileri 67.19-145.33 mg/g aralığında bulunmuştur. Üç türün EtOH fraksiyonları, diğer ekstrerilerle (242.56-620.89 mg/g) karşılaştırıldığında toplam fenol içerikleri bakımından zengindir. TBA metodundaki en önemli sonuçlar, \textit{O. macrosperma} herba (IC\(_{50}=0.13\pm0.17 \mu\text{g/mL}\)), \textit{O. sessilifolia} herba (IC\(_{50}=1.41\pm0.58 \mu\text{g/mL}\)) ve kök (IC\(_{50}=1.96\pm0.39 \mu\text{g/mL}\)) EtOH ekstraktlarında elde edildi.

Sonuç: Fenolik içerik bakımından zengin olan ellilasetat fraksiyonları, antioksidan aktivite deneylerinde de en yüksek etkili olarak tespit edildi. Su ekstrerinin antimikrobiyel aktivitesi olmasması rağmen, türlerin EtOH ekstrerileri ve n-butanol fraksiyonları, bakterileri karşı genellikle orta düzeyde etkili gösterdi. \textit{O. sessilifolia} dışında bazı EtOAc fraksiyonları, \textit{Escherichia coli}, \textit{Staphylococcus aureus}, MRSA ve \textit{Candida albicans’a} karşı daha az aktivite gösterdi.

Anahtar kelimeler: \textit{Ononis sessilifolia}, \textit{Ononis basiadnata}, \textit{Ononis macrosperma}, antioksidan kapasite, antimikrobiyal aktivite, endemic

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INTRODUCTION
Antioxidants have been recognized as potential therapeutics for preventing different human diseases (e.g., cancer, aging, cardiovascular diseases, asthma, acute central nervous system injury, neurodegenerative disease, and malaria).\(^1\) Medicinal plants and herbs have played an important role in the health care of ancient and modern cultures. They are promising as natural antioxidant sources.\(^2\) The number of studies on new natural antioxidants with plant origin is increasing.\(^3\) Ononis L. genus (Leguminosae) is represented by 18 species, 4 of which are endemic to Turkey.\(^4\) The Ononis species has various pharmacologic properties, such as antioxidant, aperient, diuretic, antimicrobial, analgesic, antiviral, cytotoxic, anti-inflammatory and anti-diarrheal activities. In Turkish folk medicine, O. spinosa L. has been used for the urinary tract, kidney stones, inflammatory diseases, wounds healing, and skin disorders.\(^5\) Süntar et al.\(^6\) reported that water and ethanol extracts of the herb O. macroasperma demonstrated the highest activity in both wound models and anti-inflammatory activity. In previous studies, some Ononis species were found to contain different component groups such as isoflavone, triterpene, sterol, pterocarpane, and resorcinol derivatives, flavonoids, isocoumarins, and hydroxycinnamic acids.\(^7\) \(^8\) A DPPH’ (1,1-diphenyl-2-picrylhydrazyl radical) radical is a stable radical with maximum absorbance at 517 nm, and when reduced to hydrazine derivatives by electron and hydrogen atom transfer from substances with antioxidant properties, its absorbance is decreased.\(^9\) Lipid peroxidation is a chain reaction that causes the deterioration of biologic systems, and is the accumulative effect of reactive oxygen species. Reactive free radicals start the reaction by the deletion of allylic hydrogen atoms from the methylene group of unsaturated fatty acids.\(^10\) Thiobarbituric acid (TBA) and DPPH methods have been used to evaluate the antioxidant capacities of the plant extracts/component.\(^11\) Medicinal plants represent potential sources of natural antioxidant and antimicrobial agents for food and medicinal purposes. The purpose of this investigation was to study antioxidant activities using TBA and DPPH [thin layer chromatography (TLC) screening], determine phenolic contents using spectrophotometry, and the antimicrobial activity with disc diffusion assays of herb and root extracts of three Ononis species. This is the first study on the antioxidant capacity and antimicrobial activities of the three Ononis species.

MATERIALS AND METHODS

Plant materials
Ononis species were gathered from different provinces of Turkey in their natural habitats. Voucher specimens were stored in the Herbarium of the Faculty of Pharmacy at the University of Ankara, Turkey (AEF). O. sessilifolia was collected from the Çamardi county of Niğde in June 2007 (AEF 23979); O. basiadnata was collected from the Gûlnar county of Içel in June 2007 (AEF 23968); and O. macroasperma was collected from the Elmali county of Antalya in May 2008 (AEF 24698).

Extraction of plants
The herb and root of the O. basiadnata and O. sessilifolia and the herb of O. macroasperma were powdered and then 50 g of the herbs and roots were macerated separately with 500 mL of water for 5 h at 60°C. Afterwards, the water extracts were filtered, frozen, and lyophilized. One hundred grams of plant material was macerated with ethanol (EtOH) for 5 h at 50°C. The extracts were then filtered and evaporated until dry. Ethanolic extracts were dispersed in methanol: water (1:9), partitioned with dichloromethane (DCM), ethyl acetate (EtOAc) and n-butanol (BuOH). Afterward, the fractions were evaporated until dry.

Determination of total phenolic content
The total phenolic contents of extracts were evaluated using the Folin-Ciocalteu assay as gallic acid equivalents (GAE).\(^12\)

Antioxidant capacities of extracts

DPPH test
The antioxidant capacity of extracts (EtOH and water extracts; DCM, EtOAc and BuOH fractions) were evaluated with rapid TLC screening.\(^13\)

TBA test (measurement of malondialdehyde value)
The amount of malondialdehyde formed in the reaction mixture was determined using the TBA reagent spectrophotometrically.\(^14\)

Antimicrobial activity

Test microorganisms
The test microorganisms used in the experiment were Gram-positive bacteria (Staphylococcus aureus ATCC 25923, Staphylococcus aureus (MRSA) (clinical isolate), Bacillus subtilis ATCC 25923), Gram-negative bacteria (Escherichia coli ATCC 25922) and fungus: (Candida albicans ATCC 10231).

Amoxicillin sulbactam (20 µg), ciprofloxacin (5 µg), fluconazole (25 µg) and cefotaxime (30 µg) were used as control drugs. ATCC strains were obtained from the culture collection of the Refik Saydam Health Institution of Health Ministry, Ankara.

Media
Mueller-Hinton agar (Difco, Detroit, MI, USA) was used for bacteria, and MHA supplemented with 2% glucose and 0.5 µg/mL methylene blue was used for C. albicans.

Disc diffusion assay
Antimicrobial activities of the extracts and fractions were evaluated by using the disc diffusion technique.\(^16\)

RESULTS
Phenolic compounds are known as a main class of active compounds determined by Folin-Ciocalteu assay. The highest total phenolic contents of all extracts and fractions of the three species were determined in the EtOAc fractions. The results of total phenolic contents of extracts and fractions are shown in Table 1.

When all DCM fractions and EtOAc fractions, excluding O. basiadnata root, were compared using propyl gallate, they were
shown to have a high radical scavenging effect with qualitative DPPH. Yellow zones on a purple ground were marked for the DCM and EtOAc fractions of all species. In DPPH test, EtOH extracts were generally active from the water extract, especially the root and herb extracts of *O. sessilifolia* (Figure 1).

The most significant results in the TBA method were obtained in EtOH extracts of *O. macrosperma* herb (OMH) (IC$_{50}$ = 0.13±0.17 µg/mL), *O. sessilifolia* herb (IC$_{50}$ = 1.41±0.58 µg/mL) and root

| Species                  | Total phenolic contents (mg gallic acid/g extract) ± SD* |
|--------------------------|---------------------------------------------------------|
|                          | Water extracts  | EtOH extracts | DCM fraction | EtOAc fraction | BuOH fraction |
| *O. sessilifolia* herb (OSH) | 37.19±1.58      | 132.01±4.16   | 208.11±3.93  | 413.67±5.50    | 124.77±0.79  |
| *O. sessilifolia* root (OSR) | 14.78±0.79      | 145.33±1.57   | 221.44±2.36  | 327.08±0.79    | 131.44±2.36  |
| *O. basiadnata* herb (OBH) | 80.33±0.79      | 111.44±0.79   | 105.33±4.16  | 620.89±12.57   | 89.77±4.71   |
| *O. basiadnata* root (OBR) | 20.15±0.64      | 91.17±5.55    | 119.22±2.36  | 242.56±4.84    | 88.11±0.79   |
| *O. macrosperma* herb (OMH) | 46.17±3.78      | 67.19±0.64    | 63.67±4.21   | 467.03±3.93    | 156.44±4.71  |

*SD: Standard deviation, EtOH: Ethanol, DCM: Dichloromethane, EtOAc: Ethyl acetate, BuOH: n-butanol

| Species                  | IC$_{50}$ value (µg/mL) ± SD* |
|--------------------------|-------------------------------|
|                          | Water ext. | EtOH ext. | DCM frac. | EtOAc frac. | BuOH frac. |
| OSH                      | NE*         | 1.41±0.58 | 14.38±1.32 | 51.18±3.31 | NE         |
| OSR                      | >1000±8.55  | 1.96±0.39 | 26.03±0.24 | 146.35±2.73 | NE         |
| OBH                      | >1000±3.11  | 24.19±2.21 | 49.73±1.41 | 532.01±5.58 | NE         |
| OBR                      | >1000±7.99  | 15.57±0.95 | 26.06±11.9 | 310±1.14 | 138.93±2.26 |
| OMH                      | NE           | 0.13±0.17  | >1000±2.85 | 61.56±3.61 | 570.41±7.14 |

*Non-effective; SD: Standard deviation, OSH: *O. sessilifolia* herb, OSR: *O. sessilifolia* root, OBH: *O. basiadnata* herb, OBR: *O. basiadnata* root, OMH: *O. macrosperma* herb, EtOH: Ethanol, DCM: Dichloromethane, EtOAc: Ethyl acetate, BuOH: n-butanol

| Extracts/Drugs OSA | S. aureus ATCC 25923 | MRSA isolate | B. subtilis ATCC 25923 | E. coli ATCC 25922 | C. albicans ATCC 10231 |
|--------------------|----------------------|--------------|------------------------|-------------------|------------------------|
| OSH EtOH ext.      | 10                   | 12           | -                      | 7                 | 13                     |
| OSR EtOH ext.      | -                    | 5            | -                      | -                 | 11                     |
| OSH DCM            | -                    | -            | -                      | -                 | -                      |
| OSR DCM            | -                    | -            | -                      | -                 | -                      |
| OSH EtOAc          | -                    | -            | 15                     | -                 | -                      |
| OSR EtOAc          | -                    | -            | -                      | -                 | -                      |
| OSH BuOH           | 11                   | 15           | 15                     | 17                | 12                     |
| OSR BuOH           | 14                   | 11           | 10                     | 16                | 17                     |
| OSH water ext.     | -                    | -            | -                      | -                 | -                      |
| OSR water ext.     | -                    | -            | -                      | -                 | -                      |

OSH: *O. sessilifolia* herb, OSR: *O. sessilifolia* root, EtOH: Ethanol, DCM: Dichloromethane, EtOAc: Ethyl acetate, BuOH: n-butanol

Figure 1. Antioxidant capacity by qualitative DPPH test on TLC of *Ononis* species

1: *O. basiadnata* root extract, 2: *O. basiadnata* herb extract, 3: *O. sessilifolia* root extract, 4: *O. sessilifolia* herb extract, 5: *O. macrosperma* herb extract, Pg: Propyl gallate

"BALDEMIR et al. Antioxidant and Antimicrobial Potentials of Ononis L. Species"
(IC$_{50}$=1.96±0.39 μg/mL) (Table 2). A survey of the published literature shows that the antioxidant activity of O. sessilifolia, O. basiadjnata and O. macrosperma has not been subjected to research so far.

In our antimicrobial activity studies, the extracts and fractions of three endemic Ononis species were examined against various bacteria and fungi. First, EtOH extracts were prepared, and then extracted with DCM, EtOAc and BuOH. The antimicrobial activity of the water extract was also examined. Water extracts of herb and root parts had no antimicrobial activity against Gram (-), Gram (+) bacteria and yeast. All of the BuOH extracts showed moderate activity compared with the standards. Some EtOAc fractions also demonstrated less activity against E. coli, S. aureus, MRSA and C. albicans. Apart from OMH, other DCM extracts showed no activity against Gram (+), Gram (-) bacteria and fungi. All EtOH extracts showed less activity against some bacteria. In addition to this, they showed moderate activity against C. albicans according to fluconazole (Table 3, 4, 5, 6).

### DISCUSSION

In the literature, there are some studies on the antioxidant activities and phenolic contents of other Ononis species. Leaf methanolic extract of O. natrix has significant total phenolic content (51 mg GAE/g DW) and flavonoid content (14.76 CE/g DW).

Table 4. Results of the antimicrobial activity of O. basiadjnata herb (OBH) and root (OBR) extracts (inhibition zones in mm)

| Extracts/Drugs | S. aureus ATCC 25923 | MRSA isolate | B. subtilis ATCC 25923 | E. coli ATCC 25922 | C. albicans ATCC 10231 |
|----------------|----------------------|--------------|------------------------|---------------------|------------------------|
| OBH EtOH ext. | 11                   | -            | 12                     | 10                  | 11                     |
| OBR EtOH ext. | 10                   | 7            | 14                     | 10                  |                        |
| OBH DCM       | -                    | -            | -                      | -                   | -                      |
| OBR DCM       | -                    | -            | -                      | -                   | -                      |
| OBH EtOAc     | 10                   | 11           | -                      | -                   | 7                      |
| OBR EtOAc     | -                    | -            | -                      | 14                  | -                      |
| OBH BuOH      | 12                   | 12           | 14                     | 14                  | -                      |
| OBR BuOH      | 11                   | 14           | 17                     | 17                  | 14                     |
| OBH water ext.| -                    | -            | -                      | -                   | -                      |
| OBR water ext.| -                    | -            | -                      | -                   | -                      |

OSR: O. sessilifolia root, OBH: O. basiadjnata herb, OBR: O. basiadjnata root, OMH: O. macrosperma herb, EtOH: Ethanol, DCM: Dichloromethane, EtOAc: Ethyl acetate, BuOH: n-butanol

Table 5. Results of the antimicrobial activity of O. macrosperma herb (OMH) extracts (inhibition zones in mm)

| Extracts/Drugs | S. aureus ATCC 25923 | MRSA isolate | B. subtilis ATCC 25923 | E. coli ATCC 25922 | C. albicans ATCC 10231 |
|----------------|----------------------|--------------|------------------------|---------------------|------------------------|
| EtOH ext.      | 10                   | 9            | -                      | 16                  |                        |
| DCM frac.      | 12                   | -            | 7                      | -                   |                        |
| EtOAc frac.    | -                    | 10           | -                      | -                   | -                      |
| BuOH frac.     | 12                   | 15           | 16                     | 13                  |                        |
| Water ext.     | -                    | -            | -                      | -                   | -                      |

OMH: O. macrosperma herb, EtOH: Ethanol, DCM: Dichloromethane, EtOAc: Ethyl acetate, BuOH: n-butanol

Table 6. Inhibition zones of standard antibiotics (inhibition zones in mm)

| Reference Substances | S. aureus ATCC 25923 | MRSA isolate | B. subtilis ATCC 25923 | E. coli ATCC 25922 | C. albicans ATCC 10231 |
|----------------------|----------------------|--------------|------------------------|---------------------|------------------------|
| Ampicillin sulbactam | 30                   | -            | 21                     | -                   | -                      |
| Ciprofloxacin        | -                    | 27           | -                      | -                   | -                      |
| Fluconazole          | -                    | -            | 32                     | -                   | -                      |
| Cefotaxime           | -                    | -            | -                      | 25                  | -                      |
The present study shows that the three Ononis species contained phenolic compounds, which inhibit the oxidation of lipids by donating hydrogen atoms to scavenge free radicals. Phenolic compounds have been shown to be more effective antioxidants than vitamin A and C. Our results showed that there seemed to be good compatibility between the phenolic content and antioxidant capacity of the extracts because the EtOAc fractions with a higher phenolic content showed higher DPPH radical scavenging capacity.

According to previous studies, the BuOH extracts of O. spinosa (4 mg/disc) had moderate antifungal activity against Aspergillus flavus, Fusarium moniliforme and C. albicans in comparison with miconazole nitrate at 40 µg/disc. Petroleum benzene, EtOH, and water extracts showed high activity against Gram (+) and Gram (-) bacteria. In another study, EtOH extract of O. spinosa demonstrated significant activity against Gram-positive (E. coli and P. aeruginosa), Gram-negative (S. aureus) bacteria and fungi (C. albicans). In our study, similar results were obtained, EtOH extracts and BuOH fractions of all species showed generally high activity against Gram (+), Gram negative (-) bacteria and C. albicans.

CONCLUSION

Further studies are being conducted to determine the characterization and identification of active components responsible for the antioxidant and antimicrobial activities. Natural products are commonly a source for active compounds that have important potential for developing new therapeutic agents. Ononis species can be introduced as new plant source for antioxidant and antimicrobial agents.

Conflict of Interest: No conflict of interest was declared by the authors.

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