International Journal of Secondary Metabolite
2018, Vol. 5, No. 2, 75–86

DOI: 10.21448/ijsm.392354

Published at http://www.ijate.net http://dergipark.gov.tr/ijsm Research Article

Analysis of phenolic compounds, antioxidant and antimicrobial properties of some endemic medicinal plants

Ersan Bektas[1], **Kaan Kaltalioglu**[1], **Huseyin Sahin**[*1], **Zafer Turkmen**[2], **Ali Kandemir**[3]

[1] Espiye Vocational School, Giresun University, Giresun, Turkey
[2] Biology Department, Science Faculty, Giresun University, Giresun, Turkey
[3] Biology Department, Science Faculty, Erzincan University, Erzincan, Turkey

Abstract: This study was designed to evaluate the phenolic compounds and the *in vitro* antioxidant and antimicrobial activities of *Onobrychis nitida*, *Hedysarum cappadocicum*, *Ebenus laguroides and Ebenus macrophylla* which are medicinal plants and endemic for the flora of Turkey. The RP-HPLC-DAD (reverse phase-high performance liquid chromatography with a diode array detector) was used to evaluate the phenolic contents. The antioxidant properties were determined to use total phenolic content (TPC), ferric reducing antioxidant power (FRAP), and DPPH• radical scavenging activity assays. Antibacterial tests were performed against 11 different microorganisms by using the microwell dilution method. Each of the plant extracts were confirmed by bioactive assays which demonstrated a significant activity due to different chemical characteristics. Especially, rutin was the dominant component in *Ebenus* species with 19.434-11.808 mg phenolic/g extract. While the highest phenolic content (101.73 mg gallic acid equivalent/g extract) was observed in *O. nitida*, the highest FRAP value was in *E. laguroides* (719.09 µM $FeSO_4.7H_2O$ equivalent), and the strongest DPPH degree was in the *E. macrophylla* extract with IC_{50}: 69.45 µg/mL, respectively. Although plant extracts didn't have efficient values for antimicrobial activity, the slight effect was arisen in O. *nitida* against *B. subtilis* and *S. aureus*. The results showed that all the extracts could be used in pharmacological or dietary applications due to their valuable properties.

ARTICLE HISTORY

Received: 25 February 2018
Revised: 05 March 2018
Accepted: 11 March 2018

KEYWORDS

Onobrychis nitida,
Hedysarum cappadocicum,
Ebenus laguroides,
Ebenus macrophylla,
Phenolics,
Bioactivity

1. Introduction

The traditional treatment of various plants is quite common and popular in the world. As it is widely known, medicinal value of plants comes from their secondary metabolites such as phenolic contents [1]. Phenolics are able to act as antioxidant and antimicrobial agent against oxidative stress and pathogen microorganisms [2, 3]. Antioxidants act a significant role in the attenuation of oxidative stress which is related to pathogenesis of various diseases [4]. Phenolics exert their antioxidant activities by various mechanisms such as free radical scavenging, single electron transfer and metal ion chelating [2]. Antimicrobial agents kill or inhibit the growth of

CONTACT: Huseyin Sahin ✉ huseyin.sahin@giresun.edu.tr Espiye Vocational School, Giresun University, Giresun, Turkey

ISSN-e: 2148-6905 /© IJSM 2018

bacteria or fungi [5]. Phenolics show potent antifungal and antibacterial activity, and through these properties they can be used for treatment of infections and diseases (such as skin diseases) [6].

Fabaceae (*Leguminosae*) contains over 18000 species widespread throughout the world, and are medicinally, economically and culturally important plants [7]. The members of this family often contain numerous chemically active constituents, such as tannins, flavonoids, and terpenes which are substances of a wide range biological activity [8, 9]. Therefore, these plants due to their therapeutic potential for the management of various conditions attract the attention of researchers. *Onobrychis nitida*, *Hedysarum cappadocicum*, *Ebenus laguroides and E. macrophylla* which are the members of *Fabaceae* are medicinal plants and endemic for Turkey flora [10].

O. nitida is popularly known as "fırat korungası" [10]. *Onobrychis* genus, the sainfoins, is used in the traditional treatment of bleeding, cuts, wounds in various countries [11, 12]. *Onobrychis* genus have rich content including phenolics such ferulic acid, benzoic acid, *p*-hydroxybenzoic acid, rutin, caffeic acid, quercetin, *p*-coumaric acid [13], and therefore it shows antioxidant [14] and antimicrobial activity [15].

H. cappadocicum is called as "tatlı tırfıl" in Turkey. Traditional medicine in Turkey and China uses the members of *Hedysarum* genus as a cure of the treatment for gingival and kidney diseases [16, 17]. This genus contains several physiologically active phytochemicals including kaempferol, rutin, chlorogenic acid [18]. Additionally, previous studies have reported that the members of *Hedysarum* genus or isolated compounds show antitumor [19] and antidiabetic [20] effects.

E. laguroides and *E. macrophylla*, also known as mor geven and ulu geven respectively, are two endemic plants for the flora of Turkey. Traditionally, *Ebenus* genus is used for the treatment of diseases such as skin, stomach and kidney problems [21]. These plants are rich in secondary metabolites, including methylinositol, rutin, catechin, hyperoside, picein, tannic acid, *p*-coumaric acid and have antioxidant [21, 22], anticancer [23], anticonvulsant [24] properties due to their chemical compositions.

However, to best of our knowledge there are no scientific reports on the effectiveness of combination of these plants to compare and show their properties. For this reason, the aim of the study is to evaluate the phenolic compounds and the *in vitro* antimicrobial and antioxidant activities of *Onobrychis nitida*, *Hedysarum cappadocicum*, *Ebenus laguroides and E. macrophylla.*

2. Material and Methods

2.1. Collection of Plant Materials

The plants were collected freshly from different locations of Erzincan during the flowering stage, in June 2015 (Table 1). Also, they were identified by Dr. Ali Kandemir. After the confirmation of taxonomic identifications of *Onobrychis nitida* Boiss., *Hedysarum cappadocicum* Boiss., *Ebenus laguroides* Boiss. var. *laguroides*, and *Ebenus macrophylla* Jaub. and Spach. were dried in sunless, their leaves and flowers were divided from the stems, and powdered, respectively and stored in tight plastic containers for further use. The voucher specimens have been collected at the Herbarium of the Espiye Vocational School, Giresu University, Giresu, Turkey.

Table 1. Collection sites and voucher number of the plant samples

Plant name	Collection site	Voucher number
Onobrychis nitida Boiss.	Iliç, north-east of Hasanova village,1391 m, Erzincan	ESPH 026
Hedysarum cappadocicum Boiss	Kemah, Kurucay roadside,1258 m, Erzincan	ESPH 027
Ebenus laguroides Boiss. var. *laguroides*	Between Kemah-Erzincan,1129 m, Erzincan	ESPH 028
Ebenus macrophylla Jaub. & Spach	Between Kemah-Erzincan,1145 m, Erzincan	ESPH 029

2.2. Plant Extract Preparation

The dried and finely powdered samples of each plant (5 g for each trial) were extracted 100 mL of methanol for 6 hours (or continued until the extract gave no coloration) using Soxhlet extractor. The extracts were then filtered by using thin paper and the homogenous solutions were evaporated until dryness by using evaporator at 40°C. Remaining extracts were divided into two parts. The first part was dissolved in distilled water for antioxidant and antimicrobial analysis. Also, the second part was prepared in pure methanol for determining of the RP-HPLC-DAD (Reverse phase-high performance liquid chromatography with a diode array detector) analyzing.

2.3. RP-HPLC-DAD Analysis

Phenolic constituents of plants were analyzed by using RP-HPLC-DAD. These analyses were achieved on Thermo Scientific Dionex Ultimate™ 3000 system (Thermo Scientific, Bremen, Germany). Chromatographic separation was performed on a Thermo Scientific™ Hypersil™ ODS C18 HPLC (250 mm × 4.6 mm x 5 µm) column (Thermo Scientific, USA) at temperature 30°C using a mobile phase (containing 2 % (v/v) acetic acid in water -A-, 70% (v/v) acetonitrile in water -B-), at a flow rate 1.2 mL/min, under gradient elution conditions. The gradient used as follows: zero-time condition was 5% B and it was increased to 60% B in 26 minutes. The eluted 10 standard phenolic acids: gallic, protocatechuic, *p*-hydroxybenzoic, chlorogenic, vanillic, caffeic, syringic, *p*-coumaric, rosmarinic, benzoic, and two flavonoids: rutin, quercetin were monitored by comparison at 280 and 315 nm.

The validation parameters of Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated for each standard according to the signal/noise (S/N) level of 3 and 9 respectively (Table 2).

Table 2. The standard chromatogram values of twelve individual phenolic compounds.

No	RT	Standards	R^2	RSD %(RT)	RSD %(Area)	LOD ($mg.L^{-1}$)	LOQ ($mg.L^{-1}$)
1	3.72±0.006	Gallic acid	0.999	0.168	4.315	0.070	0.213
2	6.74±0.019	Protocatechuic acid	0.998	0.291	5.973	0.495	1.499
3	10.13±0.029	*p*-hydroxybenzoic acid	0.999	0.290	4.817	0.224	0.680
4	11.46±0.027	Chlorogenic acid	0.997	0.239	6.177	0.512	1.550
5	13.49±0.023	Vanilic acid	0.994	0.168	6.794	0.171	0.518
6	13.84±0.032	Caffeic acid	0.999	0.235	6.861	0.058	0.175
7	14.79±0.012	Syringic acid	0.999	0.082	5.116	0.096	0.290
8	16.41±0.010	*p*-Coumaric acid	0.999	0.061	2.935	0.005	0.014
9	16.63±0.012	Rutin	0.999	0.075	2.855	0.311	0.942
10	18.41±0.013	Rosmarinic acid	0.999	0.069	3.388	0.162	0.492
11	18.84±0.014	Benzoic acid	0.999	0.076	2.721	0.550	1.665
12	21.71±0.019	Quercetin	0.999	0.087	2.268	0.335	1.014

2.4. Determination of Antioxidant Activity

2.4.1. Total Phenolic Content

Total phenolic content of analyzed plants was applied by the Folin-Ciocalteu method against the gallic acid calibration graph [25]. First, 680 µL distilled water, 20 µL of stock extracts, and 400 µL of 0.5 N Folin-Ciocalteu reagents were mixed in a tube and incubated for 10 minutes. Next, 400 µL Na_2CO_3 (10%) was supplied and incubated for 2 hours. The absorbance was calculated at 760 nm at the end of the incubation period. All the measurements were performed in triplicate. Total phenolic contents were represented as milligrams of gallic acid equivalents per gram of extract (mg GAE/g extract).

2.4.2. Ferric Reducing Antioxidant Power Assay

The FRAP method was determined according to Benzie and Strain [26]. The assay, which is based on the reduction of Fe^{3+}-TPTZ complex to Fe^{2+}-TPTZ complex under acidic condition, is used for the determination of cumulative antioxidant effects. The fresh FRAP reagent prepared with TPTZ, $FeCl_3$, and acetate buffer. Methodology consist of 3 mL FRAP reagent and 100 µL of the plant extracts. Absorbance was recorded at 593 nm after 4 min incubation at 25°C. Reagent and sample blanks were also tested, and the sum of these two measurements was subtracted from the final absorbance. The final absorbance was calculated with the $FeSO_4.7H_2O$ standard curve. The data were indicated as µM $FeSO_4.7H_2O$ equivalents/g extract. Higher $FeSO_4.7H_2O$ equivalent values means higher FRAP and thus higher antioxidant capacity.

2.4.3. DPPH Free Radical Scavenging Assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was the main reagent on scavenging activity of compounds against 517 nm. The DPPH solution was deactivated by the antioxidants [27]. Briefly, a 50 µL portion of the extract solution was mixed with 5 mL of a freshly prepared 0.004% (w/v) DPPH radical methanol solution. Reaction volume was incubated for 30 min in the dark at 25°C. The absorbance was recorded with spectrophotometer (Mapada UV-6100PC) using a positive control blank. The inhibition percentage and extract concentration were plotted for providing 50% inhibition concentration (IC_{50}) of DPPH. The assay was performed in triplicate to give standard deviation.

2.5. Antimicrobial Activity

The antimicrobial activity of extracts were evaluated with a microwell dilution method against bacterial strains and yeast isolates [28] and minimal inhibition concentration (MIC) values were expressed as µg/mL. All test microorganisms were supplied from the Department of Biology Department at Karadeniz Technical University (Trabzon, Turkey) and were as follows: *Escherichia coli* ATCC 25922, *Bacillus subtilis* subsp. *spizizenii* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumonia* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Yersinia pseudotuberculosis* ATCC 911, *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* RSKK 709, *Acinetobacter baumannii* RSKK 02026, *Enterobacter cloacea* ATCC 13047, *Candida albicans* ATCC 14053.

Stock solutions of each extracts were prepared in distilled water and sterilized by filtration by 0.45 µm Millipore filters. The final concentration was adjusted with 40 mg/mL as stock solution. A dilution concentration range from 20 to 0.039 µg/mL was obtained with starting of 100 µL of stock solution in each well containing nutrient broth. Last two wells were used as a sterility control (containing culture broth plus 100 µL of stock solution, without antimicrobial substance) and a growth control. Each test and growth control well were vaccinated with 5 µL of a bacterial suspension ($5x10^5$ CFU/well). All experiments of microdilution trays were applied in triplicate and after incubation at 30 ºC (*E. cloacea*), 28 ºC (*C. albicans*), 37 ºC (*E. coli*, *B. subtilis* subsp. *spizizenii*, *S. aureus*, *K. pneumonia*, *P. aeruginosa*, *Y. pseudotuberculosis*, *E. faecalis*, *B. cereus*, *A. baumannii*) for 18-20 h. Bacterial growth was

detected by addition of 40 µL of an INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride) alcoholic solution (0.2 mg/mL) (Sigma). The trays were again incubated at the temperatures given above for 30 min, and in those wells, where bacterial growth occurred, INT changed from yellow to purple. The MIC was defined as the concentration in the well containing lowest compound dose that observed no growth. Ampicillin, amikacin, and fluconazole were used as standard antibacterial and antifungal drugs, respectively.

3. Results and Discussion

Plant active compounds are gaining increasing popularity with their bioactive properties and they could be used as alternative medicinal sources. In our study herein, the extract of *O. nitida*, *H. cappadocicum*, *E. laguroides*, *E. macrophylla*, which had strong antioxidant and antimicrobial activity, was investigated with regard to 10 phenolic acid compounds (gallic, protocatechuic, *p*-hydroxybenzoic, chlorogenic, vanillic, caffeic, syringic, *p*-coumaric, rosmarinic, and benzoic acid) and 2 flavonoids (rutin and quercetin) Figure 1. When compared direct and indirect result from literature, the present study could be evaluated as a wide spectrum perspective with different bioactivity analysis. Also, the results of all plant samples were given as mg phenolic per g extract in Table 3.

Table 3. Phenolic constituents of the analyzed plants by RP-HPLC-DAD.

	Phenolic Compounds (mg phenolic/ g extract)	*O. nitida*	*H. cappadocicum*	*E. laguroides*	*E. macrophylla*
1	Gallic Acid	0.109	0.103	1.452	0.535
2	Protocatechuic Acid	0.953	1.668	1.935	3.745
3	*p*-hydroxybenzoic Acid	1.727	1.435	2.419	2.179
4	Chlorogenic Acid	n.d.	n.d.	n.d.	n.d.
5	Vanillic Acid	0.942	0.518	7.662	2.965
6	Caffeic Acid	0.053	0.043	0.248	0.092
7	Syringic Acid	0.077	0.271	1.231	0.668
8	*p*-coumaric Acid	0.713	0.507	5.329	2.525
9	Rutin	n.d.	n.d.	19.434	11.808
10	Rosmarinic Acid	n.d.	n.d.	n.d.	n.d.
11	Benzoic Acid	n.d.	n.d.	4.329	n.d.
12	Quercetin	n.d.	0.135	0.812	0.385

n.d.: not detected

Phenolic compounds known as secondary metabolites are separated as two groups named simple phenols (phenolic acid, coumarins) and polyphenols (flavonoids, stilbenes, lignans, tannins). Phenolic compounds are formed by some metabolic pathways which are the shikimic acid and acetic acid. These pathways can give chance for conversion and derivatization to each other. In the present study, phenolics could be seen some members of hydroxycinnamic acids, hydroxybenzoic acids, flavonols, and some ester derivatizations. Generally, ester derivatives could not be detected. Cholorogenic acid is a hydroxycinnamic acid ester and rosmarinic acid is a caffeic acid ester of 3,4-dihydroxyphenyllactic acid, they could be given as an evidence for this absence (Table 3).

With the exception of cholorogenic acid and rosmarinic acid, components were identified at various levels in sample extracts from all four types (Table 3). When the obtained results from each plant types were examined individually, different major compounds were seen as an answer. For example, rutin, the glycoside combining quercetin and rutinose, was dominate for *E. laguroides* (19.434 mg/g extract) and *E. macrophylla* (11.808 mg/g extract), protocatechuic acid for *H. cappadocicum*, and *p*- hydroxybenzoic acid for *O. nitida*.

Previous reports were generally concerning about *Onobrychis viciifolia*. It is well known as a source of proanthocyanidins which are member of tannin. There are common studies in this area. Malisch et al. [29] analyzed proanthocyanidin concentrations and simple phenolic compounds of individual plants of 27 *Onobrychis viciifolia* with Liquid chromatography-electrospray ionization triple quadrupole mass spectrometry (LC-ESI-QqQ-MS/MS). There was another study about the evaluation of polyphenolic compounds of *Onobrychis viciifolia* in order to support to its application for sustainable agriculture management in Mediterranean regions [30]. Both other actual reports which were not cited and these two studies showed us that *Onobrychis* and its subspecies had valuable bioactive sources. Although there was so many data about *Onobrychis*' studies, there was no study about the phenolic compounds of *O. nitida*. For this reason, comparisons were done with other subspecies of *Onobrychis*. Similar to our own findings, one previous study reported that *Onobrychis armena* had moderate level for *p*-hydroxybenzoic acid [13].

There is no data value about *H. cappadocicum* in scientific area. When the focus was on HPLC result, two beneficial components which were protocatechuic and *p*-hydroxybenzoic benzoic acids could be noticed. Protocatechuic acid is well known as the valuable human metabolite, combines with derivatizations of some cyanidin-glucosides. Semaming et al. [31] investigated the pharmacological properties of protocatechuic acid and their findings included antioxidant, anti-inflammatory, antihyperglycemia, antiapoptosis/proapoptosis, and antimicrobial activities. They emphasized that significant biological potential of protocatechuic acid through the modulation of cellular signals involved in the control of oxidative stress agents. Merkl et al. [32] determined the biological activities of some phenolic acids alkyl esters in the range of methyl and hexyl. It was reported that the increasing length of the alkyl chain was seen as parallel with the degree of antimicrobial effect of phenolic acid derivatives, and protocatechuic acid and *p*-hydroxybenzoic acid were possessed the bioactivity.

Besides *Onobrychis nitida* and *Hedysarum cappadocicum*, in terms of citation there wasn't any study concerning about *Ebenus laguroides* and *Ebenus macrophylla.* So, gathered results were compared with previous studies about other *Ebenus* species. Abreu et al. [22] reported some phenolic compounds from isolated of *Ebenus pinnata* aerial parts. Finally, their results showed a source of flavonoid as the present study.

There was another actual study from Turkey related to bio-properties of *Ebenus hirsute* such as some enzyme inhibitory effects, degrees of antioxidant, antimicrobial and antigenotoxic. The plant was evaluated as a remarkable biological activity source due to its rich phenolic compounds (*p*-coumaric acid, rutin, hyperoside, hesperidin, and tannic acid) which were applied by using liquid chromatography coupled with mass spectrometry (LC–MS/MS) technique [21]. Both of studies showed to us that rutin was a remarkable agent for the *Ebenus* species as in the current study.

As it is known, antioxidants mainly come from plants in the form of phenolic compounds such as flavonoid, phenolic acids. Also, phenolics have been received much attention for their effective antioxidant properties which have related with donating electrons, scavenging free radicals, and reducing power [33].

In an evaluation study written by Ince et al. [34], the antioxidant effects of the different extracts of *Onobrychis viciifolia* were designed. The antioxidant power was found 521.85±5.33 µmol/g of extract by using phosphomolybdenum method. Another study written by Karamian and Asadbegy [14] was concluded that *Onobrychis sosnovskyi* Grossh., *Onobrychis viciifolia* Scop. and *Onobrychis melanotricha* Boiss extracts represented strong antioxidant activity. In that study, Folin-Ciocalteu method, aluminum chloride colorimetric method, DPPH activity, metal-chelation activity, and ß-carotene/linoleic acid model were carried out, respectively. Although the results showed different value with the current study, because of different species

and other variable parameters (climate, region, elevation etc.), the general opinion emphasized in our study is that *Onobrychis* species suggested as antioxidant agents for special uses.

The total phenolic contents could be performed as a basis for rapid screening of antioxidant activity due to some bioactive groups such as phenolic acids, coumarins, flavonoids, stilbenes, tannins etc. known as plant secondary metabolites. Generally, total phenolic and other antioxidant assays show the strong correlation, but sometimes this situation could be change as a disadvantage. In our results, *Onobrychis nitida* had the highest value with 101.73 mg GAE/g sample. When the results were analyzed, its FRAP value showed moderate effect with 518.87 μM $FeSO_4.7H_2O$/ g extract. This uncorrelated data between TPC (Total phenolic content) and FRAP (Ferric reducing antioxidant power) could be explained with the interference of reducing ingredient such as ascorbic acid [35].

The reducing capacity of natural compounds is regarded as an impressive indicator of their potential antioxidant activity. FRAP has known as simple, quick, reproducible assay. FRAP results are calculated with linearly calibration graph related to the concentration of the concerned antioxidants. From this perspective, FRAP method was carried out to evaluate reducing capacities of the plant extracts. The results obtained showed that all samples contained high FRAP value. The highest results were in *Ebenus laguroides* with 719.09 μM $FeSO_4.7H_2O$/ g extract followed by *Ebenus macrophylla* (571.95 μM $FeSO_4.7H_2O$/ g extract), *Onobrychis nitida* (518.87 μM $FeSO_4.7H_2O$/ g extract), and *Hedysarum cappadocicum* (429.21 μM $FeSO_4.7H_2O$/ g extract). In the light of these current FRAP results, it was observed that all extracts showed nearly the same power at analyzed concentration. The main cause of small differences of these results was various bio-contents of plant species. But sometimes same species could have different bioactive values, since many factor are responsible for this changing such as storage time, geographic origin, harvesting date environment and technological factors [35].

Kedare and Singh [27] claimed that 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay is considered as precise, sensitive, simple, and commercial method to determine radical scavenging activity of antioxidants, because the radical compound is unchangeable and need not be produced. Recent studies displayed significant radical scavenging activity of natural component extracts [36, 37]. Each natural compounds show different degrees of DPPH scavenging activities depending on the capacity of antioxidant contents with losing hydrogen and converting the structural configuration [38]. *Onobrychis nitida*, *Hedysarum cappadocicum*, *Ebenus laguroides* and *Ebenus macrophylla* have various compounds, for this reason DPPH results could be changed as seen in the Table 4. According to our results, *E. macrophylla* extract confirmed the highest DPPH scavenging activity (IC_{50}: 69.45 μg/mL). Others were lined up by *O. nitida* (IC_{50}: 77.29 μg/mL), *E. laguroides* (IC_{50}: 88.00 μg/mL) and *H. cappadocicum* (IC_{50}: 108.32 μg/mL), respectively.

Results of antimicrobial activity of *Onobrychis nitida, Hedysarum cappadocicum, Ebenus laguroides and E. macrophylla* and standard antibiotics (Ampicillin, Amikacin and Fluconazole) were presented in Table 5. MIC values of the current extracts were among of 1.25-5 mg/mL, excluding *A. baumannii, E. cloacea* and *P. aeruginosa* bacteria and yeast *C. albicans*, while the analyzed extracts had no activity (MIC > 5 mg/mL). *O. nitida*'s antimicrobial activity was similar to that of other extracts against some microorganisms but was usually somewhat higher. The highest MIC values of *O. nitida* were 1.25 mg/mL against *B. subtilis* and *S. aureus*. All of the extracts showed no anticandidal activity. Some of these extracts showed antimicrobial activity (antibacterial activity), but their antimicrobial potentials were much lower than the standard antibiotics.

Table 4. Total phenolic contents, ferric reducing antioxidant power, and radical scavenging activities of plants.

	O. nitida	*H. cappadocicum*	*E. laguroides*	*E. macrophylla*
TPC (mg GAE/g extract)	101.73±0.52	50.96±1.01	64.88±1.13	56.97±1.01
FRAP (µM $FeSO_4.7H_2O$)	518.87±0.02	429.21±0.00	719.09±0.01	571.95±0.01
DPPH (IC_{50}:µg/mL)	77.29	108.32	88	69.45

When the TPC and HPLC analysis results presented in Table 3 and Table 4 were evaluated, some assumptions could be made about the better antibacterial activity of *O. nitida*. Higher total phenolic content might be responsible for higher antimicrobial activity. Moreover, low concentration of compounds (total concentration of standard phenolic compounds) identified by HPLC indicates that the phenolic or phenolics were responsible for antimicrobial activity, and it might be from unidentified phenolics. Our results showed that the Gram-positive bacteria were more susceptible for plant extracts, particularly *O. nitida*, than Gram-negative bacteria. This phenomenon might be associated with a thin peptidoglycan and extra lipopolysaccharide layer, which provides strong hydrophilic and less permeable properties [39].

Table 5. Minimal inhibitor concentrations (MIC) of plant extracts and reference antibiotics.

	O. nitida	*H. cappadocicum*	*E. laguroides*	*E. macrophylla*	Ampicillin	Amikacin	Fluconazole
E. coli	-	5000		5000	7.8	0.49	-
B. subtilis	1250	-	-	-	0.98	0.49	-
S. aureus	1250	2500	2500	2500	0.49	0.98	-
K. pneumonia	2500	2500	2500	2500	-	0.49	-
P. aeruginosa	-	-	-	-	-	0.49	-
Y. pseudo tuberculosis	2500	5000	5000	5000	125	31.2	-
E. faecalis	2500	5000	2500	-	1.9	62.5	-
B. cereus	5000	-	-	-	-	0.49	-
A. baumannii	-	-	-	-	7.8	0.98	-
E. cloacea	-	-	-	-	-	0.98	-
C. albicans	-	-	-	-	-	-	1.5

In the literature, there were no reports about the antimicrobial activity of *O. nitida, H. cappadocicum, E. laguroides and E. macrophylla*. So, results were compared with limited previous studies about other *Onobrychis, Hedysarum* and *Ebenus* species. In most of these reports, antimicrobial activities were determined by disc diffusion method, which did not provide a reliable and valid result for the determination of activity. In one of those studies, antimicrobial activity of water-insoluble and water-soluble leaves extracts of *Onobrychis armena* against *E. coli*, *S. aureus*, *B. subtilis*, and *C. albicans* were investigated and it was determined that there was no activity in water-insoluble extract. Similar to our results, water-soluble extract showed slightly antimicrobial activity against *S. aureus* (MIC 0.625 mg/mL) and *B. subtilis* (MIC >1000 mg/mL) [40]. Karakoca et al. [13] reported that the *O. armena* extract did not show antimicrobial activity against *E. coli*, *S. aureus*, *B. subtilis*, and *C. albicans*. They also determined that root extract had moderate antimicrobial activity (MBC: 22.50 mg/mL).

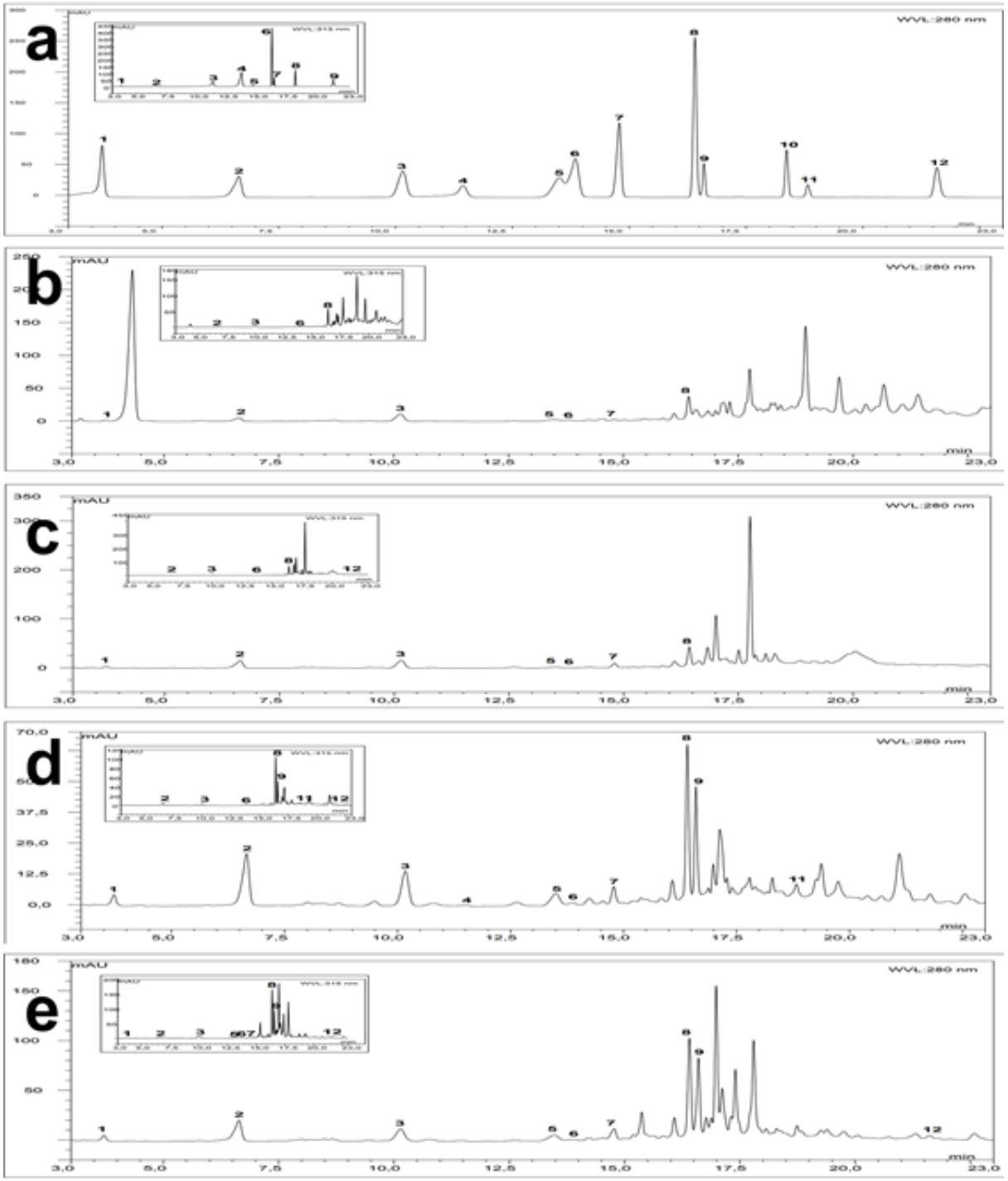

Figure 1. RP-HPLC-DAD chromatogram of plant extracts (a) standard phenolics (b) *O. nitida* (c) *H. cappadocicum* (d) *E. laguroides* (e) *E. macrophylla* (1) gallic acid (2) protocatechuic acid (3) *p*-hydroxybenzoic benzoic acid (4) chlorogenic acid (5) vanillic acid (6) caffeic acid (7) syringic acid (8) *p*-coumaric acid (9) rutin (10) rosmarinic acid (11) benzoic acid (12) quercetin

4. Conclusion

This research underlines the effort to analyze of phenolic compounds, antioxidant, and antimicrobial properties of the extracts from *Onobrychis nitida*, *Hedysarum cappadocicum*, *Ebenus laguroides* and *Ebenus macrophylla*. At this occasion, some different general parameters of bioactivity assays were combined and evaluated in four plant species for the first time. According to the results, it was noted that some species had strong characteristics in some branches of bioactive assays due to the changing of chemical components. Overall, there are broad agreements between bioactivity and chemical components that are based on types of plant characteristics. Also, there are some reasons for the decrease or increase of chemical properties of plants. The first factor is plant species and the others could be numbered as season, climate, and elevation. The efficient results of these endemic species may fill in the huge blanks of scientific reports and it may lead the way to enhance the further multidisciplinary experiments.

Conflict of Interest

The authors declare that there is no conflict of interests in this current study.

Orcid

Ersan Bektas https://orcid.org/0000-0001-9030-6908
Kaan Kaltalioglu https://orcid.org/0000-0002-4995-2657
Huseyin Sahin https://orcid.org/0000-0002-6018-1494
Zafer Turkmen https://orcid.org/0000-0001-6709-613X
Ali Kandemir https://orcid.org/0000-0003-1902-9631

5. References

[1] Chedia, A., Ghazghazi, H., Brahim, H. & Abderrazak, M. (2013) Secondary metabolite, antioxidant and antibacterial activities of *Teucrium polium* L. methanolic extract. *International Journal of Plant Production*, 4, 1790–1797.

[2] Pereira, D. M., Valentão, P., Pereira, J. A. & Andrade, P. B. (2009) Phenolics: from chemistry to biology. *Molecules*, 14, 2202–2211.

[3] Mahmoudi, S., Khali, M., Benkhaled, A., Benamirouche, K. & Baiti, I. (2016) Phenolic and flavonoid contents, antioxidant and antimicrobial activities of leaf extracts from ten Algerian *Ficus carica* L. varieties. *Asian Pacific Journal of Tropical Biomedicine*, 6, 239–245.

[4] Bhattacharyya, A., Chattopadhyay, R., Mitra, S. & Crowe, S. E. (2014) Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiological Reviews*, 94, 329–54.

[5] Wanamaker, B. P. & Massey, K. L. (2009) Antiinfective Drugs, in *Applied Pharmacology for Veterinary Technicians* 4th ed., p 227. Elsevier Health Sciences, St. Louis, Missouri, USA.

[6] Dzialo, M., Mierziak, J., Korzun, U., Preisner, M., Szopa, J. & Kulma, A. (2016) The potential of plant phenolics in prevention and therapy of skin disorders. *International Journal of Molecular Sciences*, 17, 1–41.

[7] Erbil, N., Duzguner, V., Durmuskahya, C. & Alan, Y. (2015) Antimicrobial and antioxidant effects of some Turkish fodder plants belongs to Fabaceae family (*Vicia villosa*, *Trifolium ochroleucum* and *Onobrychis altissima*). *Oriental Journal of Chemistry*, 31, 53–58.

[8] Molares, S. & Ladio, A. (2012) The Usefulness of edible and medicinal fabaceae in argentine and chilean patagonia: environmental availability and other sources of supply. *Evidence-Based Complementary and Alternative Medicine*, 2012, 1–12.

[9] Moerman, D., Pemberton, R. & Kiefer, D. (1999) A comparative analysis of five medicinal floras. *Journal of Ethnobiology,* 19, 49–67.

[10] Güner, A., Aslan, S., Ekim, T., Vural, M. & Babaç, M. (2012) Türkiye bitkileri listesi (damarlı bitkiler). Nezahat Gökyiğit Botanik Bahçesi ve Flora Araştırmaları Derneği Yayını, Istanbul.

[11] Pieroni, A. & Giusti, M. (2009) Alpine ethnobotany in Italy: traditional knowledge of gastronomic and medicinal plants among the Occitans of the upper Varaita valley, Piedmont. *Journal of Ethnobiology and Ethnomedicine,* 5(32)-1-13.

[12] Mükemre, M., Behçet, L. & Çakılcıoğlu, U. (2015) Ethnobotanical study on medicinal plants in villages of Çatak (Van-Turkey). *J. Ethnopharmacol.* 166, 361–374.

[13] Karakoca, K., Asan-Ozusaglam, M., Cakmak, Y. S. & Teksen, M. (2015) Phenolic compounds, biological and antioxidant activities of *Onobrychis armena* Boiss. & Huet flower and root extracts. *Chiang Mai University Journal of Natural of Sciences*, 42, 376–392.

[14] Karamian, R. & Asadbegy, M. (2016) Antioxidant activity, total phenolic and flavonoid contents of three Onobrychis species from Iran. *Journal of Pharmaceutical Sciences,* 22, 112–119.

[15] Usta, C., Yildirim, A. B. & Turker, A. U. (2014) Antibacterial and antitumour activities of some plants grown in Turkey. *Biotechnology & Biotechnological Equipment*, 28, 306–315.

[16] Wang, W., An, D.-G., Chen, H.-B., Tu, G.-Z. & Zhao, Y.Y. (2003) Two new pterocarpenes from *Hedysarum multijugum*. *Journal of Asian Natural Products Research,* 5, 31–34.

[17] Altundag, E. & Ozturk, M. (2011) Ethnomedicinal studies on the plant resources of east Anatolia, Turkey. *Procedia - Social and Behavioral Sciences,* 19, 756–777.

[18] Tibe, O., Meagher, L. P., Fraser, K. & Harding, D. R. K. (2011) Condensed tannins and flavonoids from the forage legume sulla (*Hedysarum coronarium*). *Journal of Agricultural and Food Chemistry,* 59, 9402–9409.

[19] Li, S., Wang, D., Tian, W., Wang, X., Zhao, J., Liu, Z. & Chen, R. (2008) Characterization and anti-tumor activity of a polysaccharide from *Hedysarum polybotrys* Hand.-Mazz. *Carbohydrate Polymers,* 73, 344–350.

[20] Hu, F., Li, X., Zhao, L., Feng, S. & Wang, C. (2010) Antidiabetic properties of purified polysaccharide from *Hedysarum polybotry*s. *Canadian Journal of Physiology and Pharmacology, 88*, 64–72.

[21] Ceylan, R., Katanić, J., Zengin, G., Matić, S., Aktumsek, A., Boroja, T., Stanić, S., Mihailović, V., Guler, G. O., Boga, M., Yılmaz, M. A. & Yilmaz, M. A. (2016) Chemical and biological fingerprints of two Fabaceae species (*Cytisopsis dorycniifolia* and *Ebenus hirsuta*): Are they novel sources of natural agents for pharmaceutical and food formulations? *Industrial Crops and Products,* 84, 254–262

[22] Abreu, P. M., Braham, H., Ben Jannet, H., Mighri, Z. & Matthew, S. (2007) Antioxidant compounds from *Ebenus pinnata*. *Fitoterapia,* 78, 32–34.

[23] Aydemir, E., Simsek, E., Imir, N., Göktürk, R., Yesilada, E. & Fiskin, K. (2015) Cytotoxic and apoptotic effects of *Ebenus boissieri* Barbey on human lung cancer cell line A549. *Pharmacognosy Magazine,* 11, 37-45.

[24] Khodaparast, A., Sayyah, M. & Sardari, S. (2012) Anticonvulsant activity of hydroalcoholic extract and aqueous fraction of *Ebenus stellata* in mice. *Iranian Journal of Basic Medical Sciences,* 15, 811–819

[25] Singleton, V.L. & Rossi, J.A. (1965) Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, 16, 144–158

[26] Benzie, I. I. & Strain, J. J. (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry*, 239(1), 70-76.

[27] Kedare, S. B. & Singh, R. P. (2011) Genesis and development of DPPH method of antioxidant assay. *Journal of Food Science and Technology*, 48, 412–422.

[28] Güllüce, M., Adigüzel, A., Oğütçü, H., Sengül, M., Karaman, I. & Sahin, F. (2004) Antimicrobial effects of *Quercus ilex* L. extract. *Phytotherapy Research*, 18, 208–211.

[29] Malisch, C. S., Lüscher, A., Baert, N., Engström, M. T., Studer, B., Fryganas, C., Suter, D., Mueller-Harvey, I. & Salminen, J.-P. (2015) Large variability of proanthocyanidin content and composition in sainfoin (*Onobrychis viciifolia*). *J. Agric. Food Chemistry,* 63, 10234–10242.

[30] Re, G. A., Piluzza, G., Sulas, L., Franca, A., Porqueddu, C., Sanna, F. & Bullitta, S. (2014) Condensed tannin accumulation and nitrogen fixation potential of *Onobrychis viciifolia* Scop. grown in a Mediterranean environment. *Journal of the Science of Food and Agriculture*, 94, 639–645.

[31] Semaming, Y., Pannengpetch, P., Chattipakorn, S. C. & Chattipakorn, N. (2015) Pharmacological properties of protocatechuic acid and its potential roles as complementary medicine. *Evidence-Based Complementary and Alternative Medicine,* 2015(4), 1–11.

[32] Merkl, R., Hrádková, I., Filip, V. & Šmidrkal, J. (2010) Antimicrobial and antioxidant properties of phenolic acids alkyl esters. *Czech Journal of Food Sciences*, *28*, 275–279.

[33] Zhao, H., Zhang, H. & Yang, S. (2014) Phenolic compounds and its antioxidant activities in ethanolic extracts from seven cultivars of Chinese jujube. *Food Science and Human Wellness*, 3, 183–190.

[34] Ince, S., Ekici, H. & Yurdakok, B. (2012) Determination of *in vitro* antioxidant activity of the sainfoin (*Onobrychis viciifolia*) extracts. *Ankara Üniversitesi Veteriner Fakültesi Dergisi*, 59, 23–27.

[35] Rabeta, M. S. & Nur Faraniza, R. (2013) Total phenolic content and ferric reducing antioxidant power of the leaves and fruits of garcinia atrovirdis and cynometra cauliflora. *International Food Research Journal*, 20, 1691–1696

[36] Da Porto, C., Calligaris, S., Celotti, E. & Nicoli, M. C. (2000) Antiradical properties of commercial cognacs assessed by the DPPH • Test. *Journal of Agricultural and Food Chemistry*, 48, 4241–4245.

[37] Soares, J. R., Dinis, T. C., Cunha, A. P. & Almeida, L. M. (1997) Antioxidant activities of some extracts of *Thymus zygis*. *Free Radical Research*, 26, 469–478.

[38] Aksoy, L., Kolay, E., Ağılönü, Y., Aslan, Z. & Kargıoğlu, M. (2013) Free radical scavenging activity, total phenolic content, total antioxidant status, and total oxidant status of endemic *Thermopsis turcica*. *Saudi Journal of Biological Sciences,* 20, 235–239.

[39] Mann, A., Abalaka, M. E., Garba S.A (1997) The antimicrobial activity of the leaf extracts of Calotropis procera. *Biomedical Letters*, 55, 205–210.

[40] Buruk, K., Sokmen, A., Aydin, F. & Erturk, M. (2006) Antimicrobial activity of some endemic plants growing in the Eastern Black Sea Region, Turkey. *Fitoterapia*, 77, 388–391.