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Investigation of Total Phenolic, Flavonoid Content and Antioxidant Activity of *Onobrychis Megataphros* Leaf Extract

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Abstract: This study aims to determine the total phenolic, flavonoid and antioxidant activity levels of *Onobrychis megataphros* plant leaves. The *O.megataphros* (Sainfoin) samples were collected, laid on blotting papers in shady places away from sunlight and dried. After that, the dried plant samples were pulverized. The powdered samples were extracted with methanol using Soxhlet device. The methanol was removed by evaporation in a rotary evaporator and stored at +4ºC until the experiment starts. Phenolic and flavonoid content and antioxidant activity properties of *O.megataphros* plant leaf extracts were examined. The determination of the Phenolic and flavonoid performed according to Zengin et al. (2015a). The sample and blank absorbance was read at 415 nm after 10 min incubation at room temperature. Absorbance of the blank was subtracted from that of the sample. Total flavonoid content was expressed as equivalent to routine. Total antioxidant activity of the samples was evaluated by phosphor molybdenum method. Sample solution (0.2mL) was combined with 2mL of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The sample absorbance was read at 695nm after 90 min incubation at 95°C. As a result, it was determined that the total phenolic content in *O.megataphros* plant leaf extracts was 20.62±0.15 mg GAE/g extract and total flavonoid content was 24.55±0.22 mg QE/g extract. At the end of this research work, it is concluded that TAS and TOS levels increased with the increase in concentration.

Keywords: Onobrychis megataphros, Total phenolic content, Total flavonoid content, TOS, TAS

Introduction

Many plant species have been used for various purposes among humans for many years across the world. According to the World Health Organization (WHO), pharmacopoeias of 91 countries and some publications on medicinal plants, the number of medicinal plants used for therapeutic purposes is over 20.000. The important properties of plants for human health have been investigated in the laboratory since 1926 (Toroglu & Cenet, 2006). The genus *Onobrychis*, which grows naturally in Turkiye consists of two subgenera, *Onobrychis* and *Sisyrosema*. Among these subgenera, *Onobrychis* is known to be divided into 3 different sections as *Dendobrychis*, *Laphobrychis* and *Onobrychis*. Fabaceae family is the second largest family after Asteraceae in Turkiye and has various uses among the people. For example, *Onobrychis gracilis* is used in the treatment of cold and flu, *Vicia faba* in gastrovascular diseases, *Vicia cracca* subsp. *stenophylla* in cold and *Vicia ervilia* in the treatment of diabetes (Demirci & Ozhatay, 2012; Sargın et al., 2013; Hayta et al., 2014).

Free radicals are formed during the functioning of normal metabolic pathways in the organism or by environmental agents such as pesticides, aromatic hydrocarbons, toxins, solvents or under the influence of various external factors such as stress and radiation (Pham Huy et al., 2008). Oxygen-centered free radicals and

reactive oxygen species, which are continuously produced in the body, can cause tissue destruction and then cell death. Oxidative damage caused by free radicals can cause aging and some diseases (Phaniendra et al., 2015). Fruits and vegetables rich in phenolic compounds protect the human body against free oxygen radicals. Epidemiological studies have shown that phytonutrients in plant sources are beneficial against reactive oxygen species and the protective effects of fruits and vegetables are due to natural compounds such as ascorbic acid (vitamin C), α-tocopherol (vitamin E), carotenoids, glutathione, flavonoids and phenolic acids (Halvorsen et al., 2002). When free radicals and reactive oxygen species cause oxidation in foods or biological systems, antioxidants can prevent or delay this process by various mechanisms (Ulusoy et al., 2010). With the measurement of antioxidant capacity in foods, people's interest in the use of natural products in pharmaceutical and cosmetic products for health purposes is increasing day by day (Lopez Alarcon & Lissi, 2006).

In recent years, indiscriminate use of antibiotics all over the world and drug resistance are closely related (Celik et al., 2010). It has been observed that the treatment of diseases and infections such as AIDS and cancer, which weaken the immune system, has become difficult due to drug-resistant pathogenic fungi and bacteria (Zazharskyi et al., 2019). This situation has encouraged scientists to search for new antimicrobial compounds from different sources. Since plants constitute a source from which new antimicrobial chemotherapeutic substances can be obtained, research has been particularly focused on medicinal plants (Zazharskyi et al., 2019). Although there is literature information that different *Onobrychis* species are used in the treatment of various diseases among humans (Demirci & Ozhatay, 2012; Sargın et al., 2013; Hayta et al., 2014), it is the first in terms of investigating the antioxidant, antimicrobial, total phenolic content of *O.megataphros* plant and bringing it to the literature.

Method

Collection and Extraction of Plant Samples

Onobrychis megataphros plant used in the study was collected from (Siverek- Hilvan) Şanlıurfa and the surrounding areas between 2021 and 2022 during the vegetation periods. After the plant samples were dried in the shade, the leaves were pulverized. The samples were subjected to methanol extraction for 6-8 hours in a Soxhlet apparatus. The extract was then filtered with Whatman blue band filter paper and evaporated at 40 °C to remove the solvent.

Biological Activity Determination

Determination of Total Phenolic Content: For total phenolic content, the sample solution (0.25 mL) was mixed with diluted Folin - Ciocalteu reagent (1 mL, 1:9) and shaken strongly. After 3 min, Na_2CO_3 solution (0.75 mL, 1%) was added and sample absorbance was read at 760 nm after incubation for 2 h at room temperature. Total phenolic content was expressed as Gallic acid equivalent (Zengin et al., 2015a).

Determination of Total Flavonoid Content: For total flavonoid content, the sample solution (1 mL) was mixed with the same volume of aluminum trichloride (2%) in methanol. Similarly, a blank was prepared by adding the sample solution (1 mL) to methanol (1 mL) without AlCl3. After incubation at room temperature for 10 min, the absorbance of the sample and the blank was read at 415 nm. The absorbance of the blank was subtracted from that of the sample. Total flavonoid content was expressed as routine equivalents (Zengin et al., 2015a).

Determination of Total Antioxidant Activity:

Total antioxidant activity of the samples was evaluated by phosphor molybdenum method. Sample solution (0.2 mL) was combined with 2 mL reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). After incubation at 95°C for 90 min, the absorbance of the sample was read at 695 nm (Zengin et al., 2015b). For 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, sample solution (1 mL) was added to 4 mL 0.004% methanol DPPH solution. After incubation for 30 min at room temperature in the dark, the absorbance of the sample was read at 517 nm (Kocak et al., 2016; Tepe et al., 2011).

For ABTS cation radical trapping activity, $ABTS^{\cdot+}$ radical cation was directly produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark for 12-16 h at room temperature. Before starting the analysis, the ABTS solution was diluted with methanol to obtain an absorbance

of 0.700 ± 0.02 at 734 nm. The sample solution (1 mL) was added to the ABTS solution (2 mL) and mixed. After incubation at room temperature for 7 min, the absorbance of the sample was read at 734 nm (Kocak et al., 2016).

For metal chelating activity on ferrous ions, briefly sample solution (2 mL) was added to FeCl2 solution (0.05 mL, 2 mM). The reaction was initiated by adding 5 mM ferrozine (0.2 mL). Similarly, a blank was prepared by adding sample solution (2 mL) to FeCl2 solution (0.05 mL, 2 mM) and ferrozine-free water (0.2 mL). After incubation at room temperature for 10 min, the absorbances of the sample and the blank were read at 562 nm (Tepe et al., 2011).

For copper ion reduction activity (CUPRAC), sample solution (0.5 mL) was added to a premixed reaction mixture containing CuCl2 (1 mL, 10 mM), neo cuproin (1 mL, 7.5 mM) and NH_4Ac buffer (1 mL, 1 M, pH 7.0). Similarly, a blank was prepared by adding sample solution (0.5 mL) to a premixed reaction mixture (3 mL) without CuCl2. Then, after incubation at room temperature for 30 min, the absorbance of the sample and the blank were read at 450 nm (Apak et al., 2006). For ferric reducing antioxidant power (FRAP), sample solution (0.1 mL) was added to FRAP reagent (2 mL) containing acetate buffer (0.3 M, pH 3.6), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) and ferric chloride (20 mM) in a ratio of 10:1:1:1 (v/v/v) in 40 mM HCl.

Inhibitory activity on α-amylase was carried out using the Caraway-Somogyi iodine/potassium iodide (IKI) method. Sample solution (25 µL) was mixed with α-amylase solution (50 µL) in phosphate buffer (pH 6.9 with 6 mM sodium chloride) in a 96-well micro plate and incubated at 37°C for 10 min. After pre-incubation, starch solution (50 µL, 0.05%) was added and the reaction was initiated. Similarly, a blank was prepared by adding sample solution to all reaction reagents without enzyme solution (α-amylase). The reaction mixture was incubated at 37°C for 10 min. The reaction was then stopped by adding HCl (25 µL, 1 M). This was followed by adding iodine-potassium iodide solution (100 µL). The absorbance of the sample and the blank was read at 630 nm. The absorbance of the blank was subtracted from that of the sample.

Tyrosinase inhibitory activity was measured using a dopachrome method modified with L-DOPA as substrate. Sample solution (25 µL) was mixed with tyrosinase solution (40 µl) and phosphate buffer (100 µl, pH 6.8) in a 96-well microplate and incubated at 25°C for 15 min. The reaction was then initiated by adding L-DOPA (40 µl). Similarly, a blank was prepared by adding sample solution to all reaction reagents without enzyme (tyrosinase) solution. Sample and blank absorbance were read at 492 nm after 10 min incubation at 25°C (Sarikurkcu et al., 2018).

Cholinesterase (ChE) inhibitory activity was measured using Ellman's method. The sample solution (50 µL) was mixed with DTNB (125 µL) and AChE solutions (25 µL) in Tris-HCl buffer (pH 8.0) in a 96-well micro plate and incubated at 25°C for 15 min. The reaction was then initiated by adding acetylthiocholine iodide (ATCI) (BTCI) (25 µL). Similarly, a blank was prepared by adding sample solution to all reaction reagents without enzyme solutions (AChE). The absorbance of the sample and the blank was read at 405 nm after 10 min incubation at 25°C. The absorbance of the blank was subtracted from the absorbance of the sample (Sarikurkcu et al., 2018).

For enzyme inhibition, radical scavenging and metal chelating assays, the sample concentration reducing the initial concentration by 50% was defined as IC50, while EC50 values were calculated as the sample concentration providing an absorbance of 0.500 for reducing power and phosphor molybdenum assays and 50% concentration providing initial inhibition for radical scavenging and metal chelation assays. The biological activities of the extracts were expressed as mg standard equivalents/g extract and compared with those of standards including trolox, ethylene di-amine tetra acetic acid (disodium salt) (EDTA), galantamine, Kojic acid and Acarbose used as positive controls.

Phyto Chemical Analysis

Phyto chemical analysis of the extract was carried out by a sensitive, rapid, simple and reproducible method previously developed and validated using LC-ESI-MS/MS (Cittan & Celik, 2018). An Agilent Technologies 6420 Infinity liquid chromatography system coupled to a 1260 triple Quadra pole mass spectrometer was used for quantitative analyses. Chromatographic separation was performed on a Poro-shell 120 EC-C18 (100 mm x 4.6 mm inner diameter, 2.7 µm) column. The mobile phase configuration (0.1% formic acid/methanol) was chosen on the basis of better chromatographic resolution of isomeric compounds. On the other hand, the selected

mobile phase configuration also provided higher sensitivity for many phenolic compounds. Consequently, the mobile phase was made of solvent A (0.1%, v/v formic acid solution) and solvent B (methanol). The gradient profile was set as follows: 0.00 min 2% B fluent, 3.00 min 2% B fluent, 6.00 min 25% B fluent, 10.00 min 50% B fluent, 14.00 min 95% B fluent, 17.00 min 95% B and 17.50 min 2% B fluent. The column temperature was maintained at 25°C. The flow rate was 0.4 mL min-1 and the injection volume was 2.0 µL.

The tandem mass spectrometer was interfaced to the LC system via an ESI source. The electro spray source of the MS was operated in negative and positive multiple reaction monitoring (MRM) mode, and the interface conditions were as follows: -3.5 kV capillary voltage, 300°C gas temperature and 11 L min-1 gas flow. The nebulizer pressure was 40 psi.

Results

Total Phenolic and Flavonoid Substance Content

Total phenolic content was 20.62±0.15 mg GAE/g extract and total flavonoid content was 24.55±0.22 mg QE/g extract (Table 1). The concentrations of selected phenolic compounds in *Onobrychis megataphros* extract are shown in Table 2. Accordingly, (+)-Catechin, Taxifolin, 2-Hydroxycinnamic acid, Luteolin and Apigenin were not detected.

Table 1. Total phenolic and flavonoid contents of *Onobrychis megataphros* extract

Total phenolics (mgGAE/g extract)	20.62±0.15
Total flavonoids (mgQE/g extract)	24.55±0.22

GAE: Gallic acid equivalent

Table 2. Concentrations of selected phenolic compounds in *Onobrychis megataphros* extract

RT	Compounds	Concentration (µg/g extract)
8.7829	Gallic acid	25.8±0.8
10.5569	Protocatechuic acid	73.1±0.4
10.8157	Pyrocatechol	13.5±1.0
10.8352	3,4-Dihydroxyphenylacetic acid	5.24±0.19
11.2863	(+)-Catechin	nd
11.7605	Chlorogenic acid	43.6±0.3
11.9714	2,5-Dihydroxybenzoic acid	6.37±0.15
12.0643	4-Hydroxybenzoic acid	42.9±0.1
12.2281	(-)-Epicatechin	9.65±0.04
12.6006	Caffeic acid	7.37±0.14
12.6904	Syringic acid	11.4±0.8
12.8289	3-Hydroxybenzoic acid	3.97±0.32
13.0044	Vanillin	7.25±0.32
13.4183	Verbascoside	0.21±0.01
13.6707	Taxifolin	nd
13.7506	p-Coumaric acid	18.5±0.1
13.8236	Sinapic acid	5.20±0.19
13.8837	Ferulic acid	39.9±0.7
14.2311	Luteolin 7-glucoside	29.8±0.3
14.4193	2-Hydroxycinnamic acid	nd
14.4289	Hesperidin	2263±2
14.4634	Hyperoside	2580±5
14.4915	Rosmarinic acid	5.84±0.14
14.7066	Apigenin 7-glucoside	93.6±1.8
14.9445	Pinoresinol	12.2±0.3
15.0470	Eriodictyol	nd
15.5459	Quercetin	2.58±0.04
15.7763	Luteolin	nd
16.0729	Kaempferol	3.34±0.11
16.2364	Apigenin	nd

RT: Retention time, nd: not detected

Antioxidant Capacity

The results of the antioxidant activities of *Onobrychis megataphros* extracts are shown in Table 3. TEs and EDTAEs refer to the equivalents of trolox and ethyl enediaminetetetra acetic acid (disodium salt), respectively. ACEs, GALAEs and CAEs refer to acarbose, galantamine and kojic acid equivalents, respectively. Values shown with the same superscripts are not significantly different after Tukey's hoc test at the 5% significance level (Table 4).

Table 3. Antioxidant activities of *Onobrychis megataphros* extract

Assays	*Onobrychis megataphros*	Trolox	EDTA
Phospho molybdenum (EC_{50}: mg/mL)	2.03±0.07	0.49±0.04	
CUPRAC Reducing (EC_{50}: mg/mL)	1.76±0.03	0.11±0.001	
FRAP Reducing (EC_{50}: mg/mL)	1.45±0.01	0.04±0.002	
DPPH Scavenging (IC_{50}: mg/mL)	6.11±0.33	0.06±0.001	
ABTS Scavenging (IC_{50}: mg/mL)	2.40±0.02	0.11±0.001	
Ferrous ion chelating (IC_{50}: mg/mL)	2.12±0.02		0.03±0.001
Phospho molybdenum (mg TEs/g extract)	242±8		
CUPRAC Reducing (mg TEs/g extract)	62.6±1.2		
FRAP Reducing (mg TEs/g extract)	30.4±0.2		
DPPH Scavenging (mg TEs/g extract)	9.34±0.51		
ABTS Scavenging (mg TEs/g extract)	45.8±0.4		
Ferrous ion chelating (mg EDTAEs/g extract)	15.6±0.2		

Table 4. Enzyme inhibition activity of *Onobrychis megataphros* extracts.

Assays	*Onobrychis megataphros*	Galanthamine	Kojic acid	Acarbose
AChE inhibition (IC_{50}: mg/mL)	1.58±0.04	0.003±0.0001		
Tyrosinase inhibition (IC_{50}: mg/mL)	2.54±0.22		0.08±0.001	
α-Amylase inhibition (IC_{50}: mg/mL)	1.60±0.01			0.79±0.01
AChE inhibition (mg GALAEs/g extract)	1.7±0.04			
Tyrosinase inhibition (mg KAEs/g extract)	31.9±2.8			
α-Amylase inhibition (mg ACEs/g extract)	490±1			

In our study, TAS, TOS and OSI values increased according to the dose (Table 5).

Table 5. TAS/TOS and OSI index results of methanol extract of *Onobrychis megataphros*

µg/ml	TAS mmol/ml	TOS µmol/ml	OSI
50	2,685	23,84	0,888
100	3,102	37,01	1,193
150	3,208	48,68	1,517
200	3,221	56,22	1,745
250	3,237	72,80	2,249
300	3,255	75,56	2,321
350	3,290	81,79	2,486
400	3,300	87,27	2,644
450	3,336	91,88	2,754
500	3,313	93,53	2,823

Discussion and Conclusion

This study aims to determine the total phenolic, flavonoid and antioxidant activity levels of *Onobrychis megataphros* plant leaves. Therefore, firstly, *O.megataphros* (Sainfoin) samples were collected, laid on blotting papers in shady places away from sunlight and dried. The completely dried plant samples were pulverized. The powdered samples were extracted with methanol using Soxhlet device. The methanol was removed by

evaporation in a rotary evaporator and stored at +4ºC until the experiment starts. Phenolic and flavonoid content and antioxidant activity properties of *O.megataphros* plant leaf extracts were investigated. To determine total phenolic content, sample solution (0.25 mL) was mixed with diluted Folin Ciocalteu reagent (1 mL, 1:9) and shaken strongly. After 3 min, Na_2CO_3 solution (0.75 mL, 1%) was added and the sample absorbance was read at 760 nm after 2 h incubation at room temperature. Total phenolic content was expressed as equivalents of gallic acid. For total flavonoid content, sample solution (1mL) was mixed with the same volume of aluminum trichloride (2%) in methanol. Similarly, a blank was prepared by adding sample solution (1mL) to methanol (1mL) without $AlCl_3$. The sample and blank absorbance were read at 415 nm after 10 min incubation at room temperature. Absorbance of the blank was subtracted from that of the sample. Total flavonoid content was expressed as equivalents of routine. Total antioxidant activity of the samples was evaluated by phosphor molybdenum method. Sample solution (0.2mL) was combined with 2mL of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The sample absorbance was read at 695nm after 90 min incubation at 95°C. As a result, it was determined that the total phenolic content in *O.megataphros* plant leaf extracts was 20.62±0.15 mg GAE/g extract and total flavonoid content was 24.55±0.22 mg QE/g extract.

As a result of our study, it was determined that TAS and TOS levels increased with the increase in concentration. Similar with our results Benchadi et al. (2022) determined that the total oxidant (TOS) and antioxidant status (TAS) levels of *O.crista-galli* increases with the concentration. Total phenolic content in our study was 20.62±0.15 mg GAE/g extract and total flavonoid content was 24.55±0.22 mg QE/g extract. Karakoca et al. (2013) investigated the phenolic compounds, biological and antioxidant activities of *Onobrychis armena* and found total phenolic content as 87.32 mg GAE/g and 128.23 mg GAE/g in flower and root methanol extracts, respectively. In a study conducted by Butkutė et al. (2016) on perennial legumes, reported that plant parts of the genus Trifolium and *Onobrychis viciifolia* contained more phenolics than Astragalus and Medicago species.

In another study investigating the phenolic content of *Onobrychis hypargyrea*, 83.25 mg GAE/g in ethyl acetate extract, 73.20 mg GAE/g in methanol extract and 69.38 mg GAE/g in water extract were determined (Zengin et al., 2015c). Godevac et al. (2008) investigated the antioxidant capacity of nine species belonging to the Fabaceae family collected from the mountains of Serbia and Montenegro. Antioxidant tests with various reaction mechanisms, including total phenolic content with Folin-Ciocalteu, DPPH radical scavenging capacity, Trolox equivalent antioxidant capacity (TEAC) values with ABTS radical cation, and inhibition of liposome peroxidation, were used in the study. As a result of the study, it was determined that the plant samples examined exhibited strong antioxidant capacity in all methods. Godevac et al (2008), determined the total phenolic content of *Onobrychis scardica* as 115.23 mg/GAE/g, which is higher than *O.megataphros* used in our study.

In our study the tyrosine inhibition and α-Amylase inhibition (IC_{50}) values were found respectively as 2.54±0.22 and 1.60±0.01 mg/mL. Similar with our results Aliyazıcıoğlu et al. (2017), in their study examining *Onobrychis oxyodonta* methanolic extract using the DPPH method, found the α-Amylase inhibition IC50 value as 1.333±0.0026 mg/mL.

In our study DPPH scavenging activity (IC_{50}) was determined as 6.11±0.33 mg/mL. Zengin et al. (2015c) investigated the antioxidant properties of *Onobrychis hypargyrea* and found DPPH activity as 0.31 ± 0.003 IC50 (mg/mL) in ethyl acetate extract, 0.29 ± 0.002 IC50 (mg/mL) in methanol extract and 0.27 ± 0.001 IC50 (mg/mL) in water extract.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPSTEM journal belongs to the authors.

Acknowledgements or Notes

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