https://doi.org/10.31925/farmacia.2022.3.20 **ORIGINAL ARTICLE**

PHYTOCHEMICAL INVESTIGATION AND ANTIOXIDANT POTENTIAL OF *ONONIS ARVENSIS* L.

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Abstract

Phytochemicals, such as polyphenols, are strong antioxidants and have an important role in the human health system. The present study investigates the content of total phenolics, flavonoids, tannins and the antioxidant activity of *Ononis arvensis* using the *in vitro* $ABTS^{\bullet+}$ antioxidant assay for the first time. Extracts were prepared with three different types of solvents: water, 50% ethanol, methanol. Among the studied parts, the 50% ethanol extract of the flower showed the highest polyphenolic content (96.33 ± 7.7 mg GAE/100 g dw), while the 50% ethanol extract of the flower (81.58 ± 3.1 mg QE/100 g dw) and the aerial part (21.15 ± 1.3 mg QE/100 g dw) showed the highest total flavonoid content. The total tannin concentration of the aerial part had a low value (0.15 ± 0.04%). The antioxidant effect was detected in all extracts obtained from flowers; however, ethanol and methanol were more effective.

Rezumat

Dintre compușii chimici, polifenolii au activitate antioxidantă puternică, având un rol important în sistemul de apărare al organismului. Prezentul studiu analizează conținutul în polifenoli, flavonoide, taninuri și precum și activitatea antioxidantă *in vitro* a speciei *Ononis arvensis* folosind metoda $ABTS^{\bullet+}$. Pentru acest studiu s-au realizat extracte, cu trei tipuri diferite de solvenți: apă, etanol 50% și metanol. Dintre produsele vegetale studiate, extractele etanolice (50%) din flori prezintă concentrații ridicate de polifenoli (96,33 ± 7,7 mg GAE/100 g produs vegetal uscat). Concentrații ridicate de flavonoide s-au determinat în extractele etanolice 50% din flori (81,58 ± 3,1 mg QE/100 g produs vegetal uscat) și din părțile aeriene (21,15 ± 1,3 mg QE/100 g produs vegetal uscat). Concentrația taninurilor este relativ scăzută (0,15 ± 0,04%). Efecte antioxidante s-au observat pentru toate extractele obținute din flori, iar dintre acestea extractele etanolice și metanolice au fost semnificativ mai potente decât extractele apoase.

Keywords: field restharrow, polyphenols, tannins, antioxidant activity

Introduction

The genus *Ononis* (restharrows), which belongs to the *Fabaceae* family, is natively present in fields, arable lands and meadows in the Canary Islands, the Mediterranean region, North Africa, North America and from Europe to Central Asia [31].

The genus is widely studied for its phytochemical compositions by various techniques. Biochemical identification and characterization of isoflavones and phenolic acids were described in *O. spinosa* L. [20], more than 20 flavonoid aglycones in *O. fruticosa* L., *O. natrix* L., *O. tridentate* L. [47], and phenolic constituents in the root of *O. vaginalis* Vahl. [1]. Isoflavone glycosides like ononin, and the 7-O-β-D-glucopyranoside of formononetin, with phyto-estrogenic properties, were identified in the root of *O. angustissima* L. [22].

From a historical and ethnomedical point of view, the *Ononis* species have been used in many countries to cure gout, skin, rheumatic and urinary diseases [16, 29]. Several studies were published on the significant biological activities of the *Ononis* species. The root of *O. angustissima* has an antioxidant and neuro-protective effect [22]. Extensive anti-inflammatory and wound healing properties have been found for the aqueous and ethanolic extracts of the aerial part of *O. macrosperma* Hub.-Mor. [44]. The aerial part of *O. natrix* has antibacterial, cytotoxic [3], and anti-inflammatory activity [8], while the aerial part of *O. natrix* ssp. *hispanica* has antioxidant, antimicrobial, DNA protective, and cytotoxic effect [48]. The root of *O. vaginalis* has been studied for its antiviral activity against herpes simplex virus type 1, as well as for cytotoxic [1], anti-inflammatory, hepatoprotective and estrogenic activity [2]. The dimethyl sulfoxide

extracts of *O. viscosa* L. were assessed against some bacterial strains [15]. Only one species of the genus, namely *O. spinosa*, has a monograph in the European Pharmacopoeia [19], and published by the European Medicines Agency [18]. The plant has been studied for diuretic, anti-inflammatory [6], analgesic [49], antioxidant [11], antibiotic [10, 30] and wound healing effect [17].

In our study, field restharrow (*Ononis arvensis*, syn.: *Ononis arvensis* subsp. *hircina* (Jacq.) Gams) [45] was selected for further analyses based on earlier reports. The plant is a perennial shrub living in humid fields across Europe. The 50 - 100 cm high erect stem is covered by trichomes. It has elliptical leaves and pink flowers [46]. The aerial part was traditionally used to treat typhus and hernia, but also an aphrodisiac drug [7]. The aerial part has been studied for its essential oil [12], phenolic coumarins acids [14, 40], flavonoids [14, 26, 41, 43], stilbenes [14] and content [39]; more isoflavonoids were identified in the root [21, 24, 26, 27], triterpenes [38, 42] and lectins [23] of the whole plant. *In vitro* antioxidant assays were performed with ECL, ORAC and DPPH assays, which studied the total antioxidant capacity of the aerial part of the plant [34]. Antimicrobial activity and minimum inhibitory concentration of the solvent fractions were earlier studied against four bacterial strains (*Escherichia coli*, *Pseudomonas aeruginosa, Salmonella typhimurium* and *Staphylococcus aureus)* and one fungus (*Candida albicans)* in the case of the aerial part [13].

To complete the previous studies, the aim of this study was to provide more details about the chemical and biological properties of *Ononis arvensis*, to determine its total phenolic, flavonoid and tannin content, as well as the antioxidant potential of the aerial part and root employing the $ABTS^{\bullet+}$ method.

Materials and Methods

Plant material

O. arvensis was collected in August 2019 on the edge of the road in Mereşti, Romania. Voucher specimens were identified by Nóra Papp (botanist), then deposited and labelled with a unique code at the Department of Pharmacognosy, University of Pécs, Hungary.

Preparation of extracts

For total phenolic and flavonoid assays, 2.5 g of the aerial part, stem, root and leaf, as well as 1.25 g of flower were powdered and extracted with 25.0 mL methanol solvent (Roth, Germany) for 20 min. The extracts were placed in an ultrasound bath for 20 minutes at 40°C (Nahita Digital Ultrasonic Bath). Then they were filtered through a 0.2 μm Nylon filter (VWR, USA) into a 25.0 mL volumetric flask, and diluted with methanol, 50% ethanol (Chimreactiv, Romania), and water to 25.0 mL. Extracts were deposited at 4 - 5°C in a refrigerator until determinations. The concentration of flower extract was 5% (5 g flower/100 mL solvent), while that of the other studied parts 10% (10 g plant material/100 mL solvent) [9].

Total polyphenol assay

20 μL of each extract was placed into tubes with 1580 μL water and 100 μL Folin-Ciocâlteu reagent (VWR Chemicals). In 5 min, 300 μL sodium carbonate (20% w/v) (Lachner, Czech Republic) was added to each; the tubes were stored for 2 hours at 20ºC. Spectrophotometric measurements (JKI UV/VIS-752N) were carried out at 765 nm for test solution compared with compensation solution (water and reagents). Polyphenol concentration was expressed in gallic acid equivalent (GAE) (Sigma Aldrich) *per* 100 gram dry weight of plant.

Total flavonoid assay

As previously presented in the Romanian Pharmacopoeia (10^{th} Edition – *Cynarae folium* monograph) [37], total flavonoid content was determined with slight modification. For 500 μL extract, 1000 μL Na-acetate (100 g/L; Reactivul, Romania), 600 μL $AlCl_3$ (25 g/L; Chimopar, Romania), 1400 μL methanol, and 1500 μL water were added. After 15 minutes, the absorbance was measured at 430 nm using an extract mixture without reagents as compensation solution. Flavonoid concentration was expressed in quercetin equivalent (Chemapol, Czech Republic) (QE) *per* 100 gram dry weight of plant.

Total tannin assay

Total tannin content was detected according to the method of the Pharmacopoeia Hungarica $VIII^{th}$ [35] using skin powder (Merck, Darmstadt, Germany) and pyrogallol (Loba Feinchemie GmbH, Austria). 0.75 g of the aerial parts were powdered and extracted with 150 mL solvent using distilled water for 30 min. Then the absorbance of total polyphenols, polyphenols which are not absorbed by skin powder, and pyrogallol standard were determined. In each case, the residue was diluted with distilled water, then phosphowolframic acid was added (Fluca, Switzerland), and finally they were diluted with sodium carbonate (150 g/L) (Alfa Aesar, Germany). Spectrophotometric measurements were performed at 715 nm in 2 min using water as compensation solution. Each analysis was performed in triplicate. Polyphenol contents were calculated with formulas from Pharmacopoeia Hungarica $VIII^{th}$ [35], expressed in % m/m of tannins.

$ABTS^{\bullet+}$ radical scavenging activity

The antioxidant capacity was studied with the $ABTS^{\bullet+}$ method (2,2′-Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid) (Sigma-Aldrich) [36] with modifications. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate (Sigma-Aldrich), then the mixture was stored in the dark at room temperature for 12 - 16 h before use. The $ABTS^{\bullet+}$ solution was diluted with methanol to an absorbance of 0.90 (± 0.02) at 734 nm. The absorbance was measured after 6 min as follows: 2.5 mL of working $ABTS^{\bullet+}$ solution and 10 - 100 μL

of sample were introduced into the spectrophotometric cuvette. Ascorbic acid (100 µM) was used as anti-oxidant standard (Roth).

Statistical analysis

Experiments were performed in triplicate, and expressed as mean ± standard deviation (mean ± SD). Analysis was performed by GraphPad Prism statistical program (paired t-test, Spearman-rank correlation). The analysis was considered to be statistically significant in case of a p value less than 0.05.

Results and Discussion

Total polyphenol content

The absorbance values of the reference solution were plotted as a function of their concentration. The polyphenol content was calculated from the straight equation of gallic acid ($y = 0.044x + 0.0057$, $r^2 = 0.9906$) and expressed in mg gallic acid equivalent (GAE)/100 g dry weight. The total polyphenol contents (mg gallic acid/100 g dry weight) are presented in Table I. The 50% ethanol proved to be the best solvent. The highest concentration of polyphenol was determined in the flower (96.33 mg GAE/100 g dw) in this extract. This was followed by the aqueous extracts; among them, the flower had the highest value of polyphenols (86.66 mg GAE/100 g dw). Methanol extract of all studied parts showed low content, among them, the flower extract had the highest concentration (65.25 mg GAE/100 g dw) (Figure 1). All in all, the highest concentration of polyphenolics was detected in the flower extract, regardless of the solvents.

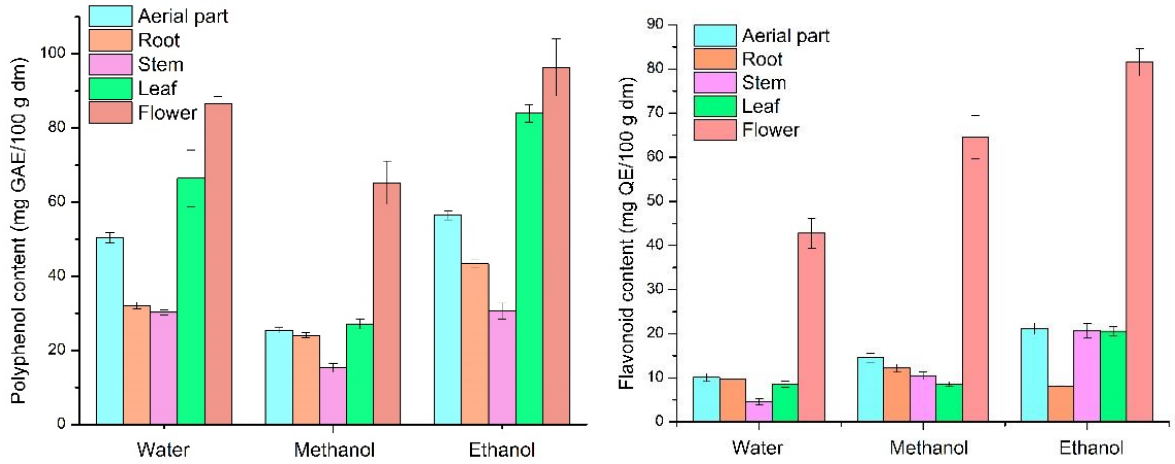

Figure 1.
Total phenolics and flavonoids in the studied extracts of *O. arvensis*

Total flavonoid content

The absorbance values of the reference solution were plotted as a function of concentration. The flavonoid concentration was calculated from the straight equation of quercetin ($y = 0.156x - 0.1676$, $r^2 = 0.9954$), and expressed in mg quercetin equivalent (QE)/100 g dry weight. The total flavonoid concentration (mg quercetin/100 g dw herbal drug) is summarized in Table I. It was the highest value detected in the 50% ethanol extract of all plant parts. The obtained results from the flower reveal a comparative rate of flavonoids in ethanol, methanol and water extracts (81.58, 64.56 and 42.78 mg GAE/g dw). However, among the tested extracts the lowest quantity of flavonoid was obtained in methanol extracts; among the tested parts the flower showed the highest value (64.56 mg QE/100 g dw). The lowest flavonoid content was detected in the water extract of the stem (4.54 mg QE/100 g dw) and the root (9.71 mg QE/100 g dw) (Figure 1). All in all, the high variability of flavonoid determination showed the highest values in the flower, regardless of the solvents used.

Total tannin content

The total tannin concentration was determined in the aerial part of the plant at 0.15 ± 0.04%, which represents a low value for this ingredient.

Antioxidant activity using the $ABTS^{\bullet+}$ method

The line equation was obtained by plotting the percentage inhibition as a function of concentration. Based on the obtained formula, 50% inhibition was calculated, and the results are summarized in Table I. For all the studied plant parts, the aqueous extract showed the lowest antioxidant capacity (IC_{50} = 20.19 - 155.46 mg/mL). The antioxidant effect of the extracts was significantly different by T-test (Figure 2). Among them, the flower has the highest antioxidant capacity in all extracts, while the leaf the lowest one. The aqueous extract of the root has an extremely low antioxidant value (IC_{50} = 155.46 mg/mL), which can be attributed to its woody structure where water is less extractible from. Ethanol and methanol were found to be more effective for the extraction of all studied plant parts, as a result of their markedly higher anti-oxidant capacities.

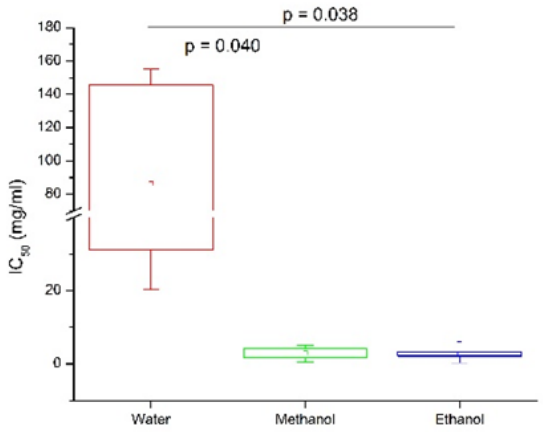

Figure 2.
IC_{50} values of the studied extracts of *O. arvensis*

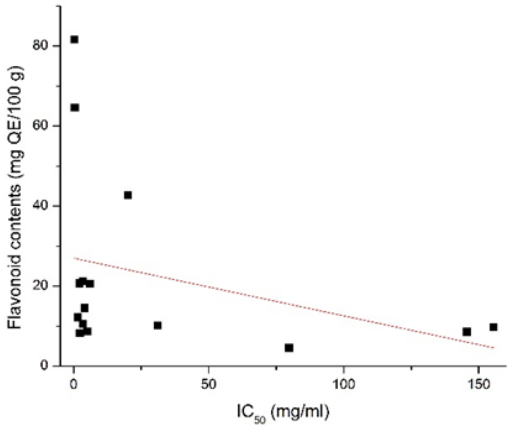

Figure 3.
Correlation analysis between IC_{50} and flavonoid content of *O. arvensis*

As no correlation was found between the total polyphenol and the $ABTS^{\bullet+}$ antioxidant values, but the total flavonoid and $ABTS^{\bullet+}$ antioxidant values showed significant correlation (Spearman correlation, p = 0.0208), we concluded that flavonoids are mainly responsible for the neutralization of the $ABTS^{\bullet+}$ cation radical (Figure 3). The antioxidant capacity was expressed in mg of ascorbic acid equivalent (AAE/g dw). AAE values were calculated for 1 g of dried plant sample (Table I). Our results showed that the extracts exhibited significant antioxidant activity which may be related to their flavonoid content. This finding is in accordance with previously reported findings that demonstrated the antioxidant properties of the extracts.

Papp *et al*. investigated the anti-oxidant activity of the ethanol extract in the aerial part of *O. arvensis* with three methods and measuring the TE value; 50% ethanol extracts showed similar antioxidant activity with DPPH (79.06 ± 7.00) and chemiluminescence determination (145.10 ± 1.40), however, the anti-oxidant capacity measured by the ORAC method showed high value (961.80 ± 64.94), data which did not correlate with the results of the chemiluminescence and the DPPH assays [39].

Table I
Total polyphenols, flavonoids and antioxidant activity of the studied parts of *O. arvensis*

Studied plant parts	Solvents	Polyphenol content (mg GAE/100 g dw)	Flavonoid content (mg QE/100 g dw)	IC_{50} (mg/mL)	AAE/g dw (µmol/g)
Aerial part	Water	50.39 ± 1.4	10.09 ± 0.9	31.09 ± 2.3	4.31 ± 0.4
	Methanol	25.51 ± 0.8	14.52 ± 1.0	4.12 ± 0.3	32.57 ± 1.3
	50% Ethanol	56.43 ± 1.2	21.15 ± 1.3	3.35 ± 0.2	40.05 ± 1.7
Root	Water	32.1 ± 0.9	9.71 ± 0.8	155.45 ± 9.8	0.86 ± 0.9
	Methanol	24.1 ± 0.7	12.2 ± 0.9	1.6 ± 0.06	82.77 ± 2.2
	50% Ethanol	43.37 ± 1.1	8.17 ± 0.08	2.30 ± 0.03	58.33 ± 3.6
Stem	Water	30.28 ± 0.7	4.54 ± 0.7	79.79 ± 6.8	1.68 ± 0.04
	Methanol	15.33 ± 1.2	10.49 ± 0.9	3.41 ± 0.4	39.25 ± 1.9
	50% Ethanol	30.6 ± 2.2	20.7 ± 1.6	2.07 ± 0.1	64.77 ± 2.1
Leaf	Water	66.43 ± 7.6	8.55 ± 0.7	145.60 ± 11.3	0.92 ± 0.06
	Methanol	27.15 ± 1.3	8.65 ± 0.5	5.10 ± 0.3	26.28 ± 1.3
	50% Ethanol	83.93 ± 2.4	20.57 ± 1.1	6.06 ± 0.4	22.15 ± 0.9
Flower	Water	86.66 ± 1.9	42.78 ± 3.4	20.19 ± 1.1	6.64 ± 0.2
	Methanol	65.25 ± 5.8	64.56 ± 4.9	0.46 ± 0.01	290.59 ± 14.8
	50% Ethanol	96.33 ± 7.7	81.58 ± 3.1	0.29 ± 0.01	462.69 ± 20.7

dw = dry weight, AAE= ascorbic acid equivalent

In another study, the total phenolic content of the aqueous extract (3.09 mg GAE/g extract) was earlier determined from *O. spinosa*, where no significant correlation was found between the total polyphenols and radical scavenging capacity using the DPPH method [33]. Numerous studies have been performed on the antioxidant activity and phenolic content of other *Ononis* species. For example, the total phenolic content of *O. natrix* was reported in Tunisia as 51 mg GAE/g, the flavonoid content as 14.76 CE/g, and high total antioxidant activity with 60.94 mg of GAE/g DW, which displayed a high DPPH scavenging ability with low IC_{50} value (29 µg/mL) and a great reducing power (EC_{50} = 100 µg/mL) [32]. In another research,

in which the total phenolic and flavonoid content in *O. natrix* was tested, the highest total phenolic and flavonoid contents of the plant were observed in the ethyl acetate extract (60.19 mg GAE/g), followed by the methanol (59.22 mg GAE/g) and aqueous ones [48]. The antioxidant capacity and phenolic content of the aerial part of the plant and the root extracts of *O. sessilifolia* Bornm., *O. basiadnata* Hub. & Mor. and *O. macrosperma* Hub. & Mor. were also investigated, where the total phenolic content of their water extracts was found to range between 14.78 - 80.33 mg/g, and that of the ethanol extracts between 67.19 - 145.33 mg/g. The most significant results in the TBA assays were obtained in the ethanol extract of the aerial part of *O. macrosperma* (IC_{50} = 0.13 ± 0.17 µg/mL) and *O. sessilifolia* (IC_{50} =1.41 ± 0.58 µg/mL), and the root of *O. sessilifolia* (IC_{50} = 1.96 ± 0.39 µg/mL) [4]. The total polyphenol and flavonoid content studied from the aerial part of *O. angustissima*, revealed a comparative rate of polyphenols in the hydro-methanolic, butanol and ethyl acetate extracts (78.11, 74.55 and 72.21 mg GAE/g), and a high total flavonoid content in the hydro-methanolic and ethyl acetate extracts (34.14, 32.01 mg CEq/g) [28]. Ghribi *et al*. investigated the antioxidant capacity of the root of *O. angustissima* with the DPPH (IC_{50} = 19.53 µg/mL) and $ABTS^{\bullet+}$ methods (IC_{50} = 28.29 µg/mL) [22]. Jaradat *et al*. investigated the aerial part and the root of *O. pubescens* L. from the Palestinian flora also using the DPPH method. The result revealed an excellent antioxidant activity, with IC_{50} = 34.67 µg/mL for n-hexane, IC_{50} = 8.67 µg/mL for acetone, IC_{50} = 19.41 µg/mL for methanol and IC_{50} = 15.14 µg/mL for aqueous fractions of the plant [25]. Besbas *et al*. determined the total phenolic content of the aerial part of *O. mitissima* L. living in the Algerian flora, expressed in gallic acid from the petroleum ether (52.11 µg GAE/mg), ethyl acetate (177.96 µg GAE/mg), and n-butanol extracts (157.10 µg GAE/mg), while the total flavonoid content expressed in quercetin was measured in the petroleum ether (27.27 µg QE/mg), ethyl acetate (132.83 µg QE/mg) and n-butanol extracts (117.95 µg QE/mg). The antioxidant activity of *O. mitissima* tested with DPPH method resulted with IC_{50} = 181.9 µg/mL in the petroleum, IC_{50} = 27.3 µg/mL in ethyl acetate, and IC_{50} = 38.3 µg/mL in n-butanol extract [5].

Our results showed that there seemed to be good compatibility between the flavonoid content and antioxidant capacity of the studied extracts of *O. arvensis*, because the ethanol and methanol extracts with high ABTS radical scavenging capacity possessed a high flavonoid content.

Conclusions

The studied extracts showed a strong correlation between the flavonoid content and their antioxidant activities in the selected parts of *O. arvensis*. Medicinal plants represent potential sources of natural anti-oxidant agents for medicinal purposes. It is necessary to identify and characterize the active components, which could be responsible for the antioxidant activity. In conclusion, based on these preliminary results, *O. arvensis* could be a potential source of new anti-oxidant agent which should be further analysed.

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Conflict of interest

The authors declare no conflict of interest.

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