Pharmaceutical Sciences, June 2016, 22, 112-119
doi: 10.15171/PS.2016.18
http://journals.tbzmed.ac.ir/PHARM

Research Article

Antioxidant Activity, Total Phenolic and Flavonoid Contents of Three *Onobrychis* Species from Iran

Roya Karamian*, Mostafa Asadbegy
Department of Biology, Faculty of Science, Bu-Ali Sina University, Hamedan, Iran

Article Info

Article History:
Received: 24 August 2015
Accepted: 06 January 2016
ePublished: 30 June 2016

Keywords:
-*Onobrychis*
-Phenol
-Flavonoid
-DPPH radical scavenging
-Metal-chelation
-ß-Carotene/linoleic acid model

ABSTRACT

Background: Plant phenolic compounds are a main group of plant natural products and flavonoids are the largest and best studied natural phenols. These substances possess a series of biological properties and act on biological systems as antioxidants. In present research, the aim is to determine in vitro total phenolics content and antioxidant activity of the methanolic extracts of three Onobrychis species belonging to the family Fabaceae, namely O. sosnovskyi Grossh., O. viciifolia Scop. and O. melanotricha Boiss. Furthermore, an attempt was made to identify any correlations between total phenolic content of the extracts with their antioxidant activities

Methods: Total phenol and flavonoid contents of the extracts were determined by Folin-Ciocalteu and aluminum chloride methods, respectively. Antioxidant activity was evaluated by three different test systems, namely 2, 2-diphenyl-1-picrylhydrazyl free radical scavenging (DPPH), metal-chelation activity and ß-carotene/linoleic acid model.

Results: Results indicated that O. viciifolia extract contains the highest total phenolic content (10.38 ± 0.33mg GAE/g of dry extract). However, the species are not remarkable different ($P < 0.05$) in total flavonoid amount. The Onobrychis species represent strong antioxidant activity in DPPH radical scavenging (IC50; 0.121 ± 0.005 to 0.129 ± 0.001 mg/ml), metal chelating (IC50; 1.10 ± 0.006 to 1.80 ± 0.003 mg/ml) and also β-carotene bleaching methods. In addition, a high correlation was found between different antioxidant assays and total phenol content of the extracts ($R2 > 0.55$ to 0.98).

Conclusion: Our results showed that the examined Onobrychis extracts represent strong antioxidant activity; hence, they can be suggested as antioxidant agents for special use in future.

Introduction

Free radicals such as reactive oxygen species (ROS) are formed naturally in the living cells that have important roles in cell signaling, also in environment some toxins may contain free radicals or stimulate the cells to produce free radicals.[1] High amount of these compounds are hazardous to the cells and damage all major its components, including nucleic acids, phospholipids, polypeptides chain and cell membranes that play a role in the development of diseases such as cancer and other health conditions.[2] Researches in the field of antioxidant advances focuses on antioxidant agents that can protect biological components from oxidative damage.[3] Antioxidants may act as scavenger, reducing, quencher agents and/or activators of cellular antioxidant enzymes to prevent from the free radical damages in biological systems.[4,5] Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) as synthetic antioxidants are used as additives in foods, but using of BHA and BHT are restricted because their toxic and carcinogenic effects.[6] Hence, there is a growing request and interest for found and product of new antioxidants without harmful side effects in food and pharmaceutical applications.[7] Plants are main sources of compounds that act as antioxidant agents.[8] The important antioxidants that exist in plants are tocopherols and tocotrienols as fat-soluble antioxidants (vitamin E), folic acid (vitamin B_9), L-ascorbic acid (vitamin C), flavonoids as the largest natural phenols, carotenoids, phenyl acrylic acids and phenyl methanoic acids.[9] The studies focused on the antioxidant effects of compounds derived from plants, because of their nutritional incidence and their role in health and disease.[10-12] Over the last few years, there has been an interest on antioxidant activity of the new compounds isolated from the Fabaceae family. The genus *Onobrychis* Miller (Fabaceae) includes about 170 annual or perennial mostly caulescent species constitutes a major group within the tribe Hedysareae DC. Members of the genus are mostly distributed in southwest Asia, Mediterranean region, and temperate Europe a few of which are cultivated as fodder or as ornamentals.[13,14] There are previous works on the chemical composition and antioxidant activity of *O.*

***Corresponding Author:** Roya Karamian, E-mail: R_karamian@basu.ac.ir

viciifolia.[15-17] However, there is no reveal any references to previous works on the antioxidant activity of other *Onobrychis*. The aim of our study is to evaluate *in vitro* total phenolic content and antioxidant activity of the methanolic extracts from three *Onobrychis* species (*O. sosnovskyi*, *O. viciifolia* and *O. melanotricha*) and assess any *correlations* between total phenolic content in the extracts and their antioxidant activities.

Materials and Methods

Plant materials

Aerial parts of three *Onobrychis* species were collected from natural distribution areas. The data of the collected species are given in Table 1. The Voucher specimens of the species representing these collections have been deposited at the Bu Ali Sina University Herbarium (BASU), Hamedan, Iran.

Preparation of extracts from Onobrychis species

Briefly, powdered aerial parts of plants (25 g) were extracted by absolute methanol (12 h), using a Soxhlet apparatus. Solvents of all extracts were removed by rotary evaporator (Lab Tech, Ev 311, Italy).

Estimation of total phenol content

Determination of total phenol content was carried out by Folin- Ciocalteu reagent method.[18] In brief, 0.5 ml of each extract (1:10 g/ml) or Gallic acid was mixed with 5 ml Folin- Ciocalteu reagent (1:10 distilled water) and 4 ml of aqueous Na_2CO_3 (1 M). The mixtures were left at room temperature for 15 min and the total phenol content was determined by colorimetric method at 765 nm (Perkin Elmer UV/visible spectrophotometer, USA). The calibration curve ($y = 0.002 x + 0.0177$, $R^2 = 0.978$) was prepared using Gallic acid solutions at concentrations of 0 to 250 µg/ml in 50% methanol. Total phenol content was calculated from calibration curve and expressed as Gallic acid equivalent (mg GAE/g).

Estimation of total flavonoids

The total flavonoid content was estimated using $AlCl_3$ method.[19] 0.5 ml of each extract (1:10 g/ml) in methanol were mixed with 1.5 ml of methanol, 0.1 ml of $AlCl_3$ (10%), 0.1 ml of KCH_3COO (1 M) and 2.8 ml of distilled water and left at room temperature for 30 min. Absorbance of the mixtures was measured at 415 nm (Perkin Elmer UV/visible spectrophotometer, USA). The calibration curve ($y = 0.0091 x + 0.0206$, $R^2 = 0.995$) was prepared using Quercetin solutions at concentrations of 12.5 to 100 µg/ml in methanol and total flavonoid content was calculated from calibration curve and expressed as Quercetin equivalent (mg QE/g extract).

Antiradical and Antioxidant activity

Free radical scavenging capacity

Free radical scavenging activity of the extracts was measured according to Mensor *et al*.[20] In order to obtain dilutions, different extract concentrations were prepared in methanol (0.2 to 1 mg/ml) and 2.5 ml of each concentration was added to 1 ml of alcohol solution of DPPH (0.3 mM). After completion the samples were first kept in a dark place at room temperature for 30 min and bleaching of DPPH was read at 517 nm against a blank (methanol). The inhibition in percent for each concentration was calculated according to the following formula, DPPH free radical scavenging (%) =

$$\left[1 - \left(A_s - A_b\right) / A_c\right] \times 100 \quad \text{Eq.(1)}$$

Where, A_s is absorbance of the reaction mixture containing 2.5 ml of extracts + 1 ml of DPPH, A_b is absorbance of the reaction mixture containing 2.5 ml of extracts + 1 ml methanol and A_c is absorbance of control sample containing 1 ml of DPPH + 2.5 ml methanol. Also IC_{50} value, which represented the concentration of the sample that caused 50% inhibition, was determined. Tests were carried out in triplicate and ascorbic acid was used as a positive control.

Iron chelating capacity

The chelation of ferrous ions by extracts in different concentration (0.2 to 1 mg/ml) was estimated by method of Dinis *et al*.[21]. Briefly, 0.05 ml of $FeCl^{2+}$ (2 mM) was added to 0.5 ml of different concentrations of the extracts. After 30 s, the reaction was initiated by the addition of 0.2 ml of ferrozine solution (5 mM). The mixture was left at room temperature and after 10 min; the absorbance at 562 nm was measured. The percentage inhibition of -Fe^{2+}-ferrozine complex formation ability was calculated with the help of the following formula: Metal-chelating activity (%)

$$= \left[\left(A_c - A_s\right) / A_c\right] \times 100 \quad \text{Eq.(2)}$$

Where, A_c was the absorbance of the control (Fe^{2+}-Ferrozine complex without extract), and A_s was the absorbance of Fe^{2+}-Ferrozine complex mixed with extract/ascorbic acid.

β-Carotene bleaching assay

Antioxidant activity of the samples was evaluated using the β-carotene/linoleic acid system.[22,23]

Table 1. The collections of the investigated *Onobrychis* species.

Species	Voucher number	Locality	Date	Altitude (m)
O. sosnovskyi	BASU14214	East Azerbaijan: 16 km to Marand	24.08.2013	1725
O. viciifolia	BASU19615	West Azerbaijan: Orumieh to Oshnavieh, 10 km after Sangar	23.08.2013	1650
O. melanotricha	BASU19263	Kordestan: Marivan to Saqqez, 60 km after Marivan	04.08.2013	1750

A mixture of β-carotene/linoleic acid was prepared by adding together of 0.5 mg β-carotene in 1 ml chloroform, 20 mg linoleic acid and 200 mg Tween 40. The chloroform was removed using a rotary evaporator apparatus under vacuum and 50 ml of oxygenated distilled water was subsequently added to the reaction mixture residue. 5 ml of the obtained emulsion added to 2 mg of extract/BHT dissolved in 0.4% (w/v) Tween 40 solution and mixed thoroughly. The test tubes were incubated in a water bath at 50 °C for 2 h together with a negative control (blank) contained 2 mg of an extract in 6 ml of Tween 40 solution. Tween 40 without extract was served as negative control. The absorbance values were measured at 470 nm (at 15 min intervals), starting immediately after sample preparation (t = 0 min). Antioxidant activity (inhibition percentage, AA%) was calculated according to the following equation:

$$AA\% = 1 - [\left(A_E^{t=0} - A_E^{t=t}\right) / \left(A_w^{t=0} - A_w^{t=t}\right) + \left(A_{BHT}^{t=0} - A_{BHT}^{t=t}\right)] \times 100 \quad \text{Eq.(3)}$$

Where, A_E is the absorbance of the extract, A_W is the absorbance of the water as negative control and A_{BHT} is the absorbance of BHT as positive control.

Statistical analysis

All data are the average of triplicate analyses. Statistical analysis of variance was performed using Student's t-test by SPSS program and p value < 0.05 was regarded as significant. Data are expressed as means ± standard deviation.

Results

Total phenol and flavonoid contents

Typical phenolic acids such as phenol and flavonoid compounds are known as antioxidant agents.[24] In this research, results indicated that the amount of total phenols varied among studied species and ranged from 6.25 ± 0.28 to 10.38 ± 0.33 mg GAE/g of dry extract. The highest total phenol content was found in *O. viciifolia* and the lowest in *O. melanotricha*. Also results showed that the three *Onobrychis* species are not remarkable different (P < 0.05) in total flavonoids content. However, *O. viciifolia* possesses a higher amount of total flavonoids (5.08 ± 0.64 mg QES/g of dry extract) in comparison with two other species (Table 2).

Free radical scavenging capacity

In foods and biological systems, antioxidants especially free radical scavenging agents are very important due to their role in the deleterious of free radicals.[25] Results of antioxidant assay of the extracts showed the excellent concentration-dependent antiradical effect in comparison with ascorbic acid (71.26%) as a synthetic antioxidant (Table 2 and Figure 1). The greater antioxidant activity is reflected in a lower IC_{50}. All studied extracts demonstrated ability to scavenge DPPH free radicals, where most of them had IC_{50} value lower than the synthetic antioxidant Vitamin C.

Table 2. Total phenol and flavonoid contents and antioxidant activity of the *Onobrychis* species.

Species	Total phenol (mg/g dw)	Total flavonoid (mg/g dw)	ß-Carotene /linoleic acid	DPPH radical scavenging		Metal chelating activity	
				IC_{50}(mg/m)	Percent (%)	IC_{50} (mg/ml)	Percent (%)
O. viciifolia	10.38 ± 0.33^a	5.08 ± 0.64^a	64.32 ± 2.2^a	0.113 ± 0.002^a	92.70	1.80 ± 0.003^b	19.13
O.melanotria	6.25 ± 0.28^c	4.92 ± 0.43^a	31.54 ± 2.5^c	0.129 ± 0.001^b	92.27	1.10 ± 0.006^a	14.62
O. sosnovskyi	8.77± 0.46^b	4.48 ± 0.26^a	55.53 ± 1.5^b	0.121 ± 0.005^b	91.38	1.26 ± 0.004^a	22.34
Ascorbic acid	-	-	-	0.143 ± 0.007^c	71.26	2.29 ± 0.009^b	12.28

Experiment was performed in triplicate and expressed as mean ± SD. Values in each column with different superscripts are significantly different (P < 0.05).

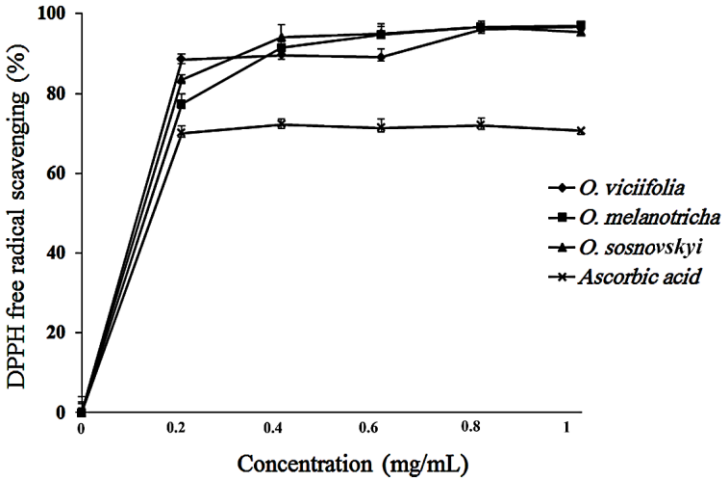

Figure 1. DPPH radical scavenging ability of the *Onobrychis* extracts in different concentrations.

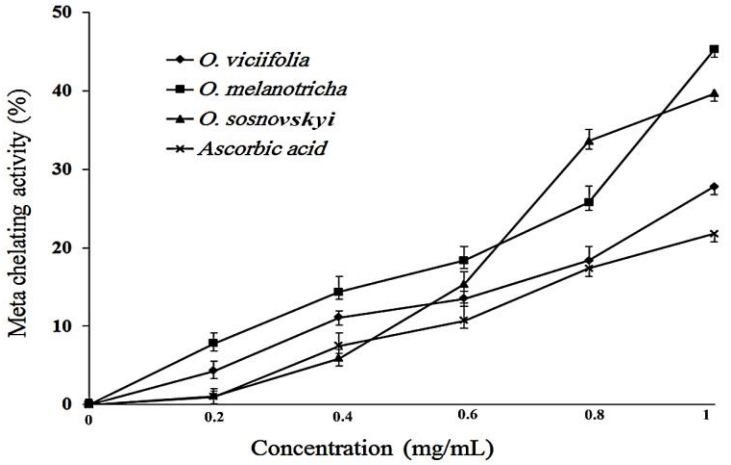

Figure 2. Metal chelating effects on ferrous ions of the *Onobrychis* extracts and ascorbic acid in different concentrations.

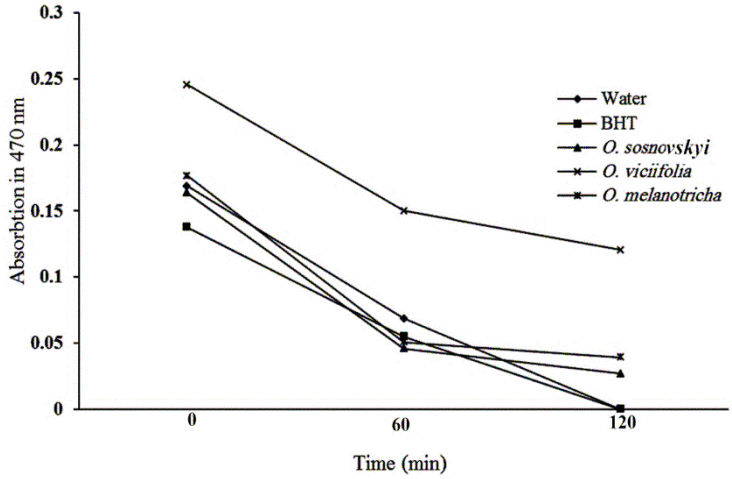

Figure 3. Inhibition of bleaching of β-carotene/linoleic acid emulsion by the *Onobrychis* extracts and BHT over 120 min.

The effectiveness of extracts as DPPH radical scavengers ranged in the following descending order: *O. viciifolia* (IC_{50}; 0.113 ± 0.002 mg/ml) > *O. sosnovskyi* (IC_{50}; 0.121 ± 0.005 mg/ml) ≥ *O. melanotricha* (IC_{50}; 0.129 ± 0.001 mg/ml) > ascorbic acid (IC_{50}; 0.143 ± 0.007 mg/ml), (Table 2).

Iron chelating capacity

All investigated extracts were capable of chelating Fe^{2+} ions. Metal chelating property of the extracts was dependent on concentration (0.2 to 01 mg/ml) and linearly increased with the concentration increase (Table 2 and Figure 2). There was no significant difference ($P < 0.05$) between metal chelating activity of *O. viciifolia* (14.62%, IC_{50}; 1.80 ± 0.003 mg/ml) and ascorbic acid (12.28%, IC_{50}; 2.29 ± 0.009 mg/ml) as a synthetic antioxidant; however the extracts from *O. melanotricha* (22.34%, IC_{50}; 1.10 ± 0.006 mg/ml) and *O. sosnovskyi* (19.13%, IC_{50}; 1.26 ± 0.004 mg/ml) showed significantly more chelating activity than that of *O. viciifolia* (Table 2).

ß-Carotene bleaching assay

Results from antioxidant activity by ß-carotene/linoleic acid system showed that *O. viciifolia* extract (64.32 ± 2.2%) was more active than other two species (Table 2). Results from Amarowicz *et al.*[23] showed that the normalized antioxidant ability by β-carotene/linoleic acid method of selected plant extracts including some legumes at t = 60 min and t = 120 min of incubation are excellent. The change in absorbance (t = 0 to t = 120) of the reaction mixture in the presence of studied extracts, BHT (as positive control) and water (absence of the extracts) are shown in Figure 3. Here the methanolic extracts showed remarkable bleaching of ß-carotene ($P < 0.05$) in comparison with control (BHT as a synthetic antioxidant).

Discussion

Phenolic compounds in their chemical structure have hydroxyl groups that are power hydrogen donors and known as antioxidant agents. Antioxidants with hydrogen-donating ability can react with reactive oxygen and nitrogen species (ROS and RNS) and or chelate metals and inhibit lipid peroxidation.[26,27] These compounds are observed in different amounts and in various parts of plants. Phenolic structures can strongly interact with proteins and inhibit activity some

enzymes involved in ROS and RNS generation, such as various cytochrome P_{450} isoforms, lipoxygenases, cyclooxygenase and xanthine oxidase.[28] Also, synergistic and anti-synergistic effects this compounds with other antioxidants and regulation of glutathione (as important antioxidant in animals, plants, fungi, and some bacteria) levels have been described.[29,30] Recent studies showed that differences in antioxidant potential of plant extracts could be related to the nature of the phenolic compounds such as flavonoids but not necessarily to their amounts.[31] Flavonoids are characterized by a phenyl benzopyran chemical structure with a pyran, pyrilium, or γ-pyrone center heterocycle.[32] Flavonoids have been classified into flavanols, flavanones, flavones, isoflavones, catechins, anthocyanins, proanthocyanidins. Pharmacological effects of flavonoids on human health are attributed to their potent antioxidant.[33] In this research, all extracts represented high total phenolic content. The highest content was detected in *O. viciifolia* (10.38 ± 0.33 mg GAE/g of dry extract) and the lowest in *O. melanotricha* (6.25 ± 0.28 mg GAE/g of dry extract). Our results indicated that the three species are not remarkable different ($P < 0.05$) in total flavonoids amount (Table 2). In other studies on *O. viciifolia* extract by different solvents, the following chemical composition were found: the phenolic glycosides; kaempferol and quercetin, sucrose; the inositol [(+)-pinitol]; L-tryptophan, several flavonol glycosides and a mixture of condensed tannins.[15-17,34,35] Ince *et al*. assessed total phenolic acid content of different extracts (methanol, acetone, distillation water and ethyl ethanoate) of *O. viciifolia* using Folin-Ciocalteu method and reported its amount between 11.35 ± 0.82 to 38.26 ± 0.15 µg Tannic acid/g of dry extract.[17] In the case of the *O. viciifolia*, the extractable compounds by acetone and methanol solvents had higher yield in comparison with ethyl ethanoate and distillation water solvents.[17,36-38]

Antioxidant activity of *Onobrychis* extracts was assessed using DPPH radical scavenging method. *In vitro* antioxidant activity of the phenolic compounds on a molar basis is higher than vitamins E and C.[39] In this research, the extracts showed excellent concentration-dependent (0.2 to 1 mg/ml) antiradical properties and also the curve of antioxidant activity (in this evaluation system) of studied samples had overlap in some concentration. However, they were higher than vitamin C as a synthetic antioxidant (Figure 1).There is no study or reveal any references to previous works on the antioxidant activity (as DPPH free radical scavenging) of *O. melanotricha* and *O. sosnovskyi*. Whereas, a study on antioxidant activity of *O. viciifolia* reported its amount between 20 to 50% that showed excellent concentration-dependent (in 0.025, 0.05 and 0.1 mg/ml concentration) with antiradical property. This is in agreement with our results. Recent studies on the plant extracts have shown a positive relationship between the amount of phenolic content of the extracts and their antioxidant properties, while there is poor or no correlation in other reports.[40-42] Here, there is a poor correlation coefficients ($R^2 > 0.55$) between total phenolic content and DPPH free radical scavenging potential of the studied species (Figure 4).

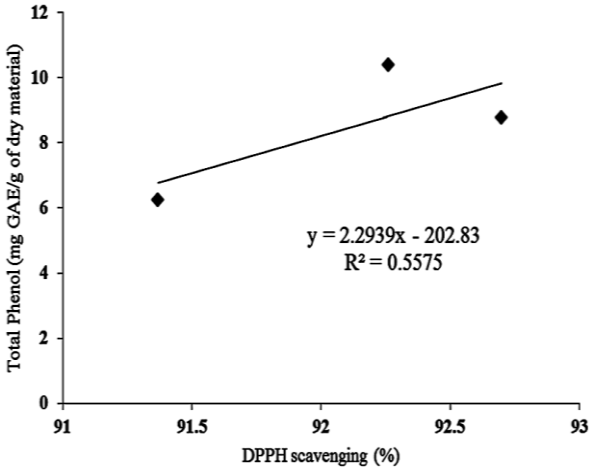

Figure 4. Correlation between DPPH free radical scavenging and total phenolic content (mg GAE/g of dry extract) of the *Onobrychis* extracts.

Our results are in agreement with Babbar *et al*. who showed phenolic compounds alone are not fully responsible for antiradical activity of plant extracts. Other constituents such as reducing carbohydrates, tocopherols, ascorbates, carotenoids, terpenes, and pigments can be responsible for antioxidant activity. Nowadays, it is detected that the synergistic or antagonistic effect of any compounds in low amount should be considered for total antioxidant activities.[43,44] In physiological processes such as oxygen transport, cell respiration and activity of many enzymes, iron plays important roles. However, among the transition metals, iron is the most important factor in lipid peroxidation and oxidative damage to DNA, polypeptide chains and other cellular components.[45] Lipid oxidation and peroxides process is accelerated by produce hydroxide free radical by breaking down of hydrogen peroxide and ferrous stateof iron, respectively ($Fe^{2+} + H_2O_2 \rightarrow F^{3+} + {}^{-}OH + {}^{-}OH$).[22] Antioxidant agents such as some of phenolic acids compounds could inactive metal ions and their dependent processes.[45] In the metal chelating assay, ferrozine can quantitatively form complexes with Fe^{2+}. Plant extracts could inhibit complete formation of Ferrozine-Fe^{2+} complex; this indicates their ability to chelate.[46] There is no report on the assessment of metal chelating properties of *Onobrychis* species studied here. Our results showed that the absorbance of Ferrozine-Fe^{2+} complex decreased linearly and the extracts showed good metal chelating properties in a dose-dependent concentration (0.2 to 1 mg/ml), (Figure 2). The activity of studied methanolic extracts as metal cheaters ranged in the following: *O. melanotricha* > *O. sosnovskyi* ≥ *O. viciifolia* > ascorbic acid (Table 2).

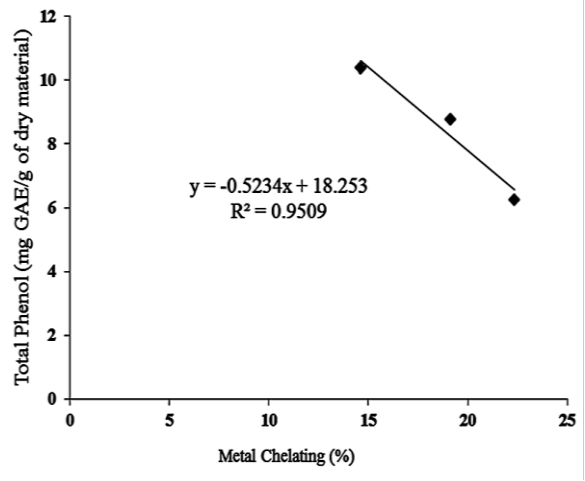

Figure 5. Correlation between metal chelating activity and total phenolic content (mg GAE/g of dry extract) of the *Onobrychis* extracts.

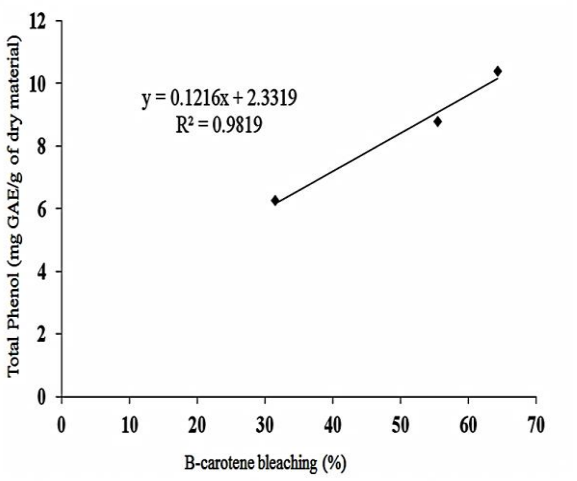

Figure 6. Correlation between β-carotene bleaching inhibition activity and total phenolic content (mg GAE/g of dry extract) of the *Onobrychis* extracts.

A high correlation ($R^2 > 0.95$) existed between total phenolic content and metal chelating potential of the studied species (Figure 5).

Our results suggest that up to 90% of the metal chelating properties of the studied species are related to phenolic compounds. In other word it can be concluded the antioxidant activity of plant extracts is not limited to phenolics (10%). The antioxidant potential of the extracts (in 5 mg/ml concentration) was assessed by ß-carotene/linoleic acid method. In this assay ß-carotene/linoleic acid emulsion because of oxidation in the absence of an antioxidant generates free radicals and rapidly discolor.[47] Oxidation of linoleic acid was effectively inhibited by *O. viciifolia* extract (64.32 ± 2.2%), followed by *O. sosnovskyi* (55.53 ± 1.5%) and *O. melanotricha* (31.54 ± 2.5%) extracts (Table 2). Ince *et al*. assessed antioxidant activity of different extracts of *O. viciifolia* using β-carotene bleaching assay and reported its amount between 32.42 ± 2.2% to 64.77 ± 4.1% in 0.05 mg/ml concentration of extracts (53.65 ± 2.4% for methanolic extract) and 57.36 ± 5.5 to 76.86 ± 5.4 in 0.1 mg/ml concentration of extracts (69.50 ± 4.3 for methanolic extract).[17] The high absorbance values or slow discoloration of β-carotene showed that the extracts have excellent antioxidant activity.

The change in absorbance (0 to 120 min) of ß-carotene/linoleic acid emulsion in the presence and absence of studied extracts and BHT as positive control are shown in Figure 3.

There is a high correlation coefficients ($R^2 > 0.98$) between total phenolic content of the extracts and β-carotene bleaching inhibition potential (Figure 6), suggesting that up to 98% of the inhibited bleaching of ß-carotene in the species are related to phenolic compounds. To conclude, this study supports that the studied *Onobrychis* extracts presented high phenolic content and antioxidant properties. This is the first report that envisages the antioxidant activity of *O. sosnovskyi* and *O. melanotricha* and also *O. viciifolia* in metal chelating assay.

Conclusion

Our work provides useful knowledge on phenolic content and antioxidant property of three *Onobrychis* species by easy, simple and reliable methods. Our observations implied that differences in the antioxidant activities between the extracts cannot adequately explain by quantitative difference in their total phenolic contents. Hence, the extracts of *Onobrychis* species include important phytochemicals that may help to develop new drug candidates for antioxidants, which might be helpful in treatment of diseases caused by different free radicals. Further studies are needed to evaluate the *in vivo* potential of these extracts in animal models.

Acknowledgments

The authors would like to thank Bu-Ali Sina University for financial support of this work.

Conflict of interests

The authors claim that there is no conflict of interest.

References

1. Aruoma OI, Cuppette SL. Antioxidant methodology, in vivo and in vitro concept. Champaign, IL: AOCS Press; 1997. p. 142-69.
2. Valko M, Leibfritz D, Moncol J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 2007;39(1):44-84. doi:10.1016/j.biocel.2006.07.001
3. Zhang HY, Yang DP, Tang GY. Multipotent antioxidants: from screening to design. Drug Discovery Today. 2006;11(15-16):749-54. doi:10.1016/j.drudis.2006.06.007
4. Yu L, Perret J, Davy B, Wilson J, Melby CL. Antioxidant properties of cereal products. J Food Chem Sci. 2002;67(7):2600-3. doi:10.1111/j.1365-2621.2002.tb08784.x
5. Prior RL, Wu XM, Schaich K. Standardized methods for the determination of antioxidant

capacity and phenolics in foods and dietary supplements. J Agric Food Chem. 2005;53(10):4290-302. doi:10.1021/jf0502698
6. Sun L, Zhang J, Lu X, Zhang L, Zhang Y. Evaluation to the antioxidant activity of total flavonoids extract from persimmon (*Diospyros kaki* L.) leaves. Food Chem Toxicol. 2011;49(10):2689-96. doi:10.1016/j.fct.2011.07.042
7. Gul M, Kulu I, Peksel A, Ocal N. Evaluation of the antioxidative properties of N-Acyl amino-substituted tricyclic imides. J Chem. 2013:920130. doi: 10.1155/2013/920130
8. Walton NJ, Brown DE. Chemicals from plants perspectives on plant secondary products. London, UK: Imperial College Press; 1999. p. 1-25.
9. Mc Call MR, Frei B. Can antioxidant vitamins materially reduce oxidative damage in humans? Free Radic Biol Med. 1999;26(7-8):1034-53. doi:10.1016/S0891-5849(98)00302-5
10. Steinmetz KA, Potter JDV. Vegetables, fruit, and cancer prevention: a review. J Am Diet Assoc. 1996;96(10):1027-39. doi:10.1016/S0002-8223(96)00273-8
11. Aruoma OI. Free radicals, oxidative stress, and antioxidants in human health and disease**.** J Am Oil Chem Soc. 1998;75(2):199-212. doi:10.1007/s11746-998-0032-9
12. Delnavazi MR, Baba-Ali F, Soufiabadi S, Sherafatmand M, Ghahremani F, Tavakoli S, et al. Essential oil composition, antioxidant activity and total phenolic content of some Lamiaceae taxa growing in northwest of Iran. Pharm Sci. 2014;20(1):22-8.
13. Mabberley DJ. Mabberley's plant-book: a portable dictionary of plants, their classification and uses. 3rd ed. Cambridge: Cambridge University Press; 2008.
14. Yakovlev GP, Sytin AK, Roskov YR. Legumes of Norhern Eurasia, a check-lis. Kew, UK:Royal Botanic Gardens; 1996.
15. Theodoridou K, Aufrere J, Andueza D, Le Morvan A, Picard F, Stringano E, et al. Effect of plant development during first and second growth cycle on chemical composition, condensed tannins and nutritive value of three sainfoin (*Onobrychis viciifolia*) varieties and lucerne. Grass Forage Sci. 2011;66(3):402-14. doi:10.1111/j.1365-2494.2011.00798.x
16. Marais JPJ, Mueuller-Harvey I, Brandt EV, Ferreira D. Polyphenols, condensed tannins and other natural products in *Onobrychis viciifolia* (Sainfoin). J Agric Food Chem. 2000;48:3440-7. doi:10.1021/jf000388h
17. Ince S, Ekici H, Yurdakok B. Determination of *in vitro* antioxidant activity of the sainfoin (*Onobrychis viciifolia*) extracts. Ank Univ Vet Fak Derg. 2012;59(1):23-7.
18. Donald SMC, Prenzler PD, Autolovich M, Robards K. Phenolic content and antioxidant activity of olive extracts. Food Chem. 2001;73(1):73-84. doi:10.1016/S0308-8146(00)00288-0
19. Chang C, Yang M, Wen H, Chern J. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Anal. 2000;10(3):178-82.
20. Mensor L, Menezes FS, Leitao GG, Reis AS, Santos TS, Coube CS. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother Res. 2001;15(2):127-30. doi:10.1002/ptr.687
21. Dinis TCP, Madeira VMC, Almeida MLM. Action of phenolic derivates (acetoaminophen, salycilate and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Arch Biochem Biophys. 1994;315(1):161-9. doi:10.1006/abbi.1994.1485
22. Miller HE. A simplified method for the evaluation of antioxidants. J Am Oil Chem Soc. 1971;48(2):91. doi:10.1007/BF02635693
23. Amarowicz R, Pegg RB, Rahimi-Moghaddam P, Barl B, Weil JA. Free radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. Food Chem. 2004;84(4):551-62. doi: 10.1016/S0308-8146(03)00278-4
24. Kahkonen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, et al. Antioxidant activity of plant extracts containing phenolic compounds. J Agric Food Chem. 1999;47(10):3954-62. doi:10.1021/jf990146l
25. Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med. 1996;20(7):933-56. doi:10.1016/0891-5849(95)02227-9
26. Shahidi F, Wanasundara PKJPD. Phenolic antioxidants. Crit Rev Food Sci Nutr. 1992;32(1):67-103. doi:10.1080/10408399209527581
27. Valentao P, Fernandes E, Carvalho F, Andrade PB, Seabra RM, Bastos ML. Hydroxyl radical and hypochlorous acid scavenging activity of small centaury (*Centaurium erythraea*) infusion. A comparative study with green tea (*Camellia sinensis*). Phytomedicin. 2003;10(6-7):517-22. doi:10.1078/094471103322331485
28. Parr AJ, Bolwell JP. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. J Sci Food Agric. 2002;80(7):985-1012. doi:10.1002/(SICI)1097-0010(20000515)80:7<985::AID-JSFA572>3.0.CO;2-7
29. Seabra RM, Andrade PB, Valentao P, Fernandes E, Carvalho F, Bastos ML. Biomaterials from Aquatic and Terrestrial organisms. In: Fingerman M, Nagabhushanam R, editors. Antioxidant compounds extracted from several plant materials. Enfield, NH:

Science Publishers; 2006. p. 115-74.
30. Cao G, Booth SL, Sadowski JA, Prior RL. Increases in human plasma antioxidant capacity after consumption of controlled diets high in fruit and vegetables. Am J Clin Nutr. 1998;68(5):1081-7.
31. Shahidi F, Marian N. Phenolics in Food and Nutraceuticals. Boca Raton, FL: CRS Press; 2003. p. 144-50.
32. Aron PM, Kennedy JA. Flavan-3-ols: Nature, occurrence and biological activity. Mol Nutr Food Res. 2008;52(1):79-104. doi:10.1002/mnfr.200700137
33. Miller AL. Antioxidant Flavonoids: Structure, Function and Clinical Usage. Alt Med Rev. 1996;1(2):103-11.
34. Theodoridoua K, Aufrerea J, Anduezaa D, Pourrata J, Morvana AL, Stringanob E, et al. Effects of condensed tannins in fresh sainfoin (*Onobrychis viciifolia*) on *in vivo* and in situ digestion in sheep. Anim Feed Sci Technol. 2010;160(1-2):23-38. doi:10.1016/j.anifeedsci.2010.06.007
35. Lu Y, Suna Y, Foo LY, Mc Nabb WC, Molanb AL. Phenolic glycosides of forage legume *Onobrychis viciifolia*. Phytochem. 2000;55(1):67-75. doi:10.1016/S0031-9422(00)00143-6
36. Xu BJ, Chang SKC. A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. J Food Sci. 2007;72(2):159-66. doi:10.1111/j.1750-3841.2006.00260.x
37. Barrau E, Fabre N, Fouraste I, Hoste H. Effect of bioactive compounds from sainfoin (*Onobrychis viciifolia* Scop.) on the *in vitro* larval migration of *Haemonchus contortus*: role of tannins and flavonol glycosides. J Parasitol. 2005;131(4):531-8. doi:10.1017/S0031182005008024
38. Bartolome B, Nunez V, Monagas M, Gomez-Cordoves C. *In vitro* antioxidant activity of red grape skins. Eur Food Res Technol. 2004;218(2):173-7. doi:10.1007/s00217-003-0833-x
39. Rice-Evans CA, Miller NJ, Paganga G. Antioxidant properties of phenolic compounds. Trends Plant Sci. 1997;2(4):152-9. doi:10.1016/S1360-1385(97)01018-2
40. Siddique NA, Mujeeb M, Najmi AK, Akram M. Evaluation of antioxidant activity, quantitative estimation of phenols and flavonoids in different parts of *Aeglemar melos*. Afr J Plant Sci. 2010;4(1):001-5.
41. Cardozo ML, Ordonez RM, Alberto MR, Zampini IC, Isla MI. Antioxidant and anti-inflammatory activity characterization and genotoxicity evaluation of *Ziziphus mistol* ripe berries, exotic Argentinean fruit. Food Res Int. 2011;44(7):2063-71. doi:10.1016/j.foodres.2011.02.040
42. Conforti F, Sosa S, Marrelli M, Menichini F, Statti GA, Uzunov D, et al. *In vivo* anti-inflammatory and *in vitro* antioxidant activities of Mediterranean dietary plants. J Ethnopharmacol. 2008;116(1):144-51. doi:10.1016/j.jep.2007.11.015
43. Babbar N, Oberoi HS, Uppal DS, Patil RT. Total phenolic content and antioxidant capacity of extracts obtained from six important fruit residues. Food Res Int. 2011;44(1):391-6. doi:10.1016/j.foodres.2010.10.001
44. Burt SA. Essential oils: their antibacterial properties and potential applications in foods - a review. Int J Food Microbiol. 2004;94(3):223-53. doi:10.1016/j.ijfoodmicro.2004.03.022
45. Finefrock AE, Bush AI, Doraiswamy PM. Current status of metals as therapeutic targets in Alzheimer's disease. J Am Geriatr Soc. 2003;51(8):1143-8. doi:10.1046/j.1532-5415.2003.51368.x
46. Yamaguchi F, Ariga T, Yoshimura Y, Nakazawa H. Antioxidative and antiglycation activity of garcinol from *Garcinia indica* fruit rind. J Agric Food Chem. 2000;48(2):180-5. doi:10.1021/jf990845y
47. Jayaprakasha GK, Singh RP, Sakariah KK. Antioxidant activity of grape seed (*Vitis vinifera*). Food Chem. 2001;73(3):285-90. doi:10.1016/S0308-8146(00)00298-3