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Determination of in vitro antioxidant activity of the sainfoin (*Onobrychis Viciifolia*) extracts

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Summary: In this study, in vitro antioxidant effects of the acetone, methanol, ethylacetate and water extracts of the aerial parts of sainfoin (*Onobrychis viciifolia*) were investigated. The phenolic content of the extracts were determined by Folin-ciocalteu method and determination of antioxidant activity were carried out by phosphomolybdenum method, β-carotene-linoleate model system, radical scavenging capacity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and reducing power based on the reduction of $Fe^{+3}/K_3Fe(CN)_6$ complex to the ferrous state. The study suggested that sainfoin could be pharmaceutically exploited in future researches for its antioxidant properties where acetone and methanol extracts exhibited high antioxidant activity.

Keywords: Antioxidant activity, free radical scavenging activity, *O. viciifolia*.

Korunga (*Onobrychis Viciifolia*) bitkisinin antioksidan etkinliğinin in vitro değerlendirilmesi

Özet: Bu çalışmada, korunga bitkisinin (*Onobrychis viciifolia*) toprak üstü kısımlarının aseton, methanol, etilasetat ve su ekstrelerinin antioksidan etkinliği araştırıldı. Ekstrelerin fenolik içeriği Folin-Ciocalteu yöntemiyle, antioksidan aktivitesinin belirlenmesi fosfomolibden metodu, beta-karoten-linoleat model sistemi, radikal süpürücü etkiye karşı 2,2-difenil-1-pikrilhidrazil (DPPH) ve demirli kompleks olan $Fe^{+3}/K_3Fe(CN)_6$ bileşiğinin indirgenmesi ile gerçekleştirildi. Bu çalışma, yüksek antioksidan özelliği gösteren korunga bitkisinin aseton ve metanollü ekstrelerinin, ileride farmasötik çalışmalar için değerlendirilebileceğini önermektedir.

Anahtar sözcükler: Antioksidan aktivite, serbest radikal süpürücü aktivite, *O. Viciifolia*.

Introduction

Polyphenols are present in a variety of plants as well as food grains, fruits and vegetables utilized as important components of both human and animal diets (9,11,12). It is widely accepted that significant antioxidant activity of food is related to high total phenolic content. Plants contain a large variety of phenolic derivatives, including simple phenols, phenylpropanoids, benzoic acid derivatives, flavonoids, stilbenes, tannins, lignans, and lignins (21,26). The flavonoids include flavones, flavanols, and condensed tannins. Flavonoids are the predominant components in some reported legumes (7,10,24), such as sainfoin (5,16,17,25).

Sainfoin (*O. viciifolia*), also known as holy grass, is a perennial forage legume with a deep taproot often grown in conjunction with forage grasses to reduce bloat hazard as well as to improve soil fertility due to its nitrogen fixing ability. Another desirable trait is that sainfoin has an early growth habit, sprouting earlier than alfalfa in spring to give good forage yields (16). The plant contains several physiologically active phytochemicals. It is a good source of flavanoids. Acetone/water extract of the fodder legume *O. viciifolia* afforded arbutin, kaempferol, quercetin, rutin, afzelin (17). The phenolic compounds were characterized as seven cinnamic acid derivatives and nine flavonoid glycosides all of which were identified by NMR spectroscopy (16).

Domestic animals just like man, are frequently exposed to oxidative stress, involving an overproduction of reactive oxygen species that cause oxidative damage to all macromolecules within the cell. In domestic animals, oxidative stress has been associated with the deterioration of many physiological functions including growth and reproduction as well as immunity. The oxidative deterioration of lipids (or lipid peroxidation) is particularly involved in this phenomenon since lipids are macromolecules which are more susceptible to peroxidative processes, especially when they are rich in n-3 PUFA diets. Therefore, it appeared that a supplement of antioxidants should be recommended to preserve the heath of animals and the oxidative stability of their products (13).

The aim of this study were to investigate and to provide a comprehensive assessment of antioxidant

properties of sainfoin, a common animal feed used also for the control of nematode parasitism, prevention of bloat and improvement of nutrients utilization (17). For this purpose, total phenolic content were assessed and multiple measuring methods to determine the antioxidant activity involving different reaction mechanisms such as β-carotene-linoleate model system, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging were carried out on ethyl acetate, acetone, methanol and water extracts of the aerial parts of the plant.

This research is the first report on the in vitro antioxidant activity of sainfoin and would serve as a good base for further pharmaceutical investigations on forage legumes for their potential antioxidant properties.

Materials and Methods

Materials: β-carotene, linoleic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and butylated hydroxyanisole (BHA) were obtained from Sigma Chemical Co. (Interlab A.S., Istanbul, Turkey). All solvents/chemicals used were of analytical grade and obtained from Merck (Merck Ilac, Ecza ve Kimya Tic. Ltd. Sti, Istanbul, Turkey). Visible spectra measurements were done using Shimadzu UV visible spectrophotometer (Spectronic Instruments Inc., NY, USA). The plant material, about 30-40 cm length (aereal parts-stem and leaves) was collected from 5 cm above the ground during the flowering period once for each month between May-July (2008). This material was obtained from Ankara University, Faculty of Agriculture, Department of Field Crops, Ankara, Turkey. Pulverized and harvested plant material, was stored in a desiccator for humid and light protection until the analysis.

Extraction: The plant extracts were prepared as described earlier by Zahin et al. with little modification (27). The plant of *O. viciifolia* was cut into pieces, dried under the sun and powdered in a grinder to 40-mesh size powder. Plant powder of 25 g was extracted with 150 ml of ethyl acetate by mixing, using a magnetic stirrer at 30 °C for 2 h. The extract was filtered through Whatman No. 41 filter paper to obtain particle free extract. The residue was re-extracted twice and filtered. The extracts were pooled and concentrated and dried under vacuum. The same procedure was followed for the other solvents such as acetone, methanol and water for antioxidant fractions and the dried extracts were used to explore their antioxidant activity.

Determination of total phenolics: The concentration of phenolic compounds in the extracts was determined according to Jayaprakasha et al. (15) and total phenolic contents were expressed as tannic acids equivalents. The extracts were dissolved in a mixture of methanol and water (6:4 v/v). Samples (0.2 ml) were mixed with 1.0 ml of tenfold diluted Folin-ciocalteu reagents and 0.8 mL of 7.5% sodium carbonate solution. After standing for 30 min at room temperature, the absorbance was measured at 765 nm by using UV-VIS spectrophotometer. The estimation of phenolic compounds in the fractions was carried out in triplicate and the results were averaged.

Evaluation of antioxidant capacity by phosphomolybdenum method: The total antioxidant capacity of ethyl acetate, methanol and water extracts of plant of *O. viciifolia* was evaluated by the method of Prieto et al. (18). An aliquot of 0.1 ml of sample solution (100 µg/ml) was combined with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank in spectrophotometer. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. For samples of unknown composition, water-soluble antioxidant capacity was expressed as equivalents of ascorbic acid (µmole/g of extract).

Antioxidant activity assay using β-carotene-linoleate model system: The antioxidant activity of plant extracts of *O. viciifolia* was evaluated using β-carotene-linoleate model system as described by Jayaprakasha et al. (15). 0.2 mg of β-carotene in 0.2 ml of chloroform, 20 mg of linoleic acid and 200 mg of Tween-40 (polyoxyethylene sorbitan monopalmitate) were mixed. Chloroform was removed at 40 °C under vacuum and the resulting mixture was diluted with 10 ml of water and was mixed well. To this emulsion, 40 mL of oxygenated water was added. 4 ml aliquots of the emulsion were pipetted into different test tubes containing 0.2 ml of extracts (50 and 100 ppm) and BHA (50 and 100 ppm) in ethanol. BHA was used for comparative purposes. Control solution, containing 0.2 ml of ethanol and 4 ml of the above emulsion was also prepared. The tubes were placed at 50 °C in a water bath and the absorbance at 470 nm was taken at zero time (t = 0). Measurement of absorbance was continued till the color of β-carotene disappeared in the control tubes *(t = 75* min) at an interval of 15 min. A mixture prepared as above without β-carotene served as blank. All measurements were carried out in triplicates. The antioxidant activity (AA) of the extracts was evaluated in terms of bleaching of the β-carotene using the following formula, $AA = 100[1 - (A_o - A_t) = (A_o^0 - A_t^0)]$, where A_o and A_o^0 are the absorbance values measured at zero time of the incubation for test sample and control, respectively. A_t and A_t^0 are the absorbance measured in the test sample and control, respectively, after incubation for 75 min. The results were expressed in % basis of preventing bleaching of β-carotene.

Radical scavenging activity assay using DPPH method: Radical scavenging activity of the extracts was determined essentially as described by Blois (8). Different concentrations (25, 50 and 100 µl equivalent to 25, 50 and 100 ppm, respectively) of *O. viciifolia* plant extracts and BHA (25, 50 and 100 ppm) were taken in different test tubes. The volume was adjusted to 100 µl by adding MeOH. 5 ml of 0.1 mM methanolic solution of DPPH was added to these tubes and shaken vigorously. The tubes were allowed to stand at 27 °C for 20 min. The control was prepared as above without any extract and MeOH was used for the baseline correction. The changes in the absorbance of the samples were measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula, % radical scavenging activity = (Control OD – sample OD/Control OD) x 100.

Reducing power activity assay: Reducing power activity of sainfoin was determined by the method which was described by Teepica Priya Darsini et.al. (23). Different concentration (25, 50, 100 mg/ml) of plant extracts were taken in test tubes and the volume was adjusted to 1 ml by the addition of dimethyl sulfoxide. Then 2.5 ml of phosphate buffer (0.2M, pH 6.6) and 2.5ml of 1 % potassium ferricyanide were added to the mixture where it was kept in a 50°C water bath for 20 minutes. The resulting solution was then cooled rapidly, spiked with 2.5 ml of 10% trichloroacetic acid and centrifuged at 3000 rpm for 10 minutes. The supernatant (5 ml) was mixed with 5 ml of distilled water and 1 ml of 0.1% ferric chloride. The absorbance was detected at 700 nm after reaction for 10 minutes. The estimation of reducing power activity in the fractions was carried out in triplicate and the results were averaged.

Statistical analysis: The results are presented as the average and standard error of three experiments. The data was analysed by using Sigma plot 10.0.

Results

The extraction rate of acetone, methanol, ethyl acetate and water extracts of dried *O. viciifolia* were 11.9%, 16.5%, 9.6% and 4.3%, respectively. The total phenolic contents of the *O. viciifolia* extracts determined by Folin-ciocalteu method are reported as tannic acid equivalents in Table 1. Antioxidant capacity of *O. viciifolia* by phosphomolybdenum method was also described in Table 1. The antioxidant activity through β-carotene-linoleate model system of *O. viciifolia* extracts at 50 and 100 µg/mL concentrations compared with BHA was presented in Table 2. The free radical scavenging activities of the extracts by DPPH method were shown in the Fig. 1 and the reducing power of the plant extract in different concentrations 25, 50, 100 µg/mL were described in Table 3.

Table 1. Phenolic contents (as tannic acid equivalent) and antioxidant capacity (as equivalent to ascorbic) of the extracts from *O. viciifolia* by phosphomolybdenum method (Mean±SD)
Tablo 1. *O. Viciifolia* ekstrelerinin fenolik içeriği (tannik asit eşdeğeri olarak) ve Fosfomolibden yöntemi ile antioksidan kapasitesi (askorbik eşdeğeri olarak) (Ortalama±SH)

Extract	Phenolics (% w/w)	Antioxidant capacity (µmol/g of extract)
Acetone	38.26±0.15	1918.78±18.70
Methanol	36.78±0.05	1739.50±17.11
Ethyl acetate	14.60±0.70	1375.63±8.13
Water	11.35±0.82	521.85±5.33

Table 2. Antioxidant activity of extracts from *O. viciifolia* and BHA by β-carotene-linoleate model system (% inhibition of bleaching of β-carotene) (Mean±SD)
Tablo 2. β-karoten-linoleat yöntemiyle *O. viciifolia* ekstrelerinin ve BHA'nın antioksidan etkinliği (β-karoten kaybolmasının % inhibisyonu) (Ortalama±SH)

Extract/BHA	50 ppm	100 ppm
BHA	93.03±0.04	96.50±0.05
Acetone	64.77±4.12	76.86±5.42
Methanol	53.65±2.38	69.50±4.36
Ethyl acetate	47.23±3.04	66.14±2.52
Water	32.42±2.24	57.36±5.51

Table 3. Reducing power levels of *O. viciifolia* extracts (Absorbance 700 nm, Mean±SD).
Tablo 3. *O. vicifolia* ekstrelerinin indirgeme kapasitesi düzeyleri (700 nm'de absorbans, Ortalama±SH).

Extract	25 ppm	50 ppm	100 ppm
Water	0,11±0,01	0,12±0,02	0,15±0,03
Acetone	0,12±0,01	0,15±0,02	0,18±0,01
Ethanol	0,24±0,01	0,26±0,01	0,34±0,03
Methanol	0,25±0,02	0,27±0,01	0,31±0,06

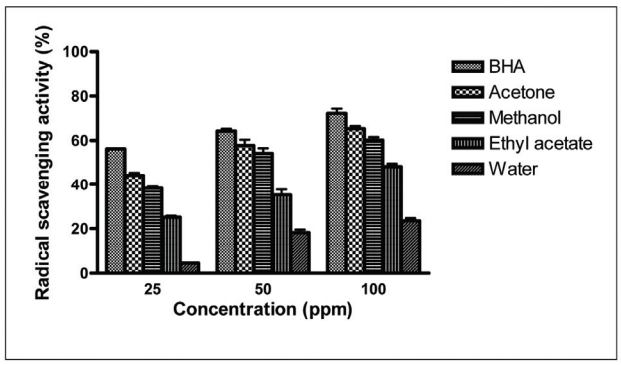

Figure 1. Radical scavenging activity of *O.viciifolia* extracts by DPPH method
Şekil 1. DPPH yöntemi ile *O.viciifolia* ekstrelerinin radikal süpürücü aktivitesi

Discussion and Conclusion

Different solvent systems have been used to extract antioxidants from plant materials such as fruits, vegetables, legumes, and other foodstuffs. Water, aqueous mixtures of ethanol, methanol, and acetone are commonly used to extract antioxidants from plant foods. In recent studies, the yield of extractable compounds was highest in acetone and methanol extract from the *O. viciifolia* in comparison with the solvents such as ethyl acetate and water (5,16,17,26). Furthermore, the extraction of phenolic compounds from the forage legume is usually provided with methanol, acetone and aqueous methanol or acetone (3,4). The levels of total phenolics determined in this way are not absolute measurements of the amounts of phenolic compounds, but are in fact based on their chemical reducing capacity relative to tannic acid. In the present study, the difference between the antioxidant activities may due to the variety and/or quantity of phenolics in different extracts. Among the four extracts, acetone and methanol extract contained the highest amount of phenolic compounds followed by ethyl acetate extract and water extract.

Antioxidants are present in foods as vitamins, minerals, carotenoids, and polyphenols, among others. The most well-known components of food with antioxidant activities are vitamins A, C, and E, *β*-carotene, the mineral selenium, and more recently, the compound lycopene (6, 14). Furthermore, phenolic compounds have ideal structural chemistry for free radical-scavenging activities and have been shown to be more effective antioxidants *in vitro* than vitamins E and C on a molar basis (20).

The antioxidant capacity of the extracts was measured spectrophotometrically through phosphomolybdenum method, which is based on the reduction of Mo (IV) to Mo (V) by the sample antioxidant constituents and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm. The antioxidant capacity of extracts of *O. viciifolia* was found to decrease in the order, acetone extract > methanol extract > ethyl acetate extract > water extract.

The addition of *O. viciifolia* extracts and BHA at 50 µg/mL concentrations prevented the bleaching of β-carotene to different degrees. β-carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. This is because of the coupled oxidation of β-carotene and linoleic acid, which generates free radicals. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated β-carotene molecules. As a result, β-carotene will be oxidized and broken down in part, subsequently the system loses its chromophore and characteristic orange color, which can be monitored spectrophotometrically (15). In our present study, the extracts from *O. viciifolia* were found to hinder the extent of β-carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in the system. Acetone, methanol, ethyl acetate and water extracts had more antioxidant activity at 100 µg/ml concentration than 50 µg/ml concentration. Threfore, antioxidant activity depends on dose.

The role of antioxidants is basically described by their interaction with oxidative free radicals. The basis of DPPH method is that the antioxidants react with the stable free radical i.e., 2,2-diphenyl-1-picrylhydrazyl (DPPH) and convert it to 2,2-diphenyl-1-picrylhydrazine (DPPH 2) with discoloration. The degree of discoloration indicates the scavenging potentials of the sample antioxidant (8). In the present study, the extracts of *O. viciifolia* were able to decolorize DPPH. The free radical scavenging potentials of the extracts according to this assay were found in the order of acetone > methanol> ethyl acetate > water. It has been found that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (hydroquinone, pyrogallol etc.), and aromatic amines (p-phenylene diamine, p-aminophenol etc.) reduce and decolorize 2,2-diphenyl-1-picrylhydrazyl by their hydrogen donating ability (8). It appears that the extracts from the *O. viciifolia* possess hydrogen donating capabilities to act as antioxidant. The extract also caused significant elevation of reducing power potential, highest in methanolic extracts, in accordance with increasing doses.

In the present study, the decreasing order of antioxidant activity among the *O. viciifolia* extracts was found as acetone extract > methanol extract > ethyl acetate extract > water extract. This order is in accordance with the phenolic contents of the extracts. These observations clearly indicated a cross linkage between phenolics and antioxidant activity. Several reports have conclusively shown close relationship between total phenolic content and antioxidative activity of the legumes, fruits and vegetables (1,2,6,19).

The chemical composition and structures of active extract components are important factors governing the efficacy of natural antioxidants. However, the antioxidant activity of an extract could not be explained only on the basis of their phenolic content, but also with its chemical characterization. For instance, phenolic compounds with ortho- and para-dihydroxylation or a hydroxy and a methoxy group are more effective than simple phenolics (22). Therefore, further pharmaceutical analysis on isolation and identification of active compounds with detailed chemical structuring needs to be done along with other in vitro-in vivo pharmacological studies to understand the mechanism of action as well as the potential capacity as antioxidant.

To conclude, this study supports the contention that traditional medicines, dietary supplements and plant feed sources remain a valuable source in the potential

discovery of natural product pharmaceuticals. This is the first report that envisages the antioxidant activities of *O. viciifolia* extracts. Hence the sainfoin (*O.viciifolia*) could be a good source of antioxidant phenolics.

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