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Research Article

Antioxidant Activity and Total Phenolic Contents of Different Alfalfa (*Medicago sativa* L.) Varieties Grown in Turkey

Hakime Hülya ORAK[*1], Magdalena KARAMAĆ[2], Adnan ORAK[3], Hazım Serkan TENİKECİER[4], Ryszard AMAROWICZ[5]

[1]Tekirdag Namık Kemal University, Vocational School of Technical Sciences, Food Technology Department, Tekirdağ, Turkey
[2,5]Polish Academy of Sciences, Institute of Animal Reproduction and Food Research, Department of Chemical and Physical Properties of Food, Olsztyn, Poland
[3,4]Tekirdag Namık Kemal University, Faculty of Agriculture, Field Crops Department, Tekirdağ, Turkey

[1]https://orcid.org/0000-0002-1803-1644 [2]https://orcid.org/0000-0001-8313-3092 [3]https://orcid.org/0000-0001-9731-0045
[4]https://orcid.org/0000-0002-1866-410X [5]https://orcid.org/0000-0001-9731-0045

*Corresponding author e-mail: horak@nku.edu.tr

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Abstract: Fifteen alfalfa varieties cultivated in Turkey were researched to compare the antioxidant potential of their seeds and find differences between the varieties. The total phenolic (TP) content, DPPH$^{\bullet}$, and ABTS$^{\bullet+}$ scavenging activities, and ferric-reducing antioxidant power (FRAP) of alfalfa seed methanol extracts were determined. TP contents of extracts were found between 37.03 and 54.04 mg GAE/g. ABTS assay results ranged between 0.100 and 0.158 mmol Trolox/g extract, and FRAP changed from 389.90 to 791.02 µmol Fe^{2+}/g extract. The results demonstrated that the differences between the alfalfa varieties were significant ($p < 0.01$) in terms of analyzed characters.

Türkiye'de Yetiştirilen Farklı Yonca (*Medicago sativa* L.) Çeşitlerinin Antioksidan Aktiviteleri ve Toplam Fenolik Madde İçeriği

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Anahtar Kelimeler

Alfalfa,
Antioksidan aktivite,
Medicago sativa L.,
Fenolik içerik,
Tohum,
Çeşit.

Öz: Bu çalışmada Türkiye'de yetiştirilen 15 yonca çeşidi, antioksidan potansiyellerini belirlemek ve çeşitler arasındaki farklılıkları karşılaştırmak amacıyla araştırılmıştır. Yonca tohumu metanol ekstraktlarında toplam fenolik madde içeriği, DPPH$^{\bullet}$, ABTS$^{\bullet+}$ giderim ve FRAP aktiviteleri belirlenmiştir. Ekstrelerin TP değerleri 37.03 mg GAE/g ile 54.04 mg GAE/g arasında bulunmuştur. TEAC değerleri 0.100 mmol Trolox/g ile 0.158 mmol Trolox/g arasında değişmiştir. FRAP aktivitesi 389.90 µmol Fe^{2+}/g'den 791.02 µmol Fe^{2+}/g aralığında değişim göstermiştir. Sonuçlar, incelenen karakterler açısından yonca çeşitleri arasındaki farklılıkların istatistiksel olarak önemli ($p < 0.01$) olduğunu göstermektedir.

1. Introduction

Alfalfa (*Medicago sativa* L.) is among the most cultivated forage legumes in Turkey and is generally recognized a suitable source of precious protein for animal feed (Gökkaya and Orak, 2021). In Poland, this species can be used in folk medicine because of its pharmacological substances to treat some diseases and strengthen immunity. Due to the valuable chemical constituents of *Medicago sativa* L., which exhibit phytobiotic action, it could be used in folk medicine and phytotherapy treatments (Gawel et al., 2017)

The *Medicago* genus is included the *Leguminosae* or *Fabaceae* family, that has a wide range of speciescan be used as human food or as an excellent pasture for animal feed (Kabtni et al., 2020). Due to the high quality of nutrient contents and its rich biological components, it has been approved as a dietary supplement by the European Food Safety Authority (Gatouillat et al., 2014). It has been reported that alfalfa is used as herbal medicine in traditional treatments in some countries such as Turkey, India, and America. (Bora and Sharma, 2011). Additionally, to its high nutritional value, alfalfa has a history of medicinal uses among conventional medicinal plants in Middle Eastern countries, in China, in America, and in India (Krakowska et al., 2017). In recent years, alfalfa sprouts can be used as salad, and alfalfa leaves or seeds are also sold as a nutritional supplement, as an herb, capsule, and tablet in health food stores (Gomathi et al., 2016). Eastern countries are also used as fodder for animals and for the treatment of different ailments (Al-Dosari, 2012). It has been reported that this plant has been used since antic time to treat fever, swelling, kidney stones, dysuria, and to relieve fluid retention by the Chinese people (Al-Dosari, 2012).

Seeds from the Fabaceae family have been attracted more attention for potential health benefits because of their antioxidant activities and phenolic content. Polyphenols are bioactive components and are produced as secondary plant metabolites in plants that deliver positive effects for humans and animals. They exhibit various biological properties that prevent oxidative stresses and degenerative diseases.Legumes can be used to produce and develop new functional foods due to their bioactive phenolics.

There are some researches related to different kinds of secondary metabolites produced by alfalfa, such as phenolic compounds, which have antioxidant activity (Rafińska et al., 2017) and play a medicinally vital role. Therefore, alfalfa can be potentially preventer against some diseases, when it is consumed or added to the diet. These potential medicinal and pharmacological properties of alfalfa have been highlighted in several studies (Rafińska et al., 2017).

Studies performed on phenolic compounds in plants demonstrated that variety is one of the most important factors contribute to differences in the quantitation of phenolic content. The aim of this study was to comparatively analyze the total phenolic content and antioxidant activity of methanolic extract of alfalfa seeds from fifteen varieties commonly grown in Turkey. Our literature studies show that there is no study showing the total phenolic content and antioxidant activities in selected cultivars and different alfalfa seed methanol extracts.

2. Materials and Methods

2.1. Plant Materials

Alfalfa (*Medicago sativa* L.) seeds used in this research were obtained from the Field Crops Department, Agricultural Faculty of Namik Kemal University in Turkey in 2013. For comparison, phenolic content and antioxidant capacity of seeds, samples of fifteen varieties were selected which named Gea, Daisy, Derby, Kayseri, Prosementi, CA 35, Bilensoy, CA B6, MA 114, Verko, Bacon-Geo, 2A 83, May 22, Plato, Aktaş were used in experiments. Alfalfa seeds were from harvested plants and were kept in paper bags under dry conditions at +5 °C. Lyophilized extracts were used for further antioxidant activity analysis, and freeze dried samples were kept in freeze conditios (± 18 °C) for analyses. Preparation of extracts, total phenolic content, and antioxidant activities analyses were carried out at the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences in Poland between 2013–

2016 years. Laboratory experiments were done according to a randomized complete block design with three replications.

2.2. Preparation of Extracts

For obtaining lyophilized extract, firstly, alfalfa seeds were grounded by using a laboratory mill. The extraction from grounded seeds was carried out with 80% methanol (v/v) as solvent. Samples were shaken with solvent in a shaking bath (SW22, Julabo, Seelbach, Germany), and the sample to solvent ratio was 1:10 (v/w). Extractions were repeated three times at 70 °C for 15 min (Karamać et al., 2018). After filtrations, solvents were removed by evaporation (Rotavapor R-200 (Büchi Labortechnik, Flawil, Switzerland). Lyophilisation was done by using a freeze dryer (Lyph Lock 6 freeze-dry system, Labconco, Kansas City, MO, USA). The yield of extract (extractable components) expressed on a dry weight basis of seed was calculated from the following equation: Percentage extraction yield (g/100 g) = (dry extract weight/dry starting material weight) x 100, where dry extract weight is the weight of the extract residue obtained after solvent removal.

2.3. Total Phenolic Content (TP)

The total phenolic (TP) content of the methanolic extracts of alfalfa seed samples was analyzed using Folin-Ciocalteu's reagent. The absorbance values of samples were read at 725 nm (DU-7500 spectrophotometer, Beckman Instruments, Brea, CA, USA). Gallic acid was used as a standard phenolic compound (Karamać et al., 2020). The TP content of the extract was calculated as mg gallic acid equivalents (GAE) per g.

2.4. $ABTS^{\bullet+}$ scavenging activity

$ABTS^{\bullet+}$ scavenging activities of alfalfa seed extracts (2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical cation; $ABTS^{\bullet+}$ scavenging activity) were evaluated as Trolox equivalent antioxidant capacity (TEAC). TEAC of samples were calculated as mmol Trolox equivalents (TE) per g of extract. The absorbance measurements of the reaction mixture with $ABTS^{\bullet+}$ and seed extracts were done at 734 nm (Re et al., 1999).

2.5. Ferric-Reducing Antioxidant Power

Ferric-reducing antioxidant power (FRAP) of alfalfa extracts was assayed according to Benzie and Strain, (1996) method. Firstly, Fe^{3+}-TPTZ complex was produced at pH 3.6 with 300 mM acetate buffer, mixing by 10 mM TPTZ (in 40 mM HCl and 20 mM ferric chloride, 1:1 v/v) for reaction conditions. The reaction was conducted by mixing 75 µL extract solution, 225 µL distilled water, and 2.25 mL FRAP solution. The absorbance reading was done at 593 nm. The results were calculated as µmol Fe^{2+} equivalents per g of the extract by using Ferrous sulphate as standard.

2.6. The 2,2-Diphenyl-1-Picrylhydrazyl ($DPPH^{\bullet}$) Radical Scavenging Activity

$DPPH^{\bullet}$ scavenging activity of alfalfa seed extracts was investigated according to the method described by Brand-Williams et al., (1995) was used. The methanolic solution of extracts was prepared (in the range 2-10 mg/mL), and 1 mM $DPPH^{\bullet}$ solution was used for determination. The absorbance reading was observed at 517 nm. The radical scavenging activity of alfalfa seed extracts was given as EC_{50} values. EC_{50} values described as the mg/mL of extract needed to scavenge 50% of the $DPPH^{\bullet}$, were calculated from the graph slope of absorbance versus extract concentration.

2.7. Statistical analysis

Data were statistically analyzed to determine differences between alfalfa varieties. Three independent extractions were performed for alfalfa varieties. At least three replications were conducted. The variance analyses was performed by using the statistical package of the MSTAT-C software program (one-way ANOVA) after the least significant difference (LSD) test. Pearson's test was applied to the correlation analysis of data.

3. Results

3.1. Extraction Yield and Total Phenolic Content

The extraction yield from seeds of alfalfa varieties ranged from 8.13% (Verko) to 22.37% (Aktaş) (Table 1). The TP content of alfalfa seeds varied between 37.03 mg GAE/g (Plato) to 54.04 mg GAE/g (Prosementi) in methanol extracts (Table 1). According to results, the varieties Derby, Daisy, and Gea were found to have high total phenolic content, and there was a significant difference between seed TPC's, statistically. According to the results, the varieties with the highest content had approximately 1.43 times more total phenolic content than the lowest ones. Differences between alfalfa seeds seem significantly important (Table 1).

Table 1. Extraction yield, the total phenolic content (TPC), and antioxidant activities of seed extracts of alfalfa varieties

Variety	Extraction yield (%)	TPC (mg GAE/g)	TEAC (mmol TE/g)	FRAP (µmol Fe^{2+}/g)	DPPH assay (EC_{50}) (mg/mL)
Gea	13.22	51.15±1.75^{a-d}	0.113±0.01^{gh}	683.01±7.74^{d}	0.71±0.03^{cd}
Daisy	12.51	52.81±1.93^{ab}	0.114±0.01^{fgh}	791.02±5.88^{a}	0.71±0.02^{cd}
Derby	15.01	52.24±1.04^{abc}	0.129±0.00^{b-g}	647.26±3.49^{e}	0.71±0.02^{cd}
Kayseri	12.37	47.41±0.13^{cde}	0.123±0.005^{c-g}	676.87±0.46^{d}	0.64±0.01^{e}
Prosomenti	15.32	54.04±0.54^{a}	0.143±0.005^{ab}	772.84±2.21^{b}	0.64±0.02^{e}
CA 35	10.02	49.08±0.25^{a-e}	0.100±0.00^{h}	541.71±1.32^{h}	0.71±0.04^{cd}
Bilensoy	11.30	45.50±1.07^{efg}	0.132±0.01^{b-f}	627.11±5.38^{f}	0.76±0.03^{bc}
CA B6	13.97	39.39±0.78^{h}	0.116±0.01^{e-h}	605.25±7.38g	0.81±0.04^{b}
MA114	15.69	48.19±1.69^{b-e}	0.133±0.01^{b-e}	680.30±7.19^{d}	0.67±0.02^{de}
Verko	8.13	46.26±0.55^{def}	0.119±0.01^{d-g}	590.47±5.42^{g}	0.81±0.03^{b}
Bacon-Geo	14.03	50.44±2.55^{a-e}	0.131±0.00^{b-g}	597.48±3.49^{g}	0.72±0.03c^{d}
2A 83	13.35	38.07±1.54^{h}	0.138±0.00^{bc}	537.40±2.85^{h}	0.71±0.04^{cd}
May22	10.87	41.44±2.44^{fgh}	0.158±0.00^{a}	722.69±6.19^{g}	0.65±0.02^{e}
Plato	12.21	37.03±2.67^{h}	0.135±0.01^{bcd}	603.19±5.62^{g}	0.72±0.02^{cd}
Aktaş	22.37	40.57±2.09^{gh}	0.121±0.01^{c-g}	389.90±3.63^{ı}	1.06±0.04$_{a}$
LSD	4.219**	5.288**	0.018**	15.267**	0.050**

Data are expressed as the mean ± standard deviation (n = 3).
Values in the same column marked different letters differ significantly ($p < 0.05$), **$p < 0.01$.

3.2. The Antioxidant Activity of Alfalfa Seeds

The antioxidant potential of alfalfa seed extracts was determined with three different assays. The $ABTS^{\bullet+}$ scavenging activity of alfalfa seed extracts was reported in terms of TEAC, as shown in Table 1. The results of the TEAC reveal that the highest antioxidant activity was determined for May 22 variety (0.158 mmol Trolox/g), and CA 35 had the lowest antiradical activity against $ABTS^{\bullet+}$ (0.100 mmol Trolox/g). According to statistical evaluations, there were significant differences between varieties ($p < 0.01$) (Table 1).

FRAP of seed methanol extracts ranged from 389.90 µmol Fe^{2+}/g (Aktaş) to 791.02 µmol Fe^{2+}/g (Daisy). Prosementi also had high FRAP with high TPC in samples. Differences between seed alfalfa varieties were found to be significant ($p < 0.01$) statistically (Table 1).

The results of the radical scavenging activity determined by the DPPH assay showed that a variety of Kayseri and Prosementi had the highest activity among varieties with an EC_{50} value of 0.64 mg/mL, and radical scavenging activity of variety May 22 did not differ significantly both of them. Otherwise, variety Aktaş had the lowest $DPPH^{\bullet}$ radical scavenging activity with EC_{50} of 1.06 mg/mL. Verko and CAB6 were found to have significantly lower activity than the other varieties (Table 1).

According to Pearson's correlation analyses, TPC of seed extracts of alfalfa varieties strongly correlated with FRAP and $DPPH^{+}$ scavenging activity. The correlation between TPC and FRAP assay ($r = 0.540$, $p < 0.01$) was found higher than that of between TPC and other antioxidant activity assays (Table 2). The highest correlation was found between FRAP and the results of the DPPH assay ($r = -0.738$, $p < 0.01$) (Table 2).

4. Discussion and Conclusion

The present study revealed that the diversity of total phenolic content in the alfalfa seed extracts from fifteen varieties in comparison with their antioxidant activities. When the compared our results with recent research, the extraction yield with methanol from alfalfa seeds was found higher than the extraction yield from vetch seed which ranged from 6.15 to 9.70% (Orak, 2019) and mung bean seed which was determined as 10.7% (Orak et al., 2018).

In recent years, legumes have attracted attention, especially in terms of their antioxidant effects and total phenolic content. When compared alfalfa's TPC (ranged from 37.03 to 54.04 mg/GAE g) with widely consumed legume seeds**,** varieties exhibited similar content to mung bean seed (47.16 mg GA eq/g extract; Orak et al., 2018). Asadi-Samani et al., (2018) reported that the TPC of *Medicago sativa* L. from Iran was 49.796 mg GAE/g dry weight of extract, similar to our results. In comparison, the phenolic content of alfalfa with the TPC of fifteen vetch genotypes determined by the same method, TPC of alfalfa was higher than those vetch genotypes which was ranged between 15.46 mg GAE/g and 31.63 mg GAE/g methanol extracts. In turn, when comparing FRAP of samples, some varieties of this legume exhibited higher activity than fifteen vetch genotypes that showed the highest activity as 0.52 mmol Fe^{2+}/g extract for a Hungarian vetch genotype (Orak, 2019). Also, differences between varieties were found to be significant.

Pearson's correlation analyses demonstrated that TPC of alfalfa varieties strongly correlated with FRAP and $DPPH^{\bullet}$ scavenging activity. Similar correlations were determined for vetch species by Orak (2019).

Table 2. Correlation coefficients values (r^2)

	TPC	**TEAC**	**FRAP**	**DPPH assay (EC_{50})**
TPC	1	-0.197ns	0.540**	-0.316*
TEAC		1	0.241ns	-0.234ns
FRAP			1	-0.738**
DPPH assay (EC_{50})				1

ns: not significant, *$p < 0.05$, **$p < 0.01$.

As a result, this study demonstrated that differences between alfalfa varieties were significant ($p < 0.01$) in terms of their total phenolic content and antioxidant activity, statistically. Therefore, the phenolic content and antioxidant activity can also be a tool to use in determining which variety will come forward to use as human food, nutritional supplement, or rich antioxidant source in meals. These results can be evaluated for animal welfare and improving the health status of the animals by feeding with high phenolic content forages. However, in order to identify, isolate, and characterize these bioactive constituents from extracts and to illustrate their structure are needed to research in further studies.

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