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Antioxidant Activity, Isolation and Identification of Some Chemical Constituents of *Sphaerophysa kotschyana*

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ABSTRACT
Sphaerophysa kotschyana, an endemic plant belonging to Fabaceae, is distributed around the Tuz Lake in the Konya region. In this study, methanolic extract of *S. kotschyana* was evaluated for antioxidant capacity. According to obtained data; free radical scavenging capacity (DPPH•) and reducing power activity were observed to be lower than standard antioxidants, cation radical scavenging activity (ABTS•+) was similar to the standard antioxidants. Total phenolic compound was found in leaf-stem and fruit 0.357 mg GAE/kg DW and 0.006 mg GAE/kg DW, respectively. D-pinitol and sucrose were isolated and identified by chromatographic and spectroscopic techniques.

Research Article

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Sphaerophysa kotschyana Bitkisinin Antioksidan Aktivitesi ve Bazı Kimyasal Bileşenlerinin Izolasyonu ve Belirlenmesi

ÖZET
Fabaceae'ye ait endemik bir bitki olan *Sphaerophysa kotschyana*, Konya bölgesindeki Tuz Gölü çevresinde dağılım gösterir. Bu çalışmada, *S. kotschyana* bitkisinden elde edilen metanol ekstrelerden antioksidan kapasitesi değerlendirildi. Elde edilen antioksidan aktivite sonuçlarına göre; serbest radikal (DPPH•) giderme aktivitesi ve indirgeme gücü aktivitesi standart antioksidanlara göre düşük olduğu gözlemlenirken, katyon radikal (ABTS•+) giderme aktivitesi standart antioksidanlara benzer bir aktivite göstermektedir. Toplam fenolik bileşiklerin tayininde sırasıyla yaprak-sapı ve meyvede, 0,357 mg GAE / kg kuru bitki ve 0,006 mg GAE / kg kuru bitki olarak bulunmuştur. D-pinitol ve sukroz kromatografik ve spektroskopik teknikler ile izole edildi ve yapısı aydınlatıldı.

Araştırma Makalesi

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INTRODUCTION

The *Sphaerophysa* genus belonging to the Fabaceae is represented by two species in the world. These are *Sphaerophysa kotschyana* Boiss. and *S. salsula* DC.(Polhill, 1981) *Sphaerophysa salsula* is widely distributed in the Middle-Asian and northwest of China. In traditional Chinese medicine, *S. salsula* is used as a phytotherapic agent for the cure of certain diseases such as nephritis, hypertension, chronic hepatitis and angioneurotic oedema. The plant is resistant to normal drought and plays an important role in improving inefficient soil environments (Lin et al., 2009; Ma et al., 2004). Phytochemical investigation on *S. salsula* resulted in the isolation of flavonoids (Ma et al., 2004), isoflavans (Jiao et al., 2018), sterols (Li et al., 2005), lignan (Ma et al., 2003) and stilbenes (Ma et al., 2002). *Sphaerophysa kotschyana* included in the list of preservation plant which is halophyte plant is endemic for the Central Anatolian province (Hamzao and Aksoy, 2009; Trouwborst, 2014; Yildiztugay et al., 2013). It can resist salt-related damages at low salt concentrations (Yildiztugay et al., 2013). This property may come from its secondary metabolites contents.

Oxygen that produces reactive oxygen species is a reactive substance with a capacity to damage living tissue. This is a contrast to metabolism (Davies, 2000; Halliwell and Gutteridge, 1984). Oxidation, takes place by electron transfer, compose an important part of metabolic activities. ATP production, the last electron acceptor in the electron transport chain (ETC) is oxygen (Gülçin et al., 2003; Halliwell and Cross, 1994). During electron flow, unpaired electrons are

formed. Unpaired electrons in free radicals want to go into a stable state and take electrons from a stable compound and convert it into a new free radical (Davies, 2000; Gülçin, 2012). This sequence of chain reactions initiated by free radicals continues until stopped by antioxidants. If the antioxidants remain insufficient, then these electrons form free radicals and cause various damages to the organism (Gulcin et al., 2008; Halliwell and Cross, 1994). The increase of free radicals can cause various diseases because it can damage the function of the cell (Attaur Rahman and Choudhary, 2001; Davies, 1995).

All aerobic organisms and human beings have defence mechanisms to preserve against these oxidant damages. These defence mechanisms are called antioxidant defence mechanisms (Davies, 2000; Vermerris and Nicholson, 2006). The antioxidant defence mechanism can act in many different stages of an oxidative sequence. Antioxidants carry out defence mechanisms such as preventing radical formation, capturing radicals, increasing elimination of damaged molecules, and destroying excess damaged DNA cells to prevent the growth of transformed cells (Halliwell and Gutteridge, 2007).

According to our literature research, we did not find any studies on isolation and antioxidant capacity of *S. kotschyana*. Therefore, we aim to work determination of antioxidant capacity of methanolic extract of the plant. The second aim of the study is isolation and identification of some chemical compounds. As a result of this study, new information about phytochemical properties of this plant will be given to the literature.

MATERIALS and METHODS

General experimental procedures

NMR spectrum analyses were performed by Bruker-400 MHz spectrometer (400 MHz for proton and 100 MHz for carbon analysis). UV measurements were executed on a Hitachi U-2900 UV-VIS spectrometer. Column chromatography was performed on Silica Gel (60-230 mesh, E. Merck, Germany). TLC was carried out on aluminium plates coated with Silica Gel (0.25 mm, E. Merck, Germany). Material detection was carried out by spraying seric sulfate solution over TLC and then heating.

BHT, BHA, Trolox, trichloroacetic acid (TCA), ABTS, Folin-Ciocalteu reagent, gallic acid, DPPH·, Potasium ferricyanide ($K_3Fe(CN)_6$), potassium persulfate ($K_2S_2O_8$) were used for antioxidant assays and All solvents and chemical compounds supplied from chemical company E. Merck (Germany).

Plant Material

The plant materials were collected from Konya, the central Anatolia region of Turkey during the fruit period in July and identified by Prof. Dr. Murad Aydın Şanda and sent to our laboratory for leaf-stem and fruit chemical analysis of the *S. kotschyana* plant which was dried in a way that would not see the sun. (Herbarium Number: Şanda 2350).

Extraction and isolation

Extraction for total phenolic compound analysis and antioxidant activity assay

The leaf-stem and fruits of arial plant were dried and powdered. 0.2 g the leaf-stem and 0.2 g fruits weighed into capped glass tubes then extracted with 10 mL methanol/dichloromethane mixture (4: 1). It was vortexed and left in the ultrasonic bath for 15 minutes and then incubated for 1 day.

Extraction for isolation

Dried and powdered leaf-stem extracted with methanol. Filtered extract was evaporated in rotary evaporator and 8 g extract were obtained from leaf-stem.

Isolation

Isolation was accomplished by adding methanol extract (8 g) to the silica gel column (5x120 cm, DxL). Fractionation was performed with increasing polarity from hexane to EtOAc and from EtOAc to MeOH. In the isolation study, a total of 373 fractions were collected by taking 100 ml into each tube. All eluates were subjected to thin layer chromatography and then the compounds having the same R_f value were combined. Pinitol (**1**) and Sucrose (**2**) were isolated from the fractions of 209 and 288, respectively.

Evaluation of Antioxidant Activities

Ferric Ions (Fe^{3+}) Reducing Antioxidant Power Assay (FRAP)

The reducing powers of *S. kotschyana* leaf-stem and fruits extracts were determined by modified Oyaizu method (Elmastaş et al., 2006; Oyaizu, 1986). To the *S. kotschyana* extract solutions (40, 80, 120 µg/mL) in 1 mL of methanol was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixtures were incubated at 50°C for 20 min. Trichloroacetic acid (2.5 mL, 10%) was added to each mixture and they were centrifuged (at 3,000 rpm for 10 min). The upper layers of solutions (2.5 mL) were mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and absorbances were measured at 700 nm. Increased absorbance of the reaction mixture indicates an increase of reduction capability.

DPPH• Free Radical Scavenging Activity

DPPH· free radical scavenging activity of *S. kotschyana* leaf-stem and fruits extracts and

standards were measured by the method of Blois (Blois, 1958). 0.1 mM solution of DPPH˙ in ethanol was prepared and 1mL of this solution was added to 3mL of the samples solution in methanol at different concentrations (60, 80, 160 µg/mL). These solutions were vortexed thoroughly and kept in the darkness for 30 min. The absorbance was measured at 517 nm by a spectrophotometer and the lower absorbance of the reaction mixture revealed the higher free radical scavenging activity. The capability to scavenge DPPH˙ radical was calculated using the following equation:

DPPH˙ scavenging effect (%) = (Absorbance of control - Absorbance of sample) / Absorbance of control x 100

ABTS+ Radical Cation Scavenging Assay

ABTS˙+scavenging activity assay was carried out according to Re method (Re et al., 1999). The process of ABTS˙+ (2.0 mM) in water with potassium persulfate ($K_2S_2O_8$) (2.45 mM) at room temperature in dark for 4 h gave the ABTS cation radical. Dilution of ABTS˙+ was applied with phosphate buffer (0.1 mol/L, pH 7.4) to measure the absorbance at 734 nm. The reactions of ABTS˙+solution (1.0 mL) with samples solution in ethanol (3.0 mL) at different concentrations (2.5, 5.0, 10.0 µg/mL) were performed. The inhibition was calculated at 734 nm for each concentration. The scavenging ability of ABTS˙+ was calculated as the following equation:

ABTS˙+ scavenging activity (%) = [(Ac – As) /Ac] x 100 where Ac is the initial concentration of ABTS˙+and as is the absorbance of the remaining concentration of ABTS˙+in the samples.

Determination of Total Phenolic Compounds

Folin-Ciocalteu reagent and gallic acid as a standard were used to determine the total phenolic constituent of *S. kotschyana* leaf-stem and fruits extracts (Slinkard and Singleton, 1977). 10-fold diluted Folin-Ciocalteu reagent (2.5 mL) and Na_2CO_3 (2 mL, 75 g/L) were added to the plant extract (0.5 mL, 1 mg/mL). After this mixture was vortexed and incubated for 2 hours. The absorbance measurement was performed at 760 nm by a spectrophotometer. Calibration graph was performed according using gallic acid. Accordingly, the results were calculated as milligram gallic acid equivalent (GAE) per kg dry plant.

Statistical analysis

The results of the study were performed by taking the average ± SD of at least three independent measurements.

RESULTS and DISCUSSION

Natural products

In this study, D-pinitol (**1**) and sucrose (**2**) are isolated from methanol extract by silica gel column chromatography (Figure 1). Their molecular formula was determined as $C_7H_{14}O_6$ (**1**) and $C_{12}H_{22}O_{11}$ (**2**) by ^{1}H-NMR, HETCOR, COSY, HMBC,^{13}C-NMR, APT, DEPT-90 and DEPT-135 spectrums.

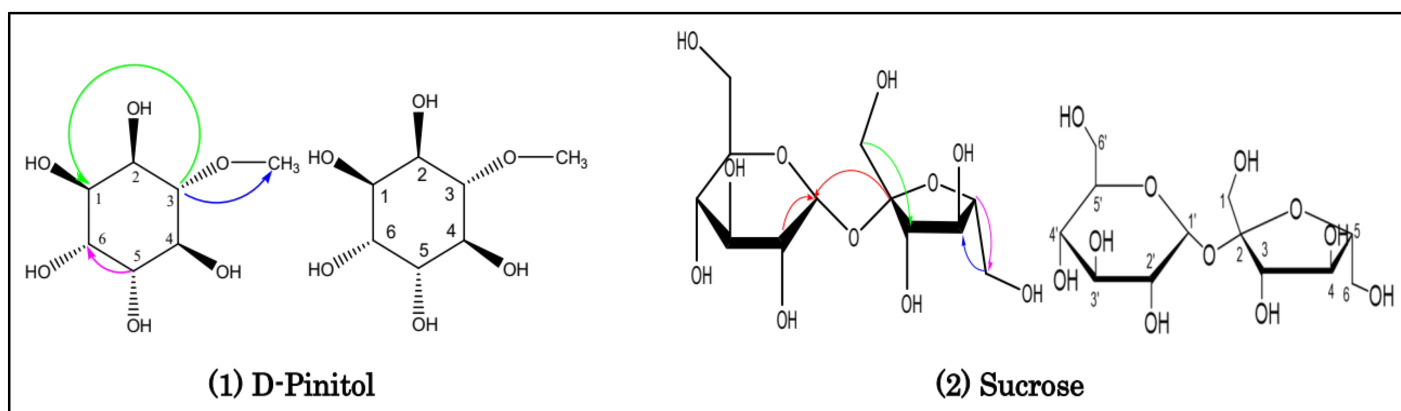

Figure 1. HMBC correlation and structures of compounds isolated from *S. kotschyana*
Şekil 1. S. kotschyana bitkisinden izole edilen bileşiklerin HMBC korelasyonu ve yapısı

D-Pinitol (3-O-Methyl-D-chiro-inositol) compound was shown to have seven carbon atoms in the ^{13}C-NMR, APT, DEPT-90 and DEPT-135 spectra (Figure 2.A). The DEPT-90 spectrum showed six methines carbons. The carbon at 60.06 ppm belongs to the methyl carbon in the methoxy group from the DEPT-135 and APT spectra. From the ^{1}H-NMR spectrum of the D-pinitol compound, the –OH protons range from 4.3 to 4.8; The H-3 proton was triplet at 3.01 (J = 9.04 Hz) and the H-4 proton at 3.50 ppm as a multiplet resonance. While H-5 and H-6 protons signal at 3.63 and 3.62 ppm, –OCH_3 protons showed singlet at 3.43 ppm (Figure.2.B). D-Pinitol In the HMBC spectrum, –OCH_3 carbon (60.07 ppm) with H-3 proton (3.01 ppm), C-6 carbon (72.85 ppm) with H-5 proton (3.63 ppm) and C-3 carbon (84.21 ppm) with H-1 proton (3.44 ppm) was seen to interact with long distance (Table 1). NMR values of D-Pinitol compound are consistent with those given in the literature (Blanco *et* al., 2008; Misra and Siddiqi, 2004; Sharma et al., 2016).

Table 1. ^{1}H-NMR, ^{13}C-NMR chemical shift values and HMBC correlations of D-Pinitol and Sucrose (400 MHz, 100 MHz, DMSO-d_6)
Çizelge 1. D-Pinitol ve Sukroz bileşiğinin ^{1}H-NMR ve ^{13}C-NMR kimyasal kayma değerleri ve HMBC korelasyonları (400 MHz DMSO-d_6)

D-Pinitol				Sucrose		
C/H	δ_C (ppm)	δ_H (ppm)	HMBC	δ_C ppm	δ_H ppm (Hz)	HMBC
1	71.38	3.44, m		62.58	3.55	H_3
2	73.04	3.35		104.18		$H_{1'}$
3	84.21	3.01, (t, J=9.04)	H_1	77.50	3.87 (t, J=8.00)	
4	70.53	3.50, m		74.45	3.76	ND
5	72.40	3.63, m		83.00	3.56	H_6
6	72.85	3.62, m	H_5	62.52	3.40	H_4
1′				92.23	5.17	
2′				72.09	3.16	$H_{1'}$
3′				73.27	3.48	$H_{5'}$
4′				70.31	3.16	
5′				73.27	3.65	$H_{3'}$
6′				60.95	3.59	
OCH_3	60.07	3.43, s	H_3			

Sucrose was isolated in crystalline form. The ^{13}C spectrum revealed the presence of twelve carbons compatible with the structure (Figure 2.C). The peaks of 4.30-5.30 ppm in the ^{1}H-NMR spectrum of sucrose compound belong to –OH protons (Figure 2.D). The triplet at δ3.87 belongs to proton H-3 (J = 8.00 Hz) (Table 1). In the HMBC spectrum, protons H-1 (5.17 ppm) with C-2 (104.18 ppm) and C-2 (72.09 ppm), H-6 (3.40 ppm) proton with C-5 (83.00 ppm) carbon, C-3′ and C-5′ carbons (73.27 ppm) with H-5 ile and H-3′ protons (3.16 ppm) was showed to interact with long distance (Table 1). The signal values of the sucrose compound are proper with the literature data (El-Domiaty et al., 2009; Popov et al., 2006).

Antioxidant activities

Antioxidant properties of functional compounds in foods are important for medical bioactivity. In this work, the antioxidant activity of methanol extract of *S. kotschyana* leaf-stem and fruits were compared to standard antioxidants (BHA and trolox). The activity of the *S. kotschyana* of the leaf-stem and fruit section was high in the determination of $ABTS^{\cdot+}$ cation free radical scavenging activity (Figure 3).

As shown in figure 3 and figure 4, *S. kotschyana* is low in DPPH, FRAP and total phenolic compounds. Phenols and their derivatives have antioxidant activity because they have hydroxyl groups. 0,357 and 0,006 mg gallic acid equivalent of phenols were detected in leaf-stem and fruit respectively. The presence of low amounts of total phenolic compounds content in this work may be the cause of these low antioxidant activities. However, the $ABTS^{\cdot+}$ cation radical scavenging activity of *S. kotschyana* showed the same activity as standard antioxidants (Figure 5). This may be due to chemical contents in the plant which react with $ABTS^{\cdot+}$ cation radical.

Sucrose and D-Pinitol isolated from the *S. kotschyana* firstly and obtained results from the study was presented new data into literature. Because sucrose and D-pinitol isolated and elucidated their structures from *S. kotschyana* first time. DPPH, superoxide scavenging activity, nitric oxide scavenging activities of D-pinitol were reported previously. It was reported that its activity was close to standard (Rengarajan et al., 2014). At the same time D-pinitol has been found to have an anti-inflammatory effect (Singh et al., 2001).

CONCLUSION

When the results are evaluated, it is seen that there is a paradox in terms of antioxidants. Because $ABTS^{\cdot+}$ is higher than other antioxidant tests. However, $ABTS^{\cdot+}$ can be used as a free radical cation clearing agent. In the future studies on *S. kotschyana* can be performed for other chemical content isolation and elucidation.

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Statement of Conflict of Interest

Authors have declared no conflict of interest.

Author's Contributions

The contribution of the authors is equal.

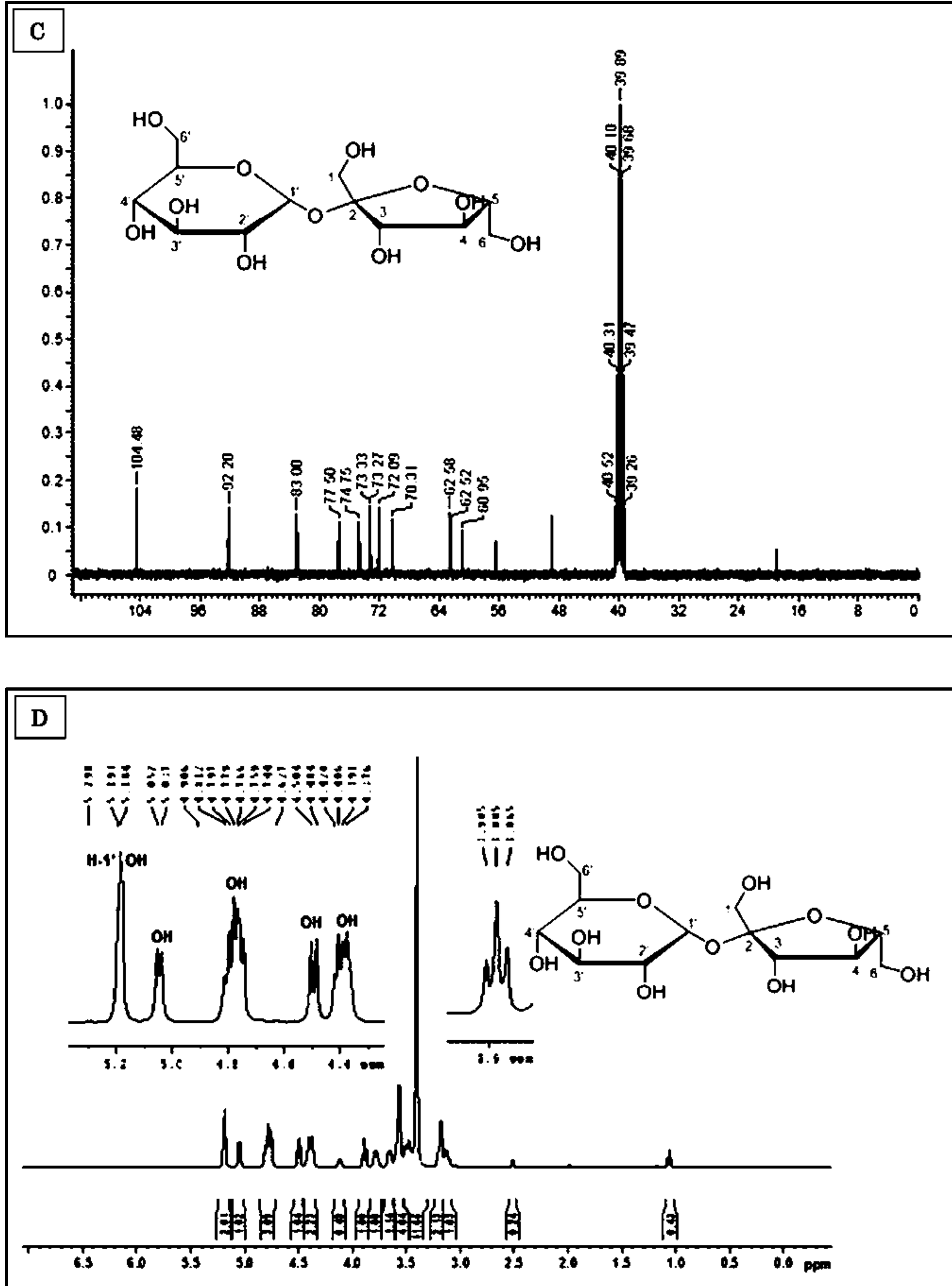

Figure 2. (A) ^{13}C-NMR spectrum of the compound D-Pinitol (100 MHz, DMSO-d_6). (B) 400 MHz ^{1}H-NMR of D-pinitol in D_2O. (C) ^{13}C-NMR spectrum of the compound Sucrose (100 MHz, DMSO-d_6). (D) 400 MHz ^{1}H-NMR of Sucrose in D_2O

Şekil *2.(A) D-Pinitol bileşiğinin ^{13}C-NMR spektrumu (100 MHz, DMSO-d_6). (B) D-Pinitol bileşiğinin D_2O'lu 400 MHz ^{1}H-NMR spektrumu. (C) Sukroz bileşiğinin ^{13}C-NMR spektrumu (100 MHz, DMSO-d_6). (D) Sukroz bileşiğinin D_2O'lu 400 MHz ^{1}H-NMR spektrumu.*

DPPH

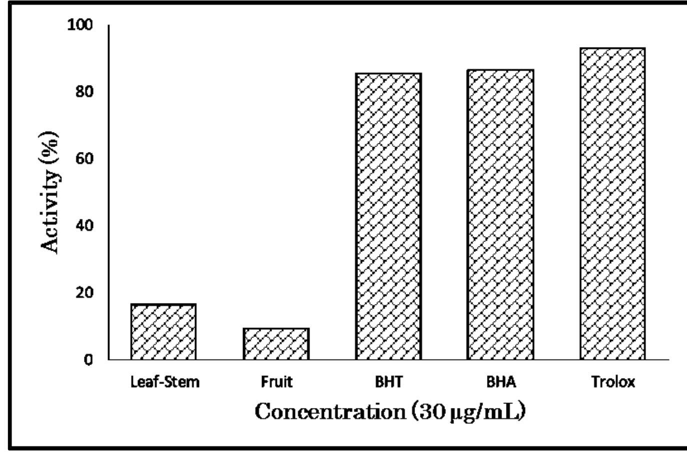

Figure 3. DPPH free radical scavenging activity of extracts and standard antioxidants

Şekil 3. Ekstraktların ve standart antioksidanların DPPH serbest radikal giderme aktivitesi

FRAP

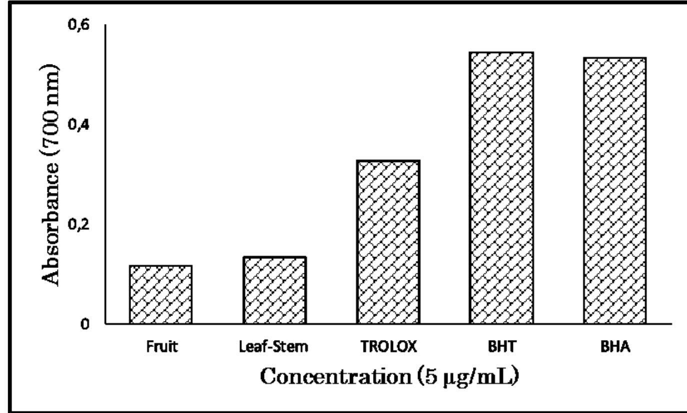

Figure 4. FRAP reducing power activity of extracts and standard antioxidants

Şekil 4. Ekstraktların ve standart antioksidanların FRAP indirme gücü aktivitesi

ABTS

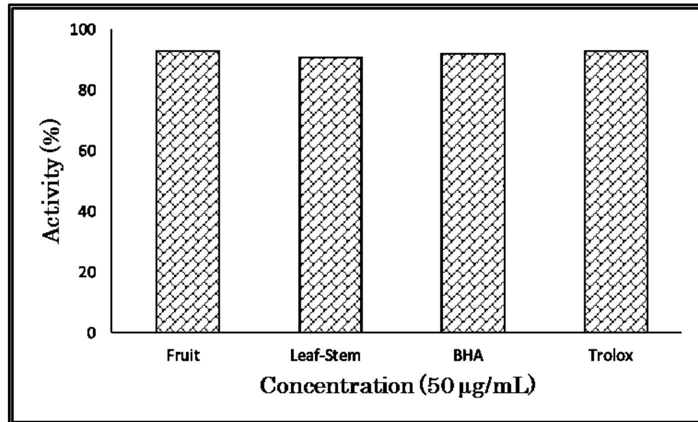

Figure 5. ABTS cation radical scavenging activity of extracts and standard antioxidants

Şekil 5. Ekstraktların ve standart antioksidanların ABTS katyon serbest radikal giderme aktivitesi

REFERENCES

Attaur Rahman, Choudhary MI 2001. Bioactive natural products as a potential source of new pharmacophores. A theory of memory. Section Title: General Biochemistry 73 (3): 555–560.

Blanco N, Flores Y, Almanza GR 2008. Secondary Metabolites From Senna Versicolor. Revista Boliviana de Química 25 (1): 36–42.

Blois MS 1958. Antioxidant determinations by the use of a stable free radical. Nature 181: 1199–1200.

Davies KJ 1995. Oxidative stress: the paradox of aerobic life. Biochemical Society symposium 61: 1–31.

Davies KJ 2000. Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. IUBMB Life 50: 279–289.

El-Domiaty MM, Aal MMA, Abou-Hashem MM, Abd-Alla RH, Wink M 2009. Antihepatotoxic Activity and Chemical Constituents of Buddleja asiatica Lour. Zeitschrift fur Naturforschung - Section C Journal of Biosciences 64 (1–2): 11–19.

Elmastaş M, Gülçin I, Beydemir Ş, Küfreviolu ÖI, Aboul-Enein HY 2006. A study on the in vitro antioxidant activity of juniper (Juniperus communis L.) fruit extracts. Analytical Letters 39: 47–65.

Gülçin I 2012. Antioxidant activity of food constituents: An overview. Archives of Toxicology 86 (3): 345–391.

Gülçin I, Oktay M, Kireçci E, Küfrevioğlu ÖI 2003. Screening of antioxidant and antimicrobial activities of anise (Pimpinella anisum L.) seed extracts. Food Chemistry 83 (1): 371–382.

Gulcin I, Tel AZ, Kirecci E 2008. Antioxidant, antimicrobial, antifungal, and antiradical activities of Cyclotrichium Niveum (BOISS.) Manden and Scheng. International Journal of Food Properties 11 (2): 450–471.

Halliwell B, Cross CE 1994. Oxygen-derived species: Their relation to human disease and environmental stress. Environmental Health Perspectives 102 (10): 5–12.

Halliwell B, Gutteridge JMC 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochemical Journal 219: 1–14.

Halliwell B, Gutteridge JMC 2007. Free Radicals in Biology and Medicine. Oxford University Press, New York 905–2015 .

Hamzaoğlu E, Aksoy A 2009. Phytosociological studies on the halophytic communities of central Anatolia. Ekoloji 14: 1–14.

Jiao LJ, Wang WD, Tao YD, Shao Y, Mei LJ, Liu ZG, Wang QL, Dang J 2018. 8-isopentenyl isoflavone derivatives from the whole herb of Sphaerophysa salsula. Natural Product Research 32 (21): 2542–2546.

Li GY, Wang JH, Li X 2005. Sterols from the pericarp of Sphaerophysa salsula DC. Journal of Asian Natural Products Research 7 (2): 165–169.

Lin X, Jiajie X, Qiaoli L, Ruimei X, Gehong W 2009. Genetic diversity in rhizobia isolated from Sphaerophysa salsula in several regions of northwestern China. Biodiversity Science 17 (1): 69–75.

Ma Z, Fujii I, Ebizuka Y, Li X, Shimomaki S, Sakano Y 2004. Flavonoids from the seeds of Sphaerophysa salsula. Journal of Asian Natural Products Research 6 (1): 69–73.

Ma Z, Li X, Li N, Wang J 2002. Stilbenes from Sphaerophysa salsula. Fitoterapia 73 (4): 313–315.

Ma Z, Tian J, Fujii I, Ebizuka Y, Li X 2003. A new lignan from the seeds of Sphaerophysa salsula. Natural Product Research 17 (6): 423–425.

Misra LN, Siddiqi SA 2004. Dhaincha (Sesbania bispinosa) leaves: A good source of antidiabetic (+)-pinitol. Current Science 87 (11): 1507.

Oyaizu M 1986. Studies on products of browning reaction. Antioxidative activities of products of browning reaction prepared from glucosamine. The Japanese Journal of Nutrition and Dietetics 44 (6): 307–315.

Polhill RM 1981. Advances in legume systematics: parts 1 & 2. Kew, Royal Botanic Gardens 2: 1–7.

Popov KI, Sultanova N, Rönkkömäki H, Hannu-Kuure M, Jalonen J, Lajunen LHJ, Bugaenko IF, Tuzhilkin VI 2006. 13C NMR and electrospray ionization mass spectrometric study of sucrose aqueous solutions at high pH: NMR measurement of sucrose dissociation constant. Food Chemistry 96 (2): 248–253.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine 26 (9): 1231–1237.

Rengarajan T, Rajendran P, Nandakumar N, Balasubramanian MP, Nishigaki I 2014. Free radical scavenging and antioxidant activity of D-pinitol against 7, 12 dimethylbenz (a) anthracene induced breast cancer in sprague dawley rats. Asian Pacific Journal of Tropical Disease 4 (5): 384–390.

Sharma N, Verma MK, Gupta DK, Satti NK, Khajuria RK 2016. Isolation and quantification of pinitol in Argyrolobium roseum plant, by 1H-NMR. Journal of Saudi Chemical Society 20 (1): 81–87.

Singh RK, Pandey BL, Tripathi M, Pandey VB 2001. Anti-inflammatory effect of (+)-pinitol. Fitoterapia 72 (2): 168–170.

Slinkard K, Singleton V 1977. Total phenol analysis: automation and comparison with manual methods. The American Journal of Enology and Viticulture 28: 49–55.

Trouwborst A 2014. Exploring the legal status of Wolf-dog hybrids and other dubious animals: International and EU law and the wildlife conservation problem of hybridization with domestic and alien species. Review of European, Comparative and International Environmental Law 23 (1): 111–124.

Venkateswarlu S, Satyanarayana B, Sureshbabu C V, Subbaraju G V 2003. Synthesis and antioxidant activity of 4-[2-(3,5-dimethoxyphenyl)ethenyl]-1, 2-benzenediol, a metabolite of Sphaerophysa salsula. Bioscience, Biotechnology and Biochemistry 67 (11): 2463–2466.

Vermerris W, Nicholson R 2006. Phenolic compounds and their effects on human health. In Phenolic Compound Biochemistry. Springer, Dordrecht 235–255.

Yildiztugay E, Ozfidan-Konakci C, Kucukoduk M 2013. Sphaerophysa kotschyana, an endemic species from Central Anatolia: Antioxidant system responses under salt stress. Journal of Plant Research 126 (5): 729–742.