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.

*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 yağışlı gösterir. Bu çalışmada, *S. kotschyana* bitkisinden elde edilen metanollü ekstrakterden antioksidan kapasitesi değerlendirildi. Elde edilen antioksidan aktivite sonuçlarına göre; serbest radikal (DPPH•) giderme aktivitesi ve indirgeme gücü aktivitesi standart antioksidanlara göre düşük olduğu gözlenmiştir, katyon radikal (ABTS**) giderme aktivitesi standart antioksidanlara benzer bir aktivite göstermektedir. Toplam fenolik bileşiklerin tayininde 0,357 mg GAE/kg DW ve 0,006 mg GAE/kg DW’lik değerler elde edilmiştir. D-pinitol ve sukroz kromatografik ve spektroskopik teknikler ile izole edildi ve yapısı açıklanmıştır.

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 phytotherapeutic 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 (Gülçin 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. kotschyanana*. 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; Potassium ferricyanide (K₃Fe(CN)₆), potassium persulfate (K₂S₂O₈) 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 Aydin Şanda and sent to our laboratory for leaf-stem and fruit chemical analysis of the *S. kotschyanana* 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 aerial 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 (6x120 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 Rf value were combined. Pinitol (1) and Sucrose (2) were isolated from the fractions of 209 and 288, respectively.

**Evaluation of Antioxidant Activities**

**Ferric Ions (Fe³⁺) Reducing Antioxidant Power Assay (FRAP)**

The reducing powers of *S. kotschyanana* leaf-stem and fruits extracts were determined by modified Oyaizu method (Elmastaş et al., 2006; Oyaizu, 1986). To the *S. kotschyanana* 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 [K₃Fe(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 FeCl₃ (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. kotschyanana* leaf-stem and fruits extracts and
standards were measured by the method of Blois (Blois, 1958). 0.1 mM solution of DPPH 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 (K2S2O8) (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 Na2CO3 (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 C22H30O11 (1) and C12H22O11 (2) by 1H-NMR, HETCOR, COSY, HMBC, 13C-NMR, APT, DEPT-90 and DEPT-135 spectrums.

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D-Pinitol (3-O-Methyl-D-chiro-inositol) compound was shown to have seven carbon atoms in the 13C-NMR, APT, DEPT-90 and DEPT-135 spectra (Figure 2A). 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 1H-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 3.55 ppm as a multiplet resonance. While H-5 and H-6 protons signal at 3.63 and 3.62 ppm, –OCH3 protons showed singlet at 3.43 ppm (Figure 2B). D-Pinitol In the HMBC spectrum, –OCH3 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).

![Figure 1](image1.png)

Figure 1. HMBC correlation and structures of compounds isolated from S. kotschyana

**Şekil 1. S. kotschyana bitkisinden izole edilen bileşiklerin HMBC korelasyonu ve yapısı**

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This study showed that the antioxidant activity of methanol extract of S. kotschyana is high in the determination of ABTS 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 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 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 cation is higher than other antioxidant tests. However, ABTS cation 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.

ACKNOWLEDGEMENTS

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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.

Table 1. 1H-NMR, 13C-NMR chemical shift values and HMBC correlations of D-Pinitol and Sucrose (400 MHz, 100 MHz, DMSO-d6).

<table>
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<th>D-Pinitol</th>
<th>Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/H</td>
<td>δC (ppm)</td>
</tr>
<tr>
<td>1</td>
<td>71.38</td>
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<tr>
<td>2</td>
<td>73.04</td>
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<tr>
<td>3</td>
<td>84.21</td>
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<td>4’</td>
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</tr>
<tr>
<td>5’</td>
<td>73.27</td>
</tr>
<tr>
<td>6’</td>
<td>60.95</td>
</tr>
<tr>
<td>OCH3</td>
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</table>

Sucrose was isolated in crystalline form. The 13C spectrum revealed the presence of twelve carbons compatible with the structure (Figure 2.C). The peaks of 4.30-5.30 ppm in the 1H-NMR spectrum of sucrose compound belong to –OH protons (Figure 2.D). The triplet at 53.87 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 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 cation radical scavenging activity of S. kotschyana showed the same activity as standard antioxidants (Figure 5).
A

B

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Şekil 2. (A) D-Pinitol bileşiginin $^{13}$C-NMR spektrumu (100 MHz, DMSO-$d_6$). (B) D-Pinitol bileşiginin D₂O'lu 400 MHz $^1$H-NMR spektrumu. (C) Sukroz bileşiginin $^{13}$C-NMR spektrumu (100 MHz, DMSO-$d_6$). (D) Sukroz bileşinin D₂O'lu 400 MHz $^1$H-NMR spektrumu.
REFERENCES


