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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Antioxidant Activity and Total Phenolic and Flavonoid Contents of *Salvia verticillata* L., *Salvia tomentosa* Mill., and *Phlomis lychnitis* L.

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Abstract: The purpose of our study was to investigate the total phenolic, total flavonoid, and antioxidant properties of methanol and ethyl acetate extracts of *Salvia verticillata* L., *S. tomentosa* Mill., and *Phlomis lychnitis* L. The antioxidant potentials of the extracts were determined using (2,2-diphenyl-1-picrylhydrazyl) DPPH free-radical scavenging activity. The total phenolic content (TPC) and total flavonoid content (TFC) were evaluated using the spectrophotometric method. The methanol extract of *S. verticillata* was the highest in TPC and TFC. The same extract revealed the most significant antioxidant effect with SC_{50} value of 0.01±0.mg/mL. Strong positive correlations were also found between DPPH and TFC ($r = 0.904$; $p < 0.01$), and TPC ($r = 0.963$; $p < 0.01$). This relationship indicated that flavonoids, together with other phenolic compounds, contribute greatly to this bioactivity. Our results further suggest that the methanol extract of *S. verticillata* L. is a high-quality antioxidant for use in the medical and food industries.

Keywords: DPPH, total flavonoid, total phenolic, *Salvia tomentosa* Mill., *Salvia verticillata* L., *Phlomis lychnitis* L..

Salvia verticillata L., *Salvia tomentosa* Mill. ve *Phlomis lychnitis* L.'nin Toplam Fenolik ve Flavonoid İçerikleri ve Antioksidan Aktivitesi

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Öz: Çalışmamızın amacı, *Salvia verticillata* L., *S. tomentosa* Mill. ve *Phlomis lychnitis* L.'nin metanol ve etil asetat ekstraklarının toplam fenolik, toplam flavonoid ve antioksidan özelliklerini araştırmaktır. Ekstraktların antioksidan potansiyelleri DPPH (2,2-difenil-1-pikrilhidrazil) serbest radikal temizleme etkinliği kullanılarak belirlendi. Toplam fenolik içerik (TPC) ve toplam flavonoid içerik (TFC) spektrofotometrik yöntem kullanılarak değerlendirildi. *S. verticillata*'nın metanol ekstraktı, TPC ve TFC'de en yüksektir. Aynı ekstrakt, 0.01 ± 0.00 mg / mL SC_{50} değeri ile en iyi antioksidan aktivite ortaya koydu. Ayrıca DPPH ve TFC ($r = 0.904$; $p < 0.01$) ve TPC ($r = 0.963$; $p < 0.01$) arasında da güçlü pozitif korelasyon bulundu. Bu ilişki, flavonoidlerin diğer fenolik bileşiklerle birlikte bu biyoaktiviteye büyük katkıda bulunduğunu gösterdi. Sonuçlarımız ayrıca *S. verticillata* L.'nin metanol ekstraktının, ilaç ve gıda endüstrilerinde kullanım için yüksek kaliteli bir antioksidan olduğunu ileri sürmektedir.

Anahtar kelimeler: DPPH, toplam fenolik, toplam flavonoid, *S. tomentosa* Mill. *Salvia verticillata* L., *Phlomis lychnitis* L..

INTRODUCTION

Salvia verticillata L., *S. tomentosa* Mill., and *Phlomis lychnitis* L. belong to the Lamiaceae family, which is an important family of the flowering plants because of the valuable essential oils and secondary metabolites contained in its various species (Baser, 2002; Flamini et al., 2007). Comprising more than 900 species, *Salvia* is considered to be one of the largest genera in Lamiaceae (Walker et al., 2004).

Members of this genus are known to be used as medicinal plants to treat various conditions, such as cold, flu, tonsillitis, diarrhea, gonorrhea, hemorrhoids, and eye diseases in many parts of Turkey (Rizk et al., 1995; Polat & Satil, 2012). Studies on this genus have revealed that *Salvia* spp. have various properties, such as antimicrobial (Ozkan et al., 2003; Tepe et al., 2004; Cardile et al., 2009; Tenore et al., 2011), antibacterial (Haznedaroglu et al., 2001; Tepe et al., 2005), antioxidant (Kolak et al., 2009), insecticidal (Copping & Menn, 2000; Karakoc¸ et al., 2006; Selvi, et al., 2019), antitumor (Cardile et al., 2009), and antidiabetic (Cardile et al., 2009) activities.

Members of this genus have been the subject of several research studies for their bioactivities and bioactive compounds, such as phenols and their derivatives, terpenes, phenolic carbonyls, acids/esters, hydrocarbons, and volatile oils (Tenore et al., 2011). Rosmarinic acid was found as a main phenolic compound in *Stevia* species (Lu & Foo 2002; Askun et al., 2009; Dincer et al., 2012). Moreover, catechin, caffeic acid, vanillic acid, ferulic acid, rutin, apigenin, quercetin, and luteolin were also identified in different *Salvia* species (Lu & Foo, 2002; Papageorgiou et al., 2008; Askun et al., 2009). Chlorogenic acids, benzoic acids, and rosmarinic acids were found as the main phenolic compounds in *Phlomis* species (Zhang & Wang, 2009; Sarikurkcu et al., 2014; Sarikurkcu et al., 2015).

The purposes of this study were to 1) determine total phenolic content (TPC), and total flavonoid content (TFC) in ethyl acetate and methanol extracts of selected plants, 2) assess the antioxidant activity of the extracts as determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 3) explore the correlation among antioxidant activity and TPC and TFC.

MATERIALS AND METHODS

Chemicals and solvents: Phenolic standards (analytical grade) were obtained from Sigma–Aldrich Corporation (St. Louis, MO, USA). Other chemicals were supplied by Merck (Darmstadt, Germany).

Sample preparation: *Salvia verticillata* L. and S. *tomentosa* Mill. were collected from Antalya, Turkey, in April and May 2018, respectively. Dried aerial parts of *P. lychnitis* L. were purchased from a market in Konya, Turkey, in May 2018.

Solvent extractions: All plant samples were air dried together and the dried samples were powdered using a blender. The resulting powder was divided into two flasks at 10 g/flask. Each sample was defatted with 100 mL chloroform at 30ºC for 30 min. Two different solvents (methanol and ethyl acetate) from each residue were extracted using 2 x 40 mL solvent at 40°C for 1 h in an ultrasonic bath (Bandelin, Germany). Extraction was repeated twice for each adequate amount of solvent, and the resulting extracts were combined. The extracts were then filtered and concentrated by rotary evaporation at 40°C. The stock solution of each crude extract was resuspended in methanol and stored at below 4°C until analyses.

Determining total phenolic content: TPC contained in the extracts were analyzed using Folin-Ciocalteu's phenol reagent (Singleton, 1985). Gallic acid and quercetin were used to generate a standard curve ranging from 0.015 to 0.250 mg/mL ($r^2 = 0.999$). Briefly, 20 µL methanolic plant extract, 400 µL 0.5 N Folin-Ciocalteu reagent, and 680 µL distilled water were combined and vortexed. After incubating for 3 min, 400 µL 10% sodium carbonate was added and vortexed. After 2 h, the absorbance of the mixture was measured at 760 nm. The concentration of TPC was calculated as mg of gallic acid equivalent (GAE) and as mg of quercetin equivalent (QE), respectively, for phenolic and flavonoid compounds/g of dried extract. All measurements were conducted in triplicate.

Determining total flavonoid content: TFC was analyzed using the aluminum chloride ($AlCl_3$) method as described by Marcucci (1998). First, 0.1 mL 10% $AlCl_3$, 0.1 mL 1 M potassium acetate, and 4.3 mL 80% ethyl alcohol were added to 0.5 mL methanolic plant extract. The samples were incubated for 40 min at room temperature, and the absorbance of the mixture was measured at 415 nm. Quercetin was used for the standard calibration curve. The concentration of TFC was calculated as QE/g dried weight (dw). All measurements were conducted in triplicate.

Assay for scavenging free radicals: Radical scavenging activity of the extracts against DPPH was spectrophotometrically studied at 517 nm (Molyneux, 2004). Briefly, different concentrations of 0.75 mL methanolic plant extracts were mixed with 0.75 mL 0.1 mM DPPH in methanol, well vortexed, and incubated for 50 min at room temperature. Gallic acid and quercetin were used as representative phenolic and flavonoid standards. The results are expressed as SC_{50} (mg/ mL), which indicates the concentration of extracts required to inhibit 50% of the radicals.

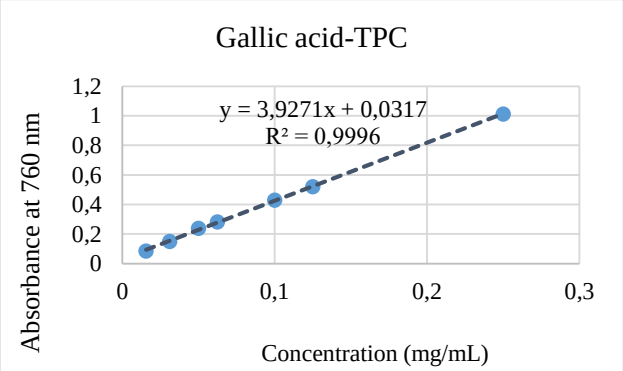
Statistical analyses: The results are presented as the mean values and standard deviations of three replicates. SC_{50} values were determined using a linear regression analysis (Microsoft Excel for Windows, v. 2010). Data were analyzed using SPSS v. 16.0 (SPSS Inc., Chicago, IL, USA) and statistical significance between mean values was assessed using Tukey's test at a 95% confidence level. $P < 0.01$ was considered significant.

RESULTS AND DISCUSSION

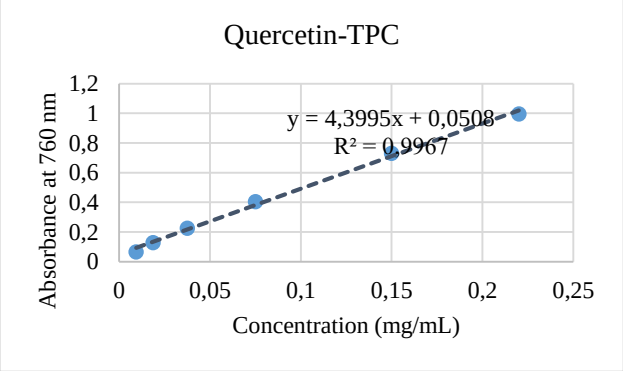
Total phenolic content: Organic solvents at different polarities, such as methanol, ethanol, ethyl acetate, acetonitrile, acetone, hexane, n-butanol, diethyl ether, and water, are normally used for the extraction of bioactive compounds from plants (Naczk & Shahidi, 2004; Stalikas et al., 2007). Among those solvents, methanol was the most efficient solvent for the extraction of phenolic compounds from the plants used in our study (Dhawan & Gupta, 2017) because the phenolic composition of these plant samples is mostly soluble and stable in this solvent. Ethyl acetate exhibited a good dissolving capacity for most of the flavonoids (flavon and flavonols) and phenolic acids in various studies (Hayder et al., 2004; Dmitrienko et al., 2012; Mokrani & Madani, 2016; Sukeksi & Sarah, 2016); therefore, plant extractions were performed using these solvents.

TPC in the methanol and ethyl acetate extracts of *S. verticillata* L., *S. tomentosa*, and *P. lychnitis* L. were determined spectroscopically (Table 1). The levels of phenolic compounds ranged from 32.01 to 347.04 mg GAE/g, and between 17.85 and 213.02 mg QE/g (r^2 = 0.999) for both phenolic standards (Supplemental Fig. 1-2). The highest TPC was in the methanol extract of *S. verticillata*; the lowest was in the ethyl acetate extract of *P. lychnitis*.

Tepe et al. (2005) have investigated different solvents for extraction of TPC in *S. tomentos*a, such as hot water, methanol, hexane, and dichloromethane, and found that TPC in this plant ranges from 10 to 275 µg GAE/mg. The highest TPC was recorded using hexane, followed by polar subfraction of methanol extract, polar subfraction of deodorized methanol extract, and polar subfraction of deodorized hot water extract. The lowest amount of TPC was obtained from the nonpolar subfraction of the deodorized methanol extract. Dinçer et al. (2013) have used cultivated and wild *S. tomentosa* plants to compare their TPC and antioxidant properties. Methanolic aqueous (80%) was used for extraction of TPC, which they determined to be between 49.27 and 63.26 mg GAE/g dw. TPC from the cultivated plants was higher than that from the wild plants. Tosun et al. (2009) have measured the TPC levels in *S. aethiopis, S. candidissima, S. limbata, S. microstegia, S. nemorosa, S. pachystachys, S. verticillat*a, and *S. virgate* using gallic acid as the standard. The results of their study showed that the highest TPC levels are from the methanol extract of *S. verticillata* (167.1 mg GAE/g dw), followed by that from *S. virgata* (101.2 mg GAE/g dw), *S. candidissima* (100.3 mg GAE/g dw), and *S. microstegia* (50.3 mg GAE/g dw).



Supplemental Figure 1. Calibration curve of standard gallic acid for determination of TPC.



Supplemental Figure 2. Calibration curve of standard quercetin for determination of TPC.

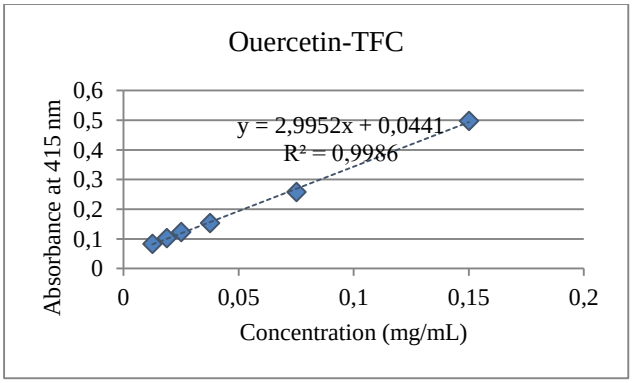
According to another TPC bioassay study using a methanol extract of *S. verticillata* L. *var. amasiaca*, and *S. microstegia*, *S. verticillata* L. *var. amasiaca* had TPC of 119.45 mg GAE/g, and *S. microstegia* had TPC at 118.08 mg GAE /g dw (Erbil et al., 2015).

Taşkın et al. (2018) have assessed the methanol extract of *P. pungens* and have found that the plant contains 30.0 mg GAE/g TPC. Previous studies have indicated that hydromethanolic extracts of *P. biloba* Desf. leaves and flowers result in 153.46 ± 1.36 µg GAE/mg from the leaves and 81.33 ± 2.29 µg GAE/mg from the flowers (Merouane et al., 2019). Compared with other species of *P. biloba* Desf., the methanolic and water extracts of *P. armeniaca* were 55.22 ± 1.95 and 54.39 ± 2.77 µg GAE/mg, respectively (Sarikurkcu et al., 2015). In another study, TPCs were 50.83 ± 1.11 and 50.90 ± 0.47, respectively, in the methanolic and water extracts from *P. nissolii*, while *P. pungens* var. *pungens* yielded 41.10 ± 2.69 and 57.68 ±

1.52 µg GAE/mg, respectively, in the methanolic and water extracts (Sarikurkcu et al., 2014).

These species showed significant differences in terms of TPC levels. For example, the TPC of *S. tomentosa* extracted using different solvents varies widely from 10 to 275 µg GAE/mg (Tepe et al. 2005; Erdogan-Orhan et al. 2010); however, we determined it to be between 62.18 and 125.44 mg GAE/g dw and suggest that the difference is related mainly to the extraction procedures. In fact, our study used methanol and ethyl acetate, while earlier studies used other solvents as well, such as hot water, hexane, and dichloromethane. In addition, the differences in the various results may be a result of different geographical locations of the plants, environmental and ecological variations (temperature, climate, diseases, etc.), harvest year, and growing conditions (Papageorgiou et al., 2008; Kallithraka et al., 2009). Dinçer et al. (2013) have reported TPC of *S. tomentosa* decreased in the harvest year, and growing condition. These differences can be explained by different climatic conditions.

Total flavonoid content: Quercetin was used as a representative standard with a linear calibration curve at r^2 = 0.999 for TFC (Supplemental Fig. 3). The highest TFC were in the methanol extract of *S. verticillata* at 51.90 ± 1.64 mg QE/g, while the lowest were in the ethyl acetate extract of *S. tomentosa* at 7.78 ± 0.20 mg QE/g ($p < 0.01$) (Table 1).



Supplemental Figure 3. Calibration curve of standard quercetin for determination of TFC.

Miliauskas et al. (2007) have investigated TFCs in *S. officinalis*, *S. sclarea*, *S. glutinosa*, and *S. pratensis* and found that *S. glutinosa* has the highest TFC at 5.7 ± 0.3 mg/g extract rutin equivalent. Tusevski et al. (2014) have reported the TFCs in methanol extracts of *S. nemorosa* L., *S. ringens Sibth. & Sm.*, and *S. sclarea* L. and found that *S. ringens Sibth. & Sm*. has the levels at 49.43 ± 1.35 mg catechin/g dw. In another report, the TFC in the methanol extract of *P. biloba* Desf. leaf (53.84 ± 0.24 µg QE/mg) was significantly higher than that in the plant's flower (14.86 ± 0.21 µg QE/mg) (Marouane et al., 2019).

Because of the differences in the procedures used to quantify TFC, reference compounds used (e.g., catechin and rutin), and different plant species used, it is not accurate to directly compare the data from the results of other studies with those from our study.

DPPH radical scavenging activity of the extracts: The free-radical scavenging activity of the extracts was determined using the DPPH test. Gallic acid and quercetin were used for the phenolic standards for the SC_{50} values 0.002 ± 0.000 and 0.003 ± 0.000 mg/mL, respectively.

The methanol extract from *S. verticillata*, the strongest DPPH scavenging activity, was recorded with the lowest SC_{50} value of 0.010 ± 0.000 mg/mL. Similarly, the same extract had the highest TFCs and TPCs. The ethyl acetate extract from *S. tomentosa* had the lowest scavenging activity with an SC_{50} value of 0.55 ± 0.01 mg/mL (Table 1).

Table 1. TPC, TFC and radical scavenging activity of the extracts

Extracts	TPC		TFC	DPPH
	mgGA/g*	mgQ/g*	mgQ/g**	SC_{50} (mg/mL)
S. verticillata -MeOH	347.04±1.42	213.02±0.92	51.90±1.64	0.01±0.00
S. verticillata - EtOAC	116.01±1.04	69.28±0.67	18.52±0.77	0.07±0.00
P. lychitis,- MeOH	56.76±0.42	33.70±0.38	21.74±0.18	0.06±0.00
P. lychitis- EtOAC	32.01±0.31	17.85±0.20	8.94±0.35	0.07±0.03
S. tomentosa- MeOH	125.44±0.65	75.41±0.42	38.73±0.31	0.03±0.00
S. tomentosa - EtOAC	62.18±0.46	37.36±0.26	7.78±0.20	0.55±0.01
Gallic acid				0.002±0.000
Quercetin				0.003±0.000

*Total phenolics are expressed in mg GAE/g dried extract and mg QE/g dried extract.
** Total flavonoids are expressed in mg QE/g dried extract.
GAE, Gallic acid equivalent; QE, quercetin equivalent; DPPH, 2,2-diphenyl-1-picrylhydrazyl; SC_{50} (mg/ mL), which indicates the concentration of extracts required to inhibit 50% of the radicals.

Examination of the antioxidant potential of 11 *Salvia* species has determined based on DPPH scavenging activity. The assessment found that *S. santolinifolia*, *S. nemorosa*, *S. atropatana*, and *S. eremophila* have the most DPPH scavenging activity with the highest TPC (Firuzi et al., 2013). Nickavar et al. (2007) have reported the DPPH scavenging activity of *S. hypoleuca* and four other *Salvia* species. All the ethanolic extracts show free-radical scavenging activity, and *S. verticillata* and *S. virgata* were the most active species with SC_{50} values of 23.53 and 27.01 µg/mL, respectively. In addition, the extracts were investigated for their TFCs, and the ethanol extracts of *S. hypoleuca* (TFC = 53.16 ± 1.95 µg/mg) and *S. reuterana* (TFC = 46.97 ± 4.43 µg/mg) had the highest TFCs. No favorable correlation was detected between free-radical scavenging potency and TFC. In another report, the methanol extracts of *S. verticillata* L. var. *amasiaca* and *S. microstegia* exhibited antioxidant effects against DPPH and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) free-radical agents (Erbil et al., 2015). In their study on the antioxidant activity of eight species of *Salvia*. Tosun et al. (2009) have reported that the antioxidant activity of eight species of *Salvia.* Methanol extracts of *S. verticillata* and *S. microstegia* showed higher DPPH free radical scavenging activity than other extracts. Another study has reported that the methanol extracts of *S. fruticosa* Mill., *S.*

pomifera Mill., and *S. tomentosa* Mill. within the Marmara region of Turkey show that *S. pomifera* collected from Topağac, Marmara, has the highest DPPH free-radical scavenging activity with an SC_{50} value of 450.51 µmol trolox equivalent/100 g dw, while the that of *S. fruticosa* from the Kumbağ forest camp, Tekirdağ, has an SC_{50} value of 287.57 µmol trolox equivalent/100 g dw (Erdogan et al., 2014). Merouane et al. (2019) have reported the antioxidant properties of *P. biloba* Desf. leaves and flowers of using 80% methanolic extract. The extract of the leaves has higher DPPH free-radical scavenging activity with an SC_{50} value of 47.78 ± 1.12 µg/mL than that of the flower with 90.85 ±1.04 µg/mL.

In this study, correlation analyses between among scavenging activity (DPPH) and TPC and TFC levels were conducted. The correlation coefficients (r) are shown in Table 2. Significant positive linear correlations (Table 2) were established between TPC and TFC ($r = 0.898$; $p < 0.01$). Strong positive correlations (Table 2) were also found between DPPH and TFC ($r = 0.904$; $p < 0.01$) and TPC ($r = 0.963$; $p < 0.01$). This relationship indicated that flavonoids together with other phenolic compounds contribute greatly to this bioactivity. In accordance with the previous study, phenolic compounds significantly contributed to the antioxidant activity in the medicinal plants used in the study (Cai, et al., 2004; Apak et al., 2006), which suggested that the methanol extract of *S. verticillata* L. had higher free-radical scavenging activity and TPC and TFC than other extracts; therefore, the methanol extract of *S. verticillata* L., has proved to be a high-quality source of natural antioxidants for use in the medical and food industries.

Table 2. Correlation analysis between scavenging activity and TPC, TFC.

r	TFC	TPC
TPC	0.898**	
DPPH	0.904**	0.963**

r, Correlation coefficient,
*The values of DPPH assay were taken as $1/SC_{50}$.
**Correlation is significant at $p < 0.01$.

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