TÜBİTAK

Turkish Journal of Agriculture and Forestry

http://journals.tubitak.gov.tr/agriculture/

Research Article

Turk J Agric For
(2013) 37: 561-567

doi:10.3906/tar-1211-72

Phenolic composition and antioxidant activity of *Salvia tomentosa* Miller: effects of cultivation, harvesting year, and storage

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Received: 26.11.2012 • **Accepted:** 03.04.2013 • **Published Online:** 28.08.2013 • **Printed:** 25.09.2013

Abstract: *Salvia tomentosa* is a common medicinal plant, and it is consumed as an herbal tea in some Mediterranean countries. It has been extensively collected from its natural habitat, and careless collection has caused the recent extinction of some plants. The present study was undertaken to cultivate *S. tomentosa* and compare the phenolic composition and antioxidant properties of wild and cultivated plants. Total phenolics, total flavonoids, and antioxidant activity of *S. tomentosa* ranged between 49.27 and 66.15 mg GAE g^{-1} dry weight (dw), 36.27 and 40.83 mg catechin g^{-1} dw, and 1.77 and 2.29 mg dw mg^{-1} DPPH, respectively. Total phenolic content of the cultivated samples was higher than that of wild samples. Seventeen different phenolic compounds, comprising 7 phenolic acids and 10 flavonoids, were identified and quantified in *S. tomentosa*. As with the many *Salvia* species, rosmarinic acid was quantified as the main component of *S. tomentosa*. It was followed by caffeic acid, morin, *p*-coumaric acid, and myricetin. Chlorogenic acid, *p*-coumaric acid, morin, kaempferol, hesperetin, and apigenin were increased through cultivation; gallic acid, caffeic acid, ferulic acid, rutin, catechin, and epicatechin were decreased. During the 6-month storage only caffeic acid changed significantly.

Key words: Antioxidant activity, cultivation, phenolics, *Salvia tomentosa,* storage

1. Introduction

Salvia species, commonly known as sage, have been used since ancient times for more than 60 different ailments ranging from aches to epilepsy, and mainly to treat colds, bronchitis, tuberculosis, hemorrhage, and menstrual disorders (Topçu 2006). Although there are around 900 species of *Salvia,* only a few (*S. officinalis* L., *S. fruticosa* Miller, and *S. tomentosa* Miller) are commercially important (Baser 2002). *S. tomentosa* is one of the most commonly consumed herbal teas, and it also has a wound-healing effect similar to that of iodine tincture (Aşkun et al. 2010).

S. tomentosa contains considerable amounts of secondary metabolites such as phenolics and terpenoids, which have antimicrobial (Haznedaroglu et al. 2001; Aşkun et al. 2010) and antioxidant (Erdogan-Orhan et al. 2010) properties. Tepe et al. (2005) reported that the total phenolic content of the aerial parts of *S. tomentosa* was 200 µg GAE mg^{-1}, while Erdogan-Orhan et al. (2010) found the following total phenolic and flavonoid contents of *S. tomentosa*: 87.87 mg GAE g^{-1} extract and 46.31 mg quercetin equivalents g^{-1} extract, respectively. These differences are generally explained by the different extraction methods, geographical coordinates, climates, and ecological conditions involved (Papageorgiou et al. 2008). There are a few studies on the phenolic composition of wild *S. tomentosa*. Rosmarinic acid, reported to be a powerful antioxidant, is the main phenolic component in the aerial part of *S. tomentosa*, as in many other *Salvia* species (Lu and Foo 2002; Askun et al. 2009; Dincer et al. 2012). Other phenolic acids and flavonoids in *Salvia* species include catechin, caffeic acid, vanillic acid, ferulic acid, rutin, apigenin, quercetin, and luteolin (Lu and Foo 2002; Papageorgiou et al. 2008; Askun et al. 2009).

As with many other medicinal plants, *S. tomentosa* has been extensively collected from its natural habitat, and this careless collection has caused the extinction of some plants. Hence, these plants have been cultivated in

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order to promote sustainable and standard agricultural production. For instance, İpek et al. (2012) comparatively studied the essential oil composition of wild and cultivated *Salvia cryptantha*. However, to the best of our knowledge no detailed comparison study has been conducted on the phenolics and antioxidant activity of wild and cultivated *S. tomentosa*. The present study therefore aimed to compare the phenolic composition and antioxidant activity of wild and cultivated *S. tomentosa* over 6 months of storage.

2. Materials and methods

2.1. Plant material

Wild *S. tomentosa* plants obtained from 3 different locations (Table 1) were cultivated in a dormant state and propagated by vegetative cutting method after adaptation. After rooting, they were transplanted to experimental plots (West Mediterranean Agricultural Research Institute, Antalya, Turkey) that were manured and periodically irrigated similar to the common agricultural practice of drip irrigation. Weed and pest control was accomplished without chemicals via organic practice. The cultivated plants were harvested separately from experimental plots during flowering season (spring). The wild plants were collected from their natural habitats (Table 1) during the same flowering season. The sampling (about 4 fresh kg of plants for each sampling) was carried out on the same plantation for 2 consecutive years. Plant species were identified at the Akdeniz University Biology Department, Antalya, and voucher specimens [R.S. Göktürk 7375 (Göynük-Lycia Plateau), 7405 (Çıralı), and 7451 (İbradı-Ormana)] were submitted to the herbarium of the department.

All the samples were dried by natural convection until they reached their equilibrium moisture content (6.61–7.31 g 100 g^{-1}) in 10–12 days. After removal of the stems and stalks, the leaves of the dried samples were divided into 2 parts; the first part was immediately analyzed for phenolics, and the other was stored in polyethylene bags under shady conditions at room temperature for storage tests at 2-month intervals over a 6-month period.

2.2. Preparation of the extracts

Extraction of the samples was accomplished according to the method of Škerget et al. (2005) with some modifications. One gram of the sample was extracted with 100 mL of aqueous methanol (80%) after crushing with a blender (Beko BKK-2155 Maxi Hand Blender, Turkey). The extraction was carried out for 2 h using an orbital shaking (150 rpm) water bath (GFL 1092, Germany) that was maintained at 40 °C. The extracts were cooled, filtered (Whatman No. 42), and kept at –18 °C until the analyses.

2.3. Determination of total phenolic content

The total phenolic content was analyzed by the Folin–Ciocalteu method as described by Škerget et al. (2005). For this purpose, 0.5 mL of extract was treated with 2.5 mL of 0.2 N Folin–Ciocalteu reagent and 2 mL of Na_2CO_3 (75 g L^{-1}). The mixture was incubated at 50 °C for 5 min and cooled immediately. Absorbance of the final solution was recorded with a spectrophotometer (Shimadzu UV-Vis 160A, Japan) at a 760-nm wavelength with respect to the blank solution (80% aqueous methanol). The standard curve was prepared using 0, 50, 100, 150, and 200 mg L^{-1} solutions of gallic acid in 80% aqueous methanol and the equation was as follows:

$y = 0.01x + 0.009$, $R^2 = 0.9999$.

Table 1. Sampling locations for *S. tomentosa*.

Samples		Sampling location	Coordinates
Wild	I.	Göynük–Lycia Plateau	36°40′50″N 30°31′33″E 70 m a.s.l.
	II.	Çıralı	36°25′2″N 30°28′32″E 16 m a.s.l.
	III.	İbradı–Ormana	37°03′43″N 31°35′28″E 890 m a.s.l.
Cultivated	West Mediterranean Agricultural Research Institute, Aksu, Antalya, Turkey		36°56′35.5″N 30°53′43.7″E 12 m a.s.l.

The results were expressed as gallic acid equivalent [milligrams of gallic acid per gram of dry weight (dw) of plant material, GAE].

2.4. Determination of total flavonoid content

Into 0.5 mL of methanolic extract of the samples, 2.5 mL of distilled water and 150 µL of 5% $NaNO_2$ solution were added. They were allowed to stand for 5 min after vortexing. Afterwards, 300 µL of 10% $AlCl_3$ solution was added to the solution and allowed to stand for 5 min; 1 mL of 1 M NaOH was then added and the final volume was increased to 5 mL with distilled water. Sample absorbance was measured at 510 nm by spectrophotometer (Shimadzu UV-Vis 160A) against a prepared blank solution (80% aqueous methanol). The calibration curve ($y = 0.0027x + 0.0066$, $R^2 = 0.9998$) was prepared by (+)-catechin solutions at concentrations of 0, 100, 200, 300, 400, and 500 mg L^{-1} in aqueous methanol (80%). The results were expressed as (+)-catechin equivalent [milligrams of (+)-catechin per gram of dw of plant material] (Chang et al. 2006).

2.5. Determination of antioxidant activity using DPPH

The antioxidant activity of the samples was analyzed by DPPH assay according to the procedure of Gadow et al. (1997) and Maisuthisakul et al. (2007). From the diluted sample extract (prepared at 4 different concentrations providing 10%–90% inhibition), 100 µL was added to 4 mL of freshly prepared DPPH (2,2 diphenyl-1-picrylhydrazyl radical) solution (6×10^{-5} M in methanol). The mixtures were shaken and kept in the dark at room temperature for 30 min. Absorbance values of the final solutions were recorded at 516 nm by spectrophotometer (Shimadzu UV-Vis 160A) with respect to the control (80% methanol in DPPH solution). The percent inhibition of the DPPH radical was calculated using the following equation:

$$IP\ (\%) = [(A_c - A_s) / A_c] \times 100,$$

where IP is the inhibition percentage, and A_c and A_s are the absorbance values of the control and test sample, respectively.

The extract concentration providing 50% inhibition [IC_{50} (milligrams of dw of plant material per milligram of DPPH)] was calculated by plotting the concentration versus IP. The IC_{50} value of Trolox solution (positive control) was also determined to compare the antioxidant activity of the samples.

2.6. Determination of phenolic compounds by HPLC

The phenolic composition of the samples was determined according to the method of Proestos et al. (2006). Extracts were prepared as follows: 40 mL of 62.5% aqueous methanol containing BHT (1 g L^{-1}) was added to 0.5 g of dried sample, to which 10 mL of 6 M HCl was carefully added by stirring. In each sample, nitrogen was bubbled for 60 s. The mixture was then sonicated for 15 min, refluxed for 2 h, and allowed to cool at room temperature. Methanol was added until the volume reached 100 mL, and it was filtered through a membrane filter (0.45 µm; Macherey Nagel, Germany) before injection into a HPLC system.

Chromatographic separation was performed on a solvent delivery system (20AD, Shimadzu) coupled with an autosampler (SIL-20A Prominence, Shimadzu), column (LiChroCART 250-4 250 × 4 mm, 5 µm; Nucleosil 100-5 C 18), and guard column (LiChroCART 4-4, Nucleosil 5 C 18) maintained at 30 °C in the column oven (CTO-20AC Shimadzu). Individual peaks were detected by a SPD-M20A Diode Array Detector (Shimadzu) controlled by LC solution software. Mixtures of water, acetic acid, and methanol in different ratios [88:2:10, v/v/v (solvent A) and 8:2:90, v/v/v (solvent B)] were used as the mobile phase in the following gradient elution at a 0.9 mL min^{-1} flow rate (Rodríguez-Delgado et al. 2001): initial, A:B 100:0; at 15 min, A:B 85:15; 25 min, A:B 50:50; 35 min, A:B 30:70; 50 min, A:B 25:75; and 55 min, A:B 100:0. Identification and quantification of individual phenolics were carried out using the method employed by Dincer et al. (2012).

2.7. Statistical analysis

The plants were grown and collected in triplicate, and measurements were performed in duplicate. The data were subjected to analysis of variance, and appropriate mean separation was conducted using Duncan's multiple range test in SAS software (SAS Institute, USA).

3. Results

3.1. Total phenolic content, total flavonoid content, and antioxidant activity

The results of total phenolic and flavonoid contents and antioxidant activity of *S. tomentosa* are shown in Table 2. The total phenolic content of the samples were determined at 49.27–66.15 mg GAE g^{-1} dw. Harvesting year and growing conditions had a significant ($P < 0.05$) effect on the total phenolic content, while storage period had no influence.

Total flavonoid content of the samples ranged between 36.27 and 40.83 mg catechin equivalent g^{-1} dw, depending on the harvesting year, growing conditions, and storage period. Although growing conditions had no influence, consecutive harvesting and longer storage caused a decrease in the total flavonoid content.

The IC_{50} values of *S. tomentosa* samples ranged between 1.77 and 2.29 mg dw mg^{-1} DPPH (Table 2). Harvesting year and growing conditions did not have significant effects on the antioxidant activity of *S. tomentosa*. However, antioxidant activity of the samples ($P < 0.05$) decreased significantly by storage period. Higher antioxidant activity, and thereby lower IC_{50} value, was estimated for the initial samples.

Table 2. Total phenolic and flavonoid contents and IC_{50} values of *S. tomentosa.*

		Phenolic content (mg GAE g^{-1} dw)	Flavonoid content (mg of CE g^{-1} dw)	IC_{50} value* (mg dw mg^{-1} DPPH)
Harvesting year (N = 24)	I	52.15 ± 2.52b	40.83 ± 1.83a	2.07 ± 0.10
	II	63.26 ± 2.33a	37.73 ± 1.45b	2.17 ± 0.12
Growing conditions (N = 24)	Wild	49.27 ± 2.00b	38.94 ± 1.72	2.15 ± 0.10
	Cultivated	66.15 ± 2.06a	39.62 ± 1.64	2.08 ± 0.12
Storage period (months) (N = 12)	0	61.90 ± 3.03	37.73 ± 1.45a	1.77 ± 0.11b
	2	56.71 ± 2.72	36.27 ± 2.58b	2.23 ± 0.20a
	4	56.16 ± 4.57	37.92 ± 1.66b	2.17 ± 0.12a
	6	56.05 ± 4.57	37.04 ± 2.38b	2.29 ± 0.12a

Results are means ± standard error; values within a column with different superscript letters are significantly ($P < 0.05$) different; N is the number of measurements.
*IC_{50} of Trolox was determined as 0.16 ± 0.01 mg mg^{-1} DPPH.

3.2. Phenolic compositions

Seventeen different phenolic compounds consisting of 7 phenolic acids and 10 flavonoids were identified in *S. tomentosa* depending on the harvesting year, growing conditions, and storage period (Table 3).

Major phenolic acids of the *S. tomentosa* were rosmarinic (8.24–10.24 mg g^{-1} dw), caffeic (2.32–3.01 mg g^{-1} dw), and *p*-coumaric (1.09–2.23 mg g^{-1} dw) acids. A few minor phenolic acids such as ferulic, chlorogenic, gallic, and vanillic acids were also identified and quantified. Only chlorogenic and caffeic acids were changed by harvesting year. The amounts of gallic, caffeic, and ferulic acids were higher in the cultivated samples than in the wild samples, while chlorogenic and *p*-coumaric acids were higher in wild *S. tomentosa*. Vanillic and rosmarinic acids content did not significantly change according to growing conditions. During the 6-month storage only caffeic acid changed significantly. In the tested samples, morin (1.41–2.06 mg g^{-1}) and myricetin (1.00–1.16 mg g^{-1}) were the main flavonols, followed by rutin (0.59–0.93 mg g^{-1}), kaempferol (0.59–0.62 mg g^{-1}), and quercetin (0.53–0.59 mg g^{-1}). While morin and kaempferol contents of the samples significantly decreased according to harvesting year, other flavonols did not change markedly. The rutin content of the *S. tomentosa* samples increased by cultivation, whereas morin and kaempferol contents decreased. Apart from flavonols, catechins (catechin and epicatechin), flavanone (hesperetin), and flavones (luteolin and apigenin) were also identified and quantified as additional flavonoids (Table 3). Among these flavonoids luteolin was found in the highest amounts (0.853–0.949 mg g^{-1} dw), followed by hesperetin (0.566 –1.002 mg g^{-1} dw), epicatechin (0.227–0.542 mg g^{-1} dw), apigenin (0.144–0.252 mg g^{-1} dw), and catechin (0.112–0.173 mg g^{-1} dw). Generally, these components were significantly ($P < 0.05$) changed by harvesting year and growing conditions; however, they did not change according to storage period.

4. Discussion

4.1. Total phenolic and flavonoid contents and antioxidant activity

Total phenolic content of *S. tomentosa* extracted with different solvents ranged broadly, between 10 and 275 µg GAE mg^{-1} (Tepe et al. 2005; Erdogan-Orhan et al. 2010). However, in our case, it was between 49.27 and 63.26 mg GAE g^{-1} dw. This enormous difference in the results was likely related to extraction procedures. The present work used aqueous methanol (80%); however, earlier works were carried out with solvents such as hot water, methanol, hexane, and dichloromethane. Additionally, results may be affected by geographical location of the plants, ecological conditions, and climate (Papageorgiou et al. 2008; Kallithraka et al. 2009).

Erdogan-Orhan et al. (2010) reported that total flavonoid content of *S. tomentosa* was 46.31 mg quercetin equivalent g^{-1}, which is consistent with our results. The flavonoid content of *S. tomentosa* decreased in the second harvesting year, whereas it did not change significantly through cultivation and storage. Differences in the flavonoid content by harvesting year can be reasoned from climatic conditions.

Table 3. Phenolic composition identified in *S. tomentosa* (mg g^{-1} dw).

Phenolics	Harvesting year (N = 24)		Growing conditions (N = 24)		Storage period (months) (N = 12)			
	1st	2nd	Wild	Cultivated	0th	2nd	4th	6th
Phenolic acids								
Vanillic	0.011 ± 0.003	0.019 ± 0.004	0.013 ± 0.005	0.017 ± 0.002	0.010 ± 0.002	0.024 ± 0.008	0.016 ± 0.004	0.011 ± 0.005
Gallic	0.040 ± 0.007	0.036 ± 0.003	0.022 ± 0.003b	0.054 ± 0.005a	0.035 ± 0.009	0.042 ± 0.007	0.040 ± 0.011	0.036 ± 0.002
Chlorogenic	0.093 ± 0.011b	0.142 ± 0.025a	0.159 ± 0.024a	0.075 ± 0.010b	0.104 ± 0.022	0.102 ± 0.020	0.134 ± 0.043	0.128 ± 0.024
Caffeic	2.502 ± 0.088b	2.716 ± 0.189a	2.318 ± 0.100b	2.900 ± 0.164a	2.440 ± 0.187b	2.465 ± 0.148b	2.524 ± 0.234b	3.007 ± 0.234a
Ferulic	0.392 ± 0.011	0.421 ± 0.022	0.355 ± 0.013b	0.459 ± 0.015a	0.400 ± 0.035	0.411 ± 0.032	0.386 ± 0.013	0.430 ± 0.013
Rosmarinic	9.751 ± 1.042	8.538 ± 0.647	8.539 ± 0.637	9.750 ± 1.048	8.242 ± 0.980	9.045 ± 1.377	9.049 ± 1.272	10.242 ± 1.328
p-Coumaric	1.774 ± 0.123	1.544 ± 0.196	2.227 ± 0.122a	1.091 ± 0.108b	1.662 ± 0.252	1.746 ± 0.237	1.453 ± 0.196	1.776 ± 0.254
Flavonoids								
Rutin	0.797 ± 0.058	0.723 ± 0.040	0.591 ± 0.034b	0.929 ± 0.037a	0.738 ± 0.058	0.752 ± 0.073	0.776 ± 0.073	0.773 ± 0.084
Myricetin	1.061 ± 0.067	1.070 ± 0.025	1.001 ± 0.019	1.130 ± 0.066	0.996 ± 0.037	1.090 ± 0.031	1.156 ± 0.082	1.019 ± 0.104
Morin	1.873 ± 0.132a	1.598 ± 0.103b	2.063 ± 0.091a	1.407 ± 0.110b	1.821 ± 0.203	1.846 ± 0.161	1.753 ± 0.206	1.520 ± 0.093
Quercetin	0.568 ± 0.026	0.544 ± 0.022	0.538 ± 0.021	0.574 ± 0.026	0.548 ± 0.024	0.557 ± 0.027	0.589 ± 0.050	0.530 ± 0.029
Kaempferol	0.614 ± 0.010a	0.596 ± 0.005b	0.623 ± 0.009a	0.587 ± 0.005b	0.603 ± 0.013	0.604 ± 0.012	0.610 ± 0.013	0.603 ± 0.009
Hesperetin	0.833 ± 0.041	0.735 ± 0.088	1.002 ± 0.056a	0.566 ± 0.048b	0.882 ± 0.108	0.756 ± 0.100	0.789 ± 0.107	0.710 ± 0.073
Luteolin	0.853 ± 0.028b	0.949 ± 0.025a	0.899 ± 0.030	0.903 ± 0.026	0.888 ± 0.048	0.855 ± 0.021	0.928 ± 0.038	0.933 ± 0.046
Apigenin	0.252 ± 0.027a	0.100 ± 0.018b	0.208 ± 0.033a	0.144 ± 0.019b	0.215 ± 0.042	0.188 ± 0.044	0.152 ± 0.042	0.148 ± 0.028
(+)-Catechin	0.169 ± 0.015a	0.127 ± 0.013b	0.123 ± 0.012b	0.173 ± 0.015a	0.170 ± 0.025	0.165 ± 0.019	0.146 ± 0.019	0.112 ± 0.016
(-)-Epicatechin	0.227 ± 0.033b	0.546 ± 0.050a	0.241 ± 0.043b	0.532 ± 0.046a	0.403 ± 0.069	0.346 ± 0.061	0.352 ± 0.083	0.446 ± 0.091

Results are means ± standard error; values within a row with different superscript letters are significantly ($P < 0.05$) different.

There are a few studies on the antioxidant properties of *S. tomentosa* accomplished by DPPH method. Bozan et al. (2002) stated that *S. tomentosa* has moderate antioxidant activity in comparison to the other *Salvia* species. They estimated that *S. tomentosa* provided almost 17% inhibition in DPPH radicals with 100 g samples, which corresponds to 1.5 mg dw mg^{-1} DPPH. The present IC_{50} values of the samples (1.77 and 2.29 mg dw mg^{-1} DPPH) were slightly higher than those of the previous study; this may be associated with the part of the plant tested. Among the dependent variables, only storage period led to a significant decrease in the antioxidant activity of *S. tomentosa*. Decreasing antioxidant activity during storage is mostly related to flavonoid content of the samples, considering the similar change in flavonoid content (Table 2) during storage.

4.2. Phenolic composition

Seventeen different phenolic components were identified and quantified in the *S. tomentosa* samples. Rosmarinic acid was the major phenolic component in all samples. Askun et al. (2009) identified 8 different phenolic components (2 phenolic acids and 6 flavonoids) in the methanolic extract of *S. tomentosa* and stated that rosmarinic acid was the major phenolic component of *S. tomentosa*. As far as we know this is the only earlier work on the phenolic composition of *S. tomentosa*. However, there are a few more studies on other *Salvia* species that identified rosmarinic acid as the main phenolic component (Skoula et al. 2000; Lu and Foo 2002; Koşar et al. 2011; Dincer et al. 2012). Only caffeic acid was significantly ($P < 0.05$) increased during the 6-month storage period, particularly after 4 months. This may be related to the degradation of flavonoids and/or catechins, which yields caffeic acids. Indeed, a similar explanation has been reported by Arunachalam et al. (2003) and Dincer et al. (2012).

Ten different flavonoid components were identified and quantified in the methanolic extracts of *S. tomentosa* leaves. Of the identified components, quercetin, catechin, apigenin, hesperidin, and luteolin were also determined by Askun et al. (2009). However, they did not report myricetin, morin, kaempferol, or epicatechin, which were additionally identified and quantified in the present study. There were significant variations in several flavonoids according to harvesting year and growing conditions. However, their noticeable changes during storage were not significant. Variations in the harvesting year and growing conditions can be attributed to climatic differences. Subsequent sampling from the same plant may also produce these types of variations (Maudu et al. 2010).

The present study found that both wild and cultivated *S. tomentosa* has considerable amounts of phenolics, which are mostly referred to as powerful antioxidants. There were also unidentified phenolics that should be studied in detail. Cultivation led to increases in the total phenolic content. With the exception of flavonoid content, all quality parameters were determined to be either higher or unchanged in the second harvesting year. Although there were slight variations in a few analyzed parameters, no remarkable changes were observed during storage. Therefore, *S. tomentosa* can be successfully cultivated for sustainable, standard medicinal plant production by the food and pharmaceutical industries.

Acknowledgments

The authors would like to thank TÜBİTAK (the Scientific and Technological Research Council of Turkey) for financial support (project no.: 106 T 747) and the Akdeniz University Research Administration Unit (Antalya, Turkey).

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