SUPPLEMENTARY MATERIAL

Essential oil composition, antioxidant activity and phenolic content of endemic *Teucrium alyssifolium* Staph. (Lamiaceae)

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Abstract: The present study was designed to examine the chemical composition of the essential oil, *in vitro* antioxidant activity and total phenolic and flavonoid content of extracts from plant parts (leaf, flower and stem) of *T. alyssifolium*. The principle components of the essential oil were trans- β -caryophyllene (16.87%), *ar*-curcumene (11.43%) and bisabolene (11.06%), representing 39.36% of the oil. The total phenolic contents ranged between 13.99 to 41.54 mg of GAE/g of extract. The concentrations of flavonoids varied from 16.82 to 49.52 mg of Ru/g of extract. Antioxidant activity was determined *in vitro* using DPPH reagent and expressed as concentration of each extract required to inhibit radical by 50% (IC_{50}) values that ranged from 13.52 to 132.55 μ g/ml. Our results have indicated that water extract of *T. alyssifolium* (part leaf) with a total content of polyphenols (41.54 mg of GAE/g) and an IC_{50} of 13.52 μ g/ml is more antioxidant.

Keywords: antioxidant activity, total phenolic, total flavonoid, essential oil, GC-MS, *Teucrium alyssifolium*

Experimental

Plant material

The plant parts of *Teucrium alyssifolium* Staph. (Lamiaceae) were collected from natural habitats in Sandras Mountain-Turkey (Figure 1), at an altitude of 1700 m in June 2014. Plant material was dried in a dry and dark place at ambient temperature. The plant was identified by Dr. Gürkan SEMİZ, a voucher specimen is deposited at Pamukkale University (Denizli, Turkey), Chemical Ecology Laboratory as no: GSE 1783.

Preparation of the extracts

The collected plant material, air-dried and fine powdered (10 g), was extracted with water (50 ml) 3 h using automatic extraction system (Gerhardt Soxtherm). The extracts were filtered through Whatman No. 1 paper and concentrated to dryness under a vacuum on a freeze dry system (Labconco FreeZone) at -105°C. The obtained extracts were kept in dark and stored at 4°C.

Extraction of the essential oil

The air-dried aerial parts of *T. alyssifolium* were subjected to steam distillation for 4 h using a Clevenger apparatus to produce essential oil in a yield of 0.03% based on the dry weight of the samples. The essential oil was dried in anhydrous sodium sulphate and after filtration stored in a sealed dark vial at 4°C until analyzed.

The yields of the essential oils were calculated by the formula:

$$\text{Yield of essential oil} = \text{volume of essential oil (g)} / \text{volume of sample (g)} \times 100\%$$

GC/MS analysis

Chemical analyses of the essential oil were performed on gas chromatography-mass spectrometry (Hewlett Packard GC type 7820A, MSD 5975; Hewlett Packard, Wilmington, DE, USA) using a 30-m long HP-5MS (ID 0.25 mm, film thickness 0.25 mm, Hewlett Packard) capillary column. The chromatographic conditions were as follows: helium was used as the carrier gas at 1.2 ml min⁻¹; the temperature program for terpenes ranged from 50°C to 250°C; the heating rate was 5°C min⁻¹; SCAN technique (mass numbers from m/z 30 to 350 were recorded; signal ions in monitoring; 93, 133, 136, 161, and 204 m/z) was used; the injected volume was 1 µl. The individual peaks were identified by comparison of their retention indices (relative to C8-C25 *n*-alkanes for HP-5MS) as well as by comparing their mass spectra with the Wiley 7 MS library (Wiley, New York, NY, USA) and NIST02 (Gaithersburg, MD, USA) mass spectral database. A series of *n*-alkanes was also injected under same analytical conditions with that of the essential oil for the calculation of Retention Indices (RI). The percentage composition of the samples was computed from the GC peak areas using the normalization method. The component percentages were calculated as mean values from duplicate GC and GC-MS analyses.

Determination of total phenolic content

Total phenolic content was determined spectrophotometrically according to the Folin-Ciocalteu colorimetric method (Singleton et al. 1999). The reaction mixture was prepared by mixing 0.5 ml of methanolic solution (1 mg/ml) of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml 7.5% NaHCO₃. The

samples were incubated at 45°C for 15 min. The absorbance was determined at $\lambda_{\text{max}} = 765$ nm. It was calibrating against acid gallic standards and expressing the results as mg gallic acid equivalents (GAE)/g extract. Data presented average of three measurements.

Determination of total flavonoid content

Flavonoid content was measured according to aluminum chloride colorimetric method (Quettier-Deleu et al. 2000). The sample contained 1 ml of methanolic solution of the extract in the concentration of 1 mg/ml and 1 ml of 2% AlCl_3 solution dissolved in methanol. The samples were incubated for an hour at room temperature. The absorbance was determined at $\lambda_{\text{max}} = 415$ nm (Stankovic et al. 2012). A calibration curve was prepared with rutin and the results were expressed in terms of rutin equivalent (mg of Ru/g of extract). Data presented average of three measurements.

Free radical scavenging activity using DPPH

The DPPH (2,2-dyphenyl-1-picrylhydrazyl) free radical scavenging activity of each sample was determined using the method described by Stankovic et al. (2012). The stock solution (1 mg/ml) of the plant extract was prepared in methanol. Dilutions were made to obtain concentrations of 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.99, and 0.97 $\mu\text{g/ml}$. Diluted solutions (1 ml each) were mixed with 1 ml of DPPH methanolic solution (80 $\mu\text{g/ml}$). After 30 min in darkness at room temperature (23°C), the absorbance was recorded at 517 nm against a blank (methanol solution). The control samples contained all the reagents except the extract. The DPPH radicals scavenging activity was calculated using the following equation:

$$\% \text{ inhibition} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

IC_{50} was obtained graphically from nonlinear regression analysis.

Statistical analysis

All measurements were performed in triplicate and results are expressed as mean $\pm$ standard deviation (SD). The data were subjected to analysis of variance, and appreciate mean separation was conducted using Duncan's multiple range test in SPSS software (SPSS 2003).

Figure S1. Location of the plant material collected in Southwestern Turkey and the plant species *Teucrium alyssifolium*.

Figure S2. Total phenolic contents in the essential oil and water extracts of *T. alyssifolium*. Results are presented as the mean from three independent experiments and expressed as relative mean $\pm$ standard error. Means having different letters within the same column are significantly different at $P < 0.05$.

Figure S3. Total flavonoid contents in the essential oil and water extracts of *T. alyssifolium*. Results are presented as the mean from three independent experiments and expressed as relative mean $\pm$ standard error. Means having different letters within the same column are significantly different at $P < 0.05$.

Figure S4. Linear correlation between the amount of total phenols and antioxidant activity. Coefficient of correlation $r = -0.77$. Correlation is significant at the $P < 0.05$ level.

Table S1. Chemical constituents of the essential oil of *T. alyssifolium*.

No	Compound name ^a	RI ^b	% of the oil
1	α -Pinene	940	0.45
2	Sabinene	975	0.87
3	1-octen-3-ol	980	0.46
4	β -Pinene	982	1.56
5	Limonene	1033	3.53
6	β -Phellandrene	1038	0.73
7	Linalool	1098	1.80
8	Camphor	1142	6.44
9	α -Terpineol	1160	0.70
10	Carvacrol	1275	0.52
11	α -Copaene	1376	0.33
12	β -Bourbonane	1380	1.59
13	β -Cubenene	1382	0.99
14	α -Cedrene	1396	0.77
15	Trans-β-Caryophyllene	1422	16.87
16	α -Humulene	1456	8.31
17	Germacrene D	1482	5.17
18	<i>ar</i> -Curcumene	1496	11.43
19	Bisabolene	1504	11.06
20	α -Elemene	1508	1.03
21	δ -Cadinene	1515	2.42
22	α -Gurjunene	1548	1.44
23	β -Eudesmol	1573	1.76
24	Isocaryophyllene oxide	1576	1.41
25	Caryophyllene oxide	1581	5.12
26	Muurolool	1615	2.82
27	δ -Cadinol	1618	2.30
<i>Monoterpene hydrocarbons</i>			7.59
<i>Oxygenated monoterpenes</i>			9.46
<i>Sesquiterpene hydrocarbons</i>			61.40
<i>Oxygenated sesquiterpenes</i>			14.66
Total			93.11

^a Compounds listed in order their elution, ^b RI: retention index measured relative to *n*-alkanes on HP-5MS column.

Table S2. The IC_{50} values of water extracts of *T. alyssifolium*.

Type of extract	IC_{50}
Flower	43.62 $\pm$ 0.56
Leaf	13.52 $\pm$ 0.23
Stem	28.30 $\pm$ 0.47
Essential oil	132.55 $\pm$ 2.13

Each value represents the mean $\pm$ SD ($n = 7$). IC_{50} values were expressed as $\mu\text{g/ml}$.

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