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Antioxidant activities of *Sideritis congesta* Davis et Huber-Morath and *Sideritis arguta* Boiss et Heldr: Identification of free flavonoids and cinnamic acid derivatives

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ABSTRACT

Different solvent extracts of endemic *Sideritis* (Labiatae) species, *Sideritis congesta* Davis et Huber-Morath and *Sideritis arguta* Boiss et Heldr, were analyzed for free flavonoids (quercetin, apigenin, myricetin and kaempferol) and cinnamic acid derivatives (rosmarinic acid, ferulic acid, caffeic acid, p-coumaric acid and chlorogenic acid) using HPLC-DAD. All the phenolics were quantified in acid-hydrolyzed extracts, except rosmarinic acid, chlorogenic acid and myricetin which were quantified in raw samples. Antioxidant activities of extracts of these two plants and many of their components in pure form were evaluated based on DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ assays. In general, *S. arguta* extracts displayed higher antioxidant activity than *S. congesta* extracts possibly due to their richness in antioxidant components of strong activity. Acetone extract of *S. arguta*, with its strikingly high TEAC value of 3.2 mM trolox and low IC_{50} value of 38.3 μg/mL showed the highest antioxidant potency among all extracts. α-tocopherol, the positive control, displayed IC_{50} and TEAC values of 33.8 μg/mL and 2.9 mM trolox, respectively. No direct correlation was found between antioxidant activities and total phenolic contents of the plant extracts studied.

1. Introduction

Extensive number of evidences has pointed out free radicals as major contributors to aging and degenerative diseases such as cancer, cardiovascular disease, cataracts and immune system decline (Ames, Shigenaga, & Hagen, 1990; Young & Woodside, 2001). However, free radical formation is controlled naturally by compounds known as antioxidants. The damage in biological systems can be cumulative when the concentration of radical species and antioxidants are not in balance (Swanson, 1998). Antioxidants are capable of neutralizing, or scavenging free radicals by hydrogen donation before the latter attack cells and other biological components. Thus, they are vital for well-being and protecting optimal health (Percival, 1998). Synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiary butyl hydroquinone (TBHQ) have been widely used as antioxidants in the food industry until recently. However, their uses have been limited for the fact that they may be responsible for liver damage and carcinogenesis (Grice, 1988; Wichi, 1986). This problem has been overcome by supplementing diets with antioxidants from natural sources, most of which are plants, fruits and vegetables (Knekt, Jarvinen, Reunanen, & Maatela, 1996).

Flavonoids and cinnamic acid derivatives, the groups of compounds that most of the antioxidant activity of plants comes from, contain phenolic structure and are widely distributed in photosynthesizing cells (Havsteen, 1983). Flavonoids can be subdivided into several classes: flavones, flavonols, flavanones, isoflavones, flavans, flavanols, and anthocyanins. Tea and herbal preparations provide the major source of these two groups of phenolic compounds in human diet (Shahidi, 2000). Investigation of the presence and activity of flavonoid and cinnamic acid derivatives as antioxidants in tea and herbs has been performed in various studies (Zaveri, 2006; Erkan, Ayranci, & Ayranci, 2008; Atoui, Mansouri, Boskou, & Kefalas, 2005). It is known that extracts obtained with organic solvents from dried leaves of such plants are of much interest due to the high capacity of these solvents in extracting antioxidant compounds.

The aerial parts of plants from the genus *Sideritis*, commonly known as 'mountain tea' are widely used as a popular folk medicine in Mediterranean countries such as Greece, Turkey and Spain. The genus Sideritis is represented in the Turkish flora by 46 species, 31 of which are endemic (Davis, Mill, & Tan, 1988). Different biological activities of *Sideritis* species, including anti-inflammatory (Hernandez-Perez & Rabanal, 2002), anti-ulcer (Aboutabl et al., 2002), antioxidant (Armata, Gabrieli, Termentzi, Zervou, & Kokkalou, 2008) and antimicrobial (Aligiannis, Kalpoutzakis, Chinou, & Mitakou, 2001) activity have been reported up to now. Many of these activities have been attributed to various phytochemicals of the plant among which flavonoids, phenolic acids and diterpenoids have been identified (Janeska, Stefova, & Alipieva, 2007; Gómez-Serranillos et al., 1998).

In this article, we report for the first time a detailed study on investigating the antioxidant activities of two endemic *Sideritis* species,

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Sideritis congesta Davis et Huber-Morath and *Sideritis arguta* Boiss et Heldr, and analyzing the extracts of these plants for free flavonoids and cinnamic acid derivatives.

2. Materials and methods

2.1. Materials

All solvents used were HPLC grade and purchased from Merck. 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical, quercetin, myricetin, apigenin, caffeic acid and p-coumaric acid were obtained from Sigma. Trolox, gallic acid, chlorogenic acid, ferulic acid and rosmarinic acid were from Aldrich. 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and kaempferol were purchased from Fluka. The standard compounds were in their highest purity and all other reagents were of analytical grade. Deionized water was used to prepare aqueous solutions.

S. congesta and *S. arguta* were collected from Akseki county of Antalya, Turkey in July, 2009. Taxonomic identification was performed by botanists in the Biology Department, Faculty of Arts and Sciences, Akdeniz University. The aerial parts of plants were washed with deionized water and dried at room temperature for 15 days. Dried samples were kept in a deep freezer at −20 °C until use.

2.2. Preparation, acid hydrolysis and analysis of plant extracts

Dried samples were blended in a blender and subjected to extraction under reflux for 3 h with 1000 mL of methanol, ethyl acetate or acetone as the extracting solvent for 35 g of aerial parts at the boiling points of the organic solvents; 64.7, 77 or 56.5 °C, respectively. The mixture was filtered through Whatman paper (GF/A, 110 mm) and its solvent was removed by a rotary evaporator (Heidolph) at 40 °C. All the extracts were in dark green color after evaporation of the solvent, due to high chlorophyll content of the plants. In order to remove chlorophyll from the extracts in the highest possible amount, repeated washing steps with hexane were applied to each extract. After filtration, the remaining extracts were kept in a vacuum oven at 35 °C for 1 day for further removal of the solvent residue. Dried samples appeared in varying colors from dark yellow to light green and were kept in the deep freezer at −20 °C until use.

An acid hydrolysis step was applied to the extracts to release aglycones of flavonoid glycosides for simplification of peak identification procedure. Acid hydrolysis was performed according to the procedure reported by Huang, Wang, Eaves, Shikany, and Pace (2007) with minor modifications. Briefly, 0.01 g of plant extract was subjected to water bath-incubation at 90 °C for 1.5 h after vortex-mixing with 4 mL of 80% methanol and 1 mL of 6 M HCl. At the end of incubation, the mixture was cooled to room temperature, made up to 5 mL with methanol and sonicated for 7 min. Then, the mixture was centrifuged at 3000 × *g* for 10 min. The supernatant was separated and its solvent was removed completely. 10 mL of methanol was added to the residue and the solution was filtered through a 0.2 μm syringe filter (Millipore, Bedford, MA) and subjected to analysis (approximate concentration, 1 mg/mL). Untreated extracts were also dissolved in methanol (1 mg/mL) and filtered prior to analysis.

HPLC analysis of *S. congesta and S. arguta* extracts were performed with an Agilent 1100 series HPLC instrument equipped with an autosampler and a diode array detector (DAD). The column was Hypersil ODS C_{18} type with a 5 μm particle size, 4.6 × 250 mm i.d. used with Hypersil ODS 4.0 × 20 mm i.d. 5 μm guard cartridges. The mobile phase was composed of 5% acetic acid in H_2O (solvent A) and methanol (solvent B). It was eluted at a flow rate of 0.9 mL/min. Gradient elution utilized was: 0 min, 5% B; 5 min, 15% B; 25 min, 30% B; 39 min, 42% B; 47 min, 55% B; 50 min, 70% B; 56 min, 75% B and 60 min, 100% B. The chromatograms were acquired at 280 and 330 nm. Column temperature and injection volume were 28 °C and 7 μL, respectively. Among the phenolic compounds analyzed, rosmarinic acid, chlorogenic acid and myricetin were quantified in raw extracts, while ferulic acid, caffeic acid, p-coumaric acid, quercetin, apigenin and kaempferol were quantified in acid-hydrolyzed extracts. All standard compounds were diluted in methanol before analysis.

Peak identification in HPLC analysis was performed by comparison of retention time with respective reference standards. Quantification of individual flavonoids was done using the peak area of identified compounds. Peak purity values, which were provided by the instrument facility, were checked in each component analysis. They were found to be within the threshold limit in each case.

2.3. DPPH radical scavenging assay

This assay was carried out as described by Blois (1958) with some modifications. 1 mL of various dilutions of the test materials (pure antioxidants or plant extracts) was mixed with 2 mL of a 0.2 mM methanolic DPPH· solution. After an incubation period of 30 min at 25 °C, the absorbances at 515 nm were recorded as A_{sample} using a Cary 100 Bio UV/VIS spectrophotometer. A blank experiment was also carried out applying the same procedure to a solution without the test material and the absorbance was recorded as A_{blank}.

The free radical scavenging activity of each solution was then calculated as percent inhibition according to the following equation:

$$\%\ \text{inhibition} = 100\left(A_{blank} - A_{sample}\right) / A_{blank} \quad (1)$$

Antioxidant activities of test compounds or extracts were expressed as IC_{50}, defined as the concentration of the test material required to cause a 50% decrease in initial DPPH· concentration. α-Tocopherol was used as the positive control.

2.4. ABTS·+ radical scavenging assay

This assay was carried out according to the procedure described by Re et al. (1999). ABTS·+ radical cation was produced by reacting 7 mM aqueous ABTS with 2.45 mM (final concentration) potassium persulfate and keeping the mixture in the dark at room temperature for 16 h. Blue-green ABTS·+ was formed at the end of this period. The solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm, wavelength of maximum absorbance in the visible region. Test materials were dissolved in and diluted with ethanol such that, after the introduction of an accurately measured volume of each dilution into the assay, they produced between 10%–90% decrease in the absorbance of the blank solution at 734 nm. After adding 100 μL of the test solution to 3.5 mL of ABTS·+ solution having A_{734} = 0.70 ± 0.02, absorbance was recorded at 6 min. The results were expressed as trolox equivalent antioxidant capacity (TEAC). TEAC is defined as the mM concentration of a trolox solution whose antioxidant activity is equivalent to the activity of 1.0 mM and 1 mg/mL test solution for pure compounds and plant extracts, respectively. In order to find TEAC values, a separate concentration response curve for standard trolox solutions was prepared. α-Tocopherol was used as the positive control.

2.5. Total phenolic content (TPC)

TPCs of *Sideritis* extracts were determined using Folin-Ciocalteu reagent (FCR) according to the procedure reported by Singleton, Orthofer, and Lamuela-Raventos (1999) with some modifications. The extracts were dissolved in deionized water to provide a concentration of 500 μg/mL. 0.5 mL aliquot of extract or deionized water (control) was mixed with 0.5 mL of FCR by manual shaking for 10–15 s. After 3 min, 0.5 mL of saturated Na_2CO_3 solution was added and the solution was diluted to 5 mL with deionized water. The reaction mixture was kept in the dark for 2 h and the absorbance was measured at 760 nm. The