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Metabolite profiling and antioxidative activity of Sage (*Salvia fruticosa* Mill.) under the influence of genotype and harvesting period

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ABSTRACT

Two cultivated accessions of *Salvia fruticosa* Mill. were investigated and evaluated for their essential oil, phenolic composition and antioxidant activity, during different harvesting time. The essential oil and its major compound 1.8 cineole, presented their higher yields during the early summer harvesting. The advanced analytical LC–MS/MS method applied in this work led to the identification of thirty five compounds with rosmarinic acid, the diterpene artefact carnosol and several flavones and flavonols as the main phenolic constituents, the concentration of which varied largely from spring to autumn. The antioxidant activity of respective methanolic extracts was determined using the Ferric Reducing Ability of Plasma (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays, as a quality control tool. High positive correlations were observed between FRAP and ABTS antioxidant activities and total phenolic/flavonoid content, and particular phenolic constituents.

1. Introduction

Salvia fruticosa Miller, Lamiaceae (syn. *S. triloba* L.) is an endemic species of the east Mediterranean basin, occurring in Greece in coastal areas of the mainland and in Ionian and Aegean islands, commonly known as Greek, or Mediterranean wild sage (Karousou et al., 1998; Skoula et al., 1999). Its common Greek name is "faskomilo" or "alisfakia" and in the international trade it is known as sage. In folk medicine aerial parts of the plant are used in the form of herbal teas against cold symptoms as antiphlogistic of the mouth and throat, cough, abdominal pains etc. (Skoula et al., 1999; Pitarokili et al., 2003). Furthermore, several studies have shown that *S. fruticosa* essential oil and alcoholic or water extracts exhibited pharmacological properties, such as antibacterial, antimicrobial, antifungal, antioxidant, anti-cholinesterase, antiproliferative etc. (Pitarokili et al., 2003; Fu et al., 2013; Hani and Bayachou, 2014). Due to these properties, which are related to the manifold presence of active compounds, originated from different secondary metabolite pathways, *S. fruticosa* became an important aromatic/medicinal and commercial species.

1.8 cineole, camphor, α- and β-pinene and borneol are the major compounds of *S. fruticosa* leaf essential oil, which was shown to have a high variability in its chemical composition and is responsible for the characteristic aroma and flavor (Catsiotis and Iconomou, 1984; Skoula et al., 1999). Rosmarinic acid, carnosic acid, carnosol and methyl carnosate are the major phenolic compounds and effective antioxidants detected in *S. fruticosa* and in the close relative and well known herbal medicine *S. officinalis* (Pizzale et al., 2002). Skoula et al. (2000) reported that the total phenolic content and particularly those of rosmarinic acid varied significantly within three *S. fruticosa* populations from Crete. Previous studies have shown that the essential oil and polyphenolic composition is largely affected by the origin, environmental and growing conditions, the ontogenetic stage of the plant, the season of harvesting, genetic factors and others (Figueiredo et al., 2008; Cheynier et al., 2013). Similarly, the amounts of secondary metabolites, responsible for the antioxidant activity of *S. fruticosa*, are beside others associated to the plant growth stage and the harvesting time (Papageorgiou et al., 2008).

Abbreviations: DPPH, 2.2-diphenyl-1-picrylhydrazyl; ABTS, 2, 2-azinobis 3-ethylbenzothiazoline 6-sulfonic acid; FRAP, Ferric Reducing Antioxidant Power; GC–MS, gas chromatography-mass spectrometry; TPTZ, 2,4,6- tripyridyl-*s*-triazine; LC–MS, liquid chromatography-mass spectrometry; UPLC, ultraperformance liquid chromatography; BA, benzoic acid; CA, caffeic acid; RA, rosmarinic acid; Ap, apigenin; CHLA, chlorogenic acid; Qu3glc, querqetin 3-*O*-glucoside; Kmf3glc, kaempferol 3-*O*-glucoside.

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The antioxidant properties of plant extracts or even specific compounds are very important, since they may cope with the harmful role of free radicals, the formation of which accelerate the oxidation of lipids in biological systems and foods, and decrease food quality and the consumers acceptance. Provided that the bioactivity and the pharmacological properties of medicinal plants are related to the presence and the content of specific constituents, which are affected by intrinsic and external parameters, the standardization of the starting plant material is essential, since it is the most important factor in manufacturing herbal products (Garg et al., 2012). Cultivation, instead of collecting wild grown plants and the determination of the optimum harvesting season, which ensure high yields of the desired constituents, might contribute to obtain uniform plant material of constant high quality, and in accordance to different market's demands and specification.

The purpose of the present study was to investigate two cultivated *S. fruticosa* populations in the same pedo-climatic conditions and acquire comprehensive data of compounds present in the essential oil and their phenolic extracts. Additionally, we aim to assess the seasonal fluctuation of these compounds and the effect on antioxidant activity during the whole vegetative period. The combination of efficient genotype, well adapted to cultivation conditions and the determination of the proper harvesting period, by using state of the art analytical tools, will pave the way standardized, high quality plant material, for multiple uses in food, phytoherapy, cosmetic industry, etc.

2. Materials and methods

2.1. Plant material and harvesting season

Leaves from upper parts of 3 years old plants, from two cultivated populations of *S. fruticosa* populations, were collected in different phenophases, from April to October in 2014. Each sample was constituted from 10 plants, per accession. Germplasm from the initial populations was originated from different districts of Greece, (1) from S. Aegean islands and (2) from W. Peloponnese. The species was authenticated as *S. fruticosa* (syn. *S. triloba*) and voucher specimens were deposited at the Herbarium of the Department of Medicinal and Aromatic Plants of IPB&GR. The populations were cultivated at the experimental field of IPB&GR (40°34′35″ N 22°57′19″ E), and the plants were equally and in regular basis drip irrigated and hand weeded as well, during cultivation period. The soil properties at the experimental site were as follows: soil type: red loam, pH 7.73%, clay: 39.0, organic matter: 1.43%, P_2O_5 (ppm): 45, K_2O (ppm): 520. The climate at the cultivation region was characterized from mild winter, warm spring and summer with high relative humidity. The fresh plant material was dried in a shady and dry place at ambient temperature for fifteen days and then leaves were separated from the stem. Only leaves were used for the essential oil isolation and the extraction of phenolic compounds.

2.2. Chemicals

The *n*-alkanes (C7-C22) were purchased from Supelco (Bellefonte, PA, USA). The highest purity pentane used for the GC–MS was purchased from Panreac Quimica S.L.U (Barcelona, Spain). All reagents for LC–MS analysis (with LC–MS grade) and assays for the determination of antioxidant activity were purchased from Sigma-Aldrich (Steinheim, Germany). Water used in sample preparation and analysis was purified by a Milli-Q water purification system. The specific standards carnosol and carnosic acid were purchased from TransMIT PlantMetaChem (Giessen, Germany) and inserted in the analytical method as described below.

2.3. Essential oil isolation

The essential oil content was determined using the European Pharmacopoeia apparatus (Clevenger-type). Fifty grams of *S. fruticosa* dried leaves were subjected to hydrodistillation for 2.30 h, with a distillation rate 3–3.5 mL/min. Three distillations for each harvesting sample were done. The oil content was estimated on the basis of dry weight plant material (mL/100 g of dried leaves). The essential oils obtained were dried over anhydrous sodium sulphate and stored at 4–6 °C.

2.4. Analysis of essential oil

The essential oils were analyzed by GC–MS on a fused silica DB-5 column, using a Gas chromatograph 17A Ver. 3 interfaced with a mass spectrometer Shimadzu QP-5050A supported by the GC/MS Solution Ver1.21 software, using the method described previously (Sarrou et al., 2013). The conditions of analysis were: injection temperature: 260 °C, interface heating: 300 °C, ion source heating: 200 °C, EI mode: 70 eV, scan range: 41–450 amu, and scan time 0.50 s. Oven temperature programs: (a) 55–120 °C (3 °C/min), 120–200 °C (4 °C/min), 200–220 °C (6 °C/min) and 220 °C for 5 min and (b) 60–240 °C at 3 °C/min, carrier gas He, 54.8 kPa, split ratio 1:30. The relative content of each compound was calculated as percent of the total chromatographic area and the results are expressed as means of three replicates.

The identification of the compounds was based on comparison of their retention indices (RI) relative to *n*-alkanes (C_7-C_{22}), with corresponding literature data and by matching their spectra with those of MS libraries (NIST 98, Willey) (Adams, 1995).

2.5. Sample preparation and extraction of phenolic compounds

The samples were randomized and a part from each leaf sample was milled to a fine powder with a mill equipped with cooling system. 100 mg of the above sample (powdered leaf tissue) was weighed and transferred into 15 mL falcon tube. A volume of 4 mL 80% methanol was added to each sample. The samples and solvent were mixed by orbital shaker for 3 h at room temperature and the extraction proceeded overnight at 4 °C in the dark. The resulting solutions where filtered on a 0.22 μm PFTE membrane into a glass vial and analyzed as described below. Three replicates for each harvesting sample were done.

2.6. Phenolic metabolite analysis

The analysis of phenolic compounds was performed using the method described previously by Vrhovsek et al. (2012). Samples were directly injected after extraction.

Ultraperformance liquid chromatography was performed on a Waters Acquity UPLC system (Milford, MA) consisting of a binary pump, an online vacuum degasser, an autosampler, and a column compartment. Separation of the phenolic compounds was achieved on a Waters Acquity HSS T3 column 1.8 μm, 100 mm × 2.1 mm (Milford, MA, USA), kept at 40 °C. Mobile phase A was water containing 0.1% formic acid; mobile phase B was acetonitrile containing 0.1% formic acid. The flow was 0.4 mL/min, and the gradient profile was 0 min, 5% B; from 0 to 3 min, linear gradient to 20% B; from 3 to 4.3 min, isocratic 20% B; from 4.3 to 9 min, linear gradient to 45% B; from 9 to 11 min, linear gradient to 100% B; from 11 to 13 min, wash at 100% B; from 13.01 to 15 min, back to the initial conditions of 5% B. The injection volume of both the standard solutions and the samples was 2 μL. After each injection, the needle was rinsed with 600 μL of weak wash solution (water/methanol, 90:10) and 200 μL of strong wash solution (methanol/water, 90:10). Samples were kept at 6 °C during the analysis.