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Antioxidant activity and phenolic compounds in 10 selected herbs from Vrujci Spa, Serbia

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ABSTRACT

There is a growing research interest on investigation of appropriate alternatives of natural origin to synthetic additives used in food products. The aim of this study was to investigate correlation of chemical composition of the best known biomarker plants from the locality of Vrujci Spa, Serbia with their antioxidant activity *in vitro*, in terms of their potential usage as feedstock materials in the food industry. Total phenolic (TP), total tannins (TT) and total flavonoid (TF) contents were determined using spectrophotometric methods. Qualitative and quantitative analyses of major phenolics by high-performance liquid chromatography (HPLC) were also used. Major phenolic acids identified in analyzed species were chlorogenic, caffeic and ferulic acid, while predominant flavonoids were flavonols (hyperoside, rutin, isoquercitrin, quercetin) and, in lesser extent flavones (luteolin, apigenin and their 7-O-glucosides). Vitexin 2-O″-rhamnoside was found only in *Crataegus* species. Antioxidant capacity was estimated by the following methods: 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) test and ferric reducing antioxidant power (FRAP). Majority of investigated plants had high levels of phenolics and exhibited noteworthy antioxidant activity. Moreover, a positive linear correlation was found between TP and TT with antioxidant capacities regarding both methods used. The plants showing the highest antioxidant activities were *Veronica officinalis*, *Mentha pulegium* and *Fragaria vesca*. *Hypericum perforatum* demonstrated by far the most potent antioxidant activity, as well as TP and TT content. Our results indicate that, in addition to their traditional usage in folk medicine, these plants represent a valuable source of natural antioxidants, and thus may be considered as great potential for the food industry, representing possible alternatives to synthetic additives.

1. Introduction

Plant species belonging to several botanical families, such as Lamiaceae, Rosaceae, Fabaceae, Moraceae, Canabinaceae, Plantaginaceae and Hypericaceae were used in ethnobotanical field survey from the locality of Vrujci Spa, Serbia. All of the investigated plants were biomarkers of aforementioned area, as well as medicinal plants popular in Serbian folk medicine for the treatment of different diseases such as diabetes, rheumatism, urinary conditions, ulcerations, hypertension, atherosclerosis, etc. (Bisset and Wichtl, 2001; Sarić, 1989). Among collected materials, 10 best known plants were chosen for further investigation: *Mellilotus officinalis* (Yellow sweet clover), *Nepeta nuda* (Hairless cat-mint), *Morus nigra* (Black mulberry), *Humulus lupulus* (Hop), *Hedera helix* (Common ivy), *Craetegus* spp. (Hawthorn), *Veronica officinalis* (Common speedwell), *Mentha pulegium* (European pennyroyal), *Fragaria vesca* (Wild strawberry) and *Hypericum perforatum* (St. John's wort). Moreover, all of the investigated plants, with the exception of *N. nuda*, *M. nigra* and *M. pulegium* have monographs in European or national pharmacopoeias and/or their usage has been approved by German Commission E for various indications (Bisset and Wichtl, 2001).

Increasing demands of consumers on safety issues regarding the use of synthetic food additives, imposes the need on search for new sources of plant's antioxidants, as feasible and natural alternatives to synthetic antioxidants. In this respect, there is a growing research interest in plants abundant in active secondary metabolites with

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antioxidant properties that could be applied in the food industry. Such compounds could prevent oxidation reactions and decomposition of oxidation products, as major causes of deterioration of various food products. Antioxidant activity of herbs is generally studied with regard to total phenolic content, using several methods of assessing antioxidant activity (Si et al., 2006).

In this context, the aim of the current study was to establish correlation of chemical composition and antioxidant activity of the investigated plants, in terms of their potential usage as feedstock materials in the food industry. Although some of the plants included in our investigation have been described elsewhere in terms of their chemical profile and/or their antioxidant capacities, to the best of our knowledge this is the first study focusing on the correlation thereof. In addition, we found it appropriate to evaluate samples from a specific locality, i.e. Vrujci Spa, Serbia, bearing in mind that environmental conditions can be effective on chemical composition of the plants, especially with respect to antioxidant contents (Simirgiotis, 2013).

Subsequently, objectives of this study were to: (1) evaluate and compare antioxidant capacity by two common antioxidant activity methods, (2) determine total phenolic (TP) content, total tannins (TT) and total flavonoid (TF) content of the investigated plants; (3) identify and quantify major phenolic compounds present in the tested species by RP-HPLC; (4) determine the relationship between antioxidant activity and phenolic compounds content.

2. Materials and methods

2.1. General

Analytical grade reagents acetate buffer, 2,4,6-tripyridyl-s-triazine (TPTZ), HCl, $FeCl_3 \cdot 6H_2O$, Folin–Ciocalteu reagent, phosphomolybdotungstic reagent, acetone, ethyl acetate, sodium bicarbonate, 1,1′-diphenyl-2-picrylhydrazyl (DPPH), methanol were purchased from Sigma–Aldrich. Acetonitrile, water, and methanol (HPLC grade) were purchased from Merck. Reference HPLC standards, hyperoside, rutin, isoquercitrin, quercetin dihydrate, chlorogenic acid, caffeic acid, ferulic acid, luteolin, apigenin, luteolin 7-O-gucoside, apigenin 7-O-gucoside, vitexin 2-O″-rhamnoside (purity > 99%) were purchased from Extrasynthese.

2.2. Plant material

Field researches of the plants in the area of Vrujci Spa have been carried out on several occasions during the year 2010 (spring, summer and autumn aspect). Aerial parts of plants and leaves were collected during the time of flowering, and the fruits during the autumn months. The plant material was air-dried and then processed in different ways, depending on the method used for qualitative/quantitative chemical analysis and antioxidant activity assays (see below). Plant material was verified and adequate voucher specimens were deposited at has been deposited at the Institute for Medicinal Plant Research "Dr Josif Pančić", Serbia.

2.3. Determination of total phenolic, tannins and flavonoids content

TP content was determined using Folin–Ciocalteu reagent. One hundred microliters of the MeOH solution of the precisely measured weight of investigated plants 1–10 (2.54, 2.58, 2.25, 4.03, 4.80, 2.13, 4.62, 1.47, 1.58, 15.05 mg/mL respectively) were mixed with 0.75 mL of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at 22 °C for 5 min; 0.75 mL of sodium bicarbonate (60 g/L) solution was added to the mixture. After 90 min at 22 °C, absorbance was measured at 725 nm. Gallic acid (0–100 mg/L) was used for calibration of a standard curve. The calibration curve showed the linear regression at $r > 0.99$, and the results were expressed as milligrams of gallic acid equivalents per gram of plant extracts dry weight (mg GAE/g DW). Triplicate measurements were taken, and data were presented as mean ± standard deviation (SD).

The percentage content of TT was calculated using the method described in the European Pharmacopoeia (Ph Eur 6.0, 2008). Briefly, decoctions prepared from the investigated extracts were treated with phosphomolybdotungstic reagent in alkaline medium after and without treatment with hide powder. The absorbance was measured by UV-VIS Spectrophotometer HP 8453 (Agilent Technologies, USA), at λ_{max} 760 nm. The percentage content of tannins expressed as pyrogallol (%, w/w), was calculated from the difference in absorbance of total polyphenols (A_1) and polyphenols not adsorbed by hide powder (A_2), using following expression:

$$\frac{62.5\,(A_1 - A_2) \times m_2}{A_3 \times m_1}$$

where m_1 represents mass of the sample to be examined, in grams; and m_2 and A_2 mass, in grams and absorbance of pyrogallol, respectively. The results represent the mean of three determinations.

The percentage content of TF expressed as hyperoside was calculated using the method described in the European Pharmacopoeia (Ph Eur 6.0, 2008). Shortly, the sample was extracted with acetone/HCl under reflux condenser; the $AlCl_3$ complex of flavonoid fraction extracted by ethyl acetate was measured by UV-VIS Spectrophotometer HP 8453 at 425 nm. The content of flavonoid, expressed as hyperoside percentage, presented the mean of three determinations.

2.4. HPLC analyses

"Fingerprinting" of the investigated phenolic compounds was achieved by an Agilent Technologies 1200 HPLC machine, equipped with Lichrospher® 100 RP 18e column, applying gradient elutions of two mobile phases, i.e. "A/B" ("A" – consisting of v/v 500.0 mL of water and 9.8 mL of 85% phosphoric acid, and "B" – being a pure acetonitrile) at flow-rates of 1 mL/min, with photodiode-array (PDA) detection (UV at 360 nm), always within 70 min. Winning combinations were 89–75% A (0–35 min); 75–60% A (35–55 min); 60–35% A (55–60 min) and 35–0% A (60–70 min). The amounts of investigated samples were 1.0000 g/25 mL methanol. Namely, 1.0000 g of homogenized, grinded plant material was measured into a flask, 10 mL of methanol was added and the sample was heated under reflux condenser for 30 min, then filtered into 25 mL volumetric flask. The procedure was repeated 2 more times with 10 and 5 mL of methanol, respectively. Volumetric flask was then supplemented with methanol to the mark. Prior to injection, samples were filtered through PTFE membrane filter. For standards used in the investigation, the concentrations were: 0.01 mg/mL for ferulic acid; 0.02 mg/mL for rutin, hyperoside, quercetin dehydrate, chlorogenic acid and caffeic acid; 0.03 mg/mL for apigenin, 0.04 mg/mL for isoquercitrin, luteolin, luteolin 7-O-gucoside and vitexin 2-O″-rhamnoside; 0.06 mg/mL for apigenin 7-O-gucoside). The volume of standard solutions being injected, as well as for the tested sample extracts, was 4 μL.

Identification was based on retention times and overlay curves. Once spectra matching succeeded, results were confirmed by spiking with respective standards to achieve a complete identification by means of the so-called peak purity test. Those peaks not fulfilling these requirements were not quantified. Quantification was performed by external calibration with standards.