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ARTICLE

# Plantain (*Plantago* L.) Species as Novel Sources of Flavonoid Antioxidants

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To examine the antioxidant properties of methanol extracts of selected *Plantago* species (*P. argentea* Chaix., *P. holosteum* Scop., *P. major* L., *P. maritima* L., and *P. media* L.), various assays that measure free radical scavenging ability were carried out: DPPH, hydroxyl radical, superoxide anion, and nitric oxide scavenger capacity tests, reducing power (FRAP) assay, and $Fe^{2+}$/ascorbate induced lipid peroxidation. In all of the tests extracts showed a potent antioxidant effect compared with BHT, a well-known synthetic antioxidant, and the extract of *P. major*, accepted as an official remedy. Besides, in examined extracts the total phenolic amount (ranging from 38.43 to 70.97 mg of GAE/g of dw) and the total flavonoid content (5.31−13.10 mg of QE/g of dw) were determined. Furthermore, the presence and content of selected flavonoids (luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, luteolin, apigenin, rutin, and quercetin) were studied using LC-MS/MS technique. LC-MS/MS analysis showed noticeable qualitative and quantitative differences between the species according to which the examined *Plantago* species could be regarded as a possible new source of natural antioxidants. In this study three of the species examined, *P. maritima*, *P. argentea*, and *P. holosteum*, have been analyzed for the first time.

**KEYWORDS: *Plantago*; antioxidant activity; flavonoids; LC-MS/MS**

## INTRODUCTION

The genus *Plantago* L. (Plantaginaceae) comprises about 275 species that can be found all over the world. The popularity of these plants for their wide application as herbal remedies has been known from ancient times, and it is due to their impressive variety of curative properties: astringent, styptic, antimicrobial, expectorant, diuretic, and demulcent (*1*, *2*). Furthermore, several previous studies have indicated that certain *Plantago* species reveal considerable cytotoxic effect on cancer cell lines (*3*) and antiviral (*4*), antiinflammatory (*5*), analgesic (*5*), and antispasmodic activities (*6*). Concerning the antioxidant activity of *Plantago* species, there are also some reports that witness this benefit (*7*−*9*). Additionally, phytochemical studies have shown that the *Plantago* genus contains a great amount of natural products such as iridoids, flavonoids, tannins, triterpenes, saponins, and sterols (*10*−*12*). On the other hand, some *Plantago* species are also included in the diet and are usually consumed as fresh salads (*1*, *2*, *9*).

Within the *Plantago* genus, the most renowned species, used in both traditional and modern medicine, is certainly *P. major* L. (common plantain, great plantain) (*10*). Medicinal applications of some other species such as *P. lanceolata* L. and *P. media* L. leaves (*13*) or *P. afra* L., *P. indica* L., *P. ovata* Forsk., and *P. asiatica* L. seeds are also known (*14*). To the best of our knowledge, no previous studies have been undertaken the biological activity of *P. media* L., *P. holosteum* Scop., *P. maritima* L., and *P. argentea* Chaix. Furthermore, there are no literature data available about the chemical constituents of *P. holosteum*, *P. maritima*, and *P. argentea* except for only one report that points out the qualitative content of a few flavonoids, performed by the TLC assay (*15*).

The reason to examine the antioxidant properties of the species mentioned above is initiated from the fact that the antioxidant supplements can contribute to the prevention of certain human diseases by reducing the levels of free radicals and allowing them to perform useful biological functions without too much damage (*16*). Additionally, antioxidant supplements have many healing effects on the human body with insignificant damage rate. Free radicals, such as reactive oxygen species (ROS), can affect DNA molecules and oxidize amino acids or polyunsaturated fatty acids in lipid membranes and so cause severe cell damage. Free radicals are associated with a risk of various diseases, such as cancer, cardiovascular diseases, diabetes, or atherosclerosis (*17*−*20*). Flavonoids, a class of polyphenols, have therapeutic properties on some of these diseases and may prevent them, due to their antiradical property toward ROS. Flavonoids act as scavengers of intermediate peroxyl and alkoxyl radicals and chelating agents for metal ions, which are of major importance for the initiation stage of radical reactions (*21*). One of the very rich sources of

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**Table 1.** LC-MS/MS Data for Standard Compounds **1**−**4**[a]

| | LC-MS/MS conditions for quantification of standard compd **1**−**4** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| compd | retention time (min) | acquisition mode | ion polarity | species | precursor ion (*m*/*z*) | product ion (*m*/*z*) | collision energy (V) | fragmentor voltage (V) |
| **1** | 16.5 | MS2SIM | positive | $(M + H)^+$ | 449 | | | 80 |
| | | | | $(M + Na)^+$ | 471 | | | |
| **2** | 18.5 | MRM | positive | $(M + H)^+$ | 433 | 271 | 10 | 100 |
| **3** | 21.4 | MRM | negative | $(M - H)^-$ | 285 | 133 | 35 | 80 |
| **4** | 22.5 | MRM | positive | $(M + H)^+$ | 271 | 153 | 40 | 80 |

[a] **1**, luteolin-7-*O*-glucoside; **2**, apigenin-7-*O*-glucoside; **3**, luteolin; **4**, apigenin.

**Table 2.** LC-MS/MS Data for Standard Compounds **5** and **6**[a]

| | LC-MS/MS conditions for quantification of standard compd **5** and **6** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| compd | retention time (min) | acquisition mode | ion polarity | species | precursor ion (*m*/*z*) | product ion (*m*/*z*) | collision energy (V) | fragmentor voltage (V) |
| **5** | 4.5 | MRM | negative | $(M - H)^-$ | 609 | 300 | 50 | 140 |
| **6** | 7.2 | MRM | negative | $(M - H)^-$ | 301 | 151 | 30 | 140 |

[a] **5**, rutin; **6**, quercetin.

flavonoids is food of plant origin such as fruits, vegetables, teas, and wine.

Antioxidant properties of natural products can be tested using various assays that measure free radical scavenging ability (*22*). With the aim of evaluating the antioxidant potential of methanol extracts of *P. argentea*, *P. holosteum*, *P. maritima*, and *P. media*, several assays related to free radicals ($DPPH^•$), reactive oxygen species ($HO^•$, $O_2^{•-}$), reactive nitrogen species ($NO^•$), lipid peroxidation (LP), and reducing power (FRAP) assay have been undertaken. Furthermore, the quantitative content of flavonoids luteolin-7-*O*-glucoside (**1**), apigenin-7-*O*-glucoside (**2**), luteolin (**3**), apigenin (**4**), rutin (**5**), and quercetin (**6**) have been studied, using an LC-MS/MS technique. To compare the activities of the extracts mentioned above, a methanol extract of *P. major* was also included in this study, although the benefits of this species are well-known (*10*).

## MATERIALS AND METHODS

**Chemicals.** The following reagents were purchased from Sigma-Aldrich Chemicals, Steinheim, Germany: gallic acid, quercetin (**6**), 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), 2-deoxy-D-ribose, NADH, and phenazine methosulfate (PMS). Folin−Ciolcateu (FC) reagent was provided by Fisher Scientific, Leicestershire, U.K. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2-thiobarbituric acid (TBA), sulfanilamide, and rutin (**5**) were obtained from Fluka Chemie GmbH (Buchs, Switzerland). Trichloroacetic acid was purchased from Lach-Ner sro (Neratovice, Czech Republic), nitroblue tetrazolium (NBT) from Alfa Aesar (Karlsruhe, Germany), and sodium nitroprusside from Reanal (Budapest, Hungary). *N*-(1-Naphthyl)ethylenediamine dihydrochloride (NEDA), ascorbic acid, and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were acquired from Merck (Darmstadt, Germany). Tween-80 was obtained from J. T. Baker (Deventer, The Netherlands), whereas luteolin (**3**) and apigenin (**4**) were from ChromaDex (Santa Ana, CA). Luteolin-7-*O*-glucoside (**1**) and apigenin-7-*O*-glucoside (**2**) were provided by Carl Roth GmbH (Karlsruhe, Germany). All other reagents used in this study were of analytical grade.

**Plant Material and Extract Preparation.** The aerial parts of *P. media* and *P. holosteum* were collected in June 2006 from the mountain of Kopaonik, Serbia, those of *P. maritima* in June 2007 in Padej, Serbia, those of *P. argentea* in April 2008 in Rtanj, Montenegro, and those of *P. major* in June 2008 from the mountain of Fruška Gora, Serbia. The voucher specimens (*P. media*, no. 2-1841; *P. holosteum*, no. 2-1842; *P. maritima*, no. 2-1827; *P. argentea*, no. 2-1833; *P. major*, no. 2-1830) were prepared and identified by Goran Anačkov and deposited at the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), Faculty of Sciences, University of Novi Sad.

Air-dried and smoothly ground herbal samples weighing 30 g were extracted by maceration with 80% aqueous methanol during 72 h at room temperature. After filtration, solvent was evaporated in vacuo at 45 °C, and crude residue was dissolved in hot, distilled water (1 g/mL). With the aim of removing nonpolar compounds, the extracts were washed exhaustively with petroleum ether (fraction 40−60 °C) and concentrated to dryness under vacuum, yielding 33.8, 10.9, 11.3, 10.1, and 17.6% for *P. argentea*, *P. holosteum*, *P. major*, *P. maritima*, and *P. media* extracts, respectively. Dried extracts were dissolved in 80% aqueous methanol for evaluation of the antioxidant activity to obtain 20% (w/v) stock solutions. Also, dried extracts were dissolved in a mixture of 1% aqueous formic acid and methanol (in a ratio of 9:1) for HPLC-MS analysis to obtain 2% (w/v) stock solutions.

**LC-MS/MS Analysis.** The analyses were performed on an Agilent Technologies 1200 series UPLC with 6410B series electrospray ionization triple-quadrupole MS/MS. The separation was achieved using a Zorbax Eclipse XDB-C18 RR 4.6 mm × 50 mm × 1.8 μm (Agilent Technologies) reversed-phase column held at 45 °C. ESI parameters were as follows: nebulizer gas ($N_2$) temperature, 350 °C; flow, 9 L/min; pressure, 40 psi; capillary voltage, 4 kV. The injection volume for all samples was 5 μL. The binary mobile phase consisted of 1% aqueous formic acid (A) and methanol (B) and was delivered at a flow rate of 0.6 mL/min.

Because the optimal conditions for different compounds required different MS acquisition modes or polarities, the quantification was performed in two runs, the first for simultaneous quantification of luteolin-7-*O*-glucoside (**1**), apigenin-7-*O*-glucoside (**2**), luteolin (**3**), and apigenin (**4**) and the second for rutin (**5**) and quercetin (**6**). Gradient elution was performed using the following solvent gradient for **1**−**4**: starting with 10% B, reaching 40% B in 15 min, then 90% B in 25 min, holding until 30 min, with post-time of 5 min. A different gradient mode was used for quantification of compounds **5** and **6**: starting with 30% B, reaching 90% B in 15 min, and holding until 18 min, with post-time of 3 min. Besides the general parameters mentioned above, other optimized parameters for LC-MS/MS analysis are given in **Tables 1** and **2**. All compounds were quantified in multiple reaction monitoring (MRM) mode, except **1**, for which single-stage MS (MS2SIM) mode was applied.

All extracts used for LC-MS/MS quantification were dissolved in starting mobile phase solvent to the concentration of 20 mg/mL. All used standards were dissolved to prepare stock solutions of 1 mg/mL. These stock solutions were serially diluted, giving working standard solutions with concentration ranging from 0.05 to 12.5 μg/mL, which were used for construction of the calibration curves. Concentrations of standard compounds in extracts were determined from the peak areas by using the equation for linear regression obtained from the calibration curves ($R^2 > 0.995$).

**Determination of Total Phenolic Content.** Determination of the total phenolic content was performed according to method of Singleton

et al. (*23*), customized for 96-well microplates. Extracts were used in concentrations of 0.125, 0.25, and 0.5 mg/mL. Gallic acid, prepared in 11 concentrations ranging from 1.25 to 100 μg/mL, was used as a standard.

Thirty microliters of each extract or standard solution, except in blank probe when only the solvent was used, was added to 150 μL of 0.1 mol/L FC reagent and mixed with 120 μL of sodium carbonate (7.5%) after 10 min. Absorbance at 760 nm was read after 2 h. The phenolics concentration was determined by comparison with the standard calibration curve of gallic acid, and results are presented as a mean value of triplicate tests. The total phenol value was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight (dw).

**Determination of Total Flavonoid Content.** The aluminum chloride colorimetric method described by Chang et al. (*24*), adapted for 96-well microplates, was used to determine the total content of flavonoids. Test samples were prepared in concentrations 1.0, 2.0, and 4.0 mg/mL, whereas quercetin solutions were prepared ranging from 1.25 to 100 μg/mL and used as a standard.

Thirty microliters of extract or standard solution was diluted with 90 μL of methanol, and 6 μL of 10% aluminum chloride (substituted with distilled water in blank probe), 6 μL of 1 mol/L potassium acetate, and 170 μL of distilled water were added. Absorbance at 415 nm was determined after 30 min. All samples were made in triplicate, and mean values of flavonoid content are expressed as milligrams of quercetin equivalents (QE) per gram of dw calculated according to the standard calibration curve.

**Reduction of DPPH Radical.** Plant extracts were tested for the scavenging effect on DPPH radical according to the method of Soler-Rivas et al. (*25*), adapted for 96-well microplates. As a positive control synthetic antioxidant BHT was used. Ten microliters of examined extract solutions, in series of different concentrations (0.08−10.0 mg/mL), was added to 100 μL of 90 μmol/L DPPH solution in methanol, and the mixture was diluted with 190 μL of methanol. In the control, the exact amount of extract was substituted with solvent, and in the blank probe, only methanol (290 μL) and extract (10 μL) were mixed. After 1 h, measurements of absorbance were done at 515 nm.

The antioxidant activity of the extracts was expressed as concentration of extract that inhibited DPPH radical formation by 50% ($IC_{50}$).

**Hydroxyl Radical Scavenger Capacity.** The deoxyribose assay (*26*) was applied to measure $HO^{\bullet}$ scavenger capacity. A 50 μmol/L solution of 2-deoxy-D-ribose in phosphate buffer pH 7.4 (0.1 mL) was mixed with 20 μL of extract (concentration ranging from 1.6 to 150 mg/mL) or solvent in control, 0.1 mL $H_2O_2$ (0.015%), and 0.1 mL of $FeSO_4$ (10 mmol/L) and diluted with 2.7 mL of phosphate buffer, pH 7.4. Three milliliters of phosphate buffer and 20 μL of extract were added in the blank probe. After incubation at 37 °C for 1 h, a 0.1 mol/L EDTA solution (0.2 mL) was added to all samples. Thiobarbituric acid reactivity was developed by adding 2 mL of aqueous mixture containing TBA (3.75 mg/mL), $HClO_4$ (1.3%), and trichloroacetic acid (0.15 g/mL), followed by heating at 100 °C for 10 min. The absorbance of the cooled mixtures was measured at 532 nm. As a positive control, BHT was used. All samples and the control were made in triplicate. $IC_{50}$ values were determined.

**Superoxide Anion Scavenger Capacity.** The capability of extracts to neutralize superoxide anion formed by the reduction of nitroblue tetrazolium (NBT) with NADH mediated by phenazine methosulfate (PMS) under aerobic conditions was conducted according to the method of Nishikimi et al. (*27*). The mixture of 0.2 mL of NBT (144 μmol/L), 10 μL of extract (concentration ranging from 0.6 to 20 mg/mL, substituted with solvent in control), 0.1 mL of NADH (0.68 mmol/L), and freshly prepared PMS (60 μmol/L) was diluted with 1.1 mL of phosphate buffer, pH 8.3. Blank probe was prepared by mixing 1.5 mL of buffer and 10 μL of extract. Absorbance was measured at 560 nm after 5 min. All samples and the control were made in triplicate. The determined $IC_{50}$ values were compared to the $IC_{50}$ values of the standard used as positive control (BHT).

**NO Scavenger Capacity.** Test of nitric oxide radical scavenging capacity was based on method of Green et al. (*28*). The reaction mixture containing sodium nitroprusside (10 mmol/L, 0.5 mL), phosphate buffer, pH 7.4 (0.5 mL), and extract (30 μL, concentration ranging from 15 to 200 mg/mL) or standard solution (BHT) was incubated at 25 °C for 90 min. One milliliter of buffer and 30 μL of extract were added in the blank probe. After incubation, an aliquot (1 mL) of solution prepared by mixing equal amounts of sulfanilamide (2% in 4% phosphoric acid) and *N*-(1-naphthyl)ethylenediamine dihydrochloride (0.2%) was added to the reaction mixture and allowed to stand for 3 min. The absorbance of these solutions was measured at 546 nm against appropriate blanks. All samples and control were made in triplicate. $IC_{50}$ values were determined.

**Lipid Peroxidation.** The extent of $Fe^{2+}$/ascorbate-induced LP was determined by TBA assay (*29*), using as a substrate polyunsaturated fatty acid methyl esters (FAME), obtained from linen semen by Soxhlet extraction and subsequent transesterification with methanol.

FAME were added to phosphate buffer, pH 7.4, in the presence of 0.25% Tween-80 to obtain a 0.035% suspension. This suspension (3.0 mL) was mixed with 20 μL of $FeSO_4$ (4.58 mmol/L), 20 μL of ascorbic acid (87 μmol/L), and 20 μL of extract (concentration ranging from 0.6 to 20 mg/mL) or solvent in control; 3.0 mL of phosphate buffer and 20 μL of extract were added in the blank probe. After incubation at 37 °C for 1 h, a 3.72% EDTA solution (0.2 mL) was added to all samples followed by 2 mL of an aqueous mixture containing TBA (3.75 mg/mL), $HClO_4$ (1.3%), and trichloroacetic acid (0.15 g/mL). Following heating at 100 °C for 15 min, cooled mixtures were centrifuged at 1866*g* for 15 min, and absorbance was measured at 532 nm. As a positive control, BHT was used. All samples and control were made in triplicate. $IC_{50}$ values were determined.

**Reducing Power (FRAP) Assay.** To evaluate the reducing power of extracts, the ferric ion reducing antioxidant power (FRAP) assay (*30*), modified for 96-well microplates, was undertaken. Extracts were prepared in concentrations of 0.5, 1.0, and 2.0 mg/mL, whereas ascorbic acid ranging from 1.25 to 160 μg/mL was used to create a standard curve.

FRAP reagent was prepared by mixing 10 mmol/L 2,4,6-tripyridyl-*s*-triazine (TPTZ) in 40 mmol/L HCl, 0.02 mol/L $FeCl_3$, and acetate buffer, pH 3.6, in a ratio of 1:1:10, respectively. Following addition of extract or ascorbic acid (10 μL) to 290 μL of FRAP reagent (substituted with distilled water in the blank probe), absorbance at 593 nm was determined after 6 min. All samples were made in triplicate, and mean values of reducing power were expressed as milligrams of ascorbic acid equivalents (AAE) per gram of dw, calculated according to the standard calibration curve.

**Statistical Analysis.** Percent of inhibition achieved by different concentration of extracts was calculated by the following equation in performed antioxidant assays: $I(\%) = (A_0 - A)/A_0 \times 100$, where $A_0$ was the absorbance of the control reaction and $A$ was the absorbance of the examined samples, corrected for the value of the blank probe. Corresponding inhibition−concentration curves were drawn using Origin software, version 8.0, and $IC_{50}$ values (concentration of extract that inhibited DPPH, $O_2^{\bullet-}$, $HO^{\bullet}$ and $NO^{\bullet}$ radicals or MDA formation by 50%) were determined. For antioxidant assays and extract composition determinations, all of the results were expressed as mean ± SD of three different trials.

A comparison of the group means and the significance between the groups were verified by one-way ANOVA. Statistical significance was set at $p < 0.05$. Correlation between total phenolic and flavonoid content and antioxidant activity was established by regression analysis (95% confidence interval).

## RESULTS AND DISCUSSION

**LC-MS/MS Analysis.** The quantification of chosen flavonoids in *Plantago* extracts was carried out using an LC-MS/MS technique. As preferred acquisition methods for accurate quantification, MRM and MS2SIM modes were applied. This type of analysis provides high sensitivity and specificity, due to the fact that only ions specific to analytes of interest are monitored.

The content of standard compounds is given in **Table 3**. Accordingly, compound **1** (luteolin-7-*O*-glucoside) was found to be present in all species, except *P. media*, and foremost in *P. holosteum* and *P. major*. Apigenin-7-*O*-glucoside (**2**) was detected in all examined extracts, with *P. argentea* having the highest content (117.77 μg/g of dw). A considerable amount of luteolin (**3**) was determined in *P. argentea*, *P. holesteum*, and *P. maritima* (121.08, 52.63, and 100.08 μg/g of dw, respectively), whereas apigenin (**4**) was present only in *P. argentea* (25.99 μg/g of dw) and *P. media* (traces). A very low concentration of rutin (**5**) was

**Table 3.** Determined Concentrations[a] of Selected Flavonoids **1**−**6**[b] in Examined *Plantago* Extracts

| | content of selected flavonoids in examined *Plantago* extracts (μg/g of dw) | | | | | |
|---|---|---|---|---|---|---|
| extract | **1** | **2** | **3** | **4** | **5** | **6** |
| *P. aregentea* | 80.07 ± 0.21 b | 117.77 ± 0.24 e | 121.08 ± 5.63 c | 25.99 ± 0.23 b | 1.55 ± 0.17 c | nd[c] |
| *P. holosteum* | 151.26 ± 0.41 c | 28.37 ± 0.68 d | 52.63 ± 1.12 a | nd | tr a[d] | nd |
| *P. major* | 213.58 ± 4.84 d | 7.66 ± 0.26 b | nd | nd | 0.80 ± 0.09 b | tr |
| *P. maritima* | 14.54 ± 0.20 a | 0.80 ± 0.05 a | 100.08 ± 0.27 b | nd | tr a | nd |
| *P. media* | nd | 9.80 ± 0.02 c | nd | tr a | 0.68 ± 0.08 b | nd |

[a] Values are means ± SD of three measurements. Means within each column with different letters (a−e) differ significantly ($p < 0.05$). [b] **1**, luteolin-7-*O*-glucoside; **2**, apigenin-7-*O*-glucoside; **3**, luteolin; **4**, apigenin; **5**, rutin; **6**, quercetin. [c] nd, not detected. [d] tr, trace (flavonoid concentration was below concentration range used for calibration curve, <0.05 μg/g).

determined in all species, and quercetin (**6**) was present in traces only in *P. major*. Evidently, all investigated *Plantago* species could be classified into flavone (flavone glycosides) chemotypes, because flavonols, quercetin and rutin, were found only sparingly and in very low amounts. Specific flavonoid composition was noted in *P. maritima* with a high content of luteolin, followed by its glucoside. Luteolin and its glucoside are also found as dominant in *P. argentea* together with the high portion of apigenin and apigenin glucoside.

The obtained results for *P. major* were in good correlation with the data previously reported (*12*), where the presence of compounds **1** and **2** and the absence of compounds **4** and **5** were also demonstrated.

However, a great disagreement between the only report about flavonoid contents in *P. holosteum*, *P. maritima*, and *P. argentea* and our results is obvious. Namely, the authors (*15*) reported the presence of glucosides and galactoside belonging to flavonol type: quercitrin, isoquercitrin, and hyperoside in the above-mentioned species. This could be the consequence of a different technique used for their determination. Thus, the advantage of LC-MS/MS over TLC technique (*15*) is evident, with LC-MS/MS providing high sensibility and specificity for the identification and quantification of certain compounds.

Remedial activity of analyzed extracts can be assumed by comparing determined concentrations of flavonoids in extracts with applied concentrations of same natural compounds in different biochemical assays. A previous paper (*3*) justifies that luteolin-7-*O*-glucoside (**1**) was the most active on breast adenocarcinoma cell line (MCF-7), with a $GI_{50}$ (concentration required to inhibit cell growth by 50%) value of 40 μg/mL, whereas luteolin (**3**) was the most active in the melanoma cell line (UACC-62) ($GI_{50}$ = 10 μg/mL). Thus, established concentrations of compounds **1** and **3** in the examined extracts are sufficient for cell growth inhibition, and it can be assumed that these extracts have anticancer activity. Similarly, the use of these extracts as antispasmodic agents can be implicated according to the content of luteolin (**3**), which is adequate for antispasmodic effect described in a paper by Fleer and Verspohl (*6*).

**Total Phenolic and Flavonoid Content.** The investigated extracts were analyzed for total phenolic and flavonoid content, compounds that are largely responsible for the antioxidant activity of plant extracts (*21*). The amount of total phenolics varied in species and ranged from 38.43 to 70.97 mg of GAE/g of dw (**Table 4**). Significant differences were not found in total phenolic content in comparisons between *P. holosteum*, *P. maritima*, and *P. media* ($p > 0.05$), as well as between *P. argentea* and *P. major* ($p > 0.05$).

Regarding the total flavonoid content (**Table 4**), the highest amount was present in *P. holosteum* (13.10 mg of QE/g of dw) and the lowest in *P. major* (5.31 mg of QE/g of dw). Significant differences in total flavonoid content were found among all species ($p < 0.05$), except between *P. holosteum* and *P. maritima* ($p > 0.05$).

**Table 4.** Total Phenolic and Flavonoid Contents[a] in Examined *Plantago* Extracts

| extract | phenolics (mg of GAE/g of dw) | flavonoids (mg of QE/g of dw) |
|---|---|---|
| *P. argentea* | 38.43 ± 2.46 a | 6.77 ± 0.26 b |
| *P. holosteum* | 68.76 ± 3.08 b | 13.10 ± 0.67 d |
| *P. major* | 42.62 ± 1.04 a | 5.31 ± 0.80 a |
| *P. maritima* | 63.32 ± 3.57 b | 12.93 ± 1.08 d |
| *P. media* | 70.97 ± 3.85 b | 8.30 ± 0.58 c |

[a] Values are means ± SD of three measurements. Means within each column with different letters (a−d) differ significantly ($p < 0.05$).

**Table 5.** $IC_{50}$ Values[a] for Evaluated Antioxidant Assays and Reducing Power of Examined Extracts and BHT

| | $IC_{50}$ values for scavenging activity of radical species | | | |
|---|---|---|---|---|
| extract | $DPPH^{\bullet}$ (μg/mL) | $HO^{\bullet}$ (μg/mL) | $O_2^{\bullet -}$ (μg/mL) | $NO^{\bullet}$ (mg/mL) |
| *P. argentea* | 7.38 ± 0.33 d | 120.47 ± 2.99 a | 50.04 ± 4.84 c | 0.34 ± 0.03 a |
| *P. holosteum* | 6.28 ± 0.13 b | 127.19 ± 6.85 a | 73.26 ± 2.46 e | 0.67 ± 0.06 b |
| *P. major* | 5.35 ± 0.29 a | 338.60 ± 9.81 e | 25.70 ± 1.52 a | 0.34 ± 0.07 a |
| *P. maritima* | 6.79 ± 0.16 c | 187.98 ± 8.04 b | 33.23 ± 1.39 b | 0.34 ± 0.06 a |
| *P. media* | 5.77 ± 0.05 a | 271.08 ± 7.63 d | 56.20 ± 3.79 d | 1.48 ± 0.20 c |
| standard, BHT | 8.28 ± 0.50 d | 233.68 ± 19.28 c | na[b] | na |

[a] Values are means ± SD of three measurements. Means within each column with different letters (a−e) differ significantly ($p < 0.05$). [b] na, 50% inhibition not achieved.

**Antioxidant Activity of Extracts.** Proven antioxidant activity can be manifested in a wide variety of actions, such as inhibition of oxidizing enzymes, chelation of transition metals, transfer of hydrogen or single electron to radicals (ROS), singlet oxygen deactivation, or enzymatic detoxification of ROS. Therefore, the total antioxidant activities should be evaluated through different methods to extensively characterize the antioxidant potential of pure compounds or extracts. Thus, our extracts were examined with regard to scavenging capacity toward DPPH, hydroxyl, superoxide anion, and nitric oxide radicals, ability to prevent lipid peroxidation, and reducing power. Determined $IC_{50}$ values are shown in **Tables 5** and **6**.

Four extracts showed considerable $DPPH^{\bullet}$ scavenger activity with $IC_{50}$ values ranging from 5.35 μg/mL (*P. major*) to 6.79 μg/mL (*P. maritima*), which was still significantly lower ($p < 0.05$) than the $IC_{50}$ of synthetic antioxidant BHT ($IC_{50}$ = 8.28 μg/mL). *P. argentea* showed the lowest activity toward DPPH radical ($IC_{50}$ = 7.38 μg/mL), comparable to that of BHT ($p > 0.05$).

The ability of extracts and BHT to neutralize hydroxyl radical increased in the following order: *P. major* < *P. media* < BHT < *P. maritima* ($p < 0.05$) < *P. holosteum* ≤ *P. argentea* ($p > 0.05$), indicating moderate activity of the examined *Plantago* species with regard to the synthetic antioxidant BHT. The scavenging effect of extracts on the superoxide anion radical decreased in

**Table 6.** $IC_{50}$ Values[a] for Lipid Peroxidation Assay and Reducing Power of Examined Extracts and BHT

| extract | $IC_{50}$ values, lipid peroxidation ($\mu$g/mL) | reducing power, FRAP (mg of AAE/g of dw) |
|---|---|---|
| *P. argentea* | 11.29 ± 0.63 a | 58.00 ± 3.16 b |
| *P. holosteum* | 18.24 ± 0.68 b | 115.99 ± 7.58 e |
| *P. major* | 31.42 ± 1.78 d | 73.89 ± 4.42 c |
| *P. maritima* | 46.69 ± 0.51 e | 93.39 ± 1.66 d |
| *P. media* | 31.95 ± 0.76 d | 120.02 ± 9.68 e |
| standard, BHT | 21.29 ± 0.40 c | 25.32 ± 2.5 a |

[a] Values are means ± SD of three measurements. Means within each column with different letters (a−e) differ significantly ($p < 0.05$).

the order *P. major* > *P. maritima* > *P. argentea* > *P. media* > *P. holosteum* ($p < 0.05$). Interestingly, the highest activity regarding neutralization of nitric oxide was accomplished by three samples (*P. argentea*, *P. major*, *P. maritima*) having equal ($p > 0.05$) $IC_{50}$ values of 0.34 mg/mL. The $IC_{50}$ value was not determined for BHT toward superoxide anion and nitric oxide radicals, because BHT reached no more than 32 and 10% of inhibition, respectively, having the concentration ranging from 0.1 to 10.0 mg/mL. This can be explained by sparing solubility of BHT in aqueous buffers. After all, one can conclude that the relatively unexplored species *P. maritima* was very efficient in the neutralization of DPPH, $O_2^{\bullet-}$, and NO radicals.

With regard to $Fe^{2+}$/ascorbate-induced lipid peroxidation (**Table 6**), all extracts exhibited notable antioxidant activity, with $IC_{50}$ values ranging from 11.29 to 46.69 $\mu$g/mL (*P. argentea* and *P. maritima*, respectively). The reducing power of extracts (**Table 6**) (determined as AAE/g of dw) was different: highly achieving by *P. media* (120.02), followed by *P. holosteum* (115.99), *P. maritima* (93.39), modest by *P. major* (73.89) and *P. argentea* (58.00), and lowest by BHT (25.32).

Correlation factors between total phenolic and flavonoid content and antioxidant activity, expressed as the reciprocal value of the calculated $IC_{50}$, except for the FRAP assay for which determined AAE values were used, are presented in **Table 7**. The strongest correlation was found between total phenolic and flavonoid content and examined activities in assays based on direct H-atom abstraction or a proton concerted electron-transfer mechanism (DPPH neutralization) and ferric reducing process (FRAP assay). Namely, considering DPPH neutralization activity, a good correlation was detected with total phenolic content ($R^2 = 0.9405$) and an inferior one with total flavonoid content ($R^2 = 0.8572$), which is in agreement with the literature (*7*). Furthermore, high correlation was shown between total phenolic and flavonoid content and FRAP activity ($R^2 = 0.9965$ and $R^2 = 0.9298$, respectively). The strongest FRAP activity was recorded in *P. media* and *P. holosteum* extracts, which contain the highest amounts of total phenolic and flavonoid contents, respectively. The obtained results can confirm the statement that flavonoids and phenolic substances, in particular, are most responsible for the reducing capacity of a certain extract.

On the other hand, the lack of correlation (**Table 7**) between total phenolic and flavonoid content in other antioxidant assays ($HO^{\bullet}$, $O_2^{\bullet-}$, and NO scavenger capacities, lipid peroxidation) can be caused by different effects. Hydroxyl radical neutralization can be strongly influenced by different Fe ion chelating agents, which can inhibit Fenton's reaction by which OH radicals are produced. Furthermore, the TBA test used for the detection of MDA, a product of reaction between OH radical and 2-deoxy-D-ribose, is nonspecific for MDA, and therefore other structurally similar substances present in plant extracts, mostly dialdehydes and carbohydrates, can react positively with TBA. These effects could

**Table 7.** Correlation Factors ($R^2$) between Total Phenolic and Flavonoid Content and Applied Assays

| assay | phenolics | flavonoids |
|---|---|---|
| reduction of DPPH radical | 0.9405 | 0.8572 |
| hydroxyl-radical scavenger capacity | 0.8107 | 0.8612 |
| superoxide anion scavenger capacity | 0.7528 | 0.6985 |
| NO scavenger capacity | 0.6752 | 0.7064 |
| lipid peroxidation | 0.6488 | 0.6420 |
| reducing power (FRAP) assay | 0.9965 | 0.9298 |

be also explanation for poor correlation between inhibition of lipid peroxidation and total content of flavonoids and phenolics. With regard to the above-mentioned, both *P. argentea* and *P. holosteum*, which were most active in OH radical neutralization, also showed the highest inhibitory effect on $Fe^{2+}$/ascorbate-induced lipid peroxidation, even though there is a great difference in their total contents of flavonoids and phenolics.

According to a weak correlation (**Table 7**) between total phenolic and flavonoid content and neutralization of NO and $O_2^{\bullet-}$ it can be assumed that other natural products present in *Plantago* extracts, besides phenolic compounds, can be involved in the scavenging of these radical species.

In conclusion, it should be emphasized that in the present study chemical and biological examinations of four *Plantago* species were undertaken for the first time. The preliminary results of their antioxidant activity undoubtedly suggest that they can be used as a very potent natural antioxidant. Especially the *P. holosteum* species, which is widely distributed in Europe, was shown to be rich in biologically active phenolic and flavonoid substances with considerable scavenging and antioxidant activities.

## ABBREVIATIONS USED

BHT, 3,5-di-*tert*-butyl-4-hydroxytoluene; DPPH, 2,2-diphenyl-1-picrylhydrazyl; dw, dry weight; EDTA, ethylenediaminetetraacetic acid; FAME, fatty acid methyl esters; FC, Folin−Ciolcateu reagent; FRAP, ferric ion reducing antioxidant power; MCF-7, breast adenocarcinoma tumoral cell line; MDA, malondialdehyde; MRM, multiple reaction monitoring; MS2SIM, single-stage mass spectrometry; NADH, nicotinamide adenine dinucleotide; NBT, nitroblue tetrazolium; NEDA, *N*-(1-naphthyl)-ethylenediamine dihydrochloride; PMS, phenazine methosulfate; ROS, reactive oxygen species; TBA, 2-thiobarbituric acid; TPTZ, 2,4,6-tripyridyl-*s*-triazine; UACC-62, melanoma tumoral cell line.

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