https://doi.org/10.31925/farmacia.2018.4.8 **ORIGINAL ARTICLE**

A COMPARATIVE PHARMACOGNOSTIC STUDY AND ASSESMENT OF ANTIOXIDANT CAPACITY OF THREE SPECIES FROM *PLANTAGO* GENUS

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Abstract

This paper presents a comparative pharmacognostic study of three species from *Plantago* genus (*Plantago media* L., *Plantago major* L., *Plantago lanceolata* L.) and assessment of their antioxidant capacity. First time reported microscopic data of *P. media* leaves revealed the presence of multicellular covering trichomes, stalked glandular trichomes, anomocytic and diacityc stomatal type. The phytochemical analysis showed that *P. media* leaves had the highest content of polysaccharides (24.43%) and total polyphenols (28.91 mg GAE/g dw), whereas *P. lanceolata* leaves were the richest source of chlorophyll (40.38 mg/L). Antioxidant activity of ethanol extracts from *Plantago* leaves were characterized by DPPH (2,2-diphenyl-2-picryl-hydrazyl-hydrate) scavenging test, CUPRAC (cupric reducing antioxidant capacity) and FRAP (ferric reducing anti-oxidant power) assays. All extracts showed significant antioxidant efficacy and among them *P. media* extracts exhibited the strongest antioxidant activity in the three methods.

Rezumat

Această lucrare prezintă studiul farmacognostic comparativ a trei specii din genul *Plantago* (*Plantago media* L., *Plantago major* L., *Plantago lanceolata* L.) și evaluarea capacității lor antioxidante. Datele microscopice prezentate privind frunzele speciei *P. media* au relevat prezența perilor multicelulari, a perilor glandulari și a stomatelor de tip anomocitic și diacitic. Analiza fitochimică a demonstrat că frunzele speciei *P. media* prezintă un conținut ridicat de polizaharide (24,43%) și polifenoli totali (28,91 mg echivalenți acid galic/g produs uscat), în timp ce frunzele speciei *P. lanceolata* reprezintă o sursă bogată de clorofilă (40,38 mg/L). Activitatea antioxidantă a extractelor etanolice vegetale ale tuturor speciilor studiate a fost evaluată prin testele DPPH, CUPRAC și FRAP. Extractele au prezentat o eficacitate antioxidantă semnificativă și, dintre acestea, cele obținute din frunzele speciei *P. media*, au evidențiat cea mai puternică activitate antioxidantă.

Keywords: *Plantago media* L.*, Plantago major* L., *Plantago lanceolata* L., pharmacognostic leaf analysis, antioxidant activity

Introduction

The genus *Plantago* belongs to the *Plantaginaceae* family and is represented by 250 perennial species growing throughout Europe and Asia [10, 16]. The leaves of some *Plantago* species are commonly used as an herbal remedy in the treatment of skin related problems, respiratory disorders, digestive system affections, inflammations of nasal and oral cavities [8, 16]. Phytochemical studies of the genus revealed the presence of phenolic compounds, polysaccharides, iridoid glycosides, vitamins and minerals [8, 10, 16].
The objects of this study are the well-known medicinal plants *Plantago major* L. (Greater plantain) and *Plantago lanceolata* L. (Ribwort plantain), and the meagrely studied herbaceous plant *Plantago media* L. (Hoary plantain). *P. lanceolata* leaves are described in the European Pharmacopoeia [23] and *P. major* leaves are included in the World Health Organization (WHO) Monographs [24]. However, so far no thorough pharmacognostic evaluation of *P. media* leaves has been reported in literature. The aim of the present work was to make microscopic, physicochemical and phytochemical evaluation of *P. media* leaves and assessment of their potential antioxidant activity. A comparative study with the closely related species *P. major* and *P. lanceolata* was also conducted in

order to ensure the proper identification and quality control of *Plantago* species.

Materials and Methods

Plant material and chemicals

The mature leaves of the studied species were collected from natural habitats in Bulgaria (Tracian valley floristic region for *P. major* and *P. lanceolata* and Rhodope Mountains floristic region for *P. media*) during vegetative season of 2015. Species identification was carried out according to Tutin *et al* [20] and Delipavlov *et al* [6]. Gallic acid, Trolox, 2,2-diphenyl-2-picryl-hydrazyl-hydrate (DPPH), 2,4,6-tris-(2-pyridyl)-s-triazine (TPTZ), butylated hydroxytoluene (BHT) were purchased from Sigma Aldrich, US. Other reagents used were of analytical grade.

Microscopic study

Qualitative, quantitative and histochemical microscopic analyses of *Plantago* leaves were conducted according to standard methods [19, 23, 25], using a light microscope Leica DM 2000 LED, Leica Microsystems, Germany, equipped with camera Leica DMC 2900 and imaging software Leica Application Suite. The samples were observed in chloral hydrate solution. Histochemical tests on cross sections of *Plantago* leaves with ferric chloride/methylene blue were performed for the localization of mucilage [7].

Physicochemical analysis

Physicochemical parameters such as the percentage of ash value, loss on drying, extractable matter and swelling index were studied in accordance to the WHO guidelines [25] and the European Pharmacopoeia [23].

Phytochemical analysis

Total polyphenolic content was assessed using the Folin-Ciocâlteu reagent according to the procedure described by Singleton and Rossi [18]. The absorbance was measured at 765 nm. The calibration was carried out against a reference standard of gallic acid (0 - 20 μg) and the results were expressed as milligram gallic acid equivalent *per* gram dry weight of plant material (mg GAE/g dw). Polysaccharide yield was determined according to the procedure described by Kratchanova *et al* [12]. For quantitative determination of chlorophyll content was used the method of Arnon [3], modified by Hiscox and Israelstam [9].

Determination of antioxidant capacity

2,2-diphenyl-2-picryl-hydrazyl-hydrate (DPPH) radical scavenging activity. In order to determine the radical scavenging activity against stable DPPH the method proposed by Brand-Williams *et al* was used with slight modifications [5]. Briefly, a volume of 50 μL of ethanol extracts of *Plantago* leaves and 2000 μL DPPH (80 μM) methanol solution were added and the decrease in the absorbance was measured at 517 nm after 15 min. The radical scavenging activity was calculated by the following equation:

$$\% \text{ Inhibition} = (AB - AA)/AB \times 100,$$

where AB was the absorption of blank sample (t = 0 min) and AA - the absorption of sample extract solution (t = 15 min) [11, 15]. BHT (0.1 mg/mL) was used as a positive control. Data were expressed by triplicate measurements with standard deviation.

Cupric Reducing Antioxidant Capacity (CUPRAC). The cupric ions (Cu^{2+}) reducing antioxidant capacity was determined by the slightly modified method of Apak *et al* [1]. Shortly, 1 mL $CuCl_2$ solution (0.01 M), 1 mL CH_3COONH_4 buffer solution (1 M, pH = 7) and 1 mL ethanolic neocuproine solution (7.5 mM) were added to a test tube, followed by mixing with 50 μL of the plants extracts and adjusting total volume of 4.1 mL with distilled water. The mixtures were incubated at room temperature for 60 min and absorbance was measured at 450 nm against a reagent blank. Trolox was used as a standard and total antioxidant capacity of extracts was expressed as μmol Trolox equivalent *per* gram dry weight of plant material (mM/TE g dw) [2, 15]. BHT (0.1 mg/mL) was used as a positive control. Values were presented by triplicate measurements with standard deviation.

Ferric reducing antioxidant power (FRAP). For determination of the ferric reducing antioxidant power was used the method of Benzie and Strain was used [4], with some modifications [15]. The freshly prepared FRAP reagent (2.85 mL) was mixed with 0.15 mL of plant extracts. The mixture was then incubated at 37°C for 10 min in dark, and absorbance was measured at 593 nm. Trolox was used as a standard solution and the results were expressed as μmol Trolox equivalents *per* gram dry weight of plant material (μM TE/g dw). BHT (0.1 mg/mL) was used as a positive control. Values were presented by triplicate measurements with standard deviation.

Results and Discussion

Microscopic study

Regardless of today sophisticated modern research techniques for evaluation of plant, drugs microscopic analysis is still one of the simplest and cheapest methods for source material identification [17]. Data on some of the microscopic features of *P. lanceolata* leaves is available in the European Pharmacopoeia [23] and microscopic description of *P. major* leaves is included in the WHO Monographs [24]. However, no information about microscopic investigation of *P. media* leaves was found. European Medicines Agency (EMA) has reported possible confusion of *P. lanceolata* leaves with *P. major*, *P. media* and *Digitalis lanata* leaves [22]. A comparative study of leaf epidermis from *P. major*, *P. media* and *P. lanceolata* was carried out to facilitate their identification and minimize the risk of substitution. The microscopic analysis showed characteristic features (Figure 1) in accordance to standard documents [23, 24]. The leaf epidermal

cells of *P. lanceolata* were polygonal with straight or slightly sinuous walls (Figure 1J), whereas those of *P. media* (Figure 1H) and *P. major* (Figure 1I) were with irregularly sinuous walls. Rosettes with striated cuticle were noted in *P. major* epidermis, while fragments from the scape with thickened outer walls and a coarsely ridged cuticle were observed in *P. lanceolata* epidermis. Stomal type was anomocytic in *P. major* (Figure 1I), mainly anomocytic and occasionally diacytic in *P. media* (Figure 1H) and diacytic (rarely anomocytic) in *P. lanceolata* (Figure 1J). The covering trichomes of the three investigated *Plantago* species were multicellular, uniseriate, unbranched (Figure 1A-1D). In *P. major*, they had an enlarged base (Figure 1C), in *P. media* some of the covering trichomes were collapsed (Figure 1B), while in *P. lanceolata* the covering trichomes were composed of a large basal cell, followed by a short cell supporting two or several elongated cells and a terminal cell with an acute apex (Figure 1D). *P. lanceolata* had stalked glandular trichomes with a multicellular, elongated head comprising of several secretory cells (Figure 1G). The glandular trichomes of *P. major* and *P. media* leaves were mainly composed of a monocellular stalk and a bicellular elongated head (Figure 1E, Figure 1F). Among the studied plantain species *P. media* covering and glandular trichomes were the largest (450 - 790 µm and 55 - 105 µm, respectively). *P. major* had the largest stomata (27 - 34 µm) and epidermal cells (58 - 100 µm). The comparative study of leaf constants showed differences among the investigated *Plantago* species with respect to stomatal number, stomatal index, vein-islet number and veinlet termination number. The results showed that *P. media* leaves had the lowest number of stomata *per* unit area (stomatal number/mm^2) and the lowest stomatal index (Table I).

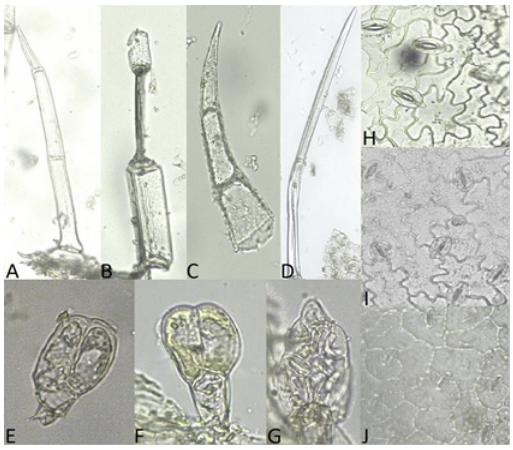

Figure 1.

Microscopic characteristics of *P. major*, *P. media* and *P. lanceolata* leaves: (A) Covering multicellular unbranched trichome of *P. media*; (B) Collapsed covering trichome of *P. media*; (C) Covering multicellular unbranched trichome of *P. major*; (D) Covering multicellular unbranched trichome of *P. lanceolata*; (E) Stalked glandular trichome of *P. media*; (F) Stalked glandular trichome of *P. major*; (G) Stalked glandular trichome of *P. lanceolata*; (H) Leaf epidermis of *P. media*; (I) Leaf epidermis of *P. major*; (J) Leaf epidermis of *P. lanceolate*

Table I

Microscopic parameters of *P. major*, *P. media* and *P. lanceolata* leaves

Microscopic parameters	*P. major*	*P. media*	*P. lanceolata*
Epidermal cells - shape and placement	polygonal with straight or slightly sinuous walls; rosettes with striated cuticle	polygonal with irregularly sinuous walls	polygonal with irregularly sinuous walls, the fragments from the scape with thickened outer walls and a coarsely ridged cuticle
Epidermal cell length range (µm)	58 - 100	54 - 88	32 - 45
Stomatal type	anomocytic	anomocytic (rarely diacytic)	diacytic (rarely anomocytic)
Stomata length range (µm)	27 - 34	21 - 33	20 - 23
Type of covering trichomes	multicellular, uniseriate, unbranched	multicellular, uniseriate, unbranched, frequently collapsed	multicellular, uniseriate, unbranched
Covering trichomes length range (µm)	207 - 310	450 - 790	220 - 632
Type of glandular trichomes	stalked, with one celled stalk and secretory head with a pair of secretory cells	stalked, with one celled stalk and secretory head with a pair of secretory cells	stalked, with one celled stalk and secretory head with several secretory cells
Glandular trichomes length range (µm)	48 - 51	55 - 105	80 - 110
Range of stomatal number	32 - 39	21 - 27	52 - 58
Range of stomatal index	22.8 - 25.6	16.6 - 20.0	21.7 - 23.0
Range of vein-islet number	7 - 9	5 - 8	7 - 10
Range of veinlet termination number	3 - 5	3 - 4	2 - 4

Values are expressed as a result from 30 measurements.

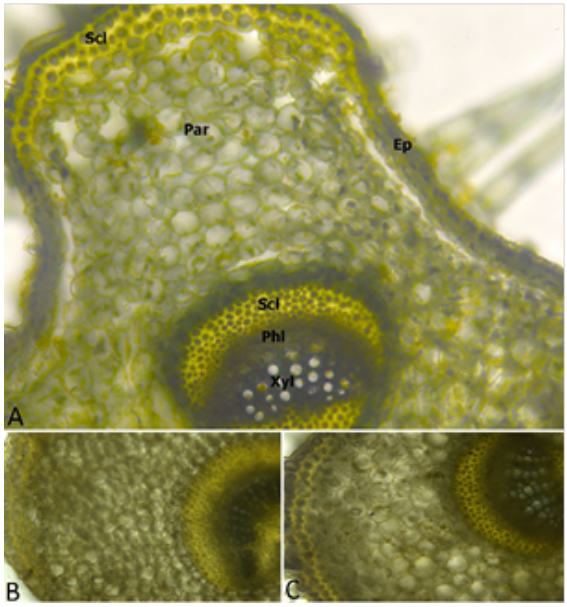

Figure 2.
Cross sections of *P. media* (A), *P. major* (B) and *P. lanceolata* leaf veins (C) after staining with ferric chloride/methylene blue. (Ep) Epidermis; (Par) Parenchyma sheath; (Scl) Sclerenchyma with thick secondary cell walls; (Phl) Phloem; (Xyl) Xylem

The histochemical staining with ferric chloride/ methylene blue is a specific qualitative test for identification of mucilage in plant tissues (yellow colouring) [7]. The conducted analysis showed that the mucilage was localized mostly in the lying beneath the leaf epidermis and around the vascular bundles secondary cell walls of the sclerenchyma tissue of the leaf veins. Minor amounts of mucilage were observed in the cell walls of parenchyma sheath around the bundles (Figure 2).

Physicochemical analysis

The physicochemical analysis is an essential part of the quality control of the plant material. The results of the studied physicochemical parameters were reported in Table II. The loss on drying value of all analysed plantain leaves was less than 10%, which minimalized the possibility of bacteria, fungi or yeast growth. The ash values provided information for the inorganic composition of the investigated plant material. The determined ash values of *P. lanceolata* (10.04%), *P. major* (10.22%) and *P. media* leaves (14.85%) corresponded to the recommendations given in the European Pharmacopoeia [23] and WHO Monographs [24]. The obtained extractable matter profiles could be used for evaluating the chemical constituents present in the crude drug and also for estimating the specific constituents soluble in a particular solvent. The extractable matter of the crude powder for the three investigated *Plantago* species was highest in water (38.79 - 45.22%), lower in ethanol (11.80 - 20.80%) and acetone (5.12 - 6.93%), and lowest in chlorophorm (3.05 - 4.56%). The high value of water extractable matter in plantain leaves revealed the presence of large amount of water soluble constituents. The swelling index values for all samples were close-range: 2.45 - 2.95 mL.

Table II
Physicochemical parameters of *P. major*, *P. media* and *P. lanceolata* leaves

Physicochemical parameters	Value		
	P. major	*P. media*	*P. lanceolata*
Ash values (%, w/w):			
Total ash	10.22 ± 0.27	14.85 ± 0.14	10.04 ± 0.39
Acid insoluble ash	2.86 ± 0.16	3.13 ± 0.09	1.82 ± 0.05
Loss on drying (%, w/w)	7.43 ± 0.45	7.57 ± 0.31	7.55 ± 0.44
Extractable matter (%, w/w):			
Water-soluble	38.79 ± 0.75	45.22 ± 1.04	39.69 ± 0.60
Ethanol-soluble	11.80 ± 0.83	20.80 ± 1.15	18.84 ± 1.33
Acetone-soluble	6.02 ± 0.49	6.93 ± 0.18	5.12 ± 0.16
Chlorophorm-soluble	3.05 ± 0.09	4.56 ± 0.21	3.40 ± 0.22
Swelling index (mL)	2.60 ± 0.16	2.95 ± 0.14	2.45 ± 0.19

Values are expressed as mean ± SD (n = 3).

Phytochemical analysis

Quantitative phytochemical analysis was carried out regarding polysaccharide, total polyphenolic and chlorophyll content in *P. media*, *P. major* and *P. lanceolata* dry leaves (Table III). Polysaccharides were isolated from plant cell walls with consecutive water and acid extraction. The polysaccharide content was highest in *P. media* leaves (24.43%), lower in *P. major* leaves (19.82%) and the lowest in *P. lanceolata* leaves (9.98%). The amount of the extracted polysaccharides from *P. major* leaves corresponded to that, recommended by WHO monographs (not less than 12%) [24]. According to EMA the percentage of mucilage in *P. lanceolata* leaves should be between 2% and 6.5% [22], while the percentage of the isolated polysaccharides from Bulgarian *P. lanceolata* leaves was much higher.

The total polyphenolic content (TPC) of ethanol extracts of the selected *Plantago* species was expressed in terms of gallic acid equivalents *per* gram dry leaves. The highest TPC was determined in *P. media* leaves (28.91 mg GAE/g dw), while in *P. major* and

P. lanceolata leaves it was close-range: 17.18 mg GAE/g dw and 17.37 mg GAE/g dw, respectively. The determined TPC in *P. major* leaves was higher than those previously reported by Mohamed *et al* [13]. On the other hand Jankovic *et al* [10] reported higher amount of total polyphenolic content in *P. lanceolata* leaves than those determined in our study. The chlorophyll content was presented as total chlorophyll, chlorophyll a and chlorophyll b. *P. lanceolata* leaves were estimated as the richest source of chlorophyll among the investigated *Plantago* species with total chlorophyll content of 40.38 mg/L (Table III).

Table III

Polysaccharides, total polyphenols and chlorophyll content of *P. major*, *P. media* and *P. lanceolata* leaves

Sample	Polysaccharides (%, w/w)	TPC (mg GAE/g dw)	Chlorophyll (mg/L)		
			Chla	Chlb	Total Chl
Plantago major	19.82 ± 2.03	17.18 ± 2.25	10.21 ± 0.51	0.83 ± 0.04	20.19 ± 1.00
Plantago media	24.43 ± 1.65	28.91 ± 3.12	16.48 ± 0.50	1.55 ± 0.05	32.89 ± 0.99
Plantago lanceolata	9.98 ± 0.84	17.37 ± 1.85	18.83 ± 0.37	0.85 ± 0.02	40.38 ± 0.81

Values are expressed as mean ± SD (n = 3).

Antioxidant capacity

Three different assays were used to evaluate the antioxidant activity of ethanol extracts from *Plantago* leaves: DPPH test (measures the ability to scavenge free radicals), CUPRAC assay (measures the reduction of cupric to cuprous ion) and FRAP assay (measures the ability to reduce ferric iron by donating an electron). The results were summarized in Table IV.

Table IV

Antioxidant activity of *P. major*, *P. media* and *P. lanceolata* leaves

Sample	DPPH (%)	CUPRAC (μM TE/g dw)	FRAP (μM TE/g dw)
Plantago major	55.21 ± 0.56	33.6 ± 0.19	97.66 ± 2.37
Plantago media	75.48 ± 0.76	69.10 ± 0.33	159.48 ± 4.16
Plantago lanceolata	59.04 ± 0.09	21.9 ± 0.58	51.85 ± 1.54
BHT	93.49 ± 0.06	573.04 ± 0.12	556.10 ± 0.09

Values are expressed as mean ± SD (n = 3).

The presented data showed that DPPH scavenging capacity increased in the order: *P. media* (75.48%) > *P. lanceolata* (59.04%) > *P. major* (55.21%), which is the same trend as the content of total polyphenols. Similarly, based on CUPRAC and FRAP assays *P. media* extracts showed strongest reducing ability, followed by *P. major* and *P. lanceolata* extracts (Table IV). Our results are in accordance with the studies of Vicaş *et al* [21], Oprică *et al* [14] and Mohamed *et al* [13] who examined *Plantago* extracts for antioxidant activity and reported that they could be regarded as possible new sources of natural antioxidants.

Conclusions

The study presents for the first time pharmacognostic evaluation of *Plantago media* leaves. The comparative pharmacognostic analysis of *P. media*, *P. major* and *P. lanceolata* leaves may serve as basis for identification and authentication of the investigated species.

The presented specific microscopic characteristics are essential for avoiding adulteration/substitution of the studied plant material. The evaluated physico-chemical parameters can be extremely beneficial to the purity and quality control of plantain species. The results from the preliminary phytochemical analysis and the assessment of antioxidant capacity may be used in future biological and pharmacological studies.

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