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Variation of free phenolic acids in medicinal plants belonging to the *Lamiaceae* family

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Abstract

Ten species belonging to the family *Lamiaceae* and representing the most popular medicinal plants used in Polish phytotherapy were examined for the content of free phenolic acids (PhAs). Two depsides, rosmarinic and chlorogenic acids, as well as eight simple PhAs, protocatechuic, gentisic, *p*-hydroxybenzoic, caffeic, vanillic, syringic, *p*-coumaric and ferulic acids, in different qualitative and quantitative proportions depending on the plant examined were determined by the rapid, selective and accurate method combining solid-phase extraction and high-performance liquid chromatography.

Keywords: *Lamiaceae* family; High-performance liquid chromatography; Phenolic acids; Solid-phase extraction; Thin-layer chromatography

1. Introduction

Based on the latest literature, the family *Lamiaceae* seems to be a rich source of plant species containing large amounts of phenolic acids (PhAs), especially their depside forms, e.g. rosmarinic acid [1–8]. Some of these cited papers dealt with isolation and quantification of rosmarinic acid, both in plant material and in cell cultures, but the exact composition and the content of other PhAs were still unknown. In our study, various parts of ten species, *Salvia officinalis* L., *Melissa officinalis* L., *Mentha piperita* (L.) Hudson, *Thymus vulgaris* L., *Lavandula officinalis* Chaix, *Rosmarinus officinalis* L., *Origanum majorana* L., *Hyssopus officinalis* L., *Ocimum basilicum* L. and *Satureja hortensis* L. (*Lamiaceae*), were analysed. The essential aim of this research was to establish the presence of PhAs showing potential immunomodulating activity (e.g. rosmarinic, gentisic, chlorogenic, caffeic acids) and to determine their concentration levels in plants examined. The results of these investigations should be helpful in the better explaining the complex pharmacological activity of some medicinal plants belonging to the *Lamiaceae* family. More and more studies carried out in numerous research centres show that this activity is strictly connected with the presence of phenolics (among others, PhAs) in these plants.

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Table 1
Plant species and their organs used in the experiments

Number	Plant	Organ examined	Symbol in the figures
1	*Salvia officinalis* L.	Leaves	*So*
2	*Melissa officinalis* L.	Leaves	*Mo*
3	*Mentha piperita* (L.) Hudson	Leaves	*Mp*
4	*Thymus vulgaris* L.	Herb	*Tv*
5	*Lavandula officinalis* Chaix	Flowers	*Lo*
6	*Rosmarinus officinalis* L.	Leaves	*Ro*
7	*Origanum majorana* L.	Herb	*Om*
8	*Hyssopus officinalis* L.	Herb	*Ho*
9	*Ocimum basilicum* L.	Herb	*Ob*
10	*Satureja hortensis* L.	Herb	*Sh*

2. Materials and methods

2.1. Chemicals

Reagents for high-performance liquid chromatography (HPLC) (methanol, acetonitrile, acetic acid) were of chromatographic grade (Merck, Darmstadt, Germany), and those for solid-phase extraction (SPE) and thin-layer chromatography (TLC) (phosphoric acid, sodium bicarbonate, benzene) of analytical grade (POCH, Gliwice, Poland). In all experiments, bidistilled water was used. Standards of PhAs were purchased from Sigma (St. Louis, MO, USA).

2.2. Plant material

As the plant material, commercial samples of ten species from the family *Lamiaceae*, purchased from 'Herbapol-Lublin S.A.' (Lublin, Poland), were used (Table 1).

2.3. Sample preparation

Dry, pulverized samples (3 g) of various organs of ten plants examined (Table 1) were refluxed with methanol (100 ml) on a water bath for 1 h. Liquid was carefully decanted and the plant material was re-extracted with the same solvent (2×100 ml). All supernatants were combined, partially evaporated under reduced pressure, filtered and placed in 25-ml volumetric flasks.

2.4. SPE procedure

Isolation of free PhAs fractions was carried out according to our method, elaborated and first applied for the selective separation of these compounds from some *Echinacea* species [8].

Samples (5 ml) of methanolic extracts were evaporated to dryness, diluted with 30% aqueous methanol and passed under vacuum through conditioned (with 10 ml methanol, followed by 10 ml bidistilled water) octadecyl BakerBond SPE-micro-columns (500 mg; J.T. Baker, Phillipsburg, NJ, USA). For SPE, a vacuum manifold processor (system spe-12G; J.T. Baker, Großgerau, Germany) was used. In the next step, eluates containing the complex of phenolics were adjusted to pH 7.0–7.2 with 5% sodium bicarbonate aqueous solution and passed (under reduced pressure, 0.01 MPa) through quaternary amine Baker-

Table 2
LOD values for PhAs, determined using UV–Vis detection

Number	PhAs	LOD (ng/ml)
1	Protocatechuic acid	46
2	*p*-Hydroxybenzoic acid	10
3	Gentisic acid	416
4	Chlorogenic acid	294
5	Syringic acid	91
6	Caffeic acid	117
7	Vanillic acid	29
8	*p*-Coumaric acid	250
9	Ferulic acid	172
10	Rosmarinic acid	91

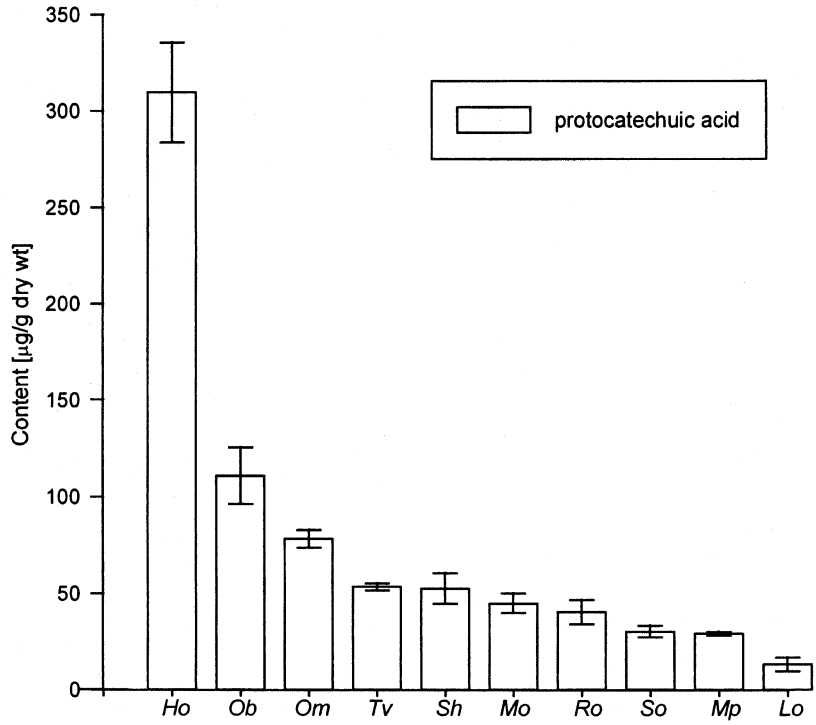

Fig. 1. Protocatechuic acid content in various medicinal species of the *Lamiaceae* family.

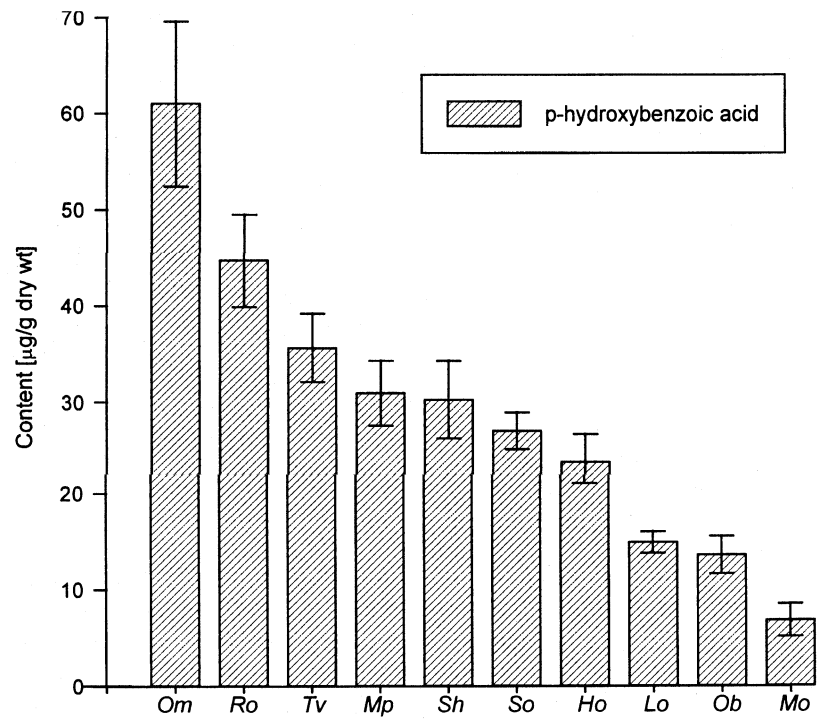

Fig. 2. *P*-Hydroxybenzoic acid content in various medicinal species of the *Lamiaceae* family.

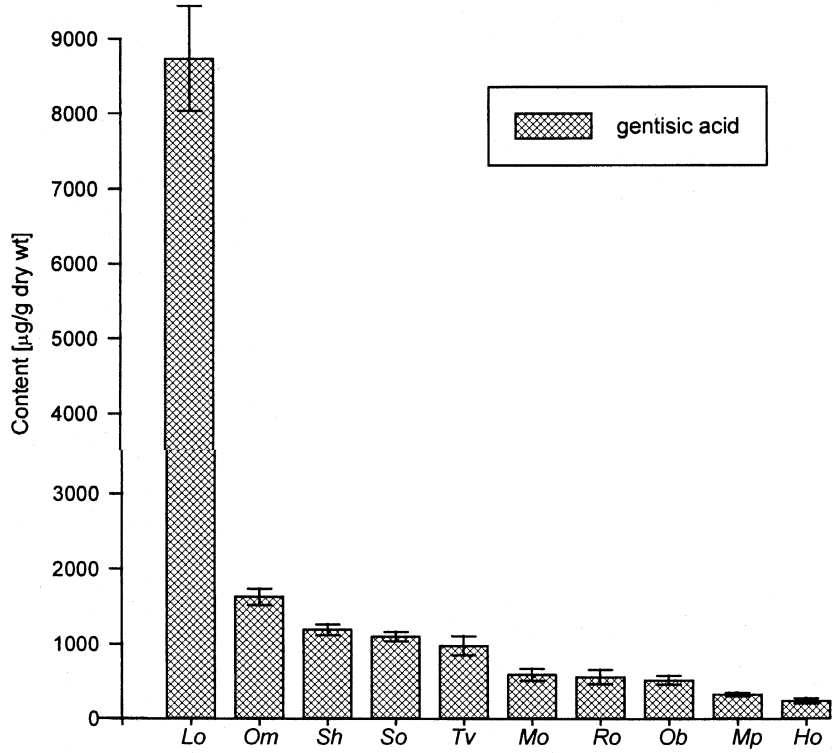

Fig. 3. Gentisic acid content in various medicinal species of the *Lamiaceae* family.

Bond SPE-microcolumns (500 mg; J.T. Baker, Phillipsburg, NJ, USA), previously conditioned with bidistilled water (10 ml) and 0.15% sodium bicarbonate aqueous solution (20 ml). Analytes (PhAs) concentrated on the sorbent beds were desorbed with 0.2 M phosphoric acid and methanol (1:1; v/v), 5 ml for each sample. The collected eluates were adjusted to pH 3 with 1 M sodium hydroxide, and subsequently qualitatively and quantitatively analysed by reverse phase-HPLC.

2.5. HPLC analysis

2.5.1. Apparatus

The HPLC system consisted of a Hewlett-Packard (Palo Alto, CA, USA) Model 1050 liquid chromatograph equipped with a Rheodyne injector with a 20-µl sample loop and a variable wavelength UV–Vis detector. A stainless-steel column (200 × 4.6 mm I.D.) packed with 5 µm ODS Hypersil (Shandon, Cheshire, UK) was used.

2.5.2. Chromatographic conditions

For quantitative analysis of rosmarinic acid, an isocratic solvent system of acetonitrile/water/acetic acid (20:80:1, v/v/v) was used and detection at 280 nm. Other PhAs were analysed with a mobile phase of methanol/water/acetic acid (25:75:1, v/v/v), detection at 254 nm and a flow rate of 1 ml/min.

2.6. TLC analysis

Simultaneously, thin-layer chromatography of SPE eluates was carried out, which was the confirmation of qualitative results of HPLC analysis. Samples (100 µl) were spotted on cellulose plates (20 × 20 cm; Merck, Darmstadt, Germany) and developed over a distance of 15 cm by a two-dimensional technique (2D-TLC) in horizontal DS chambers (CHROMDES, Lublin, Poland) using the following mobile phases: benzene–acetic acid–water (6: 7: 3, v/v/v) in the first direction, and acetic acid–water (15: 85, v/v/v) in the second. Chro-

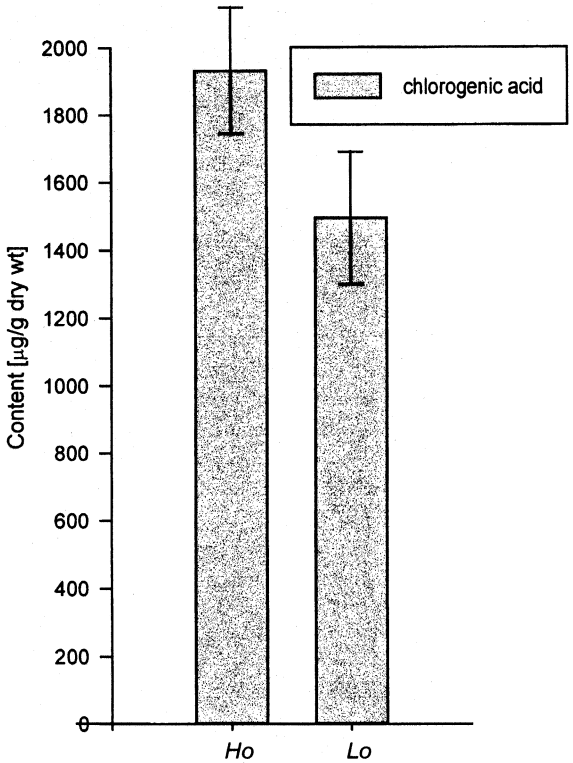

Fig. 4. Chlorogenic acid content in various medicinal species of the *Lamiaceae* family.

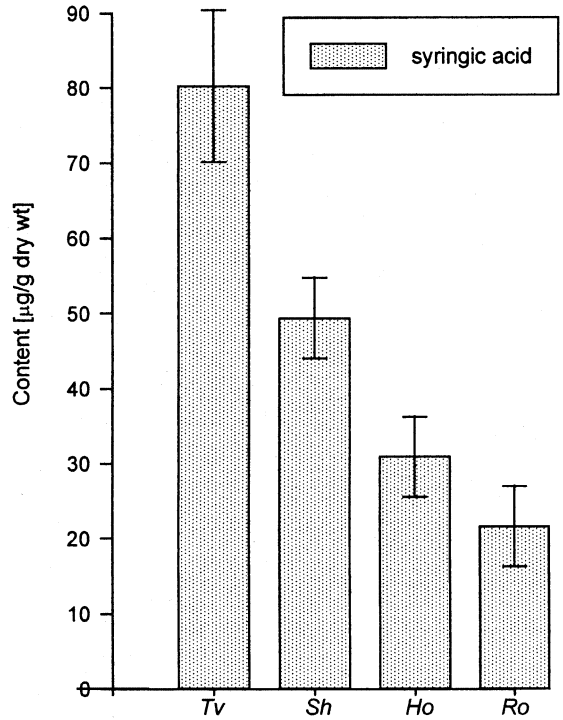

Fig. 5. Syringic acid content in various medicinal species of the *Lamiaceae* family.

matograms were examined under UV light ($\lambda =$ 254 and 366 nm). The spots of PhAs were also visualized with general sprays used for the detection of phenolic compounds, i.e. a 1:1 (v/v) mixture of diazotized sulfanilic acid and 20% aqueous solution of sodium carbonate or 5% methanolic solution of iron(III) chloride.

3. Results and discussion

3.1. Linearity, accuracy and method precision

The linearity of the HPLC method was investigated for PhAs in the range 10–100 µg/ml at five concentration levels, using the successive dilutions of mother standard solutions (100 µg/ml). Calibration plots with correlation coefficient $r^2 \geq$ 0.999 were obtained by reporting peak areas (relative units as given by the integrator) as a function of analyte concentrations.

The detection limits (LOD), calculated for a signal/noise ratio of 3, are presented in Table 2.

In earlier investigations [9], in order to establish the efficiency of the applied SPE method, recovery tests were performed for the PhA standards. Methanolic (30%) solutions (10 ml) of 1 mg caffeic and *p*-hydroxybenzoic acids (as the representatives of the derivatives of cinnamic and benzoic acids) were submitted to the SPE procedure. A percentage recovery of $98.5 \pm 0.5\%$ ($n = 5$) for these compounds was obtained.

The method precision is expressed graphically in Figs. 1–10 as vertical error bars showing values of $\pm$S.D. for $n = 5$.

3.2. Results of qualitative and quantitative analysis

The following representatives of free PhAs were identified in the particular plants examined: rosmarinic, protocatechuic, *p*-hydroxybenzoic, gentisic, chlorogenic, syringic, caffeic, vanillic,

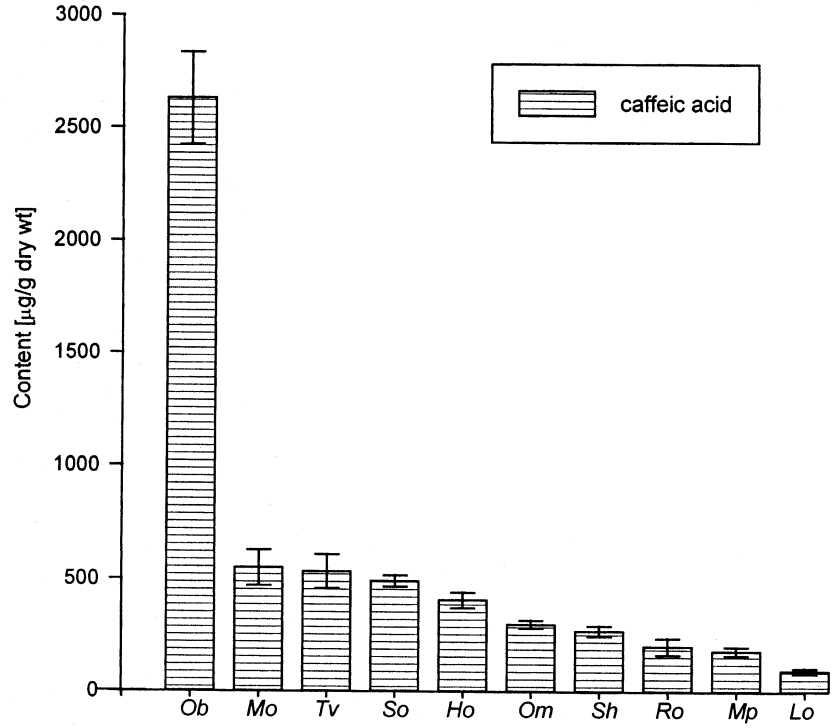

Fig. 6. Caffeic acid content in various medicinal species of the *Lamiaceae* family.

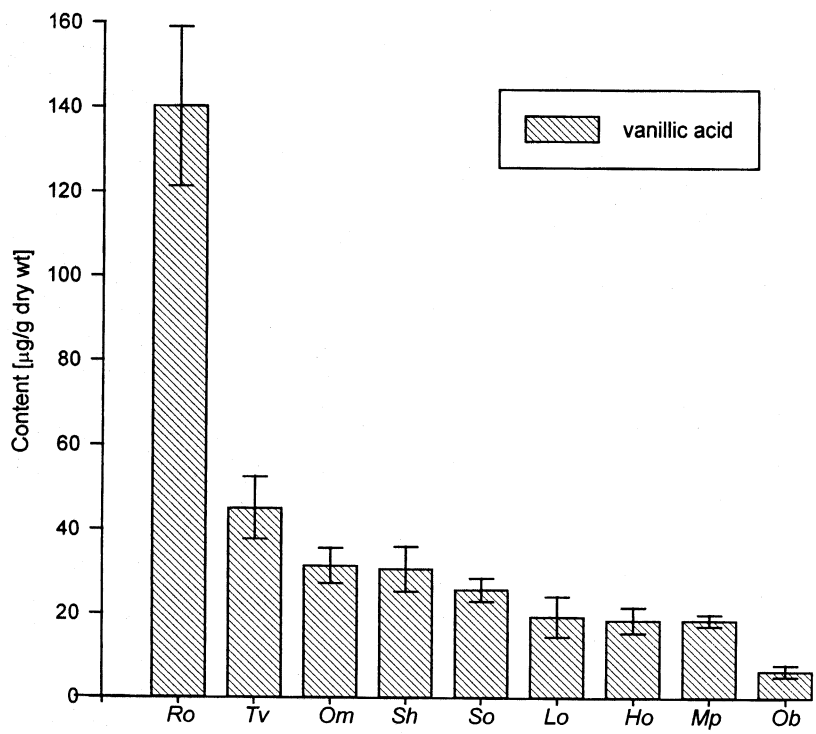

Fig. 7. Vanillic acid content in various medicinal species of the *Lamiaceae* family.

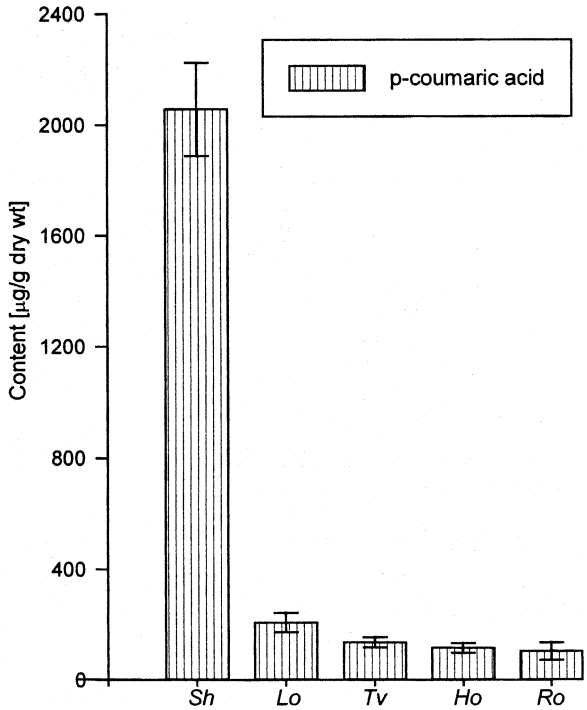

Fig. 8. *P*-Coumaric acid content in various medicinal species of the *Lamiaceae* family.

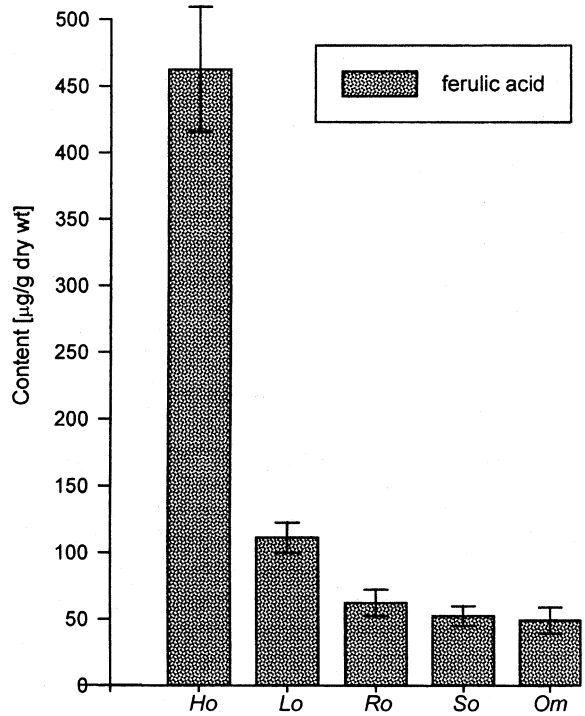

Fig. 9. Ferulic acid content in various medicinal species of the *Lamiaceae* family.

p-coumaric and ferulic acids, in various quantitative proportions (Figs. 1–10). The lowest concentration levels for *p*-hydroxybenzoic (Fig. 2) and syringic (Fig. 5) acids were established. For most of the plants examined, the middle content (approximately between 50 and 100 μg/g dry weight) for protocatechuic (Fig. 1), vanillic (Fig. 7) and ferulic (Fig. 9) acids was observed. Only in the aerial parts of *H. officinalis* and in the leaves of *R. officinalis* was the amount of these compounds significantly higher, between 150 and 450 μg/g dry weight. A similar phenomenon for *p*-coumaric acid was observed. For four plant organs (*L. officinalis* flowers, the herbs of *T. vulgaris* and *H. officinalis* and *R. officinalis* leaves), the concentration levels of this compound ranged from 100 to 200 μg/g dry weight, whereas for *S. hortensis* herb the content was above 2000 μg/g (0.2%) dry weight (Fig. 8). The results of quantitative analysis for PhAs showing potential immunotropic activity (gentisic, caffeic, chlorogenic, rosmarinic acids) were also very differentiated depending on the plant examined. The highest amounts of gentisic ($>0.85\%$ dry weight) and caffeic ($>0.25\%$ dry weight) acids were adequately established in *L. officinalis* flowers and *O. basilicum* herb, whereas in other plant organs their amounts were much more lower (Figs. 3 and 6). Chlorogenic acid (Fig. 4) was identified only in two samples examined, i.e. in *H. officinalis* herb ($\sim 0.19\%$ dry weight) and *L. officinalis* flowers ($\sim 0.15\%$ dry weight). Rosmarinic acid turned out to be the most predominant phenolic compound in all plant organs examined. The highest concentrations of this depside in the aerial parts of *Satureja officinalis* ($\sim 1.2\%$ dry weight) and *O. basilicum* ($\sim 1.1\%$ dry weight), as well as in the leaves of *M. officinalis* ($\sim 1\%$ dry weight) and *R. officinalis* ($\sim 0.7\%$ dry weight), were observed (Fig. 10). As rosmarinic acid is known as an antiviral, antibacterial, antioxidant, anti-inflammatory and immunostimulating agent [5,10–13], the results of

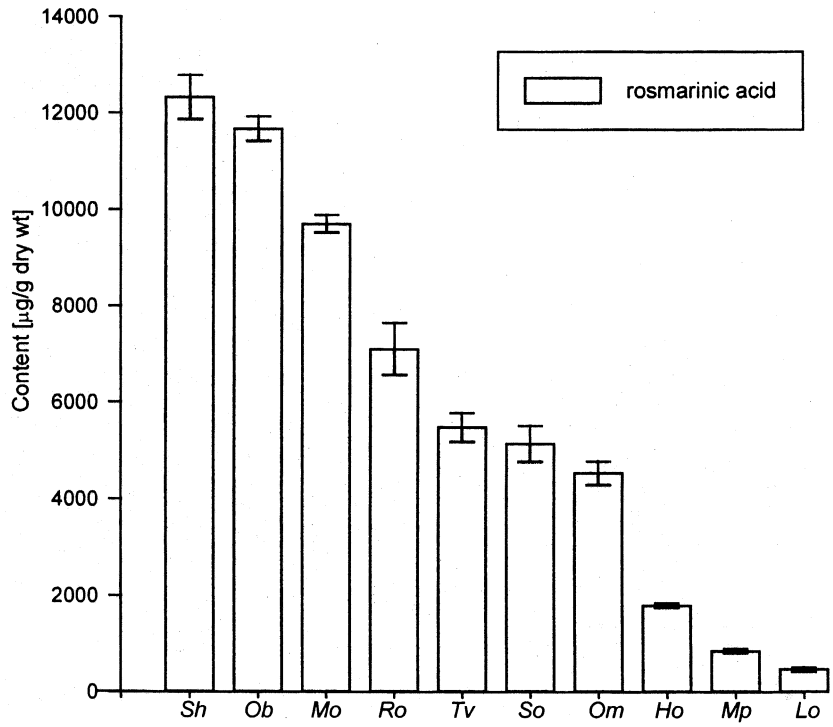

Fig. 10. Rosmarinic acid content in various medicinal species of the *Lamiaceae* family.

this work seem to be important from the point of view of additional pharmacological applications of medicinal plants belonging to the *Lamiaceae* family.

Based on the qualitative and quantitative results, the possible chemotaxonomical role of PhAs in the *Lamiaceae* family may be also discussed. Attention should be especially focused on rosmarinic acid. It is one of the most common caffeic acid esters occurring in *Lamiaceae*. The production of this compound has been investigated both in numerous lamiaceous plants and in tissue cultures of several species [1–8,14–20]. Our investigation confirmed the possible role of rosmarinic acid as the chemotaxonomic marker of the *Lamiacaae* family, especially as regarded the *Saturejoideae* sub-family [1], which comprised all our species examined. The results of our study showed that rosmarinic acid was accompanied in all these species by other hydroxycinnamic (caffeic acid) or benzoic (protocatechuic, *p*-hydroxybenzoic and gentisic acids) derivatives, which might potentially play the similar chemotaxonomic role in the *Saturejoideae* sub-family.

4. Conclusions

Our investigations confirmed the suitability of an elaborated SPE–reverse phase-HPLC method as a routine and simple procedure for isolation, qualification and quantification of free phenolic acids in plant material. The method used also enabled the efficient removal of interfering compounds (chlorophyll, waxes, polyphenols) by means of a SPE clean-up on an octadecyl sorbent and anion exchange resin.

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