https://doi.org/10.31925/farmacia.2019.6.10 **ORIGINAL ARTICLE**

HPTLC QUANTIFICATION OF POLYPHENOLIC ACIDS AND ANTIOXIDANT ACTIVITY OF *POLYGONUM HYDROPIPER* L. SPECIES FROM ROMANIAN FLORA

ROXANA KOSTICI [1], ANDREI BIȚĂ [2]*, LUDOVIC-EVERARD BEJENARU [2], CORNELIA BEJENARU [3], FLORIN-DAN POPESCU [4], GEORGE DAN MOGOŞANU [2], FLORICA POPESCU [1]

[1]*Department of Pharmacology, Faculty of Medicine, University of Medicine and Pharmacy of Craiova, 2 Petru Rareş Street, 200349 Craiova, Dolj County, Romania*
[2]*Department of Pharmacognosy & Phytotherapy, Faculty of Pharmacy, University of Medicine and Pharmacy of Craiova, 2 Petru Rareş Street, 200349 Craiova, Dolj County, Romania*
[3]*Department of Pharmaceutical Botany, Faculty of Pharmacy, University of Medicine and Pharmacy of Craiova, 2 Petru Rareş Street, 200349 Craiova, Dolj County, Romania*
[4]*Department of Allergology, Faculty of Medicine, "Carol Davila" University of Medicine and Pharmacy, 8 Eroii Sanitari Avenue, District 5, 050474 Bucharest, Romania*

**corresponding author: andreibita@gmail.com*

Manuscript received: October 2019

Abstract

For the flowering aerial parts of *Polygonum hydropiper* L. species, harvested from Romanian flora, four polyphenolic acids were separated and quantified using HPTLC-UV densitometry, with ESI-MS confirmation. Chlorogenic acid was found in the highest amount, followed by ferulic acid, *p*-coumaric acid and caffeic acid. The content of total polyphenols and caffeic acid derivatives was determined using spectrophotometric methods. *In vitro* antioxidant activity was evaluated using DPPH assay. Our studies bring new data regarding the phytochemical profile of *P. hydropiper*, as a natural source of polyphenols with antioxidant properties.

Rezumat

Părțile aeriene înflorite ale speciei *Polygonum hydropiper* L., recoltată din flora României, au fost analizate prin HPTLC–UV densitometrie, cu confirmare prin ESI–MS, fiind identificați patru acizi polifenolici. Cea mai mare concentrație s-a înregistrat pentru acidul clorogenic, urmat de acidul ferulic, acidul *p*-cumaric și acidul cafeic. Conținutul total de polifenoli și derivați ai acidului cafeic a fost determinat prin metode spectrofotometrice. Activitatea antioxidantă *in vitro* a fost evaluată folosind testul DPPH. Lucrarea completează datele fitochimice privind specia *P. hydropiper* ca sursă naturală de polifenoli cu proprietăți antioxidante.

Keywords: *Polygonum hydropiper*, HPTLC, polyphenolic acids, antioxidant activity

Introduction

Polygonum hydropiper L., water pepper, marsh pepper knotweed (*Polygonaceae*) is an herbaceous, annual, Eurasian species, common to ruderal places, wet, flooded or marshy areas, and swamps. In autumn, the stems become reddish-purple coloured and are provided with knots covered by short, ciliate ochreas. It blooms during July to September [5, 19]. *Polygoni hydropiperis herba* medicinal product contains many important active principles, such as: flavonoides (rutin, hyperoside, quercitrin) and aglycones (quercetin, kaempferol, isorhamnetin), mixed tannin, essential oil (tadeone, tadeonal - components having pungent taste, burner and irritating effect), phenylpropanoids (hydropiperosides), sesquiterpenoids (polygodial, polygonal), sterols, saponins [3, 4, 8, 15, 20]. Different extractive preparations obtained from the aerial parts of *P. hydropiper* exhibit some useful pharmacological actions, as follows: anti-inflammatory, antinociceptive, haemostatic, antihypertensive, hypoglycaemic, antibacterial, antifungal, antioxidant, anti-acetylcholinesterase, immunomodulatory, anti-angiogenic, antitumour, cytotoxic [4, 8, 11, 13, 15, 18, 20].

The aim of our paper is represented by the quantification through HPTLC-UV densitometry of polyphenolic acids and the antioxidant activity assessment of *Polygoni hydropiperis herba* medicinal product.

Materials and Methods

Chemicals and solvents

All chemicals (including chromatographic standards) and solvents (LiChrosolv®) were purchased from Sigma-Aldrich (Steinheim, Germany).

Plant material

The plant samples (aerial parts) from *P. hydropiper* species, were collected during the flowering period,

in July 2018, from the "Alexandru Buia" Botanical Garden, University of Craiova, Romania. Our research did not involve endangered or protected species. Voucher specimens (PH-1012018) are also deposited in the Herbarium of the Department of Pharmaceutical Botany, University of Medicine and Pharmacy of Craiova, Romania.

Preparation of the hydroalcoholic extract

Samples of accurately weighed, air-dried and powdered aerial parts of *P. hydropiper* species were macerated for 14 days with diluted alcohol (70% ethanol) at room temperature, according to the Romanian Pharmacopoeia, Xth edition (1993) [21]. *Polygoni hydropiperis herba* 20% hydroalcoholic extract (PHHAE) was filtered and then stored in dark bottles, in the refrigerator, until use.

HPTLC-UV densitometry analysis

High-performance thin-layer chromatography (HPTLC)–ultraviolet (UV) densitometry analysis of polyphenolic acids was performed using CAMAG TLC system (Muttenz, Switzerland), according to validated procedures highlighted in the literature [6, 7, 9, 12], using the following experimental conditions: stationary phase: HPTLC silica gel 60 F_{254} (Merck, Darmstadt, Germany) 20 × 10 cm precoated glass plates, pre-washed with chloroform–methanol (1:1, v/v) and activated by oven-drying (110°C, 30 minutes); mobile phase I: toluene:ethyl acetate:formic acid (7:2:1, v/v/v); mobile phase II: ethyl acetate:toluene:formic acid:water (13:4:3:2, v/v/v/v); in the developing twin-chamber, 10 mL of the mobile phases were added in the front through and 20 mL in the rear through, and then saturated for 20 minutes, at room temperature; sample: 20% hydroalcoholic extract of *Polygoni hydropiperis herba*; standards: 0.5% methanolic solutions of caffeic acid, chlorogenic acid, *p*-coumaric acid and ferulic acid; migration distance: 62 mm (sample application line 8 mm, solvent front 70 mm); sample (2, 3, 4, 5, 6, 7, 8, 9, 10 μL) and standards (2 μL) application: CAMAG Linomat 5 semiautomatic system - spray gas air, syringe volume 100 μL, dosage speed 150 nL/s, pre-dosage volume 0.2 μL, bands length of 8 mm; elution time: 15 minutes; plate drying: 5 minutes, at 20°C (cold air dryer); photographing the chromatographic plate: UV light (λ 254 nm); detection: CAMAG TLC Scanner 3 photodensitometer, for densitogram and *in situ* UV light (λ 280 nm) spectra, without chemical treatment (derivatization), deuterium–tungsten lamp, scanning speed 20 mm/s, data resolution 100 μm/step, measurement mode absorption; vision CATS ver. 2.5 software package (CAMAG).

Stock solutions were obtained by dissolving 0.5 mg of caffeic, ferulic, and *p*-coumaric acids and 5 mg of chlorogenic acid in 10 mL methanol. The stock solutions were used without dilution. The calibration curves were obtained by applying 2, 3, 4, 5 and 6 μL of each standard solution on the HPTLC plate. From the sample, we applied 7 μL on the plate. All measurements were performed in triplicate.

ESI-MS analysis

The mass spectrometry (MS) investigation was accomplished using the CAMAG TLC-MS Interface 2 and the Waters Acquity QDa detector coupled with the Waters 1525 binary pump. The spectra were acquired *via* the EmPower 3 software package. The detector was set in negative electrospray ionization (ESI^-) mode, capillary voltage 0.8 kV, cone voltage 15 V, probe temperature 400°C, and full scan between 100 to 1000 *m/z*. The mobile phase used for the online MS detection of polyphenolic acids was neat methanol. The flow rate of the mobile phase was 0.3 mL/min. The nebulizing and drying gas used was nitrogen [2, 9, 12].

Total polyphenolic content

Total polyphenolic content (TPC) was determined using the modified Folin-Ciocâlteu method and the results were expressed in gallic acid equivalents (GAE) (mg GAE/mL and mg GAE/g dry weight (d.w.), respectively) [1, 6, 7].

Content of caffeic acid derivatives

The content of phenolic acids, expressed as caffeic acid derivatives, was determined using Arnow spectrophotometric method and the results were expressed as in caffeic acid equivalents (CAE) (mg CAE/mL and mg CAE/g d.w., respectively) [1, 6, 7].

DPPH assay

For 2,2-diphenyl-1-picrylhydrazyl (DPPH) *in situ* qualitative assay, HPTLC plates were sprayed with a 0.5 mM methanolic solution of DPPH, in the CAMAG TLC Spray Cabinet 2, and dried at room temperature, in the dark, for 90 seconds, then heated at 60°C, in an oven, for 30 seconds. Chromatograms were documented at white light illumination [16].

DPPH radical scavenging activity was analysed spectrophotometrically, applying the method described by Olugbami *et al.* [14]. After incubating for 30 minutes in the dark, at room temperature, the absorbance was measured at 517 nm using a T70 UV/VIS Spectrometer (PG Instruments, Leicestershire, UK). The results were expressed as the concentration required to obtain 50% antioxidant effect value (EC_{50}) by determination of ascorbic acid equivalents (AAE) (μg AAE/mL and μg AAE/g d.w., respectively).

Results and Discussion

The experimental data regarding the HPTLC-UV densitometry analysis, content of polyphenols and DPPH antioxidant assay of *Polygonum hydropiper* L. species were highlighted in Figures 1-4 and Tables I and II.

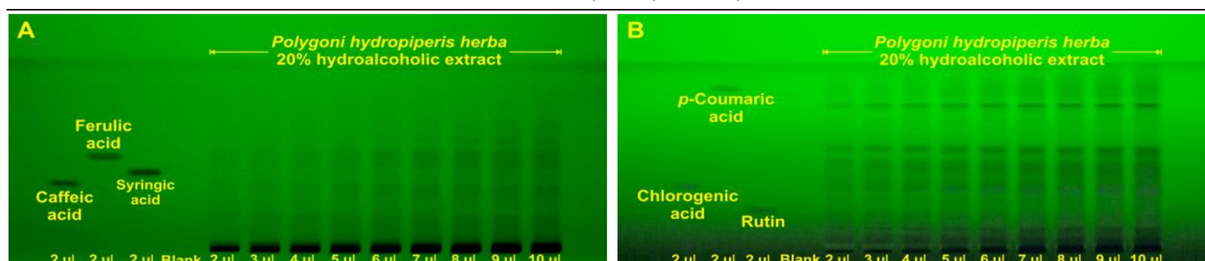

Figure 1.
HPTLC chromatograms of polyphenolic acids from *Polygoni hydropiperis herba* 20% hydroalcoholic extract (UV λ 254 nm, without derivatization): (A) Mobile phase I; (B) Mobile phase II

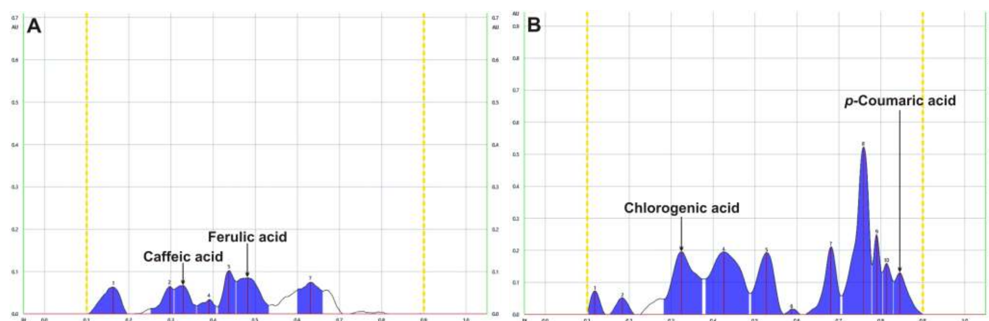

Figure 2.
Densitogram of polyphenolic acids (UV λ 280 nm, without derivatization) separated from *Polygoni hydropiperis herba* 20% methanolic extract: (A) Mobile phase I; (B) Mobile phase II

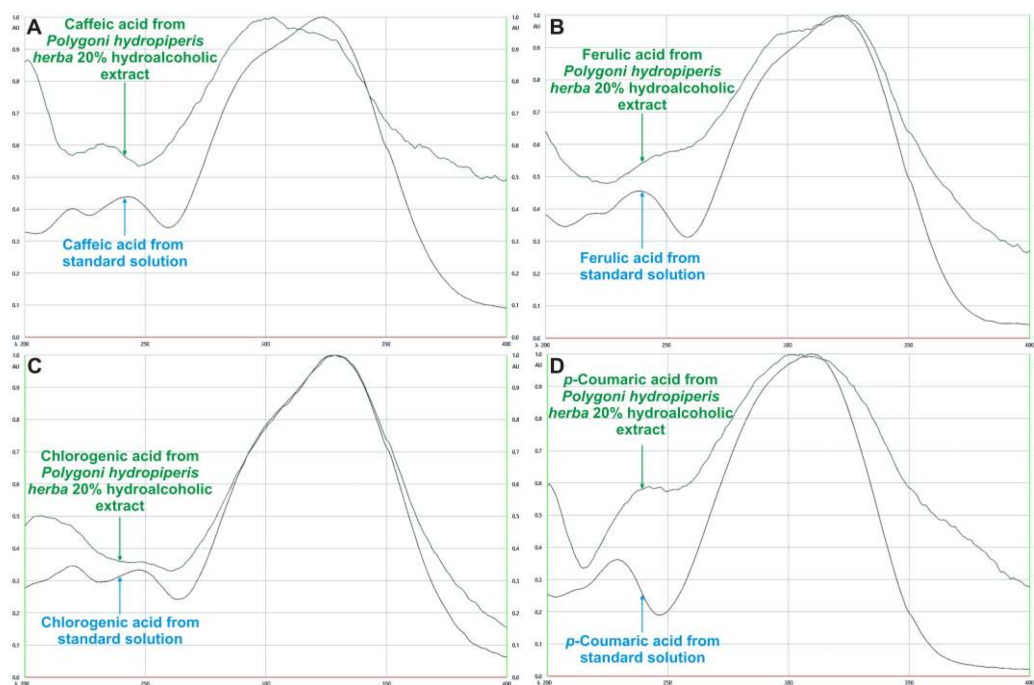

Figure 3.
In situ UV spectra (λ 280 nm) of polyphenolic acids standards and compounds separated from the analysed sample: (A) Caffeic acid; (B) Ferulic acid; (C) Chlorogenic acid; (D) *p*-Coumaric acid

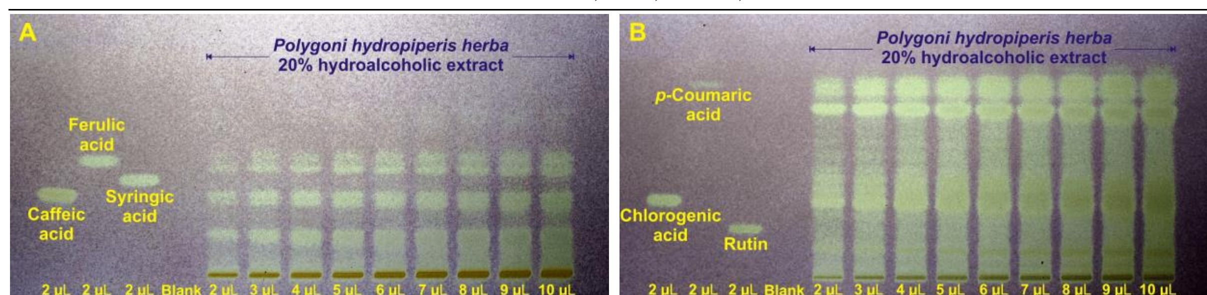

Figure 4.

HPTLC chromatograms of polyphenolic acids from *Polygoni hydropiperis herba* 20% hydroalcoholic extract (white light illumination, derivatization with 0.5 mM DPPH methanolic solution): (A) Mobile phase I; (B) Mobile phase II

Table I

HPTLC–UV densitometry analysis, with ESI–MS confirmation, for polyphenolic acids from *Polygonum hydropiper* species

Compound	R_f ± SD	m/z	PHHAE (µg/mL)	PHH (µg/g d.w.)
Caffeic acid	0.34 ± 0.006	179.1	21.5 ± 0.41	107.5 ± 2.13
Chlorogenic acid	0.35 ± 0.007	176.15; 353.21	342.3 ± 6.82	1711.5 ± 34.19
p-Coumaric acid	0.85 ± 0.015	163.08	34.75 ± 0.68	173.75 ± 3.46
Ferulic acid	0.48 ± 0.009	193.16	41.37 ± 0.82	206.85 ± 4.12

HPTLC: high-performance thin-layer chromatography; UV: ultraviolet; ESI: electrospray ionization; MS: mass spectrometry; SD: standard deviation; PHHAE: *Polygoni hydropiperis herba* hydroalcoholic extract; PHH: *Polygoni hydropiperis herba*; d.w.: dry weight. Values are expressed as mean ± SD (n = 3)

Table II

Content of polyphenols and antioxidant activity of *Polygonum hydropiper* species

Sample	TPC	CAE	DPPH EC_{50}
PHHAE	5.51 ± 0.09 mg GAE/mL	7.63 ± 0.11 mg CAE/mL	14.65 ± 0.24 µg AAE/mL
PHH	27.52 ± 0.43 mg GAE/g d.w.	38.16 ± 0.62 mg CAE/g d.w.	73.26 ± 1.28 µg AAE/g d.w.

PHHAE: *Polygoni hydropiperis herba* hydroalcoholic extract; PHH: *Polygoni hydropiperis herba*; TPC: total phenolic content; GAE: gallic acid equivalents; d.w.: dry weight; CAE: caffeic acid equivalents; DPPH: 2,2-diphenyl-1-picrylhydrazyl; EC_{50}: concentration required to obtain 50% antioxidant effect; AAE: ascorbic acid equivalents. Values are expressed as mean ± SD (n = 3)

The content of polyphenolic acids, of *P. hydropiper* species from southwestern Romanian flora, was analysed for the first time using HPTLC-UV densitometry with ESI–MS confirmation. In the decreasing order of concentrations, four polyphenolic acids have been identified and quantified (µg/mL) for 20% PHHAE: chlorogenic acid (342.3 ± 6.82) > ferulic acid (41.37 ± 0.82) > *p*-coumaric acid (34.75 ± 0.68) > caffeic acid (21.5 ± 0.41). In a recent study, using high-performance liquid chromatography (HPLC)-diode-array detection (DAD), Mihaylova *et al.* evaluated the content of polyphenolic acids of hydroalcoholic (10% tincture) from PHH [10]. As far as we know, this is the only study from the specialized literature concerning the analysis of polyphenolic acids from *Polygoni hydropiperis herba* (PHH) medicinal product. Our results are comparable to those obtained for the Bulgarian sample (10% PHHAE), regarding chlorogenic acid (as the main compound) and caffeic acid. In addition, different other polyphenolic acids were identified and quantified, such as protocatechuic acid, gallic acid and cinnamic acid [10]. Our sample (20% PHHAE) contains ferulic acid and *p*-coumaric acid, components that were not found in the herbal sample from Plovdiv (in the south of Bulgaria). This is yet another proof of the influence of pedoclimatic conditions and geographic area on the chemical composition of natural products.

For different hydroalcoholic extracts prepared from PHH, previous reports evaluated TPC over a wide range of 1.63 - 17.44 mg GAE/g d.w. [10, 17]. Taking into account the differences between the origin of the PHH medicinal product (Bulgaria, Bangladesh, Romania), the extraction methods and the ethanol concentration, for Romanian sample the value of TPC is close to that obtained in the case of the Bulgarian sample (27.52 ± 0.43 mg GAE/g d.w. for 20% PHHAE *vs*. 17.44 ± 0.24 mg GAE/g d.w. for 10% PHHAE, respectively). It was also highlighted that the maximum extraction yield for total polyphenols was obtained in the case of hydroalcoholic extracts from PHH [10, 17].

For the first time, in our research, the content of phenolic acids of PHHAE and PHH was expressed as

caffeic acid derivatives (7.63 ± 0.11 mg CAE/mL and 38.16 ± 0.62 mg CAE/g d.w., respectively). In this regard, Mihaylova *et al.* determined the total amount of the phenolic acids ranging from 1411.6 μg/g d.w. (for water extract) to 3243.74 μg/g d.w. (for 10% PHHAE); it should be mentioned that, in this case, the content of total phenolic acids was expressed differently, taking into account the specificity of the analytical method applied [10].

In situ screening of the antioxidant activity of polyphenolic acids from 20% PHHAE was determined after chromatographic separation, by HPTLC–DPPH assay. As can be seen in Figure 4, HPTLC silica gel plates stained with DPPH• radicals exhibited a light violet/purple background, with yellow bands in areas where the compounds with antioxidant activity are found. The intensity of the yellow colour of the chromatographic bands is directly correlated with the amount of polyphenolic acids (mainly chlorogenic acid) identified and quantified for the analysed sample [16]. In fact, concerning the evaluation of PHH antioxidant activity, the researches focused only on TPC, flavonoids (derivatives of quercitrin, isorhamnetin, kaemferol, quercetin) and phenylpropanoid esters of sucrose (hydropiperoside B, vanicodes A and E) [8, 10, 15, 17, 20]. Sharif *et al.* analysed the total antioxidant capacity of different extracts (in methanol, ethanol, chloroform, *n*-hexane, petroleum ether) of PHH harvested from Bangladesh. It was found that the ethanolic extract possesses the highest total antioxidant capacity (EC_{50} 12.211 μg AAE/mL) [3, 17]. A similar result was obtained for the Romanian sample (EC_{50} 14.65 μg AAE/mL). The data from the other papers regarding the antioxidant capacity of PHH are difficult to compare due to the variability of the herbal products, of the extraction techniques and solvents, as well as of the methods applied for the evaluation of radical scavenging properties [8, 10, 15, 17, 20]. However, we found that the antioxidant activity of *P. hydropiper* species from Romanian flora is correlated with the content of polyphenols (TPC and CAE).

Conclusions

As far as we are aware, our study reports for the first time, in the case of *P. hydropiper* species collected from Romanian flora, four polyphenolic acids were separated and quantified by HPTLC–UV densitometry, with ESI-MS confirmation. The highest amount was recorded for chlorogenic acid, followed by ferulic acid, *p*-coumaric acid and caffeic acid. Also, TPC and caffeic acid derivatives (CAE) were determined by spectrophotometric methods, and *in vitro* antioxidant activity was evaluated by DPPH assay. *P. hydropiper* species is a natural source of polyphenols exhibiting antioxidant activity.

References

1. Andreicuţ AD, Pârvu AE, Moţ AC, Pârvu M, Fischer-Fodor E, Feldrihan V, Cătoi AF, Cecan M, Irimie A, Anti-inflammatory and antioxidant effects of *Mahonia aquifolium* leaves and bark extracts. *Farmacia*, 2018; 66(1): 49-58.
2. Arakawa R, Yamaguchi M, Hotta H, Osakai T, Kimoto T, Product analysis of caffeic acid oxidation by on-line electrochemistry/electrospray ionization mass spectrometry. *J Am Soc Mass Spectrom.*, 2004; 15(8): 1228-1236.
3. Arora P, Lamba HS, Sharma P, Pharmacological, phytochemical, biological evaluation and future prospects of *Polygonum hydropiper*. *World J Pharm Res.*, 2018; 7(5): 539-551.
4. Ayaz M, Junaid M, Ullah F, Sadiq A, Subhan F, Khan MA, Ahmad W, Ali G, Imran M, Ahmad S, Molecularly characterized solvent extracts and saponins from *Polygonum hydropiper* L. show high anti-angiogenic, anti-tumor, brine shrimp, and fibroblast NIH/3T3 cell line cytotoxicity. *Front Pharmacol.*, 2016; 7: 1-13.
5. Ciocârlan V, Romania illustrated flora. *Pteridophyta et Spermatophyta*. 2nd ed. Revised and improved, Ed. Ceres, Bucharest, 2000; 262, (available in Romanian).
6. Hanganu D, Benedec D, Vlase L, Olah N, Damian G, Silaghi-Dumitrescu R, Moţ AC, Toma CC, Polyphenolic profile and antioxidant and antibacterial activities from two *Trifolium* species. *Farmacia*, 2017; 65(3): 449-453.
7. Hanganu D, Olah NK, Pop CE, Vlase L, Oniga I, Ciocarlan N, Matei A, Puşcaş C, Silaghi-Dumitrescu R, Benedec D, Evaluation of polyphenolic profile and antioxidant activity for some *Salvia* species. *Farmacia*, 2019; 67(5): 801-805.
8. Huq AK, Jamal JA, Stanslas J, Ethnobotanical, phytochemical, pharmacological, and toxicological aspects of *Persicaria hydropiper* (L.) Delarbre. *Evid Based Complement Alternat Med.*, 2014; 2014:1-11.
9. Krüger S, Hüsken L, Fornasari R, Scainelli I, Morlock GE, Effect-directed fingerprints of 77 botanical extracts *via* a generic high-performance thin-layer chromatography method combined with assays and mass spectrometry. *J Chromatogr A*, 2017; 1529: 93-106.
10. Mihaylova D, Vrancheva R, Ivanov I, Popova A, Phytochemical profile and antioxidant activity of water pepper (*Polygonum hydropiper* L.). *J Microbiol Biotech Food Sci.*, 2019; 8(5): 1205-1208.
11. Miyazaki Y, Immune effects and antiacetylcholinesterase activity of *Polygonum hydropiper* L. *Biosci Microbiota Food Health*, 2016; 35(2): 69-75.
12. Morlock GE, Lapin T. Effect-directed analysis of *Pimpinella saxifraga* L. root extract *via* HPTLC-UV/Vis/FLD-EDA-MS. *JPC J Planar Chromatogr.*, 2018; 31(1): 79-86.
13. Oany AR, Siddikey AA, Hossain MU, Islam R, Al Emran A, A preliminary evaluation of cytotoxicity, antihyperglycemic and antinociceptive activity of *Polygonum hydropiper* L. ethanolic leaf extract. *Clin Phytosci.*, 2016; 2: 1-6.
14. Olugbami JO, Gbadegesin MA, Odunola OA, *In vitro* free radical scavenging and antioxidant properties of

ethanol extract of *Terminalia glaucescens*. *Pharmacogn Res.*, 2015; 7(1): 49-56.
15. Peng ZF, Strack D, Baumert A, Subramaniam R, Goh NK, Chia TF, Tan SN, Chia LS, Antioxidant flavonoids from leaves of *Polygonum hydropiper* L. *Phytochemistry*, 2003; 62(2): 219-228.
16. Pozharitskaya ON, Ivanova SA, Shikov AN, Makarov VG, Separation and free radical-scavenging activity of major curcuminoids of *Curcuma longa* using HPTLC–DPPH method. *Phytochem Anal.*, 2008; 19(3): 236-243.
17. Sharif S, Shahriar M, Haque MA, Chowdhury ZS, Islam MR, Bhuiyan MA, *In-vitro* antioxidant activities, anti-nociceptive and neuropharmacological activities of *Polygonum hydropiper. J Biol Agric Healthcare*, 2013; 3(9): 61-71.
18. Tao J, Wei Y, Hu T, Flavonoids of *Polygonum hydropiper* L. attenuates lipopolysaccharide-induced inflammatory injury *via* suppressing phosphorylation in MAPKs pathways. *BMC Complement Altern Med.*, 2016; 16: 1-15.
19. Tutin TG, Burges NA, Chater AO, Edmondson JR, Heywood VH, Moore DM, Walters SM, Webb DA (eds), *Flora Europaea*. Volume 1: *Psilotaceae* to *Platanaceae*. 2nd edition, Cambridge University Press, Cambridge, 1993, 96.
20. Xiao H, Rao Ravu R, Tekwani BL, Li W, Liu WB, Jacob MR, Khan SI, Cai X, Peng CY, Khan IA, Li XC, Wang W, Biological evaluation of phytoconstituents from *Polygonum hydropiper*. *Nat Prod Res.*, 2017; 31(17): 2053-2057.
21. *** Farmacopeea Română ediţia a X-a. Ed. Medicală, Bucureşti, 1993, 335, 921-922 (available in Romanian).