

BIOINFORMATIC INSIGHT INTO *PORTULACA OLERACEA* L. (PURSLANE) OF BULGARIAN AND GREEK ORIGIN

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Portulaca oleracea L. (Portulacaceae) is used as functional food and its nutritional and therapeutic properties are related to the high levels of organic and fatty acids, polyphenols, polysaccharides and cyclo-dopa amides. This study presents a strategy based on liquid chromatography – high resolution accurate mass spectrometry method (LC – HRAMS) and bioinformatic methods to analyze 33 purslane accessions originating from 11 floristic regions in Bulgaria together with 5 accessions of Greek provenance. Extracts were obtained by microwave extraction. Based on the LC-MS metabolic “fingerprints” of assayed samples, a purslane metabolic database was developed. LC-MS data were proceeded with Software application Compound Discover 2.0 (Thermo Fischer Sci., USA). Principal Component Analysis (PCA) combined with both descriptive and differential analyses were used to find marker metabolites to distinguish different geographical regions. The differential analysis of the Bulgarian and Greek samples allowed the identification of 50 marker metabolites. Based on accurate masses, retention times, fragmentation patterns in MS/MS, comparison with commercial standards and literature data, these secondary metabolites were identified after detailed analysis of Volcano-plots. For the first time, 29 compounds are reported. The identified compounds were used to perform a study of the biosynthetic pathways of purslane secondary metabolites using Kyoto Encyclopedia of Genes and Genomes (KEGG) software platform. The statistical treatments identified marker compounds that can be used to distinguish the origin of accession set. Combining LC-MS data with multivariate statistical analysis was shown to be effective in studying the purslane metabolites, allowing for integration of chemistry with geographic origin.

Keywords: *Portulaca oleracea*, LC–HRAMS, secondary metabolites, descriptive analysis, multivariate statistics

INTRODUCTION

Portulaca oleracea L. (Portulacaceae) is an herbaceous succulent annual plant, commonly known as purslane (Delipavlov and Češmedžiev, 2003). It is a widely distributed weed, native to India and the Middle East. Nowadays, the species is naturalized in Europe, Africa, USA, China and Australia (Oliveira et al., 2009).

Purslane is one of the most common plants in the world. It is traditionally used in the human diet

of green leafy vegetables and can be consumed in raw salads, cooked in soups or sauces, as well as used for infusion (Lim and Quah, 2007; Bosi et al., 2009). The species is listed by the World Health Organization as one of the most frequently used medicinal plant, called “Global Panacea” (Lim and Quah, 2007) and the herb can substitute many additives and drugs (Farghaly et al., 2012).

The purslane is drawing increased interest from medical, phytochemical and nutritional scientists. Zhou et al. (2015) reviewed the phytochem-

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istry and pharmacological effects of the species. The plant is rich in omega-3 fatty and organic acids (Oliveira et al., 2009; Uddin et al., 2014). A variety of secondary metabolites belonging to phenolic and acylquinic acids, homoisoflavones and flavonoids, chalcones and coumarins have been reported (Yan et al., 2012; Hanan et al., 2014; Ai et al., 2015; Yang et al., 2018). Generally, the phenolic compounds content highly depends on the plant origin (Ai et al., 2015).

Cinnamic acid and cyclo-dopa amides (Kokubun et al., 2012), triterpenoid saponins (Xin et al., 2008) and polysaccharides (Zidan et al., 2014) have also been reported.

Oleraceins are representative cyclo-dopa amides of purslane. Six oleraceins A-E were isolated and structurally characterized (Xiang et al., 2005; Yang et al., 2009). In addition, eight new indoline amide glucosides (oleraceins H-O) were tentatively assigned based on their UV and MS spectra (Jiao et al., 2014). Then, the authors isolated and structurally elucidated oleraceins H, I, K, L, N-S along with the antioxidant capacity of a few compounds (Jiao et al., 2015).

Recently, the novel alkaloids oleracons and oleracimines with promising anti-inflammatory activity have been discovered (Li et al., 2016; Meng et al., 2016).

The species is reported to have also antimicrobial activity (Ramesh and Hamumantapa, 2011), hypoglycaemic effect (Sicari et al., 2018) and to improve the lipid profile (Zidan et al., 2014). The antioxidant potential of purslane has been evaluated by DPPH, ABTS and FRAP assays; moreover, the biological capacity has been determined by α -amylase and α -glucosidase inhibition tests (Uddin et al., 2012; Sicari et al., 2018).

In our previous study, the contents of total polyphenols, flavonoids, polysaccharides and

saponins, and extraction yields were determined, as well as the antioxidant potential of accessions of Bulgarian and Greek provenance (Gevrenova et al., 2016). Afterwards, Voynikov et al. (2019) developed UHPLC-HRMS method for simultaneous determination of phenolic acids and flavonoids in purslane with emphasis on the structural characterization of the oleraceins.

To the best of our knowledge, there is no metabolic database for purslane based on bioinformatic methods. For the description of secondary metabolites in a medicinal plant, the development of a common metabolic database is of great importance.

The aim of this study is to develop a method based on liquid chromatography – high resolution accurate mass spectrometry (LC – HRAMS) and bioinformatic methods to analyze 33 purslane accessions originating from 11 floristic regions in Bulgaria together with 5 accessions of Greek provenance. The object is to develop a purslane metabolic database dedicated to LC-MS metabolic “fingerprints” of the assayed samples.

MATERIALS AND METHODS

PLANT MATERIAL

Aerial parts of 33 Bulgarian *P. oleracea* accessions from 11 floristic regions and 5 accessions from Greece were collected during the full flowering stage (July-September 2016) (Table 1). The plants were identified by one of us (V. B.) according to Georgiev (1966). Voucher specimens were deposited at Herbarium Facultatis Pharmaceuticae Sophiensis, Medical University-Sofia, Bulgaria (Voucher specimen № 11 575–11 612).

TABLE 1. The investigated purslane accessions of Bulgarian and Greek origin.

Bulgarian floristic regions	№ of plant material origin	Coordinates	Collection period
Danube plain (D)	1. Komarevo	43.57° N 24.62° E	Aug 2016
	2. Pleven	43.41° N 24.62° E	Sept 2016
	3. Brest	43.63° N 24.58° E	Aug 2016
	4. Totleben	43.43° N 24.98° E	Aug 2016
	5. Koinare	43.36° N 24.15° E	Sept 2016

Bulgarian floristic regions	Nº of plant material origin	Coordinates	Collection period
Balkan foothill (Bf)	6. Mezdra	43.15° N 23.7° E	Aug 2016
	7. Ledenik	43.08° N 25.55° E	Sept 2016
	8. Samovodene	43.13° N 25.6° E	Jul 2016
	9. Ressen, Bakadzhika	42.45° N 26.68° E	Aug 2016
	10. Ressen	43.2° N 25.55° E	Aug 2016
Northeast Bulgaria (NEB)	11. Palici	42.9° N 26° E	Sept 2016
	12. Dobric	43.57° N 27.83° E	Aug 2016
Znepole region (Zn)	13. Partizani	43.02° N 27.25° E	Jul 2016
	14. Dupnica	42.27° N 23.12° E	Jul 2016
Mesta valley (M)	15. Slashten	41.5° N 24.02° E	Sept 2016
Stara Planina Mt. (East) (SpE)	16. Karnobat	42.65° N 26.98° E	Aug 2016
Sofia region (S)	17. Pirdop	42.7° N 24.18° E	Aug 2016
	18. Belopopci	42.66° N 23.82° E	Jul 2016
Thracian valley (Tv)	19. Opulchenec	42.2° N 25.12° E	Aug 2016
	20. Bratya Daskalovi	42.3° N 25.22° E	Jul 2016
	21. Plodovitovo	42.17° N 25.22° E	Sept 2016
	22. Cherna gora	42.22° N 25.23° E	Aug 2016
	23. Parvomai	42.1° N 25.22° E	Sept 2016
	24. Gradina	42.13° N 25.2° E	Sept 2016
Tundja hills region (Thr)	25. Pazhardzik	42.19° N 24.33° E	Aug 2016
	26. Straldja	42.6° N 26.68° E	Sept 2016
	27. Yambol	42.48° N 26.51° E	Jul 2016
	28. Kabelle	42.55° N 26.48° E	Jul 2016
	29. Pobeda	42.42° N 26.65° E	Jul 2016
Stranja Mt. (Sj)	30. Kubadin	42.3° N 26.95° E	Sept 2016
	31. Sredec	42.70° N 23.32° E	Aug 2016
South Black Sea Coast (SBSC)	32. Burgas	42.5° N 27.47° E	Aug 2016
	33. Ravadinovo	42.38° N 27.67° E	Aug 2016
Plant material origin (Greece)			
Kassandra, Halkidiki Peninsula	34. Possidi	39.96° N 23.38° E	Jul 2016
	35. Pefkohori	39.99° N 23.61° E	Sept 2016
	36. Lagonisi	40.23° N 23.75° E	Jul 2016
	37. Lagomandra	40.14° N 23.43° E	Jul 2016
	38. Komotini	41.12° N 25.4° E	Aug 2016

MICROWAVE EXTRACTION PROCEDURE

Air-dried powdered purslane aerial parts (100 mg) of each sample were extracted with 5 ml 0.1% formic acid in Microwave Synthesis Reactor, Microwave EDU (Anton Paar, Austria) for 5 min at 120°C. Each extraction was proceeded in triplicate and afterwards filtered through filter paper KA1 (PA-PIRNA Perštein s.r.o., Czech Republic). Then the filtrates were centrifuged for 10 min at 13 000 rpm and filtered on 0.45 mm membrane prior to LC-HRAMS analysis.

CHEMICALS

Acetonitrile and formic acid for LC-MS, and methanol for HPLC were purchased from Merck (Merck, Bulgaria).

The reference standards used for compounds identification were obtained as follows: chlorogenic acid from Extrasynthese (Genay, France); *p*-coumaric and *o*-coumaric acid, ferulic acid, quercetin, quercetin-3-*O*-glucoside, rutin were supplied from Phytolab (Germany); phenylacetic acid, cinnamic acid, *quinic acid*, nicotinic acid, nicotinamide and citric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

UHPLC-HRAMS ANALYSIS

LC-HRAMS analyses were carried out on a Q Exactive high resolution mass spectrometer (Thermo Fisher Scientific Inc., USA) equipped with TurboFlow TM Transcend chromatography system (Thermo Fisher Scientific Inc., USA) and heated electrospray ionization (HESI II) source. Data acquisition and processing were done by XCalibur® 2.4 software (Thermo Fisher Scientific Inc., USA).

UHPLC CONDITIONS

Analyses were carried out on a Synchronis C18 column of 1.7 µm, 100 x 2.1 mm, using mobile phases as follows: (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The following gradient was used: 0% B for 1 min, 0 – 60% B for 30 min, 60 – 90% B for 4 min, 90% B for 3 min. The flow rate was 300 µl/min. The injection volume was 10 µl.

MASS SPECTROMETRY CONDITIONS

The analyses were conducted on a Q Exactive Mass spectrometer in positive and negative operation modes. The instrumental parameters were as follows:

Spray Voltage – 4.0 KV, Sheath Gas – 30 AU, Auxiliary Gas – 12 AU, Capillary Temperature – 300°C, Spare Gas – 3 AU, Heater Temperature – 300°C.

Full scan experiments were carried out in a range of 120 – 1 500 m/z at 140 000 resolution. The ratio signal to noise 10:1 was used as a parameter for selecting the peak areas of the compounds subjected subsequently to parallel reaction monitoring (PRM) mode.

Analyses of selected compounds were performed using PRM mode at 17 000 resolution. The normalized collision energy (NCE) used in fragmentation experiments was set at 35. To reduce misleading positive results as well as time for calculations, some limitations were applied to certain steps in the algorithm. These restrictions were applied to: mass tolerance of 1 ppm, tolerance for retention time of 0.2 min and maximum number of results (100) per data base.

BIOINFORMATIC METHODS

Raw data files from LC-MS analyses were processed by Software application Compound Discoverer 2.0 (Thermo Fischer Sci., USA).

Individual compounds were predicted using the meta search engine ChemSpider (data bases ChEBI; EU-OpenScreen; FDA; KEGG; Natural Product Updates; Nature Chemistry; NIST; NIST Spectra; Wikipedia; WikiChem Physchim 62). The involvement of the established metabolites in potential metabolic pathways was verified using Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolite data base together with HRAM Compound Database; Endogenous Metabolites Database 4 400 Compounds; extractables and leachables HRAM Compound Database; personal data base with 650 plant secondary metabolites and 45 previously identified purslane metabolites.

Principal Component Analysis (PCA) combined with both descriptive and differential analyses were processed by Compound Discoverer application to create plots shown in two-dimensional figures.

RESULTS AND DISCUSSION

HIGH RESOLUTION ACCURATE MASS SPECTROMETRY (HRAMS) METABOLIC FINGERPRINTS

According to the common approach in reverse phase ultra high-performance liquid chromatography, the

binary mobile phase of acetonitrile-water (0.1% formic acid) was used in the chromatographic analyses. The metabolic “fingerprints” include over 1 800 chromatographic peaks in the mass spectral scan range. A representative chromatogram of purslane extract (sample 21) is presented in Supplementary material S1. Based on the parameter, the largest area of the chromatographic peaks, 70 of them were selected and analyzed in Parallel Reaction Monitoring mode. For a more accurate detection, they were included in the eloquence timeline inclusion list. Files were subjected to data processing with the Compound Discoverer application algorithm described in the Materials and Methods section.

STATISTICAL ANALYSIS OF PURSLANE SAMPLES OF DIFFERENT GEOGRAPHIC ORIGINS

For analysis of LC-HRAM data, a non-target metabolic statistical analysis PCA was used to identify possible clusters of closely related samples (Fig. 1). The visualization of the PCA score plot shows similarities/dissimilarities between (explained by PC 1) and within (explained by PC2) the sample clusters. The correlation is based on the chromatographic peak areas of the metabolites with the highest contents originating from the floristic regions of Northern Bulgaria (Danube plain and Balkan foothill), Southern Bulgaria (Thracian valley and Tundja hills region) and Greece (Gr). The floristic regions with fewer than 3 representative samples were not embedded into the set. Principal component 1 (PC1) explains 32.9% of the variation, while PC2 adds 13.8%. On the PCA score plot, the Danube plain samples were placed very close to each other in positive PC2 except for sample 1. It possesses the smallest sum of peak areas, as was seen in the Box-and-whisker plot (data not shown), and appears to be an outlier.

Among the seven samples from the Thracian valley, five were located left side of the vertical axis (negative PC1 values) while two of them were positioned to the positive side of PC1. The Balkan foothill samples were placed to the positive PC1 and PC2 except for one. The samples of Greek provenance were associated with negative values of PC1 whereas the Tundja hills region samples showed positive PC1. It appears that the geographically related Thracian valley and Greek accessions “gravitate” to the upper left quadrant of the chart, which generally speaks of a trend in the distribution of components of these origins.

The differential analysis of the samples from Bg vs Gr is presented in Fig. 2. In addition, a comparison of the following pairs of floristic regions is illustrated: the Tundja hills vs the Thracian valley, the Danube region vs the Tundja hills, the Tundja hills vs Greece, the Tundja hills vs the Balkan foothill (Supplementary material S2). Data from the analysis summary indicate that for the negative fold change (FC), 463 metabolites showed statistically significant decrease, 643 decreased and had $p \leq 0.05$ and 1 168 were with $FC \leq -1$. For the positive FC, 466 were statistically increased, 636 increased and had $p \leq 0.05$, and 1 324 – with $FC \geq 1$.

In order to identify metabolite markers that define the differences between the geographical areas of Bg/Gr, the following approach was applied. The data set excludes those that do not fall within the areas of statistically significant differences, and after applying the Compound Discoverer algorithm for the databases used, the ChemSpider search engine and the local databases, probabilistic markers are suggested.

Based on the extracted ion chromatograms, accurate masses of protonated/deprotonated molecules (a mass tolerance of up to 1 ppm), chemical formulas, fragmentation patterns in MS/MS, comparisons with retention times of available standards, data from mass spectral libraries for structural identification of small molecules from Compound Discoverer and literary data, the tentative structures of metabolites in purslane are proposed.

On the basis of the differential metabolomic analysis data and the above described approach, 50 secondary metabolites, which showed statistically significant differences in the Bg vs Gr comparative analysis (Fig. 2, Table 2), were identified or putatively annotated. Corresponding MS/MS fragmentation patterns are depicted in Supplementary material S3. They belong to the following classes: phenolic alcohols and aldehydes, hydroxycinnamic acids and alcohols, acylquinic acids, cinnamic acid amides, coumarins, flavonoids, lignans, naphthoquinones, amino acids and derivatives, vitamins, indole derivatives and tetrahydroisoquinoline.

The species exhibits a high compositional diversity. Among the 50 metabolites, 29 were identified for the first time in purslane with different MSI identification level: I level – 13 compounds; II level – 36 compounds; III level – 1 compound (Table 2). Compounds **10**, **12**, **18**, **25**, **28**, **29** and **30** were identified only in the Greek

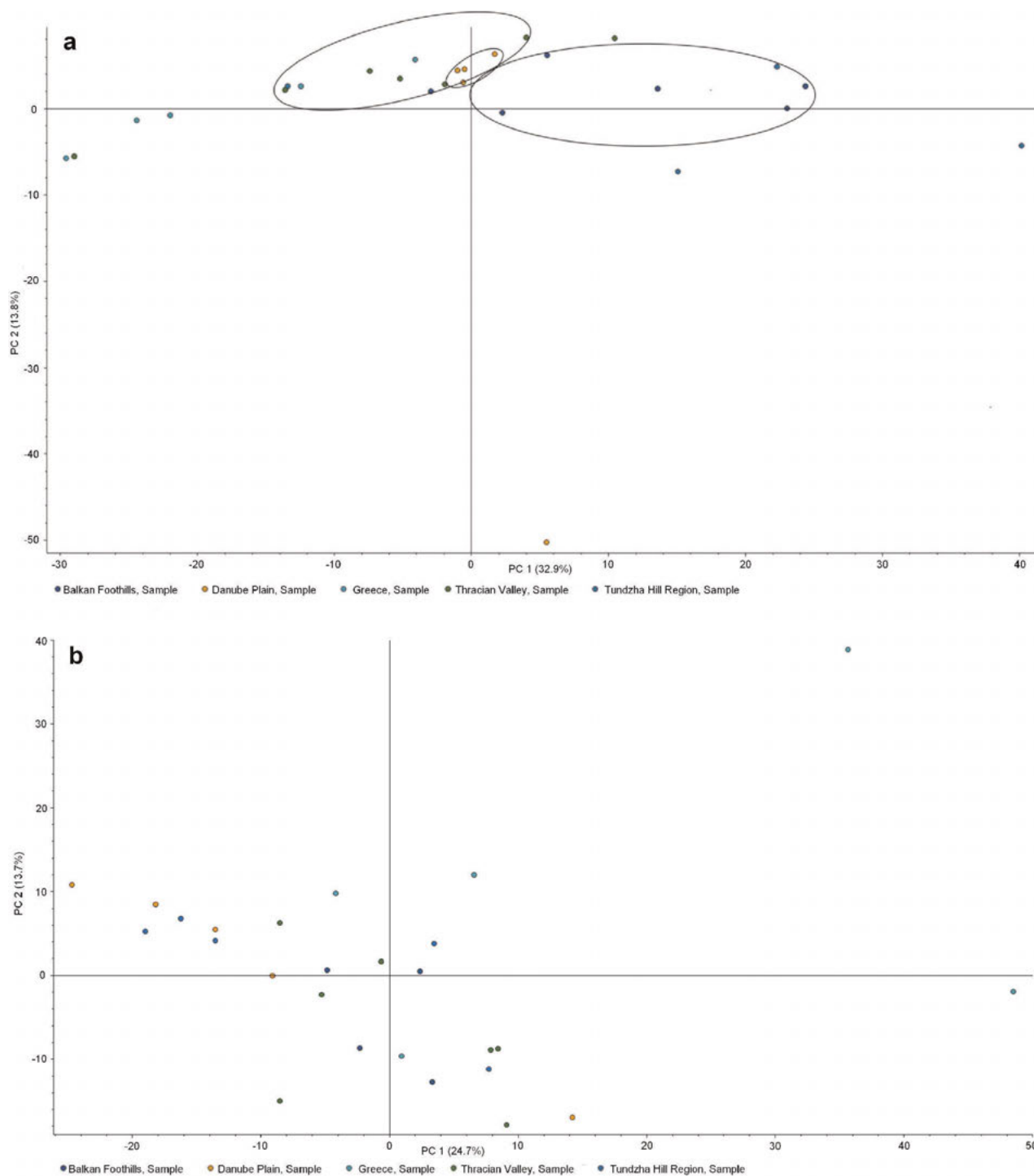


Fig. 1. Score (a) and loading (b) plots based on geographic origin of the samples. *Light blue* - Greece; *blue* - Tundzha hills region; *dark blue* - Balkan foothill; *green* - Thracian valley; *orange* - Danube plain. X-axis represents Area (PC1)/Area (average PC1) [%]; Y-axis - Area (PC2)/Area (average PC2) [%].

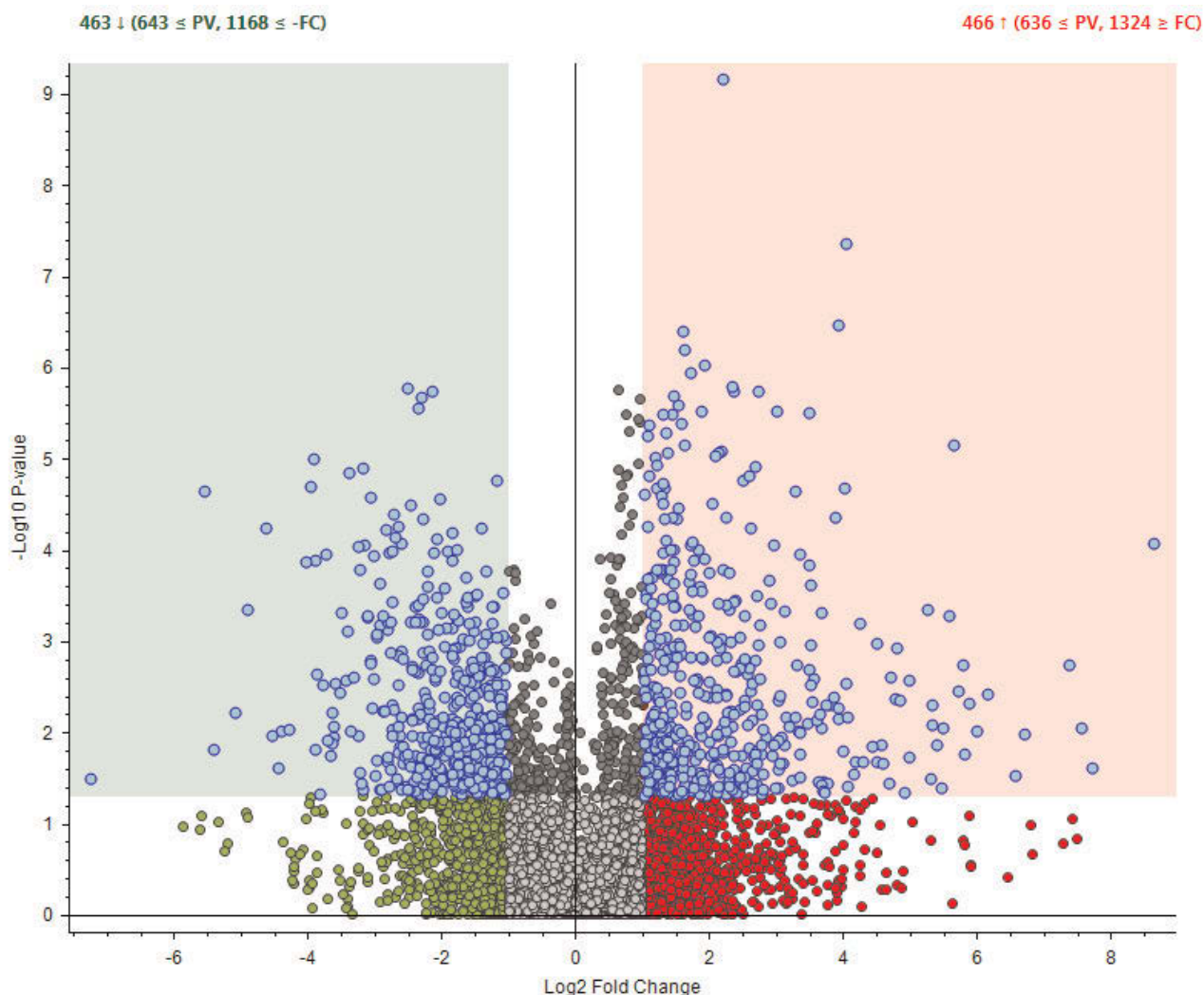


Fig. 2. Volcano-plots of differential metabolomic analysis between Bg vs Gr samples. *Light grey* – statistically nonsignificant differences, not reaching the FC threshold; *light green* – statistically nonsignificant differences, lower than the FC threshold; *light red* – statistically nonsignificant differences, higher than the FC threshold; *blue* (right) – statistically significant differences, higher than the upper FC threshold; *dark grey* – statistically significant differences ($p \leq 0.05$), not reaching the FC threshold; *blue* (left) – statistically significant differences ($p \leq 0.05$), lower than the FC threshold.

accessions; **18**, **28**, **29** and **30** were undescribed in the purslane.

Compound **13** was a characteristic of sample 26 (Thr), **21** was found for sample 31 (Sj) and **36** – for sample 33 (SBSC) and all of them are new ones for the species.

The Danube plain floristic region provenance (1–5 sample) is rich in **8**, **15**, **19**, **35** and **36** while **3**, **4**, **12** were not evidenced. The Znepole region represented by sample 13 is abounded with **8**, **12**, **16**, **19**, **20**, **29** and **32**.

The accessions from the Thracian valley (19–25 samples) were grouped by the occurrence of **11**, **14**,

17, **19**, **20**, **23**, **24**, **37**, **38**, **40** and **47**. Thus, they share common cinnamic acid amides and coumarins with the Greek samples. It appears that the Balkan foothill, the Sofia region and Northeast Bulgaria floristic regions could not be distinguished by notable compounds. Compound **15** is exclusive for the Bulgarian samples. The representative of purslane cyclo-dopa amides oleracein A–E (**31–35**) (Taha and Osman, 2015; Farag and Shakour, 2019; Voynikov et al., 2019) together with **48–50** were commonly spread in both Bulgarian and Greek samples.

The descriptive analysis visually compares the chromatogram peak area statistics of oleracein A-D

TABLE 2. Identification and annotation of the secondary metabolites in the differential metabolomic analysis of Bulgarian and Greek samples.

Class of metabolites	№	RT [min]	Accurate mass [M+H] ⁺ / [M-H] ⁻ [m/z]	Error, ppm	Exact mass [M+H] ⁺ / [M-H] ⁻ [m/z] formula	Identification	Detected in samples	MSI level
Phenolic alcohols C ₆ -C ₁	1	12.404	[M-H] ⁻ 179.07134	-0.154	179.07027 C ₁₀ H ₁₁ O ₃	coniferyl alcohol *	All samples	II
	2	1.329	[M+H] ⁺ 152.07061	-0.89	152.07061 C ₈ H ₁₀ NO ₂	(Z)-4-hydroxyphenyl- acetaldehyde oxime *	All samples	II
Hydroxybenzoic acids C ₆ -C ₁	3	7.570	[M-H] ⁻ 315.07257	-1.17	C ₁₃ H ₁₅ O ₉ 315.07106	Protocatechuic acid- hexoside	1, 2, 8, 9, 10, 20, 21, 23, 24, 25, 27, 28, 29, 35, 36, 37, 38	II
	4	1.788	[M+H] ⁺ 137.05977	-0.29	137.05971 C ₈ H ₉ O ₂	phenylacetic acid *	All samples except 3, 4, 12, 27	I
Phenolic compounds C ₆ -C ₂	5	2.115	[M+H] ⁺ 121.06486	0.069	121.06479 C ₈ H ₉ O	acetophenone *	All samples except 4, 27	II
	6	8.711	[M+H] ⁺ 149.05966	-0.31	C ₉ H ₉ O ₂ 149.05971	cinnamic acid *	11, 34, 36, 38	I
Hydroxycinnamic acids and alcohols C ₆ -C ₃	7	9.783	[M+H] ⁺ 195.06525	0.42	C ₁₀ H ₁₀ O ₄ 195.06519	isofेरulic acid	10, 38	II
	8	10.970	[M+H] ⁺ 195.06529	-0.21	C ₁₀ H ₁₀ O ₄ 195.06519	ferulic acid	3, 4, 6, 7, 8, 23, 24, 25, 26, 27, 28, 29, 34, 35, 36, 37, 38	I
	9	13.446	[M+H] ⁺ 165.05472	-0.18	C ₉ H ₉ O ₃ 165.05462	o-coumaric acid	1, 2, 3, 4, 6, 7, 8, 9, 10, 19, 20, 23, 24, 25, 26, 27, 28, 29, 34, 35, 36, 37, 38	I
	10	13.919	[M+H] ⁺ 165.05473	-0.66	C ₉ H ₉ O ₃ 165.05462	-coumaric acid	38	I
	11	5.606	[M+H] ⁺ 151.07539	0.15	C ₉ H ₁₁ O ₂ 151.07536	-coumaric alcohol	1, 5, 8, 10, 11, 12, 13, 14, 17, 18, 19, 20, 21, 22, 23, 26, 32, 33, 34, 35, 36, 38	II
	12	12.369	[M+H] ⁺ 355.10245	0.23	C ₁₆ H ₁₉ O ₉ 355.10236	chlorogenic acid	36, 38	I
Acylquinic acids	13	14.559	[M+H] ⁺	0.07	C ₁₆ H ₁₉ O ₈	p- coumaroylquinic	26	II

Class of metabolites	№	RT [min]	Accurate mass [M+H] ⁺ / [M-H] ⁻ [m/z]	Error, ppm	Exact mass [M+H] ⁺ / [M-H] ⁻ [m/z] formula	Identification	Detected in samples	MSI level	
Cinnamic acid amides	14	7.986	339.10742		339.10744	acid *			
			[M+H] ⁺	0.04	C ₁₇ H ₁₈ NO ₄	N- caffeoyl-tyramine*	25, 35, 36, 38	II	
			300.12302		300.12303				
	15	14.858	[M+H] ⁺	-0.10	C ₁₈ H ₂₀ NO ₅	N- feruloyl-octopamine	All samples except 4, 5, 14, 15, 6, 7, 9, 10, 11, 18, 19, 20, 23, 25, 28, 29, 34, 35, 36, 37, 38	II	
			330.13351		330.13360				
	16	17.058	[M+H] ⁺	0.08	C ₁₈ H ₁₉ NO ₅	feruloyl-octopamine isomer	1, 2, 4, 5, 9, 10, 11, 20, 22, 24, 25, 28, 29, 34, 35, 36, 37	II	
			330.13348		330.13360				
	17	13.360	[M+H] ⁺	0.03	C ₁₈ H ₂₀ NO ₄	N- feruloyl tyramine	1, 2, 4, 5, 6, 7, 9, 10, 11, 22, 24, 25, 28, 29, 35, 36, 39	II	
			314.13882		314.13869				
	18	22.209	[M+H] ⁺	0.13	C ₁₇ H ₁₈ NO ₃	N- coumaroyl-tyramine *	37	II	
			284.12814		314.13869				
	19	18.557	[M+H] ⁺	0.07	C ₁₉ H ₂₂ NO ₅	N- feruloyl -3-methoxy- tyramine *	4, 9, 10, 23, 13, 3, 6, 13, 21, 20, 25, 34, 35, 36, 38	II	
			344.14923		314.13869				
	Coumarin	20	12.188	[M+H] ⁺	0.31	C ₉ H ₇ O ₂	coumarin*	19, 14, 34, 36, 38	III
			147.04413		147.04406				
	Hydroxy- and methoxy- coumarins	21	9.796	[M+H] ⁺	-0.75	C ₁₀ H ₉ O ₃	herniarin*	31	II
				177.05457		177.05462			
		22	10.406	[M+H] ⁺	0.38	C ₉ H ₇ O ₃	umbelliferon*	31, 34	II
				163.03899		163.03897			
23		11.421	[M+H] ⁺	-0.94	C ₁₁ H ₁₁ O ₄	scoparon*	1, 14, 15, 6, 8, 9, 18, 19, 20, 21, 23, 25, 28, 29, 31, 32, 34, 36, 37, 38	II	
		207.06543		207.06519					
	24	11.075	[M+H] ⁺	0.02	C ₉ H ₇ O ₄	7,8- dihydroxy- coumarin *	14, 15, 13, 20, 21, 26, 32, 34, 38	II	
		179.03395		179.03389					
Flavonoids	25	14.384	[M+H] ⁺	-0.01	C ₁₅ H ₁₁ O ₇	quercetin	35	I	
			303.04993		303.04993				
	26	14.332	[M+H] ⁺	0.86	465.10275	quercetin 3- -	2, 5, 9, 10, 23, 25, 28, 32, 34, 35, 37, 38	I	
		465.10315		C ₂₁ H ₂₁ O ₁₂	glucoside*				

Class of metabolites	№	RT [min]	Accurate mass		Error, ppm	Exact mass [M+H] ⁺ / [M-H] ⁻ [m/z] formula	Identification	Detected in samples	MSI level
			[M+H] ⁺ [m/z]	[M+H] ⁺ [m/z]					
	27	13.953	[M+H] ⁺ 611.16119	[M+H] ⁺ 611.16119	-0.75	C ₂₇ H ₃₁ O ₁₆ 611.16066	rutin	1, 2, 6, 7, 8, 9, 10, 22, 23, 27, 28, 31, 34, 35, 37, 38	I
Lignans	28	14.011	[M+H] ⁺ 373.12814	[M+H] ⁺ 373.12814	-0.10	C ₂₀ H ₂₁ O ₇ 373.12818	sesamolinalol *	36	II
Quinones (naphthoquinones)	29	13.185	[M+H] ⁺ 273.11221	[M+H] ⁺ 273.11214	0.29	C ₁₆ H ₁₇ O ₄ 273.11214	deoxyshikonin*	36	II
Iridoids	30	13.058	[M+H] ⁺ 347.13382	[M+H] ⁺ 347.13382	0.55	C ₁₅ H ₂₃ O ₉	aucubin*	36	II
Cyclo-dopa amides	31	13.870	[M+H] ⁺ 504.15018	[M+H] ⁺ 504.15004	0.35	C ₂₄ H ₂₅ NO ₁₁ 504.15004	oleracein	All samples	II
	32	14.255	[M+H] ⁺ 534.16095	[M+H] ⁺ 534.16060	0.69	C ₂₅ H ₂₈ NO ₁₂ 534.16060	oleracein B	All samples except 11, 29, 17, 32	II
	33	11.008	[M+H] ⁺ 666.20306	[M+H] ⁺ 666.20286	-0.33	C ₃₀ H ₃₆ NO ₁₆ 666.20286	oleracein C	1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 19, 20, 22, 24, 25, 27, 28, 29, 34, 35, 36, 37, 38	II
	34	12.826	[M+H] ⁺ 696.21399	[M+H] ⁺ 696.21342	-0.57	C ₃₁ H ₃₈ NO ₁₇ 696.21342	oleracein D	1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 20, 22, 24, 25, 26, 28, 29, 34, 35, 36, 37, 38	II
	35	10.512	[M+H] ⁺ 220.09663	[M+H] ⁺ 220.09682	0.68	C ₁₂ H ₁₃ NO ₃	oleracein	14, 6, 8, 9, 10, 11, 18, 13, 19, 20, 21, 23, 27, 29, 32, 34, 35, 36, 37, 38	II
Terpenoids	36	15.975	[M+H] ⁺ 239.20038	[M+H] ⁺ 239.20056	0.76	C ₁₅ H ₂₇ O ₂	geranyl isopentanoate *	33	II
Amino acids and derivatives	37	4.778	[M+H] ⁺ 152.10699	[M+H] ⁺ 152.10699	-0.11	C ₉ H ₁₄ NO	methyltyramine *	25, 35	II
	38	3.813	[M+H] ⁺ 224.09164	[M+H] ⁺ 224.09173	0.64	C ₁₁ H ₁₄ NO ₄	N-acetyltirosine *	14, 13, 19, 21, 23, 35, 36, 38	II
	39	3.476	[M+H] ⁺ 152.05673	[M+H] ⁺ 152.05669	-0.37	C ₅ H ₆ N ₅ O	guanine*	38	II
	40	0.708	[M+H] ⁺ 131.12926	[M+H] ⁺ 131.12912	-0.89	C ₅ H ₁₄ N ₄	agmatine*	21, 36	II

Class of metabolites	№	RT [min]	Accurate mass [M+H] ⁺ / [M-H] ⁻ [m/z]	Error, ppm	Exact mass		Identification	Detected in samples	MSI level
					[M+H] ⁺ / [M-H] ⁻	[m/z] formula			
	41	1.264	[M+H] ⁺ 182.08110	0.46	182.08117 C ₉ H ₁₁ NO ₃		tyrosine*	4, 11, 22, 27, 31	II
	42	14.355	[M-H] ⁻ 245.09280	0.27	C ₁₃ H ₁₄ N ₂ O ₃ 245.09207		N-acetyltryptophan	1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 19, 20, 22, 24, 25, 27, 28, 29, 34, 35, 36, 37	II
Vitamines	43	1.962	[M+H] ⁺ 124.03942	-0.70	124.03930 C ₆ H ₆ NO ₂		nicotinic acid*	4, 22	I
	44	2.296	[M+H] ⁺ 123.05540	-0.98	123.05529 C ₆ H ₇ N ₂ O		nicotinamide*	27, 31	I
	45	5.768	[M+H] ⁺ 137.07101	-0.55	137.07094 C ₇ H ₉ N ₂ O		N-methylnico- tinamide *	38	II
Indole derivatives	46	1.289	[M+H] ⁺ 146.06010	-0.44	146.06004 C ₉ H ₈ NO		indole-3- carboxaldehyde *	38	II
Tetrahydro- isoquinoline	47	8.486	[M+H] ⁺ 272.12799	0.25	272.12812 C ₁₆ H ₁₈ NO ₃		norcoclaurine *	25, 35, 36, 38	II
Others	48	1.368	[M-H] ⁻ 191.01872	4.11	191.01863 C ₆ H ₇ O ₇		citric acid	3, 4, 5, 6, 7, 9, 10, 11, 19, 20, 24, 25, 27, 28, 29, 34, 35, 37	II
	49	0.981	[M-H] ⁻ 191.01878	4.06	191.01863 C ₆ H ₇ O ₇		isocitric acid	1, 2, 6, 7, 9, 10, 11, 19, 20, 22, 24, 25, 27, 28, 29, 34, 35, 36, 37	I
	50	1.049	[M-H] ⁻ 191.05489	4.66	191.05501 C ₇ H ₁₁ O ₆		quinic acid	1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 19, 20, 22, 24, 25, 27, 28, 29, 34, 35, 36, 37	I

* presented for the first time

I level - identified compound compared to reference standard

II level - putatively annotated compound

III level - putatively characterized compound classes

in Box-and-whisker-graphs. The graphics of Fig. 3 are representative of purslane samples from some floristic areas in Bulgaria (D, Bf, Thr, Tv) and Greece pointing the highest abundance of oleraceins in the Tundja hills samples (26–29).

The majority of the compounds, which show statistically significant differences ($p \leq 0.05$) in the Volcano graphs, belong to the following secondary metabolites classes: phenolic aldehydes and acetophenones, cinnamic acid and cyclo-dopa amides, hydroxybenzoic acids, hydroxycinnamic, acylquinic and amino acids (Table 2). All of the mentioned secondary metabolites suggest identical biogenic pathways with minor variations.

Literature survey shows that purslane metabolites are characterized by great variability in their structure and content (Yan et al., 2012; Hanan et al., 2014; Ai et al., 2015; Iranshahy et al., 2017). There is only one chemometric study where a combination of liquid chromatography with a diode array was used and 4 compounds were identified in samples of different origins: two flavonoid aglycones: the flavonoids myricetin and kaempferol, the flavanone glycoside hesperidin and the triterpene lupeol (Lin et al., 2012). Until now, the purslane metabolic profile has not been investigated.

Our results are in line with the studies of Liang et al. (2014) and Ai et al. (2015) where a variety of hydroxycinnamic acids, flavonoids and coumarins were evidenced. Consistent with the findings of Xing et al. (2008), hydroxycinnamic acid amides of tyramine and octopamine were characterized as marker metabolites. It is worth noting that cyclo-dopa amides A–D were remarkable for the Tundja hills floristic region. For the first time, compounds of the phenylacetic acid and acetophenone classes, lignans, naphthoquinones, iridoids, nicotinic derivatives, terpenoids, tetrahydroisoquinoline derivatives, have been reported.

BIOSYNTHETIC PATHWAYS OF PURSLANE METABOLITES

Some of the identified secondary metabolites in the differential metabolomic analysis, which show statistically significant differences ($p \leq 0.05$) and are lower or higher than the FC threshold (Fig. 4), have key roles in the biogenesis of large metabolite classes. *p*-Coumaric and ferulic acid and their esters are precursors of flavonoids and coumarins. Umbelliferone is a precursor of hydroxy- and methoxy coumarins. Coniferyl alcohol is one of the precursors

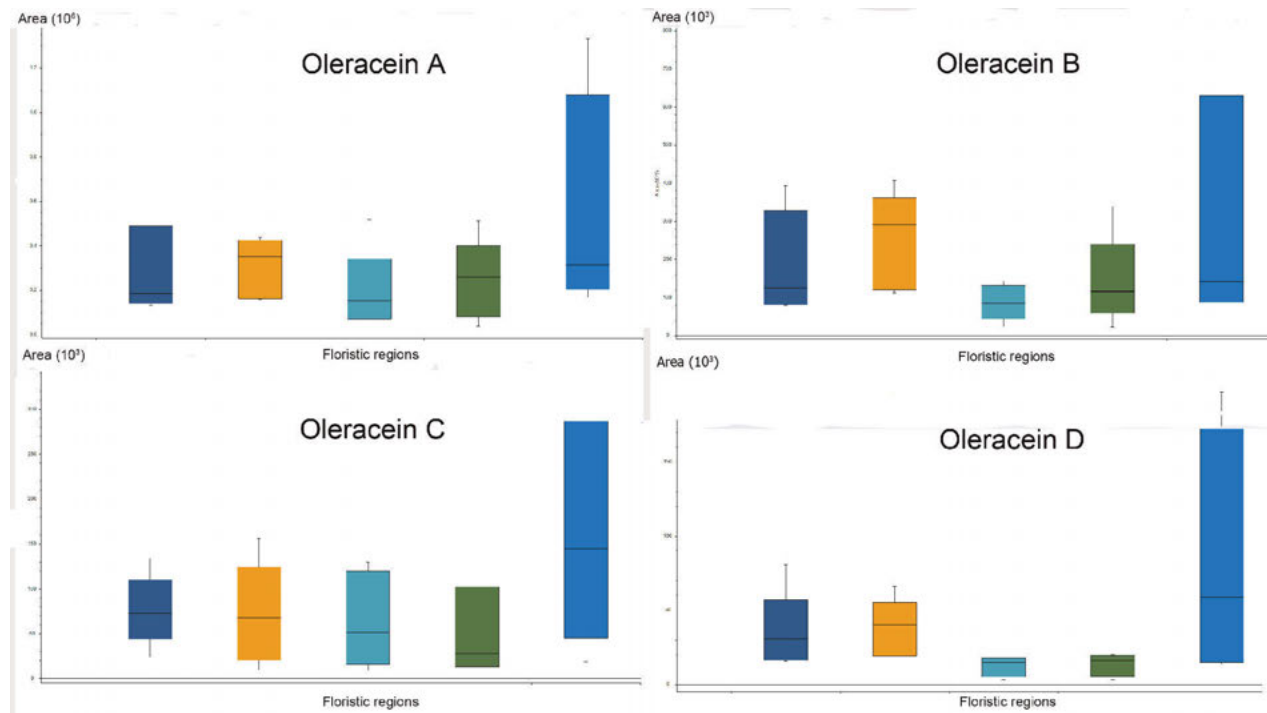


Fig. 3. Box-and-whisker graphics of descriptive statistical analysis of oleracein A-D metabolite areas of LC-HRMS analysis of purslane samples. Light blue – Greece; blue - Tundja hills region; dark blue –Balkan foothill; green – Thracian valley; orange – Danube plain.

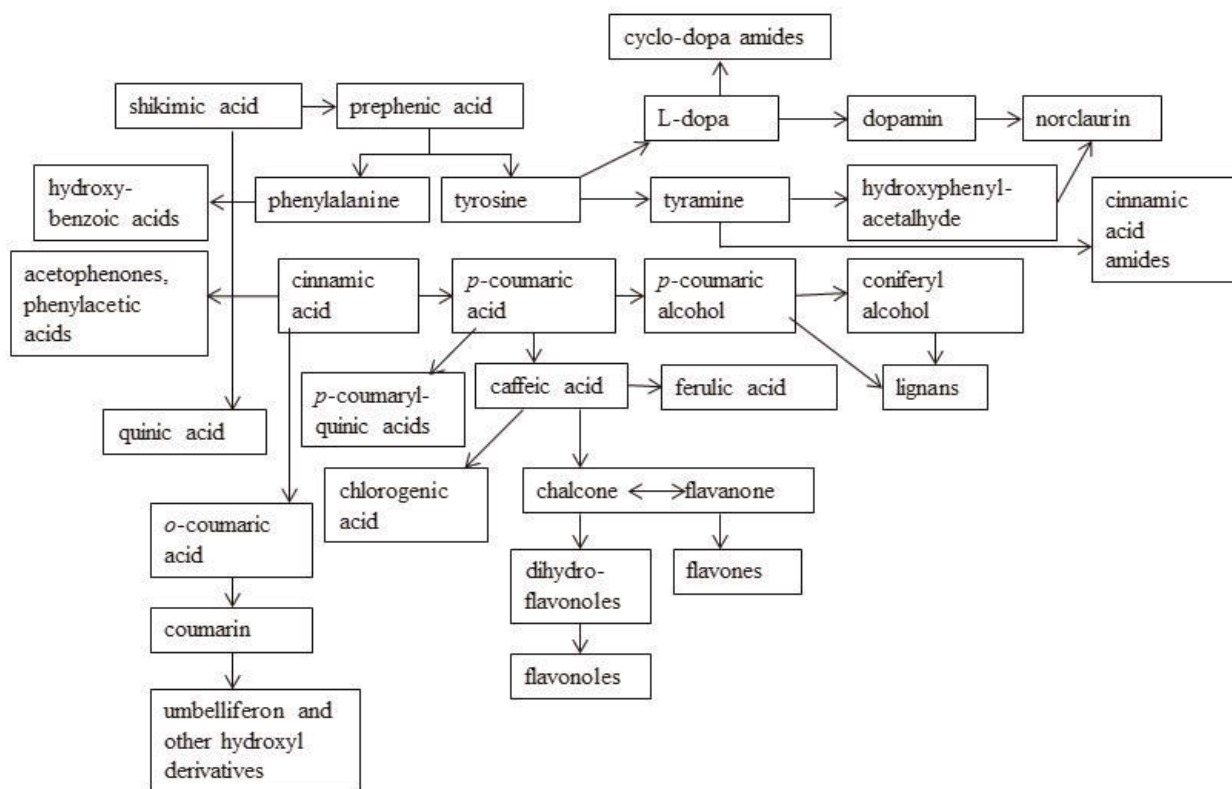


Fig. 4. Biogenetic pathway of secondary metabolites in purslane.

sors of lignans and the basis for the biogenesis of lignins, and nicotinic acid – for amides. Based on the literature on metabolites in purslane and the secondary metabolites found in the present study, biosynthetic pathways are proposed (Fig. 4). Their involvement in potential metabolic pathways was verified using the KEGG metabolic database.

There are at least four biosynthetic pathways functioning in purslane: the shikimic and aromatic amino acid pathway, the phenylpropanoid pathway, the flavonoid pathway and the alkaloid pathway (through tyrosine) (Fig. 4). Among them, the former is the first stage of biosynthesis of all 50 compounds in purslane. In this pathway, aromatic amino acid – phenylalanine – is synthesized, which is a common precursor of hydroxycinnamic and acylquinic acids, coumarins and flavonoids, while tyrosine serves as a precursor of cinnamic acid and cyclo-dopa amides. In phenylpropanoid pathway *p*-coumaric and caffeic acids could be combined with quinic acid (from shikimate pathway) and amides to yield acylquinic acids and cinnamic acid amides, respectively. Concerning alkaloid pathway, L-dopa is the precursor for the synthesis of cyclo-dopa amides and dopa-

mine. The latter is the key compound in the synthesis of isoquinoline alkaloids.

CONCLUSION

On the basis of the differential metabolomic analysis data on 38 purslane accessions originating from Bulgaria and Greece, 50 secondary metabolites were identified and 29 of them were reported for the first time. The statistical treatments determined marker compounds that can be used to distinguish the origin of the set of accessions. Combining LC-HRMS data with multivariate statistical analysis was proven effective in studying the purslane metabolites, allowing for integration of chemistry with geographic origin.

AUTHORS' CONTRIBUTIONS

The present study was accomplished with the collaboration of all authors. V. B., R. G. and D. Zh.-D. harvested the purslane samples and V. B.

identified the species. P. S., Y. H. and V. L. performed the experiments; V. L. and Y. H. designed the experiment, wrote the protocol and checked the manuscript; V. L., Y.H., V. B. and R. G. analyzed the data and wrote the first draft of the manuscript. Y. H. and V. B. have equal contribution to the work. The authors declare that they have no conflicts of interest.

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SUPPLEMENTARY MATERIAL

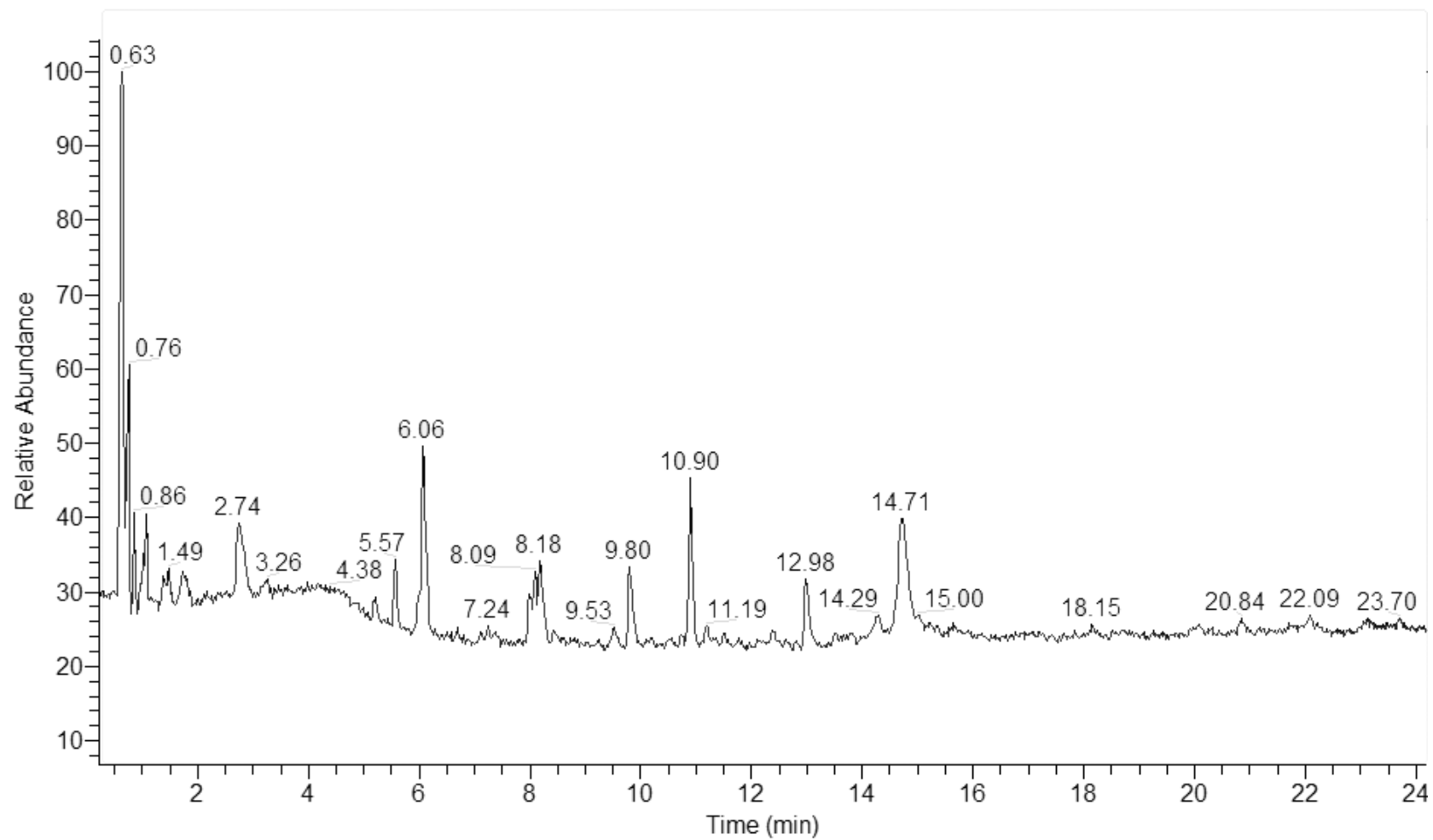
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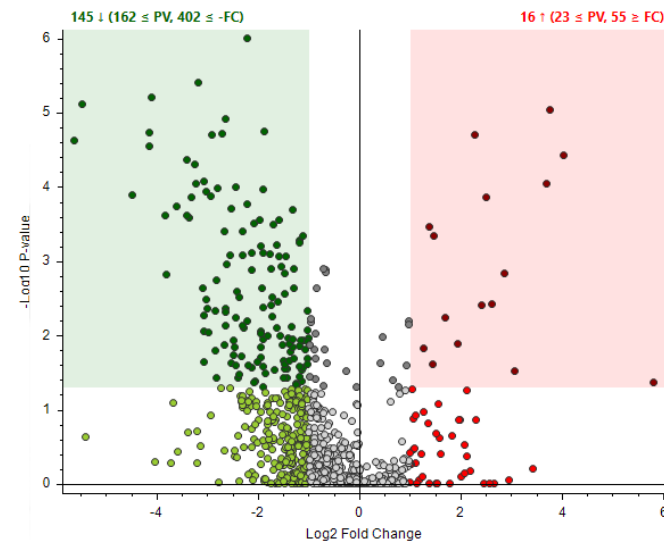
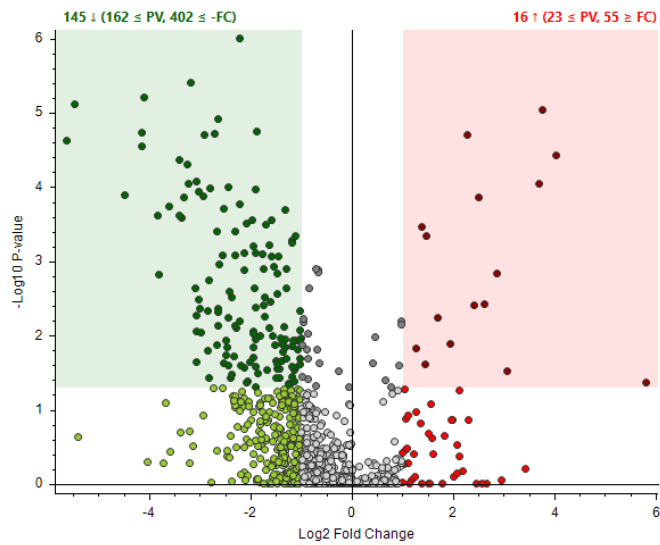
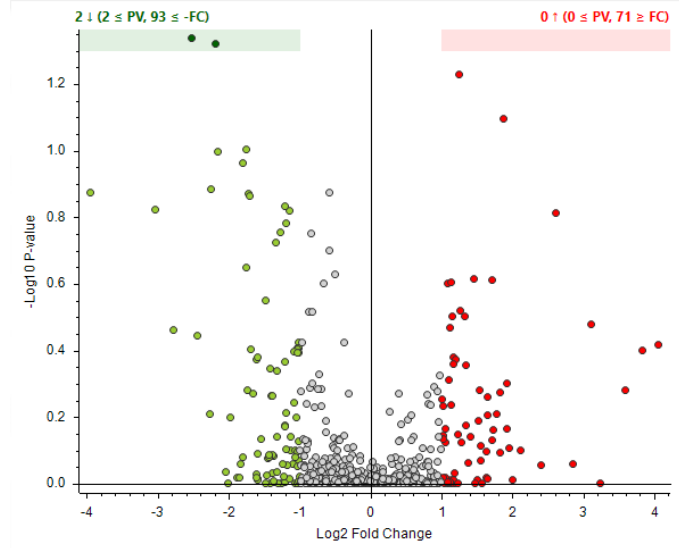
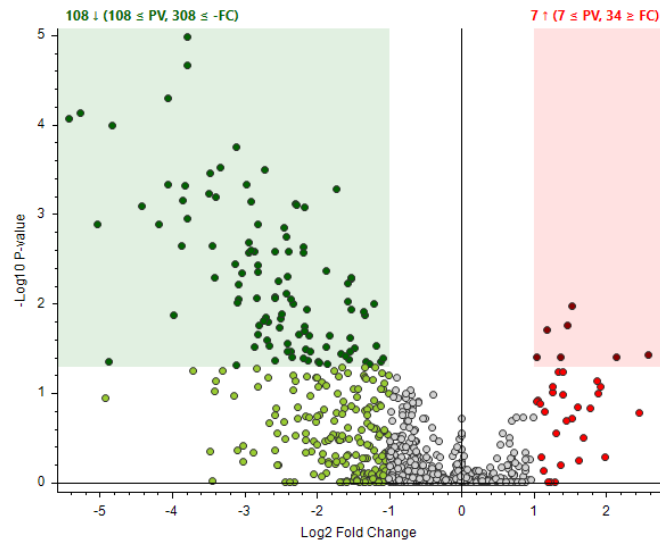
BIOINFORMATIC INSIGHT INTO *PORTULACA OLERACEA* L. (PURSLANE) OF BULGARIAN AND GREEK ORIGIN

Fig. S1. UHPLC-HRMS chromatogram of analyzed purslane extract (sample 21).

Fig. S2. Volcano-plots of differential metabolomic analysis: A. Tundja hill vs Tracian valley; B. Danube region vs Tundja hill; C. Tundja hills vs Greece; D. Tundja hills vs Balkan foothill.

Fig. S3. Fragmentation pattern of the secondary metabolites in the differential metabolomic analysis of Bulgarian and Greek samples.





Fragmentation pattern of the secondary metabolites in the differential metabolomic analysis of Bulgarian and Greek samples.

№	Identification	MS/MS fragmentation pattern
1	coniferyl alcohol *	179.0713 (59.38)**, 164.0493 (100), 135.0438 (70.81)
2	(Z)-4-hydroxyphenyl-acetaldehyde oxime *	152.0706 (100), 134.0599 (4.83), 124.0757 (23.05), 110.0604 (8.23)
3	Protocatechuic acid-hexoside	315.0715 (13.96), 153.0547 (100)
4	phenylacetic acid*	137.0597 (100), 119.0487 (21)
5	acetophenone *	121.0648 (100), 103.0545 (19.40), 93.0703 (67.70), 91.0547 (21.34)
6	cinnamic acid*	149.0597(60), 121.0497(21)
7	isoferulic acid	195.0652 (19.34), 177.0546 (100), 135.0443 (7.12)
8	ferulic acid	195.0652 (100), 177.0546 (15.05), 135.0443 (5.91)
9	<i>o</i> -coumaric acid	165.0546 (59.52), 137.0961 (23.99)
10	<i>p</i> -coumaric acid	165.0546 (34.60), 137.0961 (13.81)
11	<i>p</i> -coumaric alcohol	151.0753 (18.29), 133.0648 (6.06), 123.0807 (100), 105.0704 (18.29)
12	chlorogenic acid	355.1024 (5.49), 163.0390 (100)
13	<i>p</i> - coumaroylquinic acid*	339.1077 (11.90), 321.0952 (2.98), 177.0547 (100)
14	N- caffeoyl-tyramine*	300.1230 (71.49), 163.0390 (100), 138.0912 (3.38), 121.0649 (14.24)
15	N- feruloyl-octopamine	330.1335 (2.37), 312.1225 (17.32), 177.0548 (100), 121.0646 (1.55)
16	feruloyl-octopamine isomer	330.1335 (0.37), 177.0557 (100)
17	N- feruloyl tyramine	314.1388 (73.64), 177.0547 (100), 147.0442 (19.68), 121.0651 (8.67)
18	N- coumaroyl-tyramine *	284.1281 (54.85), 164.0705 (0.48), 147.0441 (100), 121.0652 (9.65)
19	N- feruloyl -3-methoxytyramine *	344.1492 (5.21), 177.0546 (100), 151.0754 (1.26), 145.0286 (077)
20	coumarin*	not fragmented
21	herniarin*	177.0545 (65.73), 145.0285 (100), 121.1012 (1.88)
22	umbelliferon*	163.0389 (100), 145.0285 (35.43), 135.0442 (37.70), 107.0496 (3.29)
23	scoparon*	207.0651 (94.36), 189.1283 (4.33), 175.0391 (100), 151.0751 (14.79)
24	7,8- dihydroxycoumarin *	179.0338 (58.14), 123.0444 (32.21), 105.0340 (100)
25	quercetin	303.0499 (100), 165.0124 (36.60), 137.2154 (14.33)
26	quercetin 3- <i>O</i> -glucoside*	465.1031 (100), 303.1549 (84.95)
27	rutin	611.1611 (43.12), 303.1548 (100)
28	sesamolinal *	373.1281 (61.99), 355.1182 (17.75), 341.1020 (56.59), 313.1069 (22.95), 177.0546 (100)
29	deoxyshikonin*	273.1121 (100), 255.1745 (10.77), 245.1903 (9.48), 203.1798 (1.43)
30	aucubin*	347.1338 (100), 329.1131 (31.45), 311.1010 (1.04), 167.1062 (1.13)
31	oleracein A	504.1502 (1.13), 342.0970 (9.01), 196.0606 (1.78), 178.0501 (1.02), 150.0550 (0.91), 147.0441 (100)

32	oleracein B	534.1610 (0.17), 372.1076 (5.95), 196.0604 (0.72), 178.0583 (5.07), 177.0547 (100), 150.0552 (0.24)
33	oleracein C	666.2031 (0.32), 504.1499 (6024), 342.2031 (2.60), 309.0967 (12.28), 196.0607 (0.79), 178.0499 (0.32), 150.0554 (0.32), 147.0441 (100)
34	oleracein D	696.2140 (0.23), 534.1605 (4.22), 372.1076 (6.44), 339.1074 (3.11), 196.0603 (0.73), 178.0582 (5.24), 177.0546 (100), 150.0549 (0.19)
35	oleracein E	220.0968 (100), 192.1019 (2.27), 176.0707 (2.95), 174.0914 (54.29), 156.0811 (1.96), 146.0965 (7.92)
36	geranyl isopentanoate *	239.2005 (86.04), 221.1900 (100), 203.1795 (73.99), 197.0815 (56.41), 161.1328 (10.48), 137.1327 (52.86)
37	methyltyramine *	152.1069 (59.86), 138.0551 (100), 110.0604 (62.87)
38	N-acetyltyrosine *	224.0916 (37.73), 206.1179 (1.56), 200.9724 (25.34), 182.9620 (4.69), 178.0863 (100), 107.1077 (1.09)
39	guanine*	152.0566 (100), 135.0302 (5.59), 110.0353 (1.71)
40	agmatine*	131.1293(100), 114.1001(19)
41	tyrosine*	182.0811 (10.33), 165.0547 (66.90), 136.0758 (100), 107.0502 (0.20)
42	N-acetyltryptophan	245.0928 (100), 203.0821 (9.5)
43	nicotinic acid*	124.0394 (100), 80.1100 (9)
44	nicotinamide*	123.0554 (100), 80.0010 (20)
45	N-methylnicotinamide *	137.0710 (100), 80.0500 (20)
46	indole-3-carboxaldehyde *	146.0601 (100), 118.0643 (15)
47	norcochlorine *	272.1279 (90.12), 254.2477 (100), 248.9002 (21.01), 110.7639 (1.84)
48	citric acid	191.0186 (15.10), 173.0084 (3.02), 154.9975 (1.51), 111.0074 (100), 85.0280 (22.75)
49	isocitric acid	191.0186 (13.82), 173.0084 (2.64), 154.9975 (1.02), 111.0074 (100), 85.0280 (24.12)
50	quinic acid	191.0550 (2.95), 173.0083 (0.51), 111.0074 (7.91), 85.0280 (100)

* presented for the first time

** (relative abundance of ion)