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LC-MS analysis of phenolic compounds and oleraceins in aerial parts of *Portulaca oleracea* L.

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Summary

Portulaca oleracea L. (purslane) is a well-known edible and ethnomedicinal plant and it has been called "vegetable for long life" in the Chinese herbal medicine. The plant is recognized for the high content of polyphenols, including flavonoids and phenolic acids.

In this study, hydromethanolic purslane extracts from Bulgarian and Greek locations were screened for polyphenolic content. Based on polyphenols, saponins and DPPH antioxidant activity, an orthogonal design $L_9(3^4)$ was performed in order to improve the ultrasound assisted extraction procedure of dry and fresh plant material. An UHPLC-Orbitrap-MS method in parallel-reaction monitoring mode was developed for the simultaneous identification and quantification of 14 compounds comprising hydroxybenzoic, hydroxycinnamic and caffeoylquinic acids, as well as 2 flavonol glycosides. The quantitative analysis was validated for curve fit, range, instrumental detection limit (IDL), instrumental quantification limit (IQL), LOD, LOQ, precision, recovery and accuracy. The UHPLC-MS quantification method revealed good linearity ($r^2 > 0.9950$), LOD < 925.85 ng/g dw and LOQ < 3055.31 ng/g dw. Moreover, 11 cylco-dopa amides (Oleraceins A-D, N-Q, S, U and W) were tentatively identified through UHPLC-MS and their MS^2 mass fragmentation was described.

Keywords: Portulaca; Orthogonal design; LC-MS; Orbitrap; Polyphenol; Oleracein; PCA

Introduction

Portulaca oleracea L., purslane (*Portulacaceae*) is a well-known edible and ethno-medicinal plant in Asia, Europe and the Mediterranean region. Due to its high adaptability to many adverse conditions such as drought, saline, and nutrient deficiency, nowadays purslane can be found in various locations worldwide (Sultana and Rahman, 2013; Uddin et al., 2012). The species is believed to have originated from and adapted to desert climates of the Middle East and India (Uddin et al., 2014). In folk and traditional medicine, the herb has been used as a remedy for many ailments, including high fever, diarrhea, and urinary tract infections (Brickell, 1999). Purslane can be consumed in raw salads or in teas or soups and has been named "a vegetable for long life" in the Chinese herbal medicine (Xu et al., 2006) due to its various medicinal applications.

The species contain high amount of polyphenols, including phenolic acids and flavonoids. Polyphenols are reported to exhibit various biological effects such as anti-inflammatory, antiallergic, antibacterial, antimicrobial, cardioprotective, antioxidant activities (Li et al., 2014; Payet et al., 2006). The phenolic profile is a representative indicator that contributes to the nutritional properties of plants and they are considered important antioxidants of human diet (Puigventós et al., 2015). Frequent intake of purslane has been associated with lowered risk of cancer (Zakaria and Hazha, 2013) and different chronic diseases (Tsao, 2010). Moreover, natural phenolic compounds are recognized for their therapeutic role against oxidative stress and the DPPH radical scavenging activity test is commonly used to evaluate the antioxidant potential of natural compounds or total plant extracts (Taha and Osman, 2015; YouGuo et al., 2009). Sicari et al. (2018) determined that the total polyphenolic content of hydroalcoholic extract of *P. oleraceae* leaves was 244.17 ± 4.04 mg GAE/100g. The same authors also evaluated the DPPH radical scavenging activity of the total extract to be with IC_{50} of 53.92 ± 1.3 mg/ml. Another study reported the polyphenol content of 13 different accessions of purslane and found it to be ranging from 96 ± 4.0 to 912 ± 29 mg GAE/100g, and the DPPH radical scavenging activity IC_{50} value varied between 2.52 ± 0.03 mg/ml and 3.29 ± 0.01 mg/ml (Alam et al., 2014).

Among polyphenols, a class of alkaloids called cyclo-dopa amides, or Oleraceins, which are secondary metabolites in *P. oleraceae*, are gaining increased attention (Sicari et al., 2018; Yang et al., 2009). These compounds are characterized with antioxidant activity that is comparable, and sometimes superior, to some natural antioxidants like vitamin C or vitamin E (Behravan et al., 2011; Jiao et al., 2015; Liu et al., 2011; Uddin and Hamja, 2014; Yang et al., 2009). Also, cyclo-dopa amides are potent acetylcholinesterase inhibitors (Yang et al., 2012).

Purslane aerial parts are also a rich dietary source of omega-3 and omega-6 fatty acids, vitamins, and minerals, but the unique nature of its health promoting benefits has been associated with its high levels of flavonoids (Oliveira et al., 2009; Uddin et al., 2014), homoisoflavonoids (Yan et al., 2012) hydroxycinnamic acid amides (Xing et al., 2008) and cyclo-dopa amides (Taha and Osman, 2015; Yang et al., 2009; YouGuo et al., 2009). Furthermore, *P. oleracea* seeds are effective as an alternative therapy of type 2 diabetes mellitus (El-Sayed, 2011; Gong et al., 2009). In addition to evoking modulation of glucose and insulin, purslane polysaccharides ameliorate lipid metabolism in alloxan- and streptozotocin-induced diabetic models (Gong et al., 2009). Also, purslane is reported as a rich source of the antioxidant vitamins A, C and E, as well as glutathione and amino acids (Dkhil et al., 2011).

In the present study, in order to improve the ultrasound assisted extraction conditions of valuable compounds in dry and fresh plant material, a three-level (Levels 1-3) and four-factor (A, B, C, D) orthogonal experiment $L_9(3^4)$ was performed evaluating four criteria – total extract (E), total polyphenols (P), total saponins (S), and DPPH radical scavenging activity (R) of the plant extracts. Furthermore, by employing ultra-performance liquid chromatography (UHPLC) coupled mass spectrometry with high resolution (HRMS), 14 phenolic compounds comprising hydroxybenzoic, hydroxycinnamic and caffeoylquinic acids, as well as 2 flavonol glycosides, were quantified in *Portulaca oleracea* hydromethanolic extracts. Also, 11 cyclo-dopa amides (Oleraceins A-D, N-Q, S, U and W) were tentatively identified by mass fragmentation analysis.

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Materials and methods

Plant material

Portulaca oleracea aerial parts from eight Bulgarian (Cherven bryag (43.28° N - 24.082778° E) – **Bg1**, Orizovo (42.208889° N - 25.170278° E) – **Bg2**, Pirdop (42.7° N - 24.183333° E) – **Bg3**, Kremena (43.166667° N - 23.733333° E) – **Bg4**, Nessebar (42.660833° N - 27.713889° E) – **Bg5**, Dolni Bogrov (42.7° N - 23.5° E) – **Bg6**, Burgas (42.5° N - 27.466667° E) – **Bg7**, Shumen (43.27° N - 26.924444° E) – **Bg8** and Montana (43.407778° N - 23.225° E) – **Bg9**) and three Greek (Lagonissi (40.233611° N - 23.731944° E) – **Gr1**, Possidi (39.963056° N - 23.381667° E) – **Gr2** and Komotini (41.116667° N - 25.4° E) – **Gr3**) locations were collected. The Bulgarian accessions were collected from village or city garden, while the Greek ones – from a sandy beach. The plant was identified by one of us (V. B.) (Fig. 1). Voucher specimens were deposited at the Faculty of Pharmacy, Medical University, Sofia, Bulgaria (Herbarium Facultatis Pharmaceuticae Sophiensis № 1563-1574).

Chemicals and reagents

Of the standards used for constructing quantitative calibration curves, Protocatechuic acid (**PCA**), Neochlorogenic acid (**nCGA**), Chlorogenic acid (**CGA**), and Caffeic acid (**CA**) were obtained from Extrasynthese (Genay, France). Vanillin (**V**), Ellagic acid (**EA**), Gallic acid (**GA**), Gentisic acid (**GeA**), 2-hydroxybenzoic acid (**2-HBA**), 4-hydroxybenzoic acid (**4-HBA**), *p*-coumaric acid (***p*CouA**), *m*-coumaric acid (***m*CouA**), *o*-coumaric acid (***o*CouA**), Ferulic acid (**FeA**), Rutin (**R**) and Quercetin-3-O-glucoside (**QG**) were of analytical grade (> 96%) and supplied from Phytolab (Germany). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade solvents were provided by Merck (Darmstadt, Germany). All other chemicals and solvents were of analytical grade.

Extraction and sample preparation

Air-dried powdered, (accurately weighed 3.00 g) and fresh plant material (accurately weighed 20.00 g) were extracted by sonication according to the experimental orthogonal design varying the methanol concentration (50, 70, 80%), extraction temperature (50, 70, 80 °C), extraction time (20, 30, 40 min) and solid solvent ratio (1:20, 1:30, 1:40 w/v) (Tab. 1). The extracts were concentrated in *vacuum* and subsequently lyophilized. For each extraction condition, total polyphenols (P), total saponins (S), and DPPH radical scavenging activity (R) were determined in triplicate for each extract obtained (Tab. 4 and Tab. 5).

For LC-MS experiments, air-dried powdered purslane (0.5 g) was

Tab. 1: A three-level (Levels 1-3) and four-factor (A, B, C, D) orthogonal experiment $L_9(3^4)$ evaluating four criteria – total extract (E), total polyphenols (P), total saponins (S), and DPPH radical scavenging activity (R) of the extracts.

	Factor name			
Levels	**A % MeOH**	**B T °C**	**C Extraction time (min)**	**D Solid/solvent (g/ml)**
Level 1	50	50	20	20
Level 2	70	70	30	30
Level 3	80	80	40	40

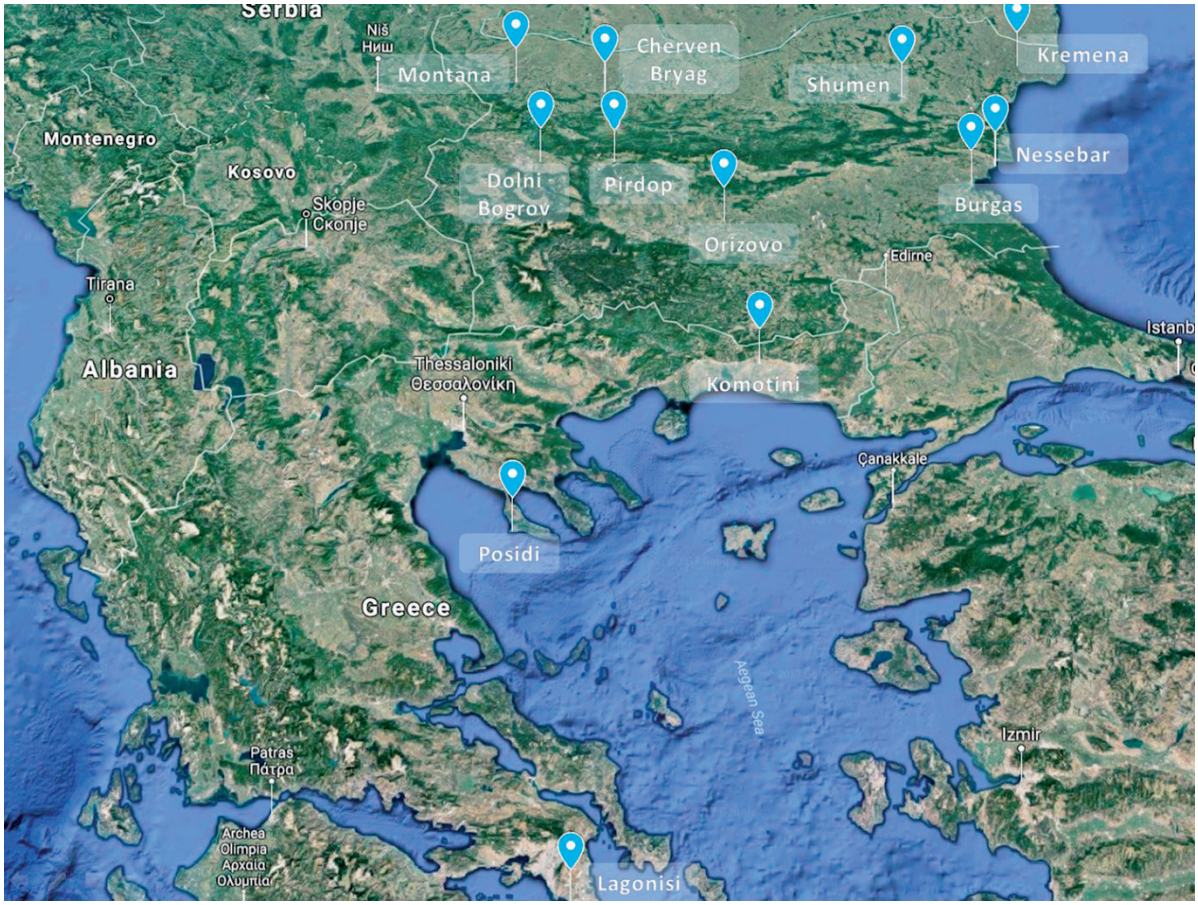

Fig. 1: Locations in Bulgaria and Greece where aerial parts of *Portulaca oleracea* L were collected.

subjected to sonication with 10 ml 50% MeOH (v/v) for 15 min at 50 °C temperature in an ultrasonic bath (× 2) (ISOLAB, 40 kHz). Afterwards, the extracts were combined, filtered and diluted with 50% MeOH to 25 ml in volumetric flasks. The solutions were filtered through a 0.22 μm syringe filter before use, and 1μL was injected into the LC instrument for LC-MS analysis.

Quantitative analysis of total saponins

The quantitative analysis of total saponins in lyophilized purslane extracts was measured by a vanillin-acetic acid method (Wu et al., 2001) with slight modifications. The lyophilized extract (0.05 g) was dissolved in 1 ml 50% MeOH by sonication (5 min), and centrifuged at 2000 rpm, for 3 min. Then 50 μl of the supernatant was evaporated to dryness, and 200 μl 5% vanillin in acetic acid (v/v) and 800 μl of 70% perchloric acid were added. The mixture was incubated at 70 °C for 15 min, cooled, and 5 ml glacial acetic acid were added. The concentration of total saponin was determined spectrophotometrically at 560 nm using a Shimadzu UV-1203 spectrophotometer (Kyoto, Japan) against a calibration curve established with aescin standard. The results are presented in mg aescin equivalent/g dry extract. All determinations were performed in triplicate (n = 3).

Quantitative analysis of total polyphenols

The quantitative analysis of total polyphenols in lyophilized purslane extracts was performed according to the European Pharmacopoeia using Folin-Chiocalteu reagent and pyrogallol as standard (European Pharmacopoeia et al., 2010). The analyses were carried out at 760 nm using a Shimadzu UV-1203 spectrophotometer (Kyoto, Japan). The content of polyphenol derivatives was calculated as pyrogallol equivalent (PE) [%] for dry weight extract. All determinations were performed in triplicate (n = 3).

DPPH-radical scavenging activity

Free radical scavenging activities of lyophilized purslane extracts were measured using DPPH method according to the procedure given by Zheleva-Dimitrova (2013). Results were evaluated as percentage scavenging of DPPH radical. The analyses were performed at 517 nm using a Shimadzu UV-1203 spectrophotometer (Kyoto, Japan). All determinations were performed in triplicate (n = 3).

Preparation of standard solutions

The stock solution of the reference standard compounds was prepared accurately in methanol and diluted in appropriate concentration according to the expected levels in matrix to yield 15 calibration levels of concentrations within the range 0.2 to 9600 ng/ml. All stocks were stored in refrigerator at -20 °C until use.

Triplicate LC-MS analyzes were performed for each concentration. Calibration curves were constructed by plotting the value of peak area versus concentrations of each analytes. Slope, intercept and other statistics of calibration curves were calculated with Xcalibur 4.1 (ThermoFisher).

Quantitative method validation of phenolic acids and flavonoids

The UHPLC-MS method was validated in respect with precision, linearity, accuracy, recovery, instrumental detection limit (IDL), instrumental quantification limit (IQL), limit of detection (LOD) and limit of quantification (LOQ).

Precision

Precision was evaluated by 5 consecutive injections per day. The precision was expressed as relative standard deviation (RSD%) between the replicate measurements.

Accuracy

The mass accuracy was calculated as Δ ppm = [(theoretical mass – measured mass)/theoretical mass] 1,000,000. A mass error limit of 5 ppm was used to distinguish true compound signals from background.

Sensitivity

The instrument detection limits (IDL) and the instrumental quantification limits (IQL) were defined as 3 times and 10 times, respectively, the standard deviation at the lowest concentration level that can be measured with less than 15% deviation from the nominal concentration value. IDLs and IQLs were calculated by the injection of the calibration solutions at the lowest calibration levels (from 0.2 to 150 ng/ml) in 5 replicates. Accordingly, LOD and LOQ were reassessed based on the weight of the dry material being analyzed (typically 0.5g dw) (Tab. 2).

Recovery

Recoveries were carried out by the method of standard additions (n = 3). The recovery was determined by analyzing separately around 0.5 g (accurately measured until the fourth digit after decimal point) of dry plant material (matrix), mixture of reference compounds (standards), and matrix spiked with solution containing the reference compounds (spiked matrix). Each spiked matrix was proceeded separately by the extraction procedure described above.

The recovery was calculated as the percentage of the area of the spiked matrix divided by the sum of the areas of standard and matrix.

The recovery was determined by the following formula:

$$\frac{\text{Area (spiked matrix)}}{\text{Area (standard)} + \text{Area (matrix)}} \times 100 = \%\ \text{recovery}$$

standard – mixture of the reference compounds,
matrix – the dry plant material,
spiked matrix – the dry plant material spiked with the mixture of the reference compounds

UHPLC-HRMS

Identification and quantification of the selected phenolic compounds were performed by an UHPLC-ESI/MS system. The LC system consisted of Dionex UltiMate 3000 RSLC HPLC, equipped with an SRD-3600 solvent rack degasser, an HPG-3400RS binary pump with solvent selection valve, a WPS-3000TRS thermostated autosampler, and a TCC-3000RS thermostated column compartment (ThermoFisher Scientific, Germany). The entire system was controlled by Chromeleon software, version 7.2.

Chromatographic separation

Separation was achieved on a reversed phase column Kromasil EternityXT C18 (1.8 μm, 2.1 × 100 mm) column maintained at 40 °C. The binary mobile phase consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile. The run time was 10 min. The following gradient was utilized: the mobile phase was held at 10% B for 4 min, gradually turned to 30% B over 3 min, increased gradually to 80% B over 1min, held at 80% B for 1 min, and the system was turned to the initial condition of 10% B in 1 min. Finally, the system was re-equilibrated over 4 min. The flow rate and the injection volume were set to 300 μL/min and 1 μL, respectively. The effluents were connected on-line with a Q Exactive Plus Orbitrap mass spectrometer where the phenolic compounds were detected.

Tab. 2: Calibration settings

Phenolic compound	Calibr range (ng/ml)	R^2	N	Curve Index	Equation	IDL (ng/ml)	IQL (ng/ml)	LOD (ng/g dw)	LOQ (ng/g dw)
Vanillin	9.370-9600	0.9989	11	Linear (1/x)	Y = -29449+8092.86*X	8.358	27.582	417.910	1379.103
Ellagic acid	108.0-1728	0.9955	5	Linear Log-Log (Equal)	log(Y) = 2.393+1.36997*log(X)	18.517	61.106	925.850	3055.305
Gallic acid	2.340-37.50	0.9950	5	Linear Log-Log (Equal)	log(Y) = 3.41118+1.13633*log(X)	0.636	2.098	31.790	104.907
Gentisic acid	9.370-1200	0.9975	8	Linear (1/x)	Y = 8130.78+34494.3*X	0.386	1.272	19.280	63.624
Protocatechuic acid	4.690-600.0	0.9992	8	Linear (1/x)	Y = -13785.2+24634*X	0.750	2.500	37.500	124.999
4-OH benzoic acid	18.750-2400	0.9993	8	Linear (1/x)	Y= 19117.4+1503.66*X	12.485	41.616	624.250	2080.813
2-OH benzoic acid	18.75-600.0	0.9984	6	Linear (1/x)	Y = 52027.6+8868.13*X	3.896	12.855	194.780	642.774
***p*-coumaric acid**	2.340-600.0	0.9965	9	Linear (1/x)	Y = 35095.8+33513.6*X	1.285	4.239	64.225	211.943
***m*-coumaric acid**	4.690-9600	0.9996	12	Quadratic Log-Log (Equal)	log(Y) = 4.12663+1.09619*log(X)-0.0287672*(log(X))^2	0.770	2.340	*	*
***o*-coumaric acid**	2.340-9600	0.9996	13	Quadratic Log-Log (Equal)	log(Y) = 4.71215+1.01852*log(X)-0.0146776*(log(X))^2	0.430	1.302	*	*
Caffeic acid	2.340-300.0	0.9991	8	Linear (1/x)	Y = -57848+55870.2*X	1.096	3.617	54.803	180.850
Ferulic acid	150.0-9600	0.9980	7	Linear (1/x)	Y = 533.22+426.758*X	1.654	5.459	82.710	272.943
Neochlorogenic acid	2.810-360.0	0.9963	8	Linear (1/x)	Y = -2406.07+1725.94*X	0.985	3.249	49.225	162.443
Chlorogenic acid	9.370-600.0	0.9963	6	Linear (1/x)	Y = -81121+12912.8*X	3.215	10.715	160.725	535.745
Rutin	0.550-426.0	0.9987	5	Linear (Equal)	Y = 15158.7+9760.22*X	0.319	1.054	15.970	52.700
Quercetin-3-O-glucoside	0.24-184	0.9995	5	Linear (Equal)	Y = 1302.24+23786.9*X	0.120	0.396	6.000	19.800

**m*- and *o*-coumaric acids were not detected in the tested samples; N – number of levels used for the calibration; IDL – instrument detection limit; IQL – instrument quantification limit; LOD – limit of detection; LOQ – limit of quantification.

LC-MS analysis

Mass analyses of the extract were carried out on a Q Exactive Plus mass spectrometer (ThermoFisher Scientific) equipped with a heated electrospray ionization (HESI-II) probe (ThermoScientific). The instrument parameters were as follows: spray voltage 3.5 kV, sheath gas flow rate 38, auxiliary gas flow rate 12, spare gas flow rate 0, capillary temperature 320 °C, probe heater temperature 320 °C, and S-lens RF level 50.

The targeted acquisition of the 14 phenolic acids and 2 flavonoids was carried out in negative ionization mode on a parallel reaction monitoring mode (PRM), with the following instrument settings: microscans at 1, resolution at 35,000, AGC target at 5e5, maximum ion time at 50 ms, MSX count at 1, isolation window at 2.0 m/z, with varying high-collision dissociation energies (HCD) (Tab. 3). Data acquisition and processing were carried out with Xcalibur 4.1 software (ThermoScientific).

Statistical analysis

Statistical analyses were carried out with Xcalibur 4.1 software (ThermoScientific), Microsoft® Excel 2010 and XLSTAT® 2014.

Identification of cyclo-dopa amides (Oleraceins) by UHPLC-MS

Chromatographic conditions

Elution was carried out on Kromasil EternityXT C18 (1.8 µm, 2.1 × 100 mm) column maintained at 40 °C.

The binary mobile phase consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile. The run time was 10 min. The following gradient was utilized: the mobile phase was held at 5% B for 0.5 min, gradually turned to 40% B over 8.5 min, and then rapidly increased to 85% B in 1min, and held at 85% B for 1 min. The system was turned to the initial condition of 5% B in 1 min and re-equilibrated over 4 min. The flow rate and the injection volume were set to 300 µL/min and 1 µL, respectively. The effluents were connected on-line with a Q Exactive Plus Orbitrap mass spectrometer where the eluting Oleraceins were detected.

LC-MS analysis

Mass spectrometric analysis of the identified Oleraceins were carried out on a Q Exactive Plus mass spectrometer (ThermoFisher Scientific) equipped with a heated electrospray ionization (HESI-II) probe (ThermoScientific) connected on-line to the chromatographic

system. The instrument parameters were as follows: spray voltage 3.5 kV, sheath gas flow rate 38, auxiliary gas flow rate 12, spare gas flow rate 0, capillary temperature 320 °C, probe heater temperature 320 °C, and S-lens RF level 50. Oleraceins were detected in positive ionization mode with the following instrument settings: microscans at 1, resolution at 70,000, AGC target at 3e6, maximum ion time at 50 ms, MSX count at 1, isolation window at 2.0 m/z, at 20 HCD (). Data acquisition and processing were carried out with Xcalibur 4.1 software (ThermoScientific).

Results and discussion

Optimization of ultrasound assisted extraction procedure

In this study, we aimed at investigating the optimal conditions of ultrasound assisted extraction of the dry and fresh purslane aerial parts by an orthogonal design $L_9(3^4)$ (Tab. 4 and Tab. 5). The impact of concentration of methanol (factor A), temperature [°C] (factor B), extraction time (factor C) and solid to solvent ratio (factor D) were investigated (Tab. 1). Total polyphenols (P), total saponins (S), and DPPH radical scavenging activity (R) were determined in triplicate for each extract obtained as shown in (Tab. 4 and Tab. 5).

In total of 18 experiments were conducted and each experiment was based on a certain combination of level values (Tab. 1). For example, **Experiment 2** was conducted by keeping the factor **A** at level 1 (extraction solvent: 50% MeOH), factor **B** at level 2 (extraction temperature: 70 °C), factor **C** at level 2 (extraction time: 30 min), and factor **D** at level 2 (extraction ratio solid/solvent: 1:30 g/ml). Likewise, **Experiment 7** was conducted by keeping factor **A** at level 3 (extraction solvent: 80% MeOH), factor **B** at level 1 (extraction temperature: 70 °C), factor **C** at level 2 (extraction time: 30 min), and factor **D** at level 3 (extraction ratio solid/solvent: 1:40 g/ml).

The **mean values** were calculated by averaging the three values obtained for any of the four criteria for determining the optimal extraction conditions, namely, total extract obtained (E), total polyphenols content (P), total saponins content (S), and DPPH radical scavenging activity (R). For example, for fresh purslane (Tab. 4), **Mean value 3 S** was calculated by averaging the three values for the Saponin content (**S**) (expressed as mg aescin equivalent /g dry extract) for Level **3** of factor **A**: (27.61 + 27.61 + 28.67)/3 = 27.96. Likewise, **Mean value 2 P** represents the mean of the three values for Polyphenols content (**P**) (expressed as % pyrogallol equivalent (PE) for dry weight extract) for level **2** of factor **C**: (3.15 + 3.09 + 3.16)/3 = 3.13. The **Range** represents the difference between the largest and the smallest **mean value** for a specific factor.

The **Optimized scheme** rows give the most favorable conditions for a particular criterion. For example, **Optimized scheme S** (for fresh purslane, Tab. 4) shows that extraction solvent of 80% MeOH (factor A), extraction temperature of 70 °C (factor B), extraction time of 20 min (factor C), and extraction ratio solid/solvent of 1:40 g/ml (factor D), are optimal for obtaining the highest Saponin (S) content (expressed as mg aescin equivalent/g dry extract), etc.

Regarding S and R criteria, the largest range of the three levels was found for the methanol concentration for both fresh and dry purslane. With respect to the dry plant, the smallest range was evaluated for factor D except for extraction yield. Factor C revealed the smallest range in the fresh plant, except for R, where the temperature showed the smallest range value.

The first level of factor A (50% methanol) was the best condition for S, P and E criteria in the dry plant witnessed by the largest average values (Tab. 5). For both P and R, 80 °C was the optimal temperature for 40 min. Solvent to solid ration 40:1 was favorable for the highest DPPH activity and extraction yield.

According to the data presented in Tab. 4 for fresh purslane, the optimal UE conditions for obtaining the highest saponin content are 50% MeOH, 70 °C, extraction time 30 min and solvent to solid ration 20:1, whereas the UE conditions that would be optimal for obtaining the highest polyphenol content are 50% MeOH, 80 °C, extraction time 40 min and solvent to solid ration 20:1.

Since multiple criteria were evaluated, compromised UE conditions were selected as follows: for dry purslane – 50% methanol, 50 °C, extraction time 30 min, solvent to solid ration 20:1 and for fresh purslane – 80% methanol, 50 °C, extraction time 30 min and solvent to solid ration 40:1.

Tab. 3: Mass spectrometric and chromatographic parameters.

№	Compound	$[M-H]^-$	Rt (min)	MS^2	HCD
1	**Gallic acid**	169.0142	1.11	169 => 125	40
2	**Neochlorogenic acid**	353.0878	1.81	353 => 191	30
3	**Gentisic acid**	153.0193	1.89	153 => 109	50
4	**Protocatechuic acid**	153.0193	3.18	153 => 123	55
5	**4-OH benzoic acid**	137.0244	3.22	137 => 93	20
6	**Chlorogenic acid**	353.0878	3.30	353 => 191	25
7	**Caffeic acid**	179.0350	4.41	179 => 135	40
8	**Vanillin**	151.0400	6.52	151 => 136	30
9	***p*-coumaric acid**	163.0400	6.71	163 => 119	25
10	**Rutin**	609.1460	7.13	609 => 300	35
11	**Ferulic acid**	193.0506	7.27, 7.41*	193 => 178	60
12	**Ellagic acid**	300.9990	7.27	301 => 284	60
13	**Quercetin-3-O-glucoside**	463.0880	7.32	463 => 300	35
14	**m-coumaric acid**	163.0400	7.36	163 => 119	40
15	***o*-coumaric acid**	163.0400	7.92	163 => 119	25
16	**2-OH benzoic acid**	137.0244	8.10	137 => 93	20

*two peaks due to *cis-trans* isomerism.

Optimization of chromatographic separation and MS detection for the identification of phenolic acids and flavonoids

When analyzing a multicomponent mixture in a complex matrix, co-elution of analytes presents an issue, which could be elegantly resolved using high resolution accuracy mass spectrometry (HRAM-MS) (De Paepe et al., 2013). The high resolution of the Orbitrap mass spectrometer allows background matrix interferences to be resolved from the ions of interest. Moreover, the superior resolution of HRAM-MS allows for the accurate and reliable determination of molecular formula in the identification of unknowns (Peng et al., 2011).

Herein we present an LC-Orbitrap-MS analytical method for the quantitation of 14 phenolics of which Vanillin (**V**), Ellagic acid (**EA**), eight hydroxybenzoic acids: Gallic acid (**GA**), Gentisic acid (**GeA**), Protocatechuic acid (**PCA**), 2-hydroxybenzoic acid (**2-HBA**), 4-hydroxybenzoic acid (**4-HBA**), *p*-coumaric acid (***p*CouA**), *m*-coumaric acid (***m*CouA**) and *o*-coumaric acid (***o*CouA**); two hydroxycinnamic acids: Caffeic acid (**CA**) and Ferulic acid (**FeA**); two Caffeoylquinic acids: Neochlorogenic acid (**nCGA**) and Chlorogenic acid (**CGA**) and two Flavonoid glycosides: Rutin (**R**) and Quercetin-3-O-glucoside (**QG**) in hydromethanolic *Portulaca* extracts from nine Bulgarian and three Greek loci (Fig. 2).

Different chromatographic conditions were investigated to obtain adequate separation of the selected phenolic compounds. The chromatographic conditions of the mobile phase system (ACN/H_2O with 0.1% formic acid), gradient program and the column temperature (40 °C) were optimized in order to obtain optimal resolution within a short analysis time. Baseline separation was achieved for all isobars

Tab. 4: Orthogonal design – fresh purslane

Experiment №	Factor name							
	A % MeOH	B T °C	C Extraction time (min)	D Solid/solvent (g/ml)	E Extract (mg)	S Saponins[1]	P Polyphenols[2]	R DPPH[3]
Experiment 1	1	1	1	1	0.4077	21.93 ± 1.16	2.97 ± 0.04	38.38 ± 2.71
Experiment 2	1	2	2	2	0.4550	25.15 ± 1.66	3.15 ± 0.04	38.96 ± 0.97
Experiment 3	1	3	3	3	0.5400	26.20 ± 3.79	3.07 ± 0.10	11.08 ± 3.50
Experiment 4	2	1	3	2	0.5035	20.15 ± 1.72	4.05 ± 0.02	52.05 ± 2.17
Experiment 5	2	2	1	3	0.4721	25.73 ± 2.75	3.11 ± 0.03	51.89 ± 0.77
Experiment 6	2	3	2	1	0.3792	22.23 ± 0.98	3.09 ± 0.03	51.57 ± 2.78
Experiment 7	3	1	2	3	0.5933	27.61 ± 1.68	3.16 ± 0.04	20.64 ± 0.91
Experiment 8	3	2	3	1	0.3429	27.61 ± 2.79	2.80 ± 0.12	21.37 ± 1.78
Experiment 9	3	3	1	2	0.5015	28.67 ± 1.05	3.42 ± 0.04	22.33 ± 1.81
Mean value 1 S	24.43	23.23	25.44	23.92				
Mean value 2 S	22.70	26.16	25.00	24.66				
Mean value 3 S	27.96	25.70	24.65	26.51				
Range S	5.26	2.93	0.79	2.59				
Optimized scheme S	80	70	20	40				
Mean value 1 P	3.06	3.39	3.17	2.95				
Mean value 2 P	3.42	3.02	3.13	3.54				
Mean value 3 P	3.13	3.19	3.31	3.11				
Range P	0.35	0.37	0.17	0.59				
Optimized scheme P	70	50	40	30				
Mean value 1 R	29.47	37.02	37.53	37.11				
Mean value 2 R	51.84	37.41	37.06	37.78				
Mean value 3 R	32.99	28.33	28.17	27.87				
Range R	22.36	9.08	9.37	9.91				
Optimized scheme R	70	70	20	30				
Mean value 1 E	0.47	0.50	0.46	0.38				
Mean value 2 E	0.45	0.42	0.48	0.49				
Mean value 3 E	0.48	0.47	0.46	0.54				
Range E	0.03	0.08	0.02	0.16				
Optimized scheme E	80	50	30	40				

[1] mg aescin equivalent /g dry extract
[2] % pyrogallol equivalent (PE) for dry weight extract
[3] % radical scavenging activity

Tab. 5: Orthogonal design – dry purslane

Experiment №	Factor name A % MeOH	B T °C	C Extraction time (min)	D Solid/solvent (g/ml)	E Extract (mg)	S Saponins[1]	P Polyphenols[2]	R DPPH[3]
Experiment 1´	1	1	1	1	0.3717	28.83 ± 2.68	1.97 ± 0.03	35.26 ± 0.37
Experiment 2´	1	2	2	2	0.3488	33.41 ± 0.46	2.09 ± 0.01	44.90 ± 0.14
Experiment 3´	1	3	3	3	0.4122	30.44 ± 2.22	2.57 ± 0.04	47.39 ± 0.78
Experiment 4´	2	1	3	2	0.3337	21.99 ± 0.15	2.07 ± 0.04	48.43 ± 0.64
Experiment 5´	2	2	1	3	0.3412	27.63 ± 2.61	1.70 ± 0.04	47.87 ± 0.37
Experiment 6´	2	3	2	1	0.3080	27.68 ± 2.88	2.55 ± 0.04	52.45 ± 0.20
Experiment 7´	3	1	2	3	0.3165	24.74 ± 1.52	1.67 ± 0.04	21.77 ± 1.69
Experiment 8´	3	2	3	1	0.3059	26.78 ± 0.33	1.91 ± 0.02	27.23 ± 2.39
Experiment 9´	3	3	1	2	0.3011	25.05 ± 2.07	2.01 ± 0.01	23.61 ± 1.10
Mean value 1 S	30.89	25.19	27.17	27.76				
Mean value 2 S	25.77	29.27	28.61	26.82				
Mean value 3 S	25.52	27.72	25.72	27.60				
Range S	5.37	4.09	2.89	0.95				
Optimized scheme S	50	70	30	20				
Mean value 1 P	2.21	1.90	1.89	2.14				
Mean value 2 P	2.11	1.90	2.10	2.06				
Mean value 3 P	1.86	2.38	2.18	1.98				
Range P	0.35	0.48	0.29	0.16				
Optimized scheme P	50	80	40	20				
Mean value 1 R	42.52	35.15	35.58	38.31				
Mean value 2 R	49.58	40.00	39.71	38.98				
Mean value 3 R	38.39	41.15	41.02	39.01				
Range R	11.19	6.00	5.44	0.70				
Optimized scheme R	70	80	40	40				
Mean value 1 E	0.38	0.34	0.34	0.33				
Mean value 2 E	0.33	0.33	0.32	0.33				
Mean value 3 E	0.31	0.34	0.35	0.36				
Range E	0.07	0.01	0.03	0.03				
Optimized scheme E	50	50	40	40				

1 mg aescin equivalent /g dry extract
2 % pyrogallol equivalent (PE) for dry weight extract
3 % radical scavenging activity

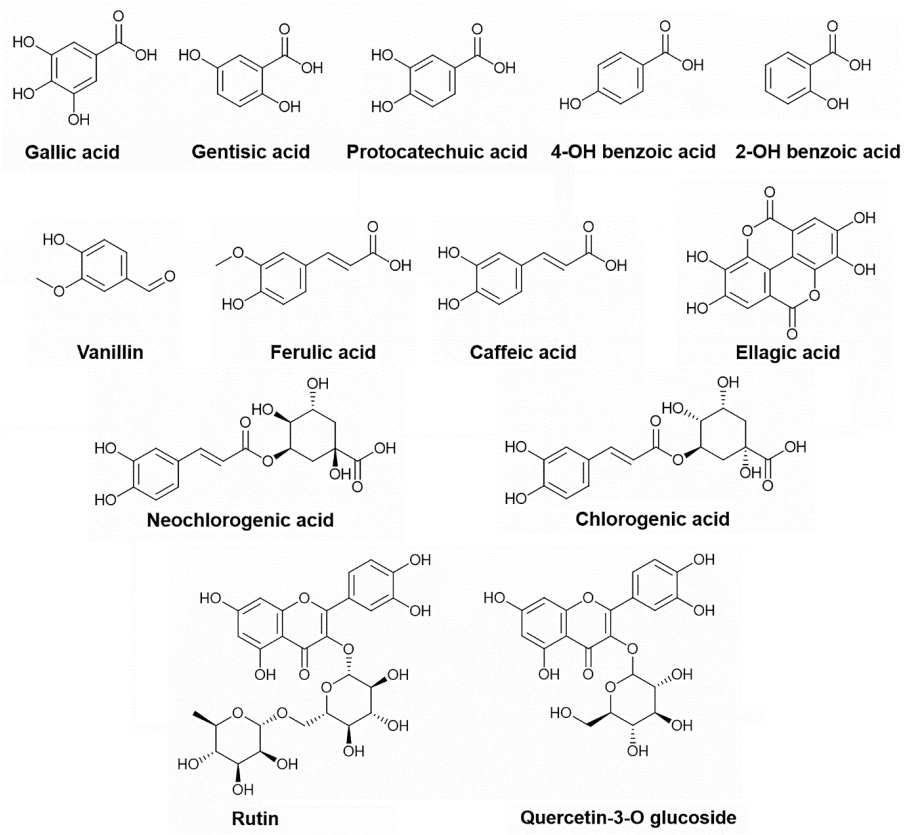

Fig. 2: Phenolic acids and flavonoid glycosides that are identified and quantified in this study.

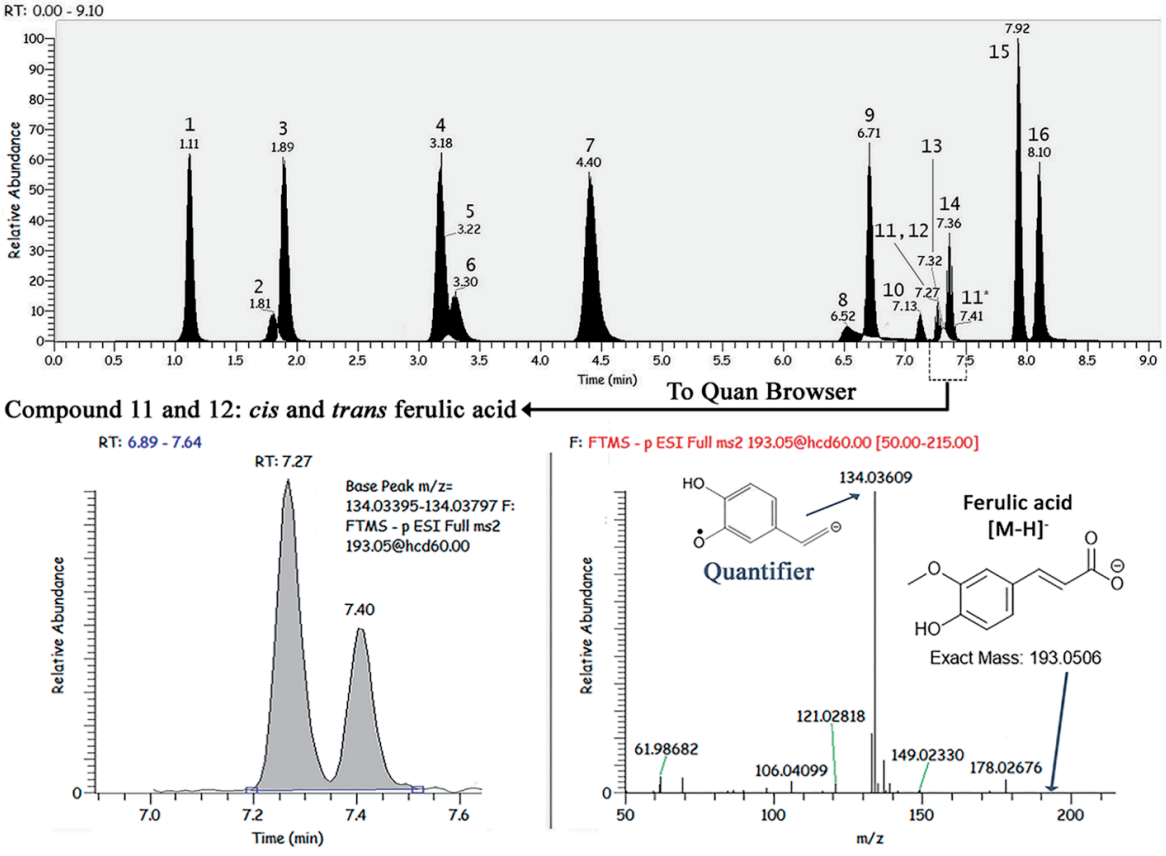

Fig. 3: LC-MS chromatogram of the quantified compounds in parallel reaction monitoring (PRM) mode. *Ferulic acid (Compound **11**) appears in two peaks due to *cis-trans* isomerism.

(like chlorogenic and neochlorogenic acids) and potential cross talk was avoided. Chromatographic and mass spectrometric parameters were optimized for fast and accurate measurements. MS detection in negative ionization mode was utilized. The total ion current (TIC) chromatogram of the studied compounds and a representative MS spectrum of ferulic acid (**FeA**) are presented in (Fig. 3).

Validation of the quantitative analysis

The established method was validated for precision, accuracy, sensitivity and recovery. Calibration settings are summarized in Tab. 2. The acceptable criteria for the calibration curves were R^2 higher than 0.9950 and a back-calculated standard concentration (from the calibration curve) within 15% deviation from the nominal value. The precision (%RSD, n = 5) was less than 10% and typically 1-3%. The intra- and inter-day precisions were <4% and <10%, respectively. With respect to the accuracy, deviation of the nominal versus the back-calculated value from the calibration curve was less than 15%. The mass errors between the theoretically calculated and measured masses were less than 5.00 ppm. A mass error limit of 5 ppm was used to distinguish true compound signals from background. IDL and IQL ranged between 0.12 ng/ml (QG) and 18.52 ng/ml (EA), and 0.40 ng/ml (QG) and 18.517 ng/ml (EA). LOD and LOQ were determined from 6.00 ng/g (QG) to 19.80 ng/g (EA), and from 925.85 ng/g (QG) to 3055.30 ng/g (EA), respectively. The recovery ranged between 95 and 108%.

Systematic characterization of phenolic compounds in purslane loci

The content of each analyte (μg/g dry weight) of different purslane loci was calculated from the corresponding calibration curve and presented in Tab. 6.

The highest content among the tested compounds was found for ferulic acid (79.998 ± 1.575 μg/g dw), followed by vanillin (11.704 ± 0.163 μg/g dw) and gentisic acid (8.984 ± 0.136 μg/g dw).

Ferulic acid was the major phenolic acid in the majority of studied plant loci, being presented up to 315.39 ± 2.349 μg/g dw and 152.38 ± 0.682 μg/g dw in Gr3 and Bg4, respectively. The ferulic acid content ranged between 30.7% (Bg6) to 91.4% (Gr3) of the total amount of assayed compounds. Vanillin was commonly found in all samples, ranging from 6.207 ± 0.170 μg/g dw (Bg1) to 21.296 ± 0.267 μg/g dw (Gr1). Gentisic acid occurred in relatively higher concentration in Bg4 and Gr2, where it reaches up to 13.7% and 20.4%, respectively. With respect to caffeoylquinic acids, the values of chlorogenic and neochlorogenic acids were low, except for Bg7 sample. Small concentrations of 2-OH benzoic acid was determined in most of the loci while the highest content was detected up to 10.116 ± 0.311 μg/g dw and 13.874 ± 0.194 μg/g dw in Bg5 and Bg7, respectively. Samples Bg4 and Gr2 were the richest in both 4-OH benzoic and caffeic acid. The highest concentration of rutin (40.135 ± 0.264 μg/g dw) and quercetin-3-O-glucoside (6.394 ± 0.090 μg/g dw) was observed in Bg6 whereas the lowest was found in Bg1 and Gr1, respectively.

The Bulgarian Bg4 and Greek Gr3 were the richest loci in total phenolic compounds due to the presence of high content of ferulic acid while Bg2 and Gr1 were the poorest (Tab. 6).

Despite of the different extraction conditions, our results were in accordance with those reported in the literature (Gatea et al., 2017; Liang et al., 2014; Silva, 2011). Liang et al. (2014) determined the content of quercetin-3-O-glucoside and rutin in *P. oleracea* samples with Chinese provenances utilizing an UPLC-MS/MS method. For quercetin-3-O-glucoside, the content ranged between 0.755 ± 0.008 and 5.55 ± 0.046 μg/g dw (Liang et al., 2014), whereas in our study, the concentration ranged between 0.080 ± 0.001 and 6.394 ± 0.090 μg/g dw. Liang et al. (2014) also determined rutin concentrations to be 1.020 ± 0.009 to 15.850 ± 0.362 μg/g dw, whereas we report levels between 0.054 ± 0.001 and 40.135 ± 0.264 μg/g dw.

In our study, the average content of rutin (4.879 ± 0.085 μg/g dw), chlorogenic acid (4.159 ± 0.095 μg/g dw) and caffeic acid (2.080 ± 0.032 μg/g dw) were lower compared to those found in a study by Gatea et al. (2017): rutin – 76.07 ± 2.97 μg/g dw; chlorogenic acid – 105.12 ± 2.02 μg/g dw; caffeic acid – 139.33 ± 0.11 μg/g dw. However, we found higher content of ferulic acid (79.998 ± 1.145 μg/g dw) compared to Gatea et al. (2017) (39.62 ± 1.78 μg/g dw).

In contrast to the finding of Silva (2011), who reported substantial concentrations of gallic acid (269.49 μg/g dw), we detected gallic acid just in one Greek sample (Gr2).

We registered a higher average concentration of gentisic acid (8.984 ± 0.136 μg/g dw) in the aerial parts compared to the values determined in purslane flowers (3.90 μg/g dw) by Silva (2011).

Likewise, higher average concentration of both chlorogenic and caffeic acid (4.159 ± 0.095 and 2.080 ± 0.032 μg/g dw, respectively) were found compared to the study by Silva (2011) (2.52 and 1.23 μg/g dw, respectively). Tab. 7 presents the sum of the content of all quantified 14 phenolic acids as well as the 2 flavonoid glycosides in each batch (plant locus).

Principal Component Analysis

Characterization and classification of plants can be accomplished from the compositional profiles as a source of analytical information. Polyphenols and other low molecular weight organic acids have been found to be efficient descriptors of some climatic and agricultural features and thus, the variability of compounds should depend on the origin of the plant (Puigventós et al., 2015; Zhao et al., 2011). Therefore, the polyphenolic profile could be a useful platform for the reliable discrimination via chemometric methods such as principal component analysis (PCA).

The performed PCA analysis in this study revealed one well-defied cluster including **Bg5**, **Bg7**, and **Bg8**. The cluster was defined by hydroxybenzoic acids, vanillin and QG. The samples shared isomeric gentisic and protocatechuic acids. Gentisic acid was presented in the range between 3.245 and 3.977 μg/g dw while the protocatechuic acid content was considerably lower (up to 0.140 μg/g dw in **Bg7**). The cluster grouped loci with high abundance of vanillin (Tab. 6). It is worth noting the high levels of 2-OH benzoic acid in **Bg5** and **Bg7**. Gallic, Chlorogenic, Neochlorogenic, Ellagic and 4-OH benzoic acids as well as Orizovo locus were excluded from the PCA analysis due to insufficient data. The two principal components with the greatest eigenvalues accounted for 64.8% of the variance. Fig. 4 displays the projection of the 11 sample locations and the variables in the plane defined by the two principal components.

Determination of Oleraceins by UHPLC-MS as contributors to the antioxidant potential of P. oleraceae

Oleraceins are characterized with a 5,6-dihydroxyindoline-2-carboxylic acid core and are acylated with cinnamic acid derivatives, like coumaric, ferulic and caffeic acids, and some are glycosylated. Oleraceins were first characterized by Xiang et al. (2005), who isolated and structurally elucidated five Oleraceins (Oleracein A, B, C, D, and E) in dried purslane plants. Later, Yang et al. (2009) sought to determine the antioxidant potential of three Oleraceins – A, B and E. The DPPH radical scavenging activities, expressed as EC_{50} μM, of Oleraceins A, B and E (8.96 ± 0.19 μM, 5.56 ± 0.11 μM and 9.87 ± 0.08 μM, respectively) were slightly lower than that of caffeic acid (4.97 ± 0.09 μM), but higher than that of ascorbic acid and α-Tocopherol (11.70 ± 0.22 μM and 13.14 ± 0.11 μM, respectively). In 2011 (Liu et al., 2011) isolated two new Oleraceins named Oleracein F and G and evaluated their DPPH radical scavenging ac-

Tab. 6: Concentration of 14 phenolic acids and 2 flavonoids as μg/g dry weight in *Portucala oleracea* samples from 12 different loci from Bulgaria and Greece.

	V	EA	GA	GeA	PCA	4-HBA	2-HBA	*p*CouA	*m*CouA	*o*CouA	CA	FeA	nCGA	CGA	R	QG
Bg1	18.478 ±0.268	NQ	NQ	4.380 ±0.021	0.111 ±0.005	NQ	5.016 ±0.095	2.067 ±0.009	ND	ND	0.442 ±0.021	50.692 ±1.336	0.555 ±0.025	2.304 ±0.051	0.054 ±0.001	0.408 ±0.007
Bg2	6.325 ±0.044	NQ	NQ	0.720 ±0.008	NQ	4.210 ±0.208	5.108 ±0.073	1.978 ±0.030	ND	ND	NQ	14.713 ±1.285	NQ	NQ	NQ	NQ
Bg3	9.981 ±0.131	NQ	NQ	8.652 ±0.054	0.246 ±0.001	3.342 ±0.058	4.380 ±0.035	2.250 ±0.068	ND	ND	0.850 ±0.010	53.069 ±1.212	NQ	0.759 ±0.008	0.053 ±0.002	0.474 ±0.009
Bg4	19.357 ±0.144	NQ	NQ	32.060 ±0.189	1.056 ±0.017	11.613 ±1.411	4.084 ±0.101	4.527 ±0.012	ND	ND	6.371 ±0.039	152.38 ±0.682	NQ	0.536 ±0.006	0.066 ±0.003	2.724 ±0.038
Bg5	12.034 ±0.133	NQ	NQ	3.977 ±0.028	0.140 ±0.004	2.152 ±0.065	10.116 ±0.311	1.486 ±0.400	ND	ND	0.956 ±0.003	70.715 ±2.304	NQ	NQ	NQ	1.231 ±0.017
Bg6	11.609 ±0.039	NQ	NQ	3.828 ±0.030	0.208 ±0.006	NQ	4.033 ±0.020	1.144 ±0.032	ND	ND	1.991 ±0.034	30.489 ±1.277	0.168 ±0.022	3.193 ±0.023	40.135 ±0.264	2.671 ±0.035
Bg7	8.449 ±0.109	8.605 ±0.189	NQ	3.231 ±0.030	0.082 ±0.001	6.461 ±0.092	13.874 ±0.194	2.632 ±0.045	ND	ND	0.191 ±0.006	40.999 ±0.809	9.727 ±0.533	22.915 ±0.284	5.767 ±0.035	1.004 ±0.033
Bg8	13.180 ±0.174	NQ	NQ	3.245 ±0.045	0.110 ±0.003	3.822 ±0.504	2.397 ±0.029	1.701 ±0.022	ND	ND	0.510 ±0.008	56.791 ±2.406	0.238 ±0.024	0.800 ±0.012	1.701 ±0.027	0.574 ±0.012
Bg9	6.903 ±0.054	NQ	NQ	3.701 ±0.055	0.083 ±0.015	NQ	1.018 ±0.011	1.936 ±0.431	ND	ND	0.606 ±0.004	51.092 ±0.810	0.306 ±0.024	1.585 ±0.027	0.085 ±0.003	2.144 ±0.029
Gr1	21.296 ±0.267	NQ	NQ	4.700 ±0.017	0.074 ±0.002	3.617 ±0.150	2.299 ±0.060	2.383 ±0.072	ND	ND	0.186 ±0.014	52.090 ±1.962	0.514 ±0.019	0.965 ±0.044	0.177 ±0.005	0.080 ±0.001
Gr2	6.207 ±0.170	23.822 ±0.132	0.333 ±0.009	35.590 ±0.419	0.272 ±0.009	12.958 ±0.439	2.244 ±0.033	3.062 ±0.043	ND	ND	6.426 ±0.080	71.549 ±0.996	0.401 ±0.023	4.528 ±0.054	0.438 ±0.006	6.394 ±0.090
Gr3	6.632 ±0.205	NQ	NQ	3.723 ±0.026	0.279 ±0.003	NQ	2.972 ±0.067	2.108 ±0.051	ND	ND	4.354 ±0.042	315.39 ±2.349	0.912 ±0.024	4.006 ±0.043	0.313 ±0.001	4.635 ±0.050
Average values	11.704 ±0.163	ND	ND	8.984 ±0.136	0.242 ±0.008	6.022 ±0.561	4.795 ±0.119	2.273 ±0.174	ND	ND	2.080 ±0.032	79.998 ±1.575	1.602 ±0.190	4.159 ±0.095	4.879 ±0.085	1.862 ±0.036

NQ - less than LOQ; ND – not determined/not enough data. Bg# - Bulgarian locations; Gr# - Greek locations. **Bg1**, Cherven bryag; **Bg2**, Orizovo; **Bg3**, Pirdop; **Bg4**, Kremena; **Bg5**, Nessebar; **Bg6**, Dolni Bogrov; **Bg7**, Burgas; **Bg8**, Shumen; **Bg9**, Montana; **Gr1**, Lagonissi; **Gr2**, Possidi; **Gr3**, Komotini. **V**, Vanillin; **EA**, Ellagic acid; **GA**, Gallic acid; **GeA**, Gentisic acid; **PCA**, Protocatechuic acid; **4-HBA**, 4-hydroxybenzoic acid; **2-HBA**, 2-hydroxybenzoic acid; ***p*CouA**, *p*-coumaric acid; ***m*CouA**, *m*-coumaric acid; ***o*CouA**, *o*-coumaric acid; **CA**, Caffeic acid; **FeA**, Ferulic acid; **nCGA**, Neochlorogenic acid; **CGA**, Chlorogenic acid; **R**, Rutin; **QG**, Quercetin-3-O-glucoside.

Tab. 7: Total antioxidant compounds in the different batches.

Plant locus	Abbrev.	Sum of all determined polyphenols (µg/ g dw)
Cherven bryag	**Bg1**	84.507 ± 1.839
Orizovo	**Bg2**	33.054 ± 1.648
Pirdop	**Bg3**	84.056 ± 1.588
Kremena	**Bg4**	234.774 ± 2.642
Nessebar	**Bg5**	102.807 ± 3.265
Dolni Bogrov	**Bg6**	99.469 ± 1.782
Burgas	**Bg7**	123.937 ± 2.360
Shumen	**Bg8**	85.069 ± 3.266
Montana	**Bg9**	69.459 ± 1.463
Lagonissi	**Gr1**	88.324 ± 2.613
Possidi	**Gr2**	174.224 ± 2.503
Komotini	**Gr3**	345.324 ± 2.861

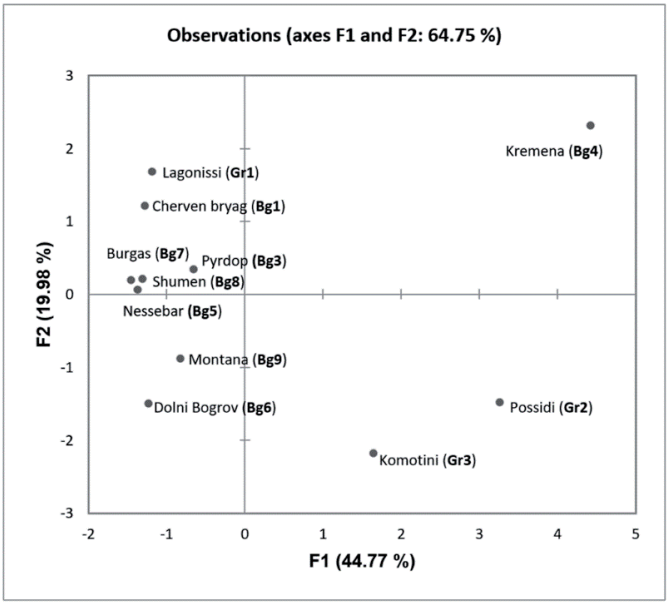

Fig. 4: PCA plot of the polyphenol content of nine Bulgarian (Cherven bryag, Pirdop, Kremena, Nhessebar, Dolni Bogrov, Burgas, Shumen and Montana) and three Greek (Lagonisi, Posidi and Komotini) loci of purslane.

tivities. The EC_{50} values were 21.00 ± 0.10 µM and 37.69 ± 0.75 µM which were comparable but slightly lower than that of the control – ascorbic acid (16.44 ± 0.44 µM). Later, (Jiao et al., 2014) tentatively identified and characterized based on UV spectra and MS and MS/MS fragment analysis eight new indoline amide glucosides (Oleraceins H-O). A year later, the same team isolated and structurally characterized Oleraceins H, I, K, L, N-S. These ten Oleraceins together with Oleraceins A-D were tested for their antioxidant potential and exhibited potent DPPH radical scavenging activities (Jiao et al., 2015). From all the tested fourteen indoline amide glycosides, Oleraceins K and L were found to be the most potent radical scavengers of the DPPH radical with EC_{50} values of 15.30 and 16.13 µM, respectively. Oleraceins K and L were almost twice as potent as the natural antioxidant vitamin C with EC_{50} = 30.15 µM, used as a control. The respective antioxidant activities of the other twelve Oleraceins were comparable to that of ascorbic acid and ranged from 27.64 to 43.52 µM (Jiao et al., 2015). Recently, (Farag and Shakour, 2019) identified through MS/MS fragmentation analyses the Oleraceins A-D, K, N, O, T, U, V and W in aerial parts of the plant *P. oleraceae*.

Herein, by employing UHPLC-HRMS, we sought to determine Oleracein compounds in the hydromethanolic samples derived from *P. oleraceae* flower beads (Tab. 8). Fig. 5 depicts the Full-scan and extracted ion chromatogram, and Fig. 6 shows the chemical structure of the tentatively identified Oleraceins A-D, N-Q, S, U and W. MS^2 fragmentation data of identified Oleraceins are provided in the Supplementary material (Suppl. 1-9) and are discussed below.

Oleracein A (See Suppl. 1) displayed its molecular ion at m/z 504.1893 $[M+H]^+$ and a characteristic MS^2 fragment ion at m/z 342 $[M\text{-hex}+H]^+$ resulting from the loss of a hexose moiety. A fragment ion corresponding to a protonated 5,6-dihydroxy indoline 2-carboxylic acid ($[Ind+H]^+$), characteristic for every Oleracein, appeared at m/z 196, along with fragment ions resulting from subsequent losses of water, at m/z 178 ($[Ind\text{-}H_2O+H]^+$), and formic acid, at m/z 150 ($[Ind\text{-}HCOOH+H]^+$). The fragment ion at m/z 147 indicated the presence of a coumaroyl moiety. Hence the compound appearing at 5.26 min with m/z 504.1500, having a molecular formula of $C_{24}H_{25}NO_{11}$, was assigned as Oleracein A (Fig. 6).

Oleracein B (See Suppl. 2) exhibited a $[M+H]^+$ ion at m/z 534.1564. In general, the MS^2 fragmentation of Oleracein B was identical to that of Oleracein A differing in that instead of a coumaroyl moiety, Oleracein B has a feruloyl one attached to 1st position in the indoline core (Fig. 6). Fragment ion at m/z 372 $[M\text{-hex}+H]^+$ resulted from the cleavage of the hexose moiety and fragment ion at m/z 196 ($[Ind+H]^+$) indicated the presence of the indoline moiety. The fragment ion at m/z 177 was derived from the feruloyl moiety. Hence, the substance appearing at 5.47 min with $[M+H]^+$ at m/z 534.1606, having a molecular formula of $C_{25}H_{27}NO_{12}$ was identified as Oleracein B.

Oleracein C (See Suppl. 3) showed a molecular ion at m/z 666.2018 $[M+H]^+$ followed by a fragment ion at m/z 504 $[M\text{-hex}+H]^+$ indicating a loss of a hexose. The presence of the 7-glycosyl coumaroyl moiety was confirmed by the fragment ion at m/z 309. Fragment ions at m/z 196 and m/z 147 revealed the presence of the indoline and the coumaroyl moieties, respectively. Hence, the substance appearing at 4.03 min with $[M+H]^+$ at m/z 666.2018, having a molecular formula of $C_{30}H_{35}NO_{16}$ was assigned as Oleracein C (Figure 6).

Oleracein D (See Suppl. 4) displayed a molecular ion $[M+H]^+$ at m/z 696.2177. The fragment ions at m/z 534 $[M\text{-hex}+H]^+$ and m/z 372 $[M\text{-}2hex+H]^+$ revealed two consecutive losses of hexoses from the molecular ion. Noteworthy, the fragment ion $[M\text{-hex}+H]^+$ of Oleracein D is identical to the molecular ion $[M+H]^+$ of Oleracein B, since structurally Oleracein D has an additional glucose moiety at 7´, compared to Oleracein B (Fig. 6). The fragment ion at m/z 339 revealed the presence of the 7-glycosyl feruloyl moiety, similarly to the fragment ion at m/z 309 for Oleracein C. Fragment ions at m/z 196 and m/z 147 were indicative for the indoline and coumaroyl moieties, respectively. Hence, the substance appearing at 4.23 min with $[M+H]^+$ at m/z 696.2177, having a molecular formula of $C_{31}H_{37}NO_{17}$ was identified as Oleracein D.

According to the mass fragmentation analysis of isobars with molecular ions at m/z 842.2508 and at m/z 842.2505 eluting at 5.64 min and 6.33 min, respectively, having a chemical composition of $C_{40}H_{43}NO_{19}$, can be tentatively assigned to either Oleracein N or Oleracein S. The structural difference between Oleracein N or Oleracein S is that Oleracein N has 2´´´- feruloyl, and Oleracein S has 6´´´-feruloyl moiety (Fig. 6). Both MS/MS spectra (See Suppl. 5) showed characteristic fragment ions for Oleraceins but in different rations to one another.

Molecular ions at m/z 872.2599 and m/z 872.2616, having a chemical composition of $C_{41}H_{45}NO_{20}$ corresponding to Oleracein O, were observed eluting at 5.77 min and 6.37 min, respectively. Both spectra

Tab. 8: Mass spectrometric and chromatographic data for identified Oleraceins by UHPLC-MS.

Name	Molecular formula	Exact mass $[M+H]^+$	MS2	t_R (min)	Δ ppm
Oleracein A	$C_{24}H_{25}NO_{11}$	504.1500	504.1400 (0.3), 342.0969 (8.3), 196.0605 (1.8), 178.0499 (0.8), 150.0550 (0.8), 147.0441 (100), 119.0495 (0.3)	5.26	1.2
Oleracein B	$C_{25}H_{27}NO_{12}$	534.1606	534.1616 (0.7), 372.1078 (4.5), 196.0602 (0.5), 177.0548 (100), 145.0286 (0.9)	5.47	0.9
Oleracein C	$C_{30}H_{35}NO_{16}$	666.2029	666.2018 (0.2), 504.1497 (6.2), 342.0974 (2.4), 309.0966 (12.5), 291.086 (3.3), 196.0607 (0.9), 165.0542 (0.8), 147.0441 (100)	4.03	1.8
Oleracein D	$C_{31}H_{37}NO_{17}$	696.2134	696.2117 (0.6), 534.1606 (4.1), 372.1076 (5.9), 339.1074 (2.9), 321.0967 (1.8), 196.0607 (0.7), 177.0547 (100), 145.0289 (0.6)	4.23	2.0
Oleracein N/ Oleracein S	$C_{40}H_{43}NO_{19}$	842.2502	504.1472 (0.8), 342.097 (10.8), 339.1073 (100), 321.0967 (14.8), 261.0755 (1.3), 196.0607 (1.3), 177.0546 (81), 147.0441 (29.3)	5.64	-0.7
Oleracein N/ Oleracein S	$C_{40}H_{43}NO_{19}$	842.2502	504.1507 (4.6), 342.0969 (45.7), 339.1065 (5.4), 321.0978 (1.3), 196.0603 (6.1), 195.065 (4.2), 177.0546 (46.5), 147.044 (100)	6.33	-0.4
Oleracein O/ isomer	$C_{41}H_{45}NO_{20}$	872.2608	534.1641 (0.5), 515.1557 (0.8), 372.1079 (7.2), 339.1073 (82.4), 321.0967 (13.9), 196.0605 (0.9), 195.0653 (1.9), 177.0547 (100), 147.0441 (0.9)	5.77	1.0
Oleracein O/ isomer	$C_{41}H_{45}NO_{20}$	872.2608	534.1622 (2.7), 515.1549 (0.3), 372.1075 (19.2), 339.1071 (4.1), 321.0965 (1.3), 196.0602 (2.8), 195.0651 (2.9), 177.0546 (100), 147.0442 (1.6)	6.37	-1.0
Oleracein P	$C_{36}H_{45}NO_{21}$	828.2557	666.2023 (4.8), 504.1497 (25.1), 471.1494 (1.2), 358.112 (0.7), 342.0969 (5.3), 309.0967 (32.7), 291.086 (5.7), 196.0605 (4.2), 147.0441 (100)	3.65	1.0
Oleracein Q	$C_{37}H_{47}NO_{22}$	858.2663	696.2139 (3.3), 534.1608 (15.8), 372.1076 (11.6), 339.1073 (8.7), 321.0968 (3.6), 196.0605 (3.4), 177.0547 (100), 145.0493 (0.5)	3.87	0.9
Oleracein U	$C_{18}H_{15}NO_6$	342.0972	342.0962 (1.2), 324.0871 (0.4), 296.0929 (0.1), 282.0759 (0.5), 196.0602 (0.7), 178.0499 (1.1), 150.0553 (0.5), 147.0441 (100), 132.0445 (0.2), 97.6962 (0.2)	4.66	-1.9
Oleracein W	$C_{18}H_{15}NO_7$	358.0921	358.0923 (4.5), 298.0963 (0.6), 196.0605 (14.5), 163.0390 (100), 137.0600 (1.5)	4.85	1.0

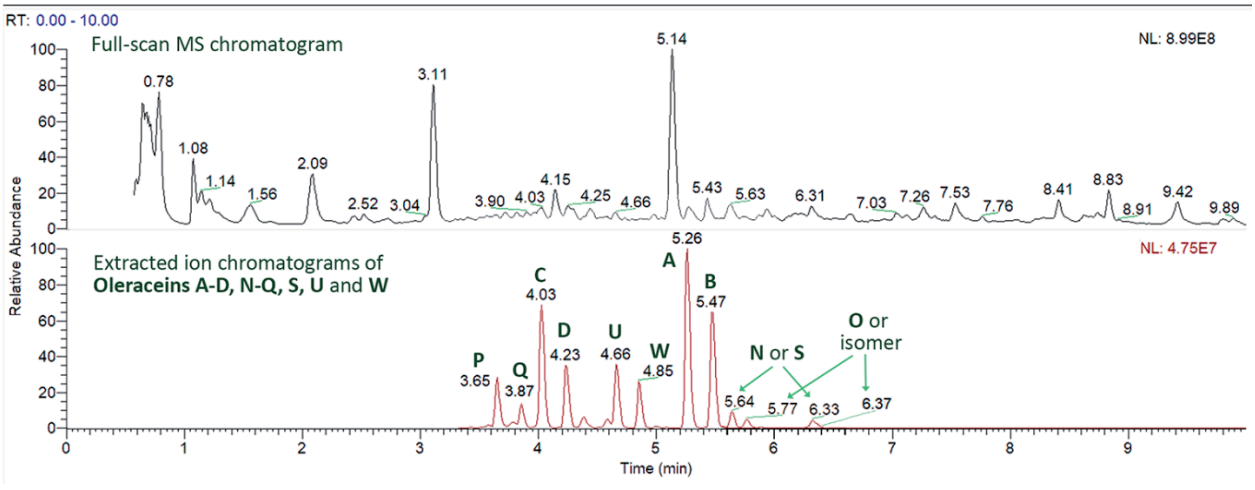

Fig. 5: Full-scan MS chromatogram and extracted ion chromatogram of tentatively identified Oleraceins by UHPLC-MS

displayed identical and characteristic fragment ions for Oleraceins, however, by the MS^2 analysis it was not possible to state which of the abovementioned two chromatographic peaks corresponds to Oleracein O.

The fragmentation behavior of a peak eluting at 3.65 min with molecular ion $[M+H]^+$ at m/z 828.2549 was identical to that of Oleracein C, except that this compound is heavier with 162.05 Da which corresponds to a hexose. After analysis of the mass fragmentation, the

Num	Name	R_1	R_2
1	Oleracein A	H	H
2	Oleracein B	H	OCH_3
3	Oleracein C	Glu	H
4	Oleracein D	Glu	OCH_3

Num	Name	R_1	R_2	R_3	R_4
5	Oleracein N	H	H	H	Feruloyl
6	Oleracein O	H	OCH_3	H	Feruloyl
7	Oleracein P	Glu	H	H	H
8	Oleracein Q	Glu	OCH_3	H	H
9	Oleracein S	H	H	Feruloyl	H

Num	Name	R_1
6	Oleracein U	Coumaroyl
11	Oleracein W	Caffeoyl
12	Oleracein C	Glu
13	Oleracein D	Glu

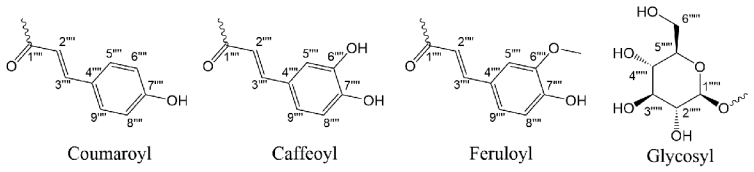

Fig. 6: Chemical structure of tentatively identified Oleraceins A-D, N-Q, S, U and W

compound with $[M+H]^+$ at m/z 828.2549 was assigned as Oleracein P (See Suppl. 6), which compared to Oleracein C has an additional glycosyl moiety at 6´´ (Fig. 6).

Oleracein Q (See Suppl. 7) showed a molecular ion $[M+H]^+$ at m/z 858.2655 eluting at 3.87 min. The MS^2 data showed that the molecule undergoes two consecutive hexose losses resulting in fragment ions at m/z 696 and at m/z 534. Another loss of a neutral hexose (162.05 Da) results in a fragment ion at m/z 372 being a protonated *N*-feruloyl-5,6-dihydroxy indoline 2-carboxylic acid. A fragment ion indicating a 7´-glycosyl feruloyl moiety was evident at m/z 339. The protonated 5,6-dihydroxy indoline 2-carboxylic acid and a feruloyl fragment ion were observed at m/z 196 and at m/z 177, respectively (Fig. 6).

Oleracein U (See Suppl. 8) eluted at 4.66 min and was observed as a molecular ion $[M+H]^+$ at m/z 342.0979. Structurally, Oleracein U essentially is *N*-coumaroyl-5,6-dihydroxy indoline 2-carboxylic acid. The MS^2 fragmentation analysis revealed several characteristic fragment ions as m/z 282 $[M-C_2H_4O_2+H]^+$. The 5,6-dihydroxy indoline 2-carboxylic acid moiety in the molecule was confirmed with several characteristic fragment ions as m/z 178 and m/z 150, and the coumaroyl moiety was confirmed by the presence of fragment ion at m/z 147 (Fig. 6).

Oleracein W (See Suppl. 9) appeared at 4.85 min with a molecular ion $[M+H]^+$ at m/z 358.0918. Structurally, Oleracein W is *N*-caffeoyl-5,6-dihydroxy indoline 2-carboxylic acid, similar to Oleracein U, but with a *N*-caffeoyl moiety instead of a *N*-coumaroyl one (Fig. 6). The 5,6-dihydroxy indoline 2-carboxylic acid moiety was confirmed by the presence of a fragment ion at m/z 298 and at m/z 196, and a characteristic fragment ion for the caffeoyl moiety was observed at m/z 163.

Conclusion

In order to establish chemical profiles of *Portulaca* extracts from 12 different loci in Bulgaria and Greece, an UHPLC coupled with a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer were utilized for the simultaneous identification and quantification of 14 phenolic acids and 2 flavonoids. The results confirmed that the developed method is suitable for quantifications of the analyzed phenolic compounds as it was successfully applied in twelve different *Portulaca* samples derived from Bulgarian and Greek loci. The validation of the method, including linearity, sensitivity (LOD and LOQ), precision and accuracy, was carried out and demonstrated to satisfy the requirements of quantitative analysis. Moreover, 11 known Oleraceins (A-D, N-Q, S, U and W) were tentatively identified by analyzing their mass fragmentation. Furthermore, a three-level and four-factor orthogonal design $L_9(3^4)$ was performed to determine the optimal conditions for the ultrasound assisted extraction (UE) in regard to extraction yields (E), polyphenols (P) and saponins (S) content, and DPPH radical scavenging activity (R).

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References

Alam, M., Juraimi, A.S., Rafii, M., Abdul Hamid, A., Aslani, F., Hasan, M., Zainudin, M., Asraf, M., Uddin, M., 2014: Evaluation of antioxi-

dant compounds, antioxidant activities, and mineral composition of 13 collected purslane (*Portulaca oleracea* L.) accessions. Biomed Res Int. 296063. DOI: 10.1155/2014/296063

Behravan, J., Mosafa, F., Soudmand, N., Taghiabadi, E., Razavi, B.M., Karimi, G., 2011: Protective Effects of Aqueous and Ethanolic Extracts of Portulaca oleracea L. Aerial Parts on H2O2- Induced DNA Damage in Lymphocytes by Comet Assay. J. Acupunct. Meridian Stud. 4, 193-197. DOI: 10.1016/j.jams.2011.09.008

Brickell, C., 1999: The Royal Horticultural Society new encyclopedia of plants and flowers. Dorling Kindersley.

European Pharmacopoeia Commission, E.P., Medicines, E.D.F.T.Q.O., Healthcare, 2010: European pharmacopoeia. Council of Europe..

De Paepe, D., Servaes, K., Noten, B., Diels, L., De Loose, M., Van Droogenbroeck, B., Voorspoels, S., 2013: An improved mass spectrometric method for identification and quantification of phenolic compounds in apple fruits. Food Chem. 136, 368-375. DOI: 10.1016/j.foodchem.2012.08.062

Dkhil, M.A., Moniem, A.E.A., Al-Quraishy, S., Saleh, R.A., 2011: Antioxidant effect of purslane (*Portulaca oleracea*) and its mechanism of action. J. Med. Plants Res. 5, 1589-1593.

El-Sayed, M.-I.K., 2011: Effects of *Portulaca oleracea* L. seeds in treatment of type-2 diabetes mellitus patients as adjunctive and alternative therapy. J. Ethnopharmacol. 137, 643-651. DOI: 10.1016/j.jep.2011.06.020

Farag, M.A., Shakour, Z.T.A., 2019: Metabolomics driven analysis of 11 Portulaca leaf taxa as analysed via UPLC-ESI-MS/MS and chemometrics. Phytochem. 161, 117-129. DOI: 10.1016/j.phytochem.2019.02.009

Gatea, F., Teodor, E.D., Seciu, A.M., Nagodă, E., Radu, G.L., 2017: Chemical constituents and bioactive potential of *Portulaca pilosa* L vs. *Portulaca oleracea* L. Med. Chem. Res. 26, 1516-1527. DOI: 10.1007/s00044-017-1862-5

Gong, F., Li, F., Zhang, L., Li, J., Zhang, Z., Wang, G., 2009: Hypoglycemic effects of crude polysaccharide from purslane. Int. J. Mol. Sci. 10, 880-888. DOI: 10.3390/ijms10030880

Jiao, Z.-Z., Yue, S., Sun, H.-X., Jin, T.-Y., Wang, H.-N., Zhu, R.-X., Xiang, L., 2015: Indoline Amide Glucosides from *Portulaca oleracea*: Isolation, Structure, and DPPH Radical Scavenging Activity. J. Nat. Prod. 78, 2588-2597. DOI: 10.1021/acs.jnatprod.5b00524

Jiao, Z., Wang, H., Wang, P., Sun, H., Yue, S., Xiang, L., 2014: Detection and quantification of cyclo-dopa amides in *Portulaca oleracea* L. by HPLC-DAD and HPLC-ESI-MS/MS. J. Chin. Pharmaceut. Sci. 23(8). DOI: 10.5246/jcps.2014.08.069

Li, A.-N., Li, S., Zhang, Y.-J., Xu, X.-R., Chen, Y.-M., Li, H.-B., 2014: Resources and biological activities of natural polyphenols. Nutrients 6, 6020-6047. DOI: 10.3390/nu6126020

Liang, X., Li, L., Tian, J., Wu, Y., Gao, P., Li, D., Zhang, Q., Song, S., 2014: A rapid extraction and analysis method for the simultaneous determination of 26 bioflavonoids in *Portulaca oleracea* L. Phytochem. Anal. 25, 537-543. DOI: 10.1002/pca.2524

Liu, D., Shen, T., Xiang, L., 2011: Two Antioxidant Alkaloids from *Portulaca oleracea* L.. HCA 94, 497-501. DOI: 10.1002/hlca.201000250

Oliveira, I., Valentão, P., Lopes, R., Andrade, P.B., Bento, A., Pereira, J.A., 2009: Phytochemical characterization and radical scavenging activity of *Portulaca oleraceae* L. leaves and stems. Microchem. J. 92, 129-134. DOI: 10.1016/j.microc.2009.02.006

Payet, B., Shum Cheong Sing, A., Smadja, J., 2006: Comparison of the concentrations of phenolic constituents in cane sugar manufacturing products with their antioxidant activities. J. Agricult. Food Chem. 54, 7270-7276. DOI: 10.1021/jf060808o

Peng, J.-B., Jia, H.-M., Liu, Y.-T., Zhang, H.-W., Dong, S., Zou, Z.-M., 2011: Qualitative and quantitative characterization of chemical constituents in Xin-Ke-Shu preparations by liquid chromatography coupled with a LTQ Orbitrap mass spectrometer. J. Pharmaceut. Biomed. Anal. 55, 984-995. DOI: 10.1016/j.jpba.2011.03.045

Puigventós, L., Navarro, M., Alechaga, É., Núñez, O., Saurina, J., Hernández-Cassou, S., Puignou, L., 2015: Determination of polyphenolic profiles by liquid chromatography-electrospray-tandem mass spectrometry for the authentication of fruit extracts. Anal. Bioanal. Chem. 407, 597-608. DOI: 10.1007/s00216-014-8298-2

Sicari, V., Loizzo, M.R., Tundis, R., Mincione, A., Pellicano, T.M., 2018: *Portulaca oleracea* L.(Purslane) extracts display antioxidant and hypoglycaemic effects. J. Appl. Bot. Food Qual. 91, 39-46. DOI: 10.5073/JABFQ.2018.091.006

Silva, R., 2011. Antioxidant properties and phenol content of *Portulaca oleracea* L. leaf, stems and flowers infusions: health benefits.

Sultana, A., Rahman, K., 2013: *Portulaca oleracea* L. A global Panacea with ethno-medicinal and pharmacological potential. Int. J. Pharm. Pharm. Sci. 5, 33-39.

Taha, H., Osman, A., 2015: Assessment of antioxidant capacity of ethanolic extract of *Portulaca oleracea* leaves in vitro and in vivo. J. Med. Plants Res. 9, 335-342. DOI: 10.5897/JMPR2014.5757

Tsao, R., 2010: Chemistry and biochemistry of dietary polyphenols. J Nutrients 2, 1231-1246. DOI: 10.3390/nu2121231

Uddin, M., Juraimi, A., Hossain, M., Anwar, F., Alam, M., 2012: Effect of salt stress of *Portulaca oleracea* on antioxidant properties and mineral compositions. Aus. J. Crop Sci. 6, 1732-1736. DOI: 10.1111/j.1744-7348.2008.00272.x

Uddin, M.K., Juraimi, A.S., Hossain, M.S., Nahar, M.A.U., Ali, M.E., Rahman, M., 2014: Purslane weed (*Portulaca oleracea*): a prospective plant source of nutrition, omega-3 fatty acid, and antioxidant attributes. Sci. World J. 2014.

Uddin, M.S., Hamja, M.A., 2014: Ultra high speed coherent optical communication using digital signal processing techniques along with advanced modulation system. 2014 International Conference on Electrical Engineering and Information & Communication Technology. 1-6. DOI: 10.1109/ICEEICT.2014.6919032

Wu, J., Lin, L., Chau, F.-T.J.U.S., 2001: Ultrasound-assisted extraction of ginseng saponins from ginseng roots and cultured ginseng cells. 8, 347-352. DOI: 10.1016/S1350-4177(01)00066-9

Xiang, L., Xing, D., Wang, W., Wang, R., Ding, Y., Du, L., 2005: Alkaloids from *Portulaca oleracea* L. Phytochem. 66, 2595-2601. DOI: 10.1016/j.phytochem.2005.08.011

Xing, J., Yang, Z., Lv, B., Xiang, L., 2008: Rapid screening for cyclo-dopa and diketopiperazine alkaloids in crude extracts of *Portulaca oleracea* L. using liquid chromatography/tandem mass spectrometry. Rapid Com. Mass Spec. 22, 1415-1422. DOI: 10.1002/rcm.3526

Xu, X., Yu, L., Chen, G., 2006: Determination of flavonoids in *Portulaca oleracea* L. by capillary electrophoresis with electrochemical detection. J. Pharm. Biomed. Anal. 41, 493-499. DOI: 10.1016/j.jpba.2006.01.013

Yan, J., Sun, L.-R., Zhou, Z.-Y., Chen, Y.-C., Zhang, W.-M., Dai, H.-F., Tan, J.-W., 2012: Homoisoflavonoids from the medicinal plant *Portulaca oleracea*. Phytochem. 80, 37-41. DOI: 10.1016/j.phytochem.2012.05.014

Yang, Z., Liu, C., Xiang, L., Zheng, Y., 2009: Phenolic alkaloids as a new class of antioxidants in *Portulaca oleracea*. Phytotherapy Res. 23, 1032-1035. DOI: 10.1002/ptr.2742

Yang, Z., Zhang, D., Ren, J., Yang, M., Li, S., 2012: Acetylcholinesterase inhibitory activity of the total alkaloid from traditional Chinese herbal medicine for treating Alzheimer's disease. Med. Chem. Res. 21, 734-738. DOI: 10.1007/s00044-011-9582-8

YouGuo, C., ZongJi, S., XiaoPing, C., 2009: Evaluation of free radicals scavenging and immunity-modulatory activities of Purslane polysaccharides. Int. J. Biol. Macromol. 45, 448-452. DOI: 10.1016/j.ijbiomac.2009.07.009

Zakaria, A., Hazha, J., 2013: Cytogenetic toxicity effects of local purslane (*Portulaca oleracea*) leaf crude extracts on normal and cancer cell lines *in vitro*. Int. J. Drug Discovery 5, 173. DOI: 10.9735/0975-4423.5.1.173-180

Zhao, Y., Chen, P., Lin, L., Harnly, J., Yu, L.L., Li, Z., 2011: Tentative identification, quantitation, and principal component analysis of green pu-erh, green, and white teas using UPLC/DAD/MS. Food Chem. 126, 1269-1277. DOI: 10.1016/j.foodchem.2010.11.055

Zheleva-Dimitrova, D.Z.J.P.M., 2013: Antioxidant and acetylcholinesterase inhibition properties of *Amorpha fruticosa* L. and *Phytolacca americana* L. Pharmacogn Mag. 9(34), 109-113.
DOI: 10.4103/0973-1296.111251

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