Polyphenols, Organic Acids and Antioxidant Activity in Unexplored *Phemeranthus Confertiflorus L*

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Some aspects of the chemical composition were studied in extracts of unexplored Phemeranthus confertiflorus L, an alien plant collected from Bucharest delta. 16 polyphenols and 10 short-chain organic acids were analysed and quantified by capillary electrophoresis in Phemeranthus extracts and antioxidant activity was investigated. The results obtained could promote Phemeranthus as an eligible plant with potential in food industry and for human health, because of the high antioxidant activity, high content of flavonoids (naringenin, rutin, daidzin) and lactic acid.

Keywords: polyphenols; antioxidant activity; short-chain organic acids; Phemeranthus confertiflorus; capillary electrophoresis.

Phemeranthus confertiflorus belongs to Montiaceae family and was reported as a new alien species to Europe [1]. It is native to North America and used as decorative in rock gardens. The *Phemeranthus* species are almost completely North American, with one exception for *P. punae* found in northern Argentina. The centre of diversity of this genus is northern Mexico and the south-western United States [2].

In Romania was recorded a single population of *Phemeranthus confertiflorus*, in Bucharest, on the north part of an area named Bucharest delta or Balta Vãcãresti, with about 175 individuals in 2013 (N44°24'16", E26°08'02") [1]. The species extended in the last years and is accompanied in its habitat mainly by *Portulaca pilosa* (fig. 1) and was collected from the arid, marginal area of the mentioned territory.

Long time *Phemeranthus* was considered as belonging to Portulacaceae family; the species of this family are more studied (especially *Portulaca oleracea* L), are considered dietary valuable and are used for consumption (in salads).

Fig. 1 *Phemeranthus confertiflorus* sorrounded by *Portulaca pilosa* in Bucharest delta

Phemeranthus confertiflorus is practically unstudied. There are only some papers, already mentioned, and others with brief considerations about occurrences of this species [3]. The importance of polyphenolic compounds is known and they are further studied as antioxidants with the capacity to protect cells and bio-macromolecules [4], as neutralizing free radicals and providing protection from oxidative degradation in some human diseases (cancer, inflammatory diseases, neurological degeneration, heart diseases, etc. [5]. The chemical study of organic acids, as part of metabolomics analysis, provides biochemical information on cellular functioning and the organic acids are also responsible for the taste, the flavour and the microbial stability of plants and their derived products [6].

This is the first study about chemical constituents of *Phemeranthus confertiflorus*. UV-Vis spectrometry and capillary zone electrophoresis (CZE) were used for chemical investigation and antioxidant activity. Polyphenols, antioxidant activity and short chain organic acids of different fractions extracted from *Phemeranthus* were assessed with the aim to explore the potential of this species for health and nutrition.

Experimental part

Materials and methods

Reagents

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid diammonium salt (ABTS) and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) were purchased from Sigma–Aldrich (Steinheim, Germany). The caffeic acid (Sigma, C0625), quercetin (Sigma, Q4951), kaempferol (BioChemika, 60010), rutin (Sigma, R5143), luteolin (Fluka, 72511), ferulic acid (Aldrich, 128708), chlorogenic acid (Aldrich, C3878), gallic acid (Fluka, 48630), izoquercitrin (Sigma–Aldrich, 00140585), sinapic acid (Sigma, D7927), daidzin (Sigma, 30408), p-coumaric acid (Fluka, 28200), syringic acid (Fluka, 86230), umbelliferone, naringenin (BioChemika,71155), and cinnamic acid (Fluka, 96340) stock solutions, 1 mg mL^{-1}, were prepared by dissolving in methanol the appropriate amount of substance. Sodium tetra borate and sodium phosphate were purchased from Sigma (Germany) and sodium dodecyl sulphate from Fluka (Switzerland).

Organic acids (CZE): all the reagents were of analytical reagent grade (purity > 98 %): D-lactic acid sodium salt from Fluka (Switzerland), L-(+)-tartaric acid, citric acid, oxalic acid, succinic acid, malic acid, acetic acid and butyric acid from Sigma–Aldrich (USA). Phosphoric acid 85 mass % was purchased from Merck (Germany), cetyltrimethylammonium bromide (CTAB) from LobaChemie (Austria).

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Ultra-pure water, 0.1M and 1M sodium hydroxide solutions were purchased from Agilent Technologies (Germany). Solvents (Merck, Germany) and solutions were filtered on 0.2 μm membranes (Millipore, Bedford, MA, USA) and degassed prior to use. Stock solutions for each standard were stored at +4°C. Working solutions were prepared daily by diluting the stock solutions in background electrolyte (BGE).

Samples preparation

The plant was collected in July 2015 from the so called Bucharest *delta* or Balta Vacaresti (http://www.themidlandhostel.com/the-uncommon-history-of-the-bucharest-delta-and-the-former-monastery-of-Vacaresti/); *Phemeranthus confertiflorus* (Greene) Hershkovitz (voucher ID BUC404604) was identified by biologist Petronela Comanescu, PhD from the Bucharest University, Faculty of Biology. The plants were dried up to 10% from initial weight in a Memmert oven with aeration and then were finely ground using a Grindomix GM200 grinder.

The extraction of polyphenols was performed by sonication at RT (the flask was immersed in ice to avoid overheating), over 1 hour, with water and a mixture of ethanol: water (30 vol. %, 50 vol. % and 70 vol. %) in 1: 10 ratio (g mL^{-1}). Next, the extracts were centrifuged for 10 min at 5000 rpm and the supernatants were collected, adjusted to 10 mL, filtered (0.2μm Millipore PTFE), and distributed for analyses of total contents of polyphenols and flavonoids (data not shown), polyphenols analysis by CZE and radicals scavenger activities.

For quantification of the organic acids from the herb collected, aqueous extracts were obtained (infusion and decoction). For infusions, 0.5 g of plant was mixed with 100 mL of hot distilled water (100°C) and allowed to infuse for 5 min; when the solution was cold it was filtered through a 0.2μm Millipore PTFE filter and injected undiluted into the instrument (CE). For decoction, a similar amount of the sample, 0.5 g of plant, was added to boiling water, boiled for 5 min, then left to cool; the volume was adjusted to 100 mL, filtered (0.2μm Millipore PTFE) and injected undiluted into the instrument.

Radical scavenger activity on DPPH and ABTS

The antioxidant activity of the extracts was assessed by measuring their scavenging abilities to DPPH stable radicals. The DPPH assay was performed after a method detailed previously [7,8]. The sample (0.1 mL) was mixed with 1 mL of 25 mM DPPH solution and adjusted to a final volume of 3 mL with methanol. The absorbance of the resulting solutions and the blank were recorded at 517 nm, after 30 min at RT. For each sample, three replicates were recorded. Inhibition of free radical by DPPH in percent (%) was calculated using the equation: $I = 100[(A_b - A_s)/A_b]$, where A_b is the absorbance of the control reaction mixture excluding the test compounds and A_s is the absorbance of the assessed compounds.

The 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS•+) scavenging activity was measured according to the method described by Erel (2004) [9], with some modifications. 2.5 mL of this ABTS•+ solution (ABTS radical cation was produced by reaction between ABTS stock solution 7mM in water and 2.45 mM potassium persulfate over 12h) was added to 0.1 mL of extract solution and 0.4 mL distilled water; the absorbance decrease was recorded for 6 min at 734 nm. Control sample contained 0.1 mL of methanol instead of extract sample. TEAC values were calculated using a calibration curve of Trolox: $y = 20327x - 0.035$, $R^2 = 0.997$ ($p < 0.0001$).

Polyphenols and short-chain organic acids separation by CZE

Capillary electrophoresis (CE) has proved a good alternative to HPLC for the investigation of different analytes in samples, with high efficiency and minimal sample pre-treatment [10]. The electrophoretic separation was carried out using an Agilent CE instrument (software ChemStation) with diode array detector (DAD) and CE standard bare fused-silica capillary (Agilent Technologies, Germany) with internal diameter of 50 μm and effective length of 72 cm for all the electrophoretic separations. Prior to use, the capillary was washed successively with basic solutions: 10 min with 1 M NaOH, 10 min with 0.1 M NaOH followed by ultra-pure water for 10 min and buffer for 20 min. The capillary was flushed between runs with 0.1M NaOH for 1 min, H_2O for 1 min and background electrolyte for 2 min. The electrolyte was refreshed after 3 consecutive runs. Sample injection was performed using the hydrodynamic mode (35 00 Pa, 12 s) while the capillary was maintained at a constant temperature of 30 °C.

The simultaneous separation of the polyphenols (polyphenolic acids and flavonoids) was obtained using 45 mM tetra borate buffer with 0.9 mM SDS (*p*H=9.35 adjusted with HCl 1 M) as background electrolyte[11]. BGE was filtered on 0.2 μm membranes (Millipore, PTFE, Bedford, MA, USA) and degassed prior to use. The applied voltage was 30 kV; direct UV absorption detection was carried out at 280 nm. The method was validated for selectivity, linearity, precision, accuracy (recovery), limit of detection and limit of quantification.

For the analysis of short-chain organic acids the CZE method selected belongs in the reversed polarity category with direct UV detection and is largely based on the method detailed by Galli and Barbas (2004) and modified by Gatea et al. (2015) [12,13]. The applied voltage was –25 kV and the best UV detection at 200 nm. Sample injection was performed using the hydrodynamic mode (3500 Pa, 12 s) while the capillary was maintained at a constant temperature of 25 °C. The background electrolyte finally used contained 0.5 M H_3PO_4, 0.5 mM CTAB (pH adjusted with NaOH to 6.24) and with 15 vol. % of methanol as organic modifier, filtered on 0.2 ìm membranes (Millipore PTFE, Bedford, MA, USA) and degassed prior to use. The order of elution of organic acids was formic, oxalic, succinic, malic, tartaric, acetic, citric, propionic, lactic and butyric acid and the analysis time of 17 min.

Statistical analysis

All the assays were carried out in triplicate in three different samples, at each level (dry plant, extracts, electrophoresis and cytotoxicity assays) and the results are expressed as mean values ± standard deviation (SD). The other statistical data were obtained with MaxStat version 3.60.

Results and discussions

Individual polyphenols content and antioxidant activity

The phenolics variation in plants are determined by various factors such as temperature, light, humidity, soil state, gathering period, maturity at harvest, chemical composition, the solvent used for the extraction, etc.[14,15]. Using our validated CZE method detailed described previously [11], 16 polyphenolic compounds were quantified, 8 flavonoids and 8 phenolic acids, and the results obtained are presented in Table 1. For identification of compounds the method of standard additions was used,

	aqueous extract	30% ethanolic extract	50% ethanolic extract	70% ethanolic extract
Polyphenols				
Daidzin	41.38±1.04	*546.76±4.16*	*394.17±5.20*	*430.58±5.20*
Rutin	22.11±0.19	*936.95±2.57*	*1862.86±3.22*	152.39±4.83
Naringenin	*948.79±5.86*	*3351.68±8.38*	*4503.13±6.28*	*4626.68±8.38*
Izoquercitrin	nd	nd	nd	916.76±4.98
Umbelliferone	nd	165.73±4.63	232.82±3.86	*362.19±1.93*
Cinnamic acid	*787.41±2.05*	*325.74±1.15*	*294.84±2.88*	*309.79±1.64*
Ferulic acid	34.24±2.31	180.18±0.51	127.63±1.28	*210.90±5.12*
Kaempferol	nd	nd	117.06±1.04	119.13±2.07
Luteolin	21.49±0.34	61.94±0.68	59.20±2.54	55.82±2.54
Coumaric acid	17.62±0.27	79.75±1.09	95.58±2.04	nd
Quercetin	181.00±1.61	118.91±1.43	*364.21±2.68*	166.09±0.89
Antioxidant activity				
IC_{50}mg g^{-1}DW	0.48±0.03	0.42±0.03	*0.29±0.01*	0.30±0.02
μM TEAC g^{-1}DW	22.31±0.19	22.44±0.41	*36.47±0.32*	33.62±0.41

Table 1
THE INDIVIDUAL POLYPHENOL CONTENT (μg g^{-1} DW**)** AND ANTIOXIDANT ACTIVITY IN *PHEMERANTHUS CONFERTIFLORUS*

comparing the migration time of polyphenolic compounds from samples with the migration times obtained for 16 standard polyphenols (fig. 2).

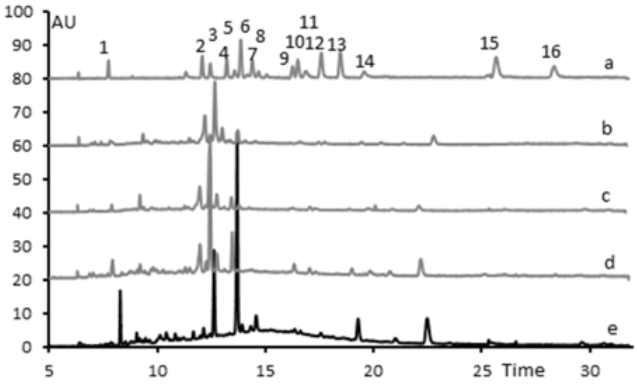

Fig. 2 Comparison of electrophoregrams obtained for polyphenol standards (a): 1- Daidzin; 2- Rutin; 3- Naringenin; 4- Izoquercitrin; 5- Umbelliferone; 6- Cinnamic acid; 7- Chlorogenic acid; 8-Sinapic acid; 9- Syringic acid; 10- Ferulic acid; 11- Kaempferol; 12- Luteolin; 13- Coumaric acid; 14- Quercetin; 15- Caffeic acid; 16- Gallic acid and different extracts of *Phemeranthus*: (b)-70% ethanolic extract diluted 5 time; (c) -50% ethanolic extract diluted 5 time; (d)- 30% ethanolic extract diluted 2 time; (e) – aqueous extract.

Looking at the results, the plant studied is rich in active compounds despite the fact that the soil contains little organic matter, provides by sediments deposited on the damp along the time [1]. In correlation with the results obtained for total polyphenols and flavonoids (data not shown), *Phemeranthus* 50% ethanolic extract indicated higher content of polyphenols, respectively high concentrations of rutin and naringenin (1862.86±3.22 and 4503.13±6.28 μg g^{-1}DW**)**, noticeable amounts of daidzin (394.17 ± 5.20 μg g^{-1} DW), quercetin (364.21 ± 2.68 μg g^{-1}DW), and cinnamic acid (294.84±2.88 μg g^{-1}DW), and low concentrations of umbeliferone, ferulic acid, kaempferol, coumaric acid and luteolin. The 70% ethanolic extract contains similar amounts of compounds with 50% ethanolic extract, with exception of rutin, which was found in low concentrations in 70% ethanolic extract. We can say that *Phemeranthus* is characterized by a *high level of flavonoids, especially in 50% ethanolic extract, correlated with a high antioxidant activity.*

Another observation is that naringenin seems to be the most predominant polyphenolic compound in *Phemeranthus*. Naringenin is known for antioxidant and anti-inflammatory activities, and for low toxicity, having potential as a therapeutic agent [16-18]. It is found especially in grapefruit and other citruses and is one of the mains flavonoids consumed by humans, being easily detected in the human serum after its intake [19]. The analgesic, anti-inflammatory and neuroprotective effects of naringenin were reported by different teams in models of neuropathic pain and neurodegenerative disorders [18, 20-22].

Recently, naringenin was found in various medicinal plants. Tang et al. (2016) [23] found low concentrations of naringenin in a species of *Mentha* from Australia, and some of our previous studies reported naringenin in noticeable amounts in 70% ethanolic extracts of *Mentha aquatica* (from the same habitat that is in discussion in this paper) and in ethanolic extract of *Calendula officinalis*, *Gallium verum* and *Origanum vulgare* from the Romanian market [11, 24].

Another preponderant flavonoid found in *Phemeranthus* extracts was rutin, which is commonly found in many foods and traditional medicines. Rutin has a wide range of pharmacological effects, including anti-radical, anti-inflammatory, antimicrobial, antiviral, anti-carcinogenic, and cytoprotective properties [25, 26].

Table 2

CONCENTRATIONS OF SHORT-CHAIN ORGANIC ACIDS IN AQUEOUS INFUSION (I) AND DECOCTION (D) OF *PHEMERANTHUS CONFERTIFLORUS* μg g^{-1} DW (PLANT)

Oxalic acid		Succinic acid	Tartaric acid	Citric acid	Lactic acid	Butyric acid
I	1191.91±4.8	15.85±0.05	918.38±3.22	1215.41±6.93	1392.05±8.80	522.08±8.13
D	1630.97±5.90	15.27±0.24	1151.44±11.15	1196.15±6.67	2344.26±6.99	562.45±8.13

Formic, malic, acetic and propionic acids were not detected

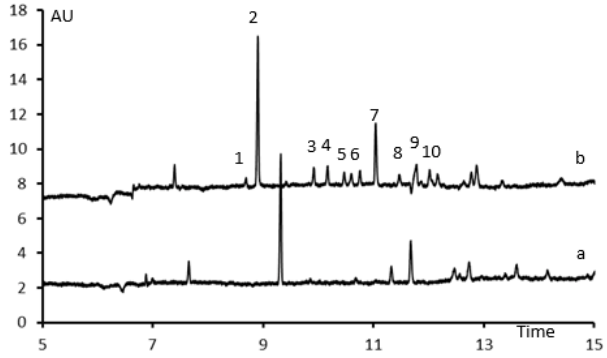

Fig. 3 Electrophoregrams of infusion sample obtained from *Phemeranthus confertiflorus* (a) and (b) the same sample spiked with 10μg mL^{-1} of 1-formic acid, 2-oxalic acid, 3-succinic acid, 4-malic acid, 5-tartaric acid, 6- acetic acid, 7-citric, acid 8-propionic acid, 9-lactic acid and 10-butyric acid

Short-chain organic acids content

Short-chain organic acids are important compounds in plants, because they influence some characteristics (taste, stability) of the plant extracts, and their metabolism is closely related to a controlling adaptation to the environment [6].

The standard addition was used for the identification of organic acids, owing to the presence of other components in the infusions and decoctions of plant samples; their migration times were compared with the migration times obtained for 10 organic acids standards.

The results obtained from the CZE analysis of aqueous samples, infusion and decoction are presented in table 2; figure 3 shows the electropherograms for an undiluted *Phemeranthus confertiflorus* decoction and for the same sample spiked with a known concentration of each analysed organic acid.

As it can see in table 2, formic acid, malic, acetic and propionic were not found. Succinic acid was perceived in very low amounts, butyric acid in medium concentrations (522.08±8.13 to 562.45±8.13 μg g^{-1}) and oxalic, tartaric, citric and lactic acids were found in higher concentrations (between 1151.44±11.15 μg g^{-1} for tartaric acid and 2344.26±6.99 μg g^{-1} for lactic acid in decoctions).

Our previous studies about organic acids in plants reported different levels and different organic acids in medicinal teas from the market or in invasive plants from Bucharest *delta* [27,28]. As is reported by Frankowski (2016) [29], the organic anions are not found in the soils, and it is difficult to clearly explain the variability of organic anions in different organs of the plants, and the variability in different plants. Organic acids are involved as intermediate or end products in different fundamental pathways in plant metabolism and catabolism. We can notice in the case of *Phemeranthus*, the absence of malic acid (a ubiquitous compound in plants) and *the high levels of lactic acid* (important in food and pharmaceutical industries as natural conserving, acidulate and flavour enhancer) [30] and tartaric acids, both unreported or in low concentrations, usually .

Conclusions

The study of the chemical compounds in *Phemeranthus* evidenced the presence of phenolic acids (cinnamic and ferulic acids) and flavonoids (naringenin, rutin and quercetin) which are correlated with high antioxidant activity of plant aqueous and ethanolic extracts. We can emphasize that *naringenin* is major phenolic compound in *Phemeranhus.* Comparing the results obtained on *Phemeranthus* with others data previously obtained in the same habitat on others plants (see Teodor et al., 2015) we can say that the content of *Phemeranthus* in phenolics and the antioxidant activity is higher. After this first study, *Phemeranthus confertiflorus* could be appreciated as favourable for pharmacological properties determined by antioxidant activity, which is associated with its diverse chemical constituents, including phenolic acids, flavonoids, and organic acids. Nevertheless, more mechanistic studies are required before *Phemeranthus confertiflorus* can be considered for further pharmacological use.

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