Phytochemical Screening: Antioxidant and Antibacterial Properties of Potamogeton Species in Order to Obtain Valuable Feed Additives

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Abstract: The alcoholic extracts from three submerged perennial plants *Potamogeton crispus* L., *P. pusillus* L. and *P. pectinatus* L. were analyzed by gas chromatography-mass spectrometry coupled with solid phase microextraction (SPME-GC/MS) and by High Performance Liquid Chromatography (HPLC) and their volatile fingerprint and polyphenols composition was mutually compared. Twenty-nine chemical compounds were detected and identified in ethanolic and methanolic extracts; the highest abundance (over 5%) in descending order, was detected for 9,9-dimethyl-8,10-dioxapentacyclo (5,3,0(2,5)0(3,5,)0 (3,6) decane (21.65%), phenol 2,6 bis (1,1 dimethylethyl) 4-1-methylpropil (20.8%), pentadecanoic acid (14.3%), 2-(5-chloro-2-Methoxyphenyl) pyrrole (8.66%), propandioic (malonic) acid 2-(4-methylphenyl) sulfonyl ethylidene (5.77%), 2 hydroxy-3 tert butyl-5-isopropyl-6 methyl phenyl ketone (5.76%). The highest total polyphenols and flavonoids content was found in the methanolic extract of *P. crispus* (112.5±0.5 mg tannic acid/g dry extract; 64.2±1.2 mg quercitin/g dry extract). Antioxidant activities (2,2-difenil-1-picrilhidrazil, hydrogen peroxide and reducing power assays) of obtained extracts are comparable with the standard compounds, butylated hydroxytoluene, rutin and ascorbic acid. Antibacterial efficiency of methanolic extracts was notably demonstrated against Gram negative (*Escherichia coli, Enterobacter hormaechei*) and Gram positive bacteria (*Enterococcus casseliflavus*). The data reported for the first time for Romanian *Potamogeton* species, provides extensive support for the chemical investigations of these plants of the aquatic anthropogene ecosystems in order to obtain valuable bioadditives for animal feed and/or pharmaceutical/food industry.

Key words: bioadditives, *P. crispus*, *P. pusillus*, *P. pectinatus*

1 INTRODUCTION

Terrestrial and aquatic plants are a valuable source of bioadditives that influence forage quality required in feed. The Romanian flora has approximately twenty aquatic plant species from the *Potamogeton* (Potamogetonaceae) genus with both floating and submerged foliage which spreads widely in freshwater lakes, ponds and rivers, along the Danube Delta and across the country up to the mountain zones. Several phytochemical studies on the importance and potential use of extracts obtained from *Potamogeton* species, were published. Some authors have isolated and identified the flavonoid pigments from *Potamogeton perfoliatus* L. and *P. richardsonii* and others brought up new information on some flavonoids never reported in *Potamogeton* genus, such as luteolin 3′-O-glucoside, a chrysoeriol glucuronide and isomers of apigenin 7-O-glucoside and others isolated from *P. crispus* with antioxidative effects and previous investigations on different species of *Potamogeton* have indicated the presence of alkaloids. An acyclic sesquiterpene glycoside (crenulatoside A) found in *P. crispus* demonstrated antioxidant activity. Furthermore, the
methanolic extracts of cultured plants (*Potamogeton pectinatus*) were efficient against gram-positive bacteria belonging to the *Micrococcus, Staphylococcus, Streptococcus, Bacillus, Aerococcus, Mycobacterium and Corynebacterium* genera and against some gram-negative bacteria belonging to the *Vibrio, Listonella and Pasteurella* genera. Many researchers have reported the macrophytic role of *Potamogeton* genus as biofilters for the aquatic ecosystems as scavenger of heavy metals, phthalic acid esters, and the potential phytoremediation activity of the *P. crispus*. *P. pusillus* has the ability to accumulate substantial amounts of Cu and Cr from aqueous solutions. Some chemicals (labdanes) from *P. pectinatus* play an ecological function as allelochemicals and also fenols of these type of aquatic plants have been reported as algicidal.

*P. crispus* is an important primary producer in freshwater ecosystems, providing good foulder source for herbivorous fishes and poultry. Moreover, Wang et al. found that ducks fed with *P. crispus* could lay natural red-yolk eggs with good quality. Chemical analytical study and feeding trials proved carotenoids in the red-yolk eggs mainly derived from *P. crispus*.

However, the research regarding the volatile compounds analysis and the antioxidant and antibacterial potential of extracts of *Potamogeton* species is not fully investigated. In our research we describe for the first time the chemical investigation of three submersed herbaceous perennial Romanian plants, harvested from aquatic anthropogene ecosystems: *Potamogeton crispus* L., *P. pusillus* L. and *P. pectinatus* L. In addition, this study aims to determinate antioxidant and antibacterial properties in order to obtain valuable bioadditives for animal feed and/or Pharmaceutical/Food Industry.

### 2 EXPERIMENTAL

#### 2.1 Collection and preparation of plants

*P. crispus* (curly-leaf pondweed), *P. pusillus* (small pondweed) and *P. pectinatus* (sago pondweed) were collected in aquatic anthropogene ecosystems (45° 16′ 457″ N; 028° 17′ 098″ E; altitude 21 m, of Dobrogea-Romania), in May-June 2014. Voucher specimens (*P. crispus* no. 145042; *P. pusillus* no. 145043; *P. pectinatus* no. 145044) of the plant material were taxonomically identified and are deposited at the Herbarium-Botanic Garden of Galati Natural Sciences Museum Complex. Harvesting plant materials were washed free of debris by tap water to remove epiphytes, sand, shells and later by deionized water, and dried at 25°C in darkness (15 days). The powder samples were kept in freezer at −20°C for future application use.

#### 2.2 Extract preparation

The extraction was made with ethanol (70%) and methanol (95%) under ultrasounds. Briefly, 10 g of powdered biomass were mixed with 100 mL solvent and sonicated for two hours at 50°C with Bandelin (Sonorex Digi-tech) device. Further, all the extracts were filtered through Whatman No. 1 filter paper and concentrated under vacuum (Buchi R215, heating bath B-491, rotation 280 rpm, vacuum controller V-850 of 290 mbar) at 50°C ± 1°C. The residues were dried to constant weights and stored in the dark at 4°C to prevent degradations until use. For HPLC analyses, the chlorophyll was removed.

#### 2.3 Chemicals

All the solvents were of chromatographic grade. Standards, tannic acid (T), quercitin (Q), butylated hydroxytoluene (BHT), ascorbic acid (AA), rutin (RUT) and galic acid (G) were purchased from Sigma-Aldrich. Stock working solutions of the standards were stored in darkness at −18°C. DPPH (2,2-difenil-1-picrilhidrazil), trolox (6 hidroxi-2,5,7,8-tetrametilcromat-2-acid carboxilic), TCA (tricloracetic acid), TBA (tiobarbituric acid) and (K3Fe(CN)6) were purchased from Merck.

#### 2.4 SPME-GC/MS analyses

An amount of 250 mg of sample (extract) was transferred to a glass vial (10 mL), to which 200 mg saturated (NH4)2 SO4 was added; 0.5 mL of 2-octanol was added as an internal standard. The vials were tightly closed (screw-caps with PTFE/silicone septum seal) and homogenized, after which they were placed in the autosampler. All the volatile compounds (VOC) were analyzed on a Trace GC-MS Ultra, ITQ 900 from Thermo. The GC was coupled to a mass selective detector (MS) with an ion trap (Thermo). The method of VOCs analysis with a SPME fibre type allowed automated incubation in a thermostat and control of the extraction time and temperature on GC and MS. This method was optimized beforehand for fingerprinting a wide range of vegetal origin volatiles. Volatile compounds were monitored according to the modified protocol of Qin et al. In a first step, each vial was equilibrated in the incubator at 50°C for 25 min under agitation. Next, the plant volatile compounds in the headspace of the vial were sampled for 10 min by means of a solid phase microextraction (SPME) fiber with CAR/PDMS 50/30 μm carboxen/polydimethylsiloxane (CAR/PDMS). Each fiber was preconditioned according to the manufacturer’s guidelines before its first use. After headspace extraction, the fiber was transferred to the GC injection port, where the adsorbed compounds were thermally desorbed for 2 min at 200°C. After each run, the fiber was thermally cleaned for 20 min at 220°C in the conditioning station of the autosampler. The volatiles were injected in splitless mode and subsequently separated on a TG-WAX capillary column (60 m × 0.25 mm i.d., 0.25 μm), using...
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helium as carrier gas at a constant flow rate of 1 mL/min. The column oven was programmed at a starting temperature of 50°C, which was set for 1 min, after which a temperature ramp was programmed from 50-230°C with rate 10°C/min and holding at 230°C for 10 min. Afterwards, the oven was cooled again to the initial temperature. The temperature of the transfer line in MS was set to 270°C. Mass spectra were obtained by electron ionisation (EI) at 200 eV, with a scanning range of 50 to 650 m/z. The volatile compounds were identified comparing the mass spectra with the information provided by the Wiley and Nist08 library database available with Xcalibur software provided. The linear retention index (LRI) of the components was determined with Kovats equation with an alkane C₆-C₄₀ series.

2.5 HPLC assay
The HPLC data was recorded using a HPLC Thermo Scientific system, using a C18 BDS column (150 × 4.6 mm², 5 µm), and a gradient mobile phase with formic acid/methanol/water.

2.6 Quantification of total polyphenols and flavonoids content
Total polyphenolic content (TPC) was measured using the Folin-Ciocalteu assay and T as standard
d. Results were expressed as T equivalents based on dry mass. The total flavonoids content were estimated according to Florea et al.28. Q and T were used as reference compounds to obtain the standard curve and the results were expressed as mg of polyphenol equivalents (Q or T)/g dry mass.

2.7 DPPH radical scavenging activity
The scavenging capacity assay was evaluated according to the modified methods of Bozin et al.29 and Ganesan et al.30 using the stable DPPH. The samples (1.0 mL of pondweed extracts, dilution 1:100) were mixed with 1 mL of 90 µM DPPH and were brought with MeOH at final volume of 4 mL. At the same time blank test was performed. The solutions were maintained for 1 hour at room temperature and the absorbance were recorded at 517 nm. Each test was performed three times. AA, BHT and RUT were used as standards. The radical scavenging capacity (RSC) was calculated as a percentage of DPPH discoloration using the equation:

\[ \text{RSC}(\%) = 100 \times \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \]

where \(A_{\text{blank}}\) and \(A_{\text{sample}}\) are the absorbance values of the blank sample and of the test sample respectively, after 30 min.

2.7.1 Reducing power assay
The reducing power of the extracts was evaluated according to methods by Athukorala et al.31. 1 mL of the different concentrations of various extracts was mixed with 2.5 mL potassium ferricyanide and 2.5 mL phosphate buffer (pH 6.6). The mixture was incubated at 50°C for 20 minutes. After the incubation, 2.5 mL of TCA (10%) was added and centrifuged at 3000 rpm for 10 minutes. 2.5 mL of the supernatant was taken then, 2.5 mL water and 0.5 mL of ferric chloride (0.1%) were added. The absorbance of the solution was measured at 700 nm. The increasing of the absorbance indicated the increasing of reducing power. Each test was performed in triplicate and BHT, RUT and AA were used as standards solutions (0.01% in suitable solvents).

2.8 Hydrogen peroxide scavenging capacity assay
The hydrogen peroxide (H₂O₂) scavenging capacity of the pondweed extracts was estimated by the method of Ruch et al.26. A solution of H₂O₂ was prepared in phosphate buffer (pH 7.4). To 3.4 mL pondweed extracts (dilution 1:100) 0.6 mL of phosphate buffer was added. The absorbance value of the reaction mixture was recorded at 230 nm. Three replicates were made for each sample in comparisons with the same standard solutions (BHT, RUT, AA). The percentage of H₂O₂ scavenging capacity was calculated as:

\[ \% \text{ scavenged } \text{H}_2\text{O}_2 = \left( \frac{A_0 - A_1}{A_0} \right) \times 100 \]

where \(A_0\) is the absorbancy of the control and \(A_1\) is the absorbancy in the presence of the sample or standards.

2.9 Determination of antibacterial properties
All strains were obtained from the Clinic Laboratory of Faculty of Medicine and Pharmacy, Dunărea de Jos University Galați. Antibacterial activity tests were carried out by Kirby-Bauer testing with disk diffusion assay described by Brauer et al.32 employing the Gram negative bacteria Enterobacter hormaechei ATCC 700323, Pseudomonas aeruginosa ATCC 17934, Escherichia coli ATCC 4350 and the Gram positive ones Streptococcus pyogenes ATCC 19615, Staphylococcus aureus ATCC 25923, Enterococcus casseliflavus ATCC 12755. The strains were cultured in nutrient agar and tryptic soy agar following the suggestions given by ATCC protocols. The standard antibiotics CN-10 ciprofloxacin, TE-20 tetracycline, E-15 erythromycin and P-10 penicillin were used to control the sensitivity of the tested bacteria. Sterilized paper disks having a 6 mm diameter were impregnated with 40 µL of each extract and placed on the surface of previously inoculated petri dishes. The antibacterial assay plates were incubated at 37°C for 48 h. The diameters of the inhibition zones were measured in mm including the thickness of the disc and compared to the blank, under the same standardized conditions. The experiments were performed in triplicate.

2.10 Statistical analysis
All data are expressed as means ± SD of triplicate mea-
3 RESULTS AND DISCUSSION

3.1 Volatile compounds analyses by a SPME-GC/MS

The volatile fingerprint of *P. crispus*, *P. pusillus* and *P. pectinatus* from the aquatic ecosystems of Dobrogea County, Romania in both methanolic and ethanolic extracts showed a wide range of compounds. The qualitative analysis based on the difference in volatility of each individual component and the comparison of the mass spectra with the library database (Wiley and NIST) resulted in identification of different structures distributed in six chemical classes: heterocyclic compounds, acids, alcohols, phenols, ketones and hydrocarbons. In total 29 chemical components were present in both extracts and their retention time (RT), linear retention index (LRI), chemical formula and peak area are listed in Table 1. The percentage of the volatiles that significantly contributed to the total compounds profile extracted in methanol (52.37% *P. crispus*, 42.8% *P. pusillus* and 42.48% *P. pectinatus*) was less abundant and less diverse compared to extracts (74.21% *P. pectinatus*, 61.3% *P. crispus*, 53.55% *P. pusillus*) in ethanol. The major constituents of the volatile fingerprint with a relative peak area higher than 5% were: 9,9-dimethyl-8,10-dioxa-pentacyclododecane (26; 21.65%) - present only in the ethanolic extracts; phenol 2,6-bis(1,1-dimethylethyl) 4-1-methylpropil (20; 20.8%) - present in all extracts; pentadecanoic acid (12; 14.3%); 2-(5-chloro-2-methoxyphenyl) pyrrole (8.66%) - present only in the ethanolic extracts; propanoic acid (malonic) acid 2-(4-methylphenyl) sulfonyl ethylidene (9; 5.77%) - present in all extracts and 2-hydroxy-3-tertbutyl-5-isopropyl-6-methyl phenyl ketone (25; 5.76%) - present only in the ethanolic extracts (Table 1). Particular relevance should be also given to the presence of minor, but not negligible amount, compounds detected in the extracts such as N-phthaloyl-4-aminobenzoic acid (10; 1.46%), pentanoic acid, 10-undecenyl ester (11; 1.21%), phosphoric acid (14; 1.71%), linoleic acid (15; 1.22%).

The presence of N-phthaloyl-4-aminobenzoic acid (10) is associated with folate synthesis in plants and PABA (para amino benzoic acid) derivatives in *Potamogeton* could be the result of chemical contamination. Moreover, the presence of pentadecanoic acid (12) in high concentration in the ethanolic extracts of *P. pectinatus* and in low concentration in the rest could be linked with an increased metabolic activity in *P. pectinatus* caused by fungi presence. The total volatile chromatographic profiles of the analyzed *Potamogeton* species was similar, and many constituents were common to all the investigated species, i.e. the peaks of compounds 2, 7, 16, 20-21, 25, 29 of the EtOH extracts (Table 1) and the peaks of compounds 5-6, 8-10, 17-18, 20-22, 24, 28 of the MeOH extracts (Table 2). It can be noticed that at 20.76 RT (20), the fingerprint of *P. pusillus* displayed a tall peak identified as phenol (2,6-bis (1 1-dimethylethyl) 4-1-methyl propyl). The concentration displayed by this phenolic compound had the following distribution in descending order: *pusillus* > *pectinatus* > *crispus* in the plants. This compound is present in all the *Potamogeton* species studied, in all the extracts (ethanolic and methanolic), and have a calculated LRI of 1269. An even distribution in concentration of the phenolic compound 2-(3,7-dimethyloct-2,6-dienyl) in all *P. crispus*, *P. pusillus* and *P. pectinatus* extracts is noticed at LRI value of 1404 (21). The same phenolic compound was identified by other authors in the chloroform extract of flower plants detected by GC-MS without silylation. The profile chromatograms of the ethanolic extracts (Table 1) showed that seven volatile compounds are the most abundant ones of the total components. The phenolic compound (20) has the highest yield with a peak area of 20.8% in *P. pusillus* species. The profile chromatograms of the methanolic extracts (Table 1) showed that from the total volatile compounds, phenols are the most abundant ones (20; 22). The phenolic compound 2 tert butyl-4-isopropyl 5 methyl phenol (22) had the highest yield with a peak area of 12.06% in *P. crispus* species. The analysis of the extracts’ fingerprint showed that the phenolic compounds had the highest yield in comparison with other chemical classes. In accordance with these results other authors highlighted, as well, high concentration of polyphenols in aquatic plants from *Myriophyllum* genus. The presence of phenols was reported also in *Potamogeton malaianus* aqueous extracts, together with the benzoic acid. In this research the presence of benzoic acid 2,6 bis (trimethylsilyl) oxy (8) was identified, though, only in the methanolic extracts for all 3 species analyzed.

Some phenolic compounds are extremely widespread while others are specific to certain plant families or found only in certain plant organs or at certain development
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Table 1 Phytochemical analyses (SPME-GC/MS) of ethanolic and methanolic extracts.

| Chemical classes | No. compounds/ chemical formula | P. crispus | P. pusillus | P. pectinatus |
|------------------|---------------------------------|-----------|------------|--------------|
|                  | EIOH PA RT LRI | MeOH PA RT LRI | EIOH PA RT LRI | MeOH PA RT LRI |
| Phenols          |                  |            |            |              |
| 1/ C₆H₄(3,4,5)   | 5.76* 21.50 1290 | – – – – | – – – – | – – – – |
| 2/ C₆H₄(3,4,5)   | 3.67 24.99 1397 | – – – – | – – – – | – – – – |
| 3/ C₆H₄(3,4,5)   | 3.67 24.99 1397 | – – – – | – – – – | – – – – |
| 4/ C₆H₄(3,4,5)   | 0.04 23.00 1362 | – – – – | – – – – | – – – – |
| 5/ C₆H₄(3,4,5)   | 1.36 12.27 988 | – – – – | – – – – | – – – – |
| 6/ C₆H₄(3,4,5)   | 1.36 12.27 988 | – – – – | – – – – | – – – – |
| 7/ C₆H₄(3,4,5)   | 0.04 23.00 1362 | – – – – | – – – – | – – – – |
| Ketones          |                  |            |            |              |
| 1/ C₆H₄(3,4,5)   | 1.36 12.27 988 | – – – – | – – – – | – – – – |
| 2/ C₆H₄(3,4,5)   | 1.36 12.27 988 | – – – – | – – – – | – – – – |
| 3/ C₆H₄(3,4,5)   | 1.36 12.27 988 | – – – – | – – – – | – – – – |
| 4/ C₆H₄(3,4,5)   | 0.04 23.00 1362 | – – – – | – – – – | – – – – |
| 5/ C₆H₄(3,4,5)   | 1.36 12.27 988 | – – – – | – – – – | – – – – |
| 6/ C₆H₄(3,4,5)   | 1.36 12.27 988 | – – – – | – – – – | – – – – |
| 7/ C₆H₄(3,4,5)   | 0.04 23.00 1362 | – – – – | – – – – | – – – – |
| Hydrocarbons     |                  |            |            |              |
| 1/ C₆H₄(3,4,5)   | 1.36 12.27 988 | – – – – | – – – – | – – – – |
| 2/ C₆H₄(3,4,5)   | 1.36 12.27 988 | – – – – | – – – – | – – – – |
| 3/ C₆H₄(3,4,5)   | 1.36 12.27 988 | – – – – | – – – – | – – – – |
| 4/ C₆H₄(3,4,5)   | 0.04 23.00 1362 | – – – – | – – – – | – – – – |
| 5/ C₆H₄(3,4,5)   | 1.36 12.27 988 | – – – – | – – – – | – – – – |
| 6/ C₆H₄(3,4,5)   | 1.36 12.27 988 | – – – – | – – – – | – – – – |
| 7/ C₆H₄(3,4,5)   | 0.04 23.00 1362 | – – – – | – – – – | – – – – |

Legend: 1/2-(1-chloro-1-methoxyphenyl) Pyridine; 2/ Ethyl (4-chloro methyl) 2-2 diphenyl-3 oxazoline 5 carbosylate; 3/ Methyl-3H- 1-acenaphthen-1,2-(1)-pyrrole-7 carboxylic Acid; 4/3 dimethylphosphate; 5/ Acetamide, 2-(4 morpholyl) N-(4,5,6,7-tetrahydro-2-cyano-benzothiene-3-yl); 6 Benzothiophene - 2 carbosyl acid, 4,5,6,7-tetrahydro-7-hydroximino-3H-[2(4)-morphyl] (toxothioborin); 7/14 hydroxy-3,5-di - tert-butylpheny) 2 methyl-3-morpholinophenol-1 one; 8 Benzoic Acid 2,6 bis (trimethylsilyl)oxy; 9 Propanedioic (malonic) Acid2-(4 methylphenyl) sulfonyl ethylidene; 10 N (Phthalonyl-4-aminobenzoic Acid; 11 Pentosinic Acid, 10 undecyloxyl ester; 12 Pentadecanoid Acid; 13 Hexadecanolide (adipic Acid); 14 Phosphoric Acid; 15 Hexadecanol; 16 Hexadecanolide; 17 1-Hexadecanol; 18 1-Hexadecanolide; 19 1-Hexadecanolide; 20 (2-hydroxy-3 tert butyl-5 isopropyl-6 methyl phenyl ketone; 21 Phenol, 2-(4 methyl-2,6-dimethoxyphenol); 22 Tert butyl-4-isopropyl 5 methyl phenol; 23 Benzene-1,2,3-triol; 24 2-(3,7-dimethylocta-2,6 diene); 25 Benzene-1,2,3-triol; 26 2-(3,7-dimethylocta-2,6 diene); 27 2-(3,7-dimethylocta-2,6 diene); 28 2-(3,7-dimethylocta-2,6 diene); 29 2-(3,7-dimethylocta-2,6 diene); 29 2-(3,7-dimethylocta-2,6 diene). 

* volatile compound non detectable in the peak areas; peak area (PA) %; retention time (RT); linear retention index (LR)
erocyclic compounds also have an important contribution to the total variation. *P. pusillus* is associated with high heterocyclic compounds, while for *P. pectinatus* acids are explaining the variation in EtOH extracts. For the MeOH extracts (Fig. 2) the phenols content is explaining the variation in *P. crispus*. However, the total variation explained by PC2 is relatively small compared to heterocyclic compounds that are explained by PC1. In this case the highest content was registered for *P. pectinatus*. The PCA analysis for EtOH and MeOH extracts showed differences and while for EtOH, *P. pusillus* has the highest influence on PC1, in the case of MeOH, *P. pusillus* and *P. pectinatus* are similar and placed in the same quadrant, with a strong influence on PC1. In the case of EtOH each *Potamogeton* species is placed in a different quadrant and has its own specific characteristics.

Following SPME-GC/MS analysis we found that *P. crispus*, *P. pusillus* and *P. pectinatus* harvested from Romania are sources of volatile compounds, the phenolic compounds proved to be in a significant yield and higher in comparison with other chemical compounds. The presence of these molecules could also help to verify specific biologic activities important from health and pharmaceutical point of view. The phenolic compounds are an important indicator in the characterization of volatile compounds and in the possibility of showing both antioxidant and antibacterial activity.

### 3.2 Determination the Polyphenols content (qualitative and quantitative)

The presence of polyphenols in alcoholic extracts of *Potamogeton* sp. is shown through HPLC analysis in Figs. 3 and 4. The qualitative screening of the extracts showed the presence of important polyphenols such as T and flavonoids such as Q and RUT. From the graphs it can be observed that the chromatographic profile of the three extracts show slight differences depending on the solvent, methanolic extracts being richest in these compounds (Figs. 3 and 4). Also, it can be seen that only the ethanolic extracts (*P. crispus*, *P. pusillus*) have a small quantity of RUT (68.15 min) in their mixture (Fig. 3). Instead, all extracts contain a good quantity of Q (77.60 min) and also a small quantity of T (75.43 min). The presence of these compounds may be an argument for obtaining a satisfactory antioxidant activity for all the extracts. The quantitative evaluations of these polyphenols were determined by the TPC and flavonoids spectrophotometric methods (Table 2). The best results showed the *P. crispus* extracts. So, the TPC of the *P. crispus* extracts showed a significant variation ranging from 94.6 ± 1.2 (ethanolic extract) to 112.5 ± 0.5 mg T/g dry extract (methanolic extract) followed by the extract of *P. pectinatus* (99.8 ± 0.2 and 100.1 ± 0.4 mg T/g dry extract). The methanolic extracts contain higher amounts of TPC. The flavonoids content of the *P. crispus* varies between 63.0 ± 0.4 (ethanolic extract) and 64.2 ± 1.2
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Our results (Figs. 3 and 4; Table 2) are fully in agreement with the literature data. Durdevic et al.\textsuperscript{8} reported 47.96 ± 0.10 mg RUT/g dry extract of flavonoids and 22.74 ± 0.12 mg G/g dry extract (of Potamogeton nodosus). Other authors reported for some aquatic plants: 56.5 mg T/g dry mass\textsuperscript{40} of polyphenols (Miriophillum spicatum extract); 3.20 g G/100 g extract of polyphenols (Pistia stratiotes extract) and not detected flavonoids\textsuperscript{41}. The differences may be due to the solvents used and/or to a low extraction efficiency.

Fig. 3 The HPLC chromatographic profiles of ethanolic extracts of P. crispus, P. pusillus and P. pectinatus.

Fig. 4 The HPLC chromatographic profiles of methanolic extracts of P. crispus, P. pusillus and P. pectinatus.
yield. Polyphenol compounds are reducing agents, free radical scavengers and quenchers of singlet oxygen. Flavonoids play important roles in the control of human diseases, reducing the risk of cardiovascular disease or as antioxidants. Due to their importance in plants and human health, it would be useful to know the concentration of the polyphenolic compounds. The results of the present study suggest that methanolic extracts of P. crispus, P. pusillus and P. pectinatus may be considered an important source of polyphenols.

3.3 Antioxidant activity

The extracts of Potamogeton genus were processed through DPPH assay, in order to evaluate the antioxidant properties of the plant’s phytocomplex. The results expressed by percentage of DPPH scavenging are shown in Fig. 5 and varies thus: 45% to 46.8% for P. crispus, 45.2% to 45.58% for P. pusillus and 29.63% for P. pectinatus. The hydrogen peroxide scavenging activity was studied and results are presented in Fig. 6. The scavenging capacity varies with the different sample. Our results show that the percentage inhibition capacity ranged from 72% to 76.19% (P. crispus), 78% to 78.06% (P. pusillus) and 68.7% to 70.36% (P. pectinatus). The reducing power assay gave good results for Potamogeton extracts with values: 0.193 to 0.21 (Fig. 7).

The chemical complexity of volatile compounds, expressed as quality and abundance of compounds and chemical properties involved a used, in particular. The antioxidant activity (DPPH assay) was measured in comparison with three standards BHT, AA and RUT (Fig. 5). Thus, we can observe significant differences (p < 0.05) between standards and our samples. However, among those 3 analyzed samples, it was proved that the P. crispus and P. pu-

Table 2 Content of total polyphenols and flavonoids in the extracts.

| Extracts          | TPC (mgT/g dry extract) | Flavonoids (mgQ/g dry extract) |
|-------------------|-------------------------|---------------------------------|
|                   | P. crispus | P. pusillus | P. pectinatus | P. crispus | P. pusillus | P. pectinatus |
| methanolic        | 112.5 ± 0.5 | 98.0 ± 0.9 | 100.1 ± 0.4 | 64.2 ± 1.2 | 58.4 ± 0.8 | 60.1 ± 1.4 |
| ethanolic         | 94.6 ± 1.2 | 98.2 ± 0.4 | 99.8 ± 0.2 | 63.0 ± 0.4 | 58.9 ± 0.2 | 54.6 ± 0.1 |

Each value was obtained by calculating the average of three analyses ± SD.

Fig. 5 Antioxidant activity (results of DPPH assay). Standards: BHT, RUT, AA. All the data are in triplicate and the p values of <0.05 were considered significant.

Fig. 6 Antioxidant activity (results of H₂O₂ assay). Standards: BHT, RUT, AA. All the data are in triplicate and the p values of <0.05 were considered significant.

Fig. 7 Antioxidant activity (reducing power assay). Standards: BHT, RUT, AA. All the data are in triplicate and the p values of <0.05 were considered significant.
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sillus extracts have a higher antioxidant activity in comparison with the P. pectinatus extracts. Furthermore, methanolic extracts from P. pectinatus have significant risen values as against the ethanolic ones. Our data are fully in agreement with the other researchers who proved the antioxidative efficiency of the ethanol extract of the Potamogeton nodosus. Different aquatic plants extracts showed similar antioxidant effect being able to reduce the stable radical DPPH with 40.4% for Ranunculus rionii; over 40% for Chaetomorpha antennina and over 49.94% for Trapa natans.

The phenolic compounds present in the analyzed plant extracts are a class of antioxidants that occur in nature. Similar results were obtained by Nagavani et al. who proved that there are more antioxidant volatile components and act both at the beginning and during the propagation of oxidative processes. The antioxidant activity of methanolic and ethanolic extracts can be attributed to the fact that there are more antioxidant volatile components present in these extracts which could react rapidly with DPPH radicals and reduce most of DPPH radical molecules.

The highest values for P. pusillus extract mean a high inhibition capacity to of the hydrogen peroxide. The results (Fig. 6) are comparable with the used standards. The ability to reduce Fe(III) may be attributed to hydrogen donation from phenolic compound which is also related to the presence of reducing agents. On the other hand, Kartal et al. got similar results from other aquatic plants, such Ceratophyllum demersum and Ranunculus rionii.

3.4 Antibacterial activity

Antibacterial activity of Potamogeton extracts was analyzed as inhibition zones and results were related to that expressed by positive control ciprofloxacin, tetracycline, erythromycin and penicillin known as synthetic antibiotics (Table 3). Antibacterial activity of the plant extracts showed inhibitory activities against some Gram negative bacteria and Gram positive bacteria with significant differences according to the extraction solvent. The extracts showed antimicrobial activities with a significantly differences (p < 0.05). Methanolic extract of P. crispus (0.22 ± 0.001 mg TPC/50 μL/disk) exhibited a clear inhibition zones against Enterococcus casseliflavus with values within the range of 25.0 ± 0.5 mm. Both extracts of P. pectinatus (0.20 ± 0.00 mg TPC/50 μL/disk and 0.19 ± 0.00 mg TPC/50 μL/)

Table 3 Antibacterial activity expressed as zones of inhibition (mm) against pathogens.

| Microorganism                | Plant extracts |
|------------------------------|----------------|
|                              | P. crispus   | P. pusillus | P. pectinatus |
|                              | EtOH | MeOH | EtOH | MeOH | EtOH | MeOH |
| **Gram negative bacteria**   |      |      |      |      |      |      |
| Enterobacter hormaechei      | 15.0 ± 0.0 | 31.0 ± 0.1 | 18.0 ± 0.0 | 20.0 ± 0.2 | 18.0 ± 0.5 | 19.0 ± 0.4 | 9.0 ± 0.0 | 9.0 ± 0.0 |
| (ATCC 700323)                |      |      |      |      |      |      |      |      |
| Pseudomonas aeruginosa       | 20.0 ± 0.0 | 18.0 ± 0.0 | 13.0 ± 0.0 | 13.0 ± 0.0 | 14.0 ± 0.0 | 15.0 ± 0.0 | 13.0 ± 0.4 | 13.0 ± 0.0 |
| (ATCC 17934)                 |      |      |      |      |      |      |      |      |
| Escherichia coli             | 16.0 ± 0.0 | 32.0 ± 0.3 | 19.0 ± 0.6 | 21.0 ± 0.0 | 14.0 ± 0.0 | 17.0 ± 0.0 | 9.0 ± 0.0 | 12.0 ± 0.0 |
| (ATCC 4350)                 |      |      |      |      |      |      |      |      |
| **Gram positive bacteria**   |      |      |      |      |      |      |
| Streptococcus pyogenes       | 16.0 ± 0.1 | 18.0 ± 0.2 | 19.0 ± 0.2 | 10.0 ± 0.0 | 16.0 ± 0.0 | 16.0 ± 0.0 | 15.0 ± 0.1 | 14.0 ± 0.0 |
| (ATCC 19615)                |      |      |      |      |      |      |      |      |
| Staphylococcus aureus        | 17.0 ± 0.0 | 25.0 ± 0.2 | 17.0 ± 0.0 | 18.0 ± 0.3 | 13.0 ± 0.3 | 11.0 ± 0.0 | 8.0 ± 0.0 | 9.0 ± 0.0 |
| (ATCC 25923)                |      |      |      |      |      |      |      |      |
| Enterococcus casseliflavus   | 20.0 ± 0.0 | 21.0 ± 0.4 | 22.0 ± 0.1 | 25.0 ± 0.5 | 15.0 ± 0.0 | 20.0 ± 0.0 | 25.0 ± 0.2 | 26.0 ± 1.0 |
| (ATCC 12755)                |      |      |      |      |      |      |      |      |

Standards: CN-10 ciprofloxacin (10 μg/disk); TE-30 tetracycline (30 μg/disk); E-15 erythromycin (15 μg/disk); P-10 penicillin (10 μg/disk). Mean value ± SD, n=3 (determinations). Extract mass 0.5 mg/50 μL disk. Each value was obtained by calculating the average of three analyses ± SD.
Ceratophyllum genus such as which are and. Some researchers reported the S. aureus and Nymphaea lotus P. Pistia stratiotes have methanolic extract of P. crispus (in comparison with standard CN-10) and methanolic extract of P. pusillus (in comparison with standard CN-10). However, curiously the P. crispus methanolic extract has a decreased antibacterial activity particularly for Streptococcus pyogenes, in comparison with all the other analyzed extracts. These results are fully in agreement with the literature data. Durdevic et al. obtained favorable results regarding the antibacterial activity against Pseudomonas aeruginosa of some plants from the Potamogeton genus, harvested in the western Serbia. Other authors proved the antagonistic effect exercised by the aqueous, methanolic and ethanolic extracts of some aquatic plants (P. crispus, P. pectinatus, Ceratophyllum demersum, Eichhornia crassipes) harvested at the southern coast of lake Manzalah (Egypt), against many bacterial microorganisms such as Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and also against fungus such as Aspergillus niger and Fusarium oxysporum. These authors reported inhibition zones of 11.0 ± 0.00 mm (methanolic extract) versus 14.0 ± 0.00 mm (ethanolic extract) against E. coli. for P. crispus. The differences compared with our data (Table 3) may be due to the applied method or to a low extracted yield. In addition, the results of Daboor and Haroon suggested that the studied aquatic extract plants possess some natural compounds that could be used as antimicrobial agents. The same authors notify also the absence of the antimicrobial activity of Pistia stratiotes and Nymphaea lotus against P. aeruginosa and S. aureus. Some researchers reported the low antioxidant activity of many aquatic plants, among which are Potamogeton genus such as Ceratophyllum demersum with RSC of 22.6% or Chaetomorpha anten- nina with the percentage of inhibition capacity ranged from 38% to 63%. Kumar et al. reported lowest antibacterial activity (7 mm) for Trapa natans against E. coli and S. aureus; Abu Ziada et al. reported, also, low antibacterial activity from methanolic extract of Myriophyllum spicatum against E. coli (17 mm) and S. aureus (13 mm).

We described here, for the first time, a phytochemical screening (methanolic versus ethanolic extract) of Potamogeton genus, harvested from aquatic anthropogene ecosystems (Romania). P. pusillus highlights as new source of biocompounds especially with antioxidant properties.

The ability of vegetal extracts to inhibit bacterial growth varies significantly, predominantly depending on the analyzed Potamogeton species and the extract type (methanolic or ethanolic) but also on the sensibility of the bacterial species. Our results emphasize an antibacterial activity much stronger in the case of methanolic extracts and an moderate antibacterial activity in the case of ethanolic extracts. Some hydroalcoholic extracts are capable of inhibiting the spreading of the pathogenic germs, even in the case of antibiotics resistant forms. A variety of microorganisms lead food into spoilage that is encountered as one of the most important matter concerning the food industry and many pathogenic microorganisms (e.g. Escherichia coli, Staphylococcus aureus) have been reported as the causative agents of diseases and/or food spoilage. Therefore, our studies offer useful information regarding antibacterial activity of the obtained methanolic extracts from P. crispus, P. pusillus and P. pectinatus which might represent an alternative for the conventional antimicrobial additives.

4 CONCLUSIONS

The volatile compounds of the three Potamogeton sp. are very similar but can be distinguished by differences in phenolic profiles and antioxidant and antibacterial properties. We were noted three major phenolic compounds representing 32.03% (methanolic extract of P. crispus) from total compounds. By the qualitative screening (HPLC) of the extracts, we found the presence of important polyphenols, such as tannic acid and flavonoids: quercetin and rutin. The highest content of total polyphenols and flavonoids was detected in methanolic extract of P. crispus (112.5 ± 0.5 mg T/g dry extract; 64.2 ± 1.2 mg Q/g dry extract). The antioxidant activity evaluated using the hydrogen peroxide and reducing power assays indicated that Potamogeton genus methanolic extracts was the most powerful antioxidant from all the studied samples and correlated with the TPC. The antibacterial tests of methanolic extracts revealed an important activity against Gram negative (Escherichia coli, Enterobacter hormaechei) and Gram positive (Enterococcus casseliflavus) bacteria. This research, first realized in Romania with these three species of aquatic plants, demonstrate that the methanolic extracts of P. pusillus, P. pectinatus and mainly P. crispus could be considered as potential alternatives for synthetic bactericides and natural sources of antioxidants for use in the food industry (bioadditives), for animal feed or in the pharmaceutical industry.

ACKNOWLEDGMENT

The authors are grateful for the financial support granted through the SOPHRD/159/1.5/S/138963-, Sustainable performance in doctoral and post doctoral research-PERFORM” and thank the specialists of the Botanic Garden of the Natural Sciences Complex Museum of Galati.
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