INTRODUCTION

Polygonum hydropiper L. (Persicaria hydropiper, water pepper) is one of the most numerous genuses in the family Polygonaceae, which has a long history of use in folk medicine as a anti-cancer and anti-rheumatic agent, against diarrhoea, positively influencing cerebral ischemia, Parkinson’s disease, and with neuroprotective effects. The aim of the current study was to evaluate and compare the total polyphenol content and antioxidant activity of water (infusion, decoction and microwave) and alcohol (tincture) extracts of Polygonum hydropiper. The total flavonoid content, total monomeric anthocyanin content and detailed phenolic acids profile were additionally assessed. The polyphenol content was established to be in range from 5.02 ± 0.07 to 17.44 ± 0.24 mg GAE/g dw, the total flavonoids from 3766.27 ± 118.25 to 6909.66 ± 66.03 µM QE/g dw and total monomeric anthocyanins from 0.26 ± 0.14 to 3.17 ± 0.44 mg/l. The in vitro antioxidant activity was evaluated by four common procedures, the highest results were established for the decoction, and tincture extracts. The phenolic acids profile revealed the highest yield, in respect of total phenolic acids, in the water pepper tincture. As a result, the consumption of P. hydropiper extracts could be recommended as a good source of biologically active substances with potential benefit effects.

Keywords: Polygonum hydropiper, antioxidant potential, water extracts, tincture, phenolic acids profile

MATERIAL AND METHODS

Extraction preparation

The samples of Polygonum hydropiper L. (stems of water pepper) were obtained from a local pharmacy (Plovdiv, Bulgaria) in 2016. The plant parts were dried, ground and stored at ambient temperature in air-tight containers prior to extraction. Four extraction procedures were performed as follows: water (infusion, decoction, microwave extraction) and 70 % ethanol (v/v, tincture). Two extraction solvents (water and 70 % ethanol) were used by applying the extraction methods - infusion, decoction, microwave extraction, and tincture in particular.

The infusion was obtained by pouring 50 mL boiling water into 2.5 g of plant material and left for 30 min to cool down. Water decoction was retrieved by boiling 2.5 g of plant material in 50 mL water for 30 min; Microwave-assisted extract (MAE) experiment was carried out in a domestic microwave oven (LG MB4047C) where 2.5 g of plant material was subjected to 2450 MHz frequency waves for 30 seconds, at 800W output power with 50 mL water.

The tincture was obtained with ethanol (70 %, v/v) with periodic manual agitation for a period of seven days, left in the dark, at room temperature. The material/solvent ratio used was 1:10 (v/w). All extracts were filtered after preparation and stored at 4 °C without adding any preservatives until analyses.
The TPC was analyzed following the method of Kujala et al. (2000) with some modifications. Each extract (0.1 ml) was mixed with 0.5 ml Folin-Ciocalteu reagent and 0.4 ml 7.5% NaCO₃. The mixture was vortexed and left for 5 min at 50 °C. After incubation, the absorbance was measured at 765 nm. The TPC was expressed as mg gallic acid equivalents (GAE) per g dry weight (dw).

### Total flavonoid content

The total flavonoid content was evaluated according to the method described by Kivrac et al. (2009). An aliquot of 0.5 ml of the sample was added to 0.1 ml of 10 % Al(NO₃)₃, 0.1 ml of 1M CH₃COOK and 3.8 ml of ethanol. After incubation at room temperature for 40 min, the absorbance was measured at 415 nm. Quercetin was used as a standard and the results were expressed as µg QE/g dw.

### Total monomeric anthocyanin content

The total monomeric anthocyanin content was determined using the pH-differential method (AOAC Official Method 2005.02). Properly diluted samples were mixed with KCl (0.025 M, pH 1.0) and CH₃COONa (0.4 M, pH 4.5) with an appropriate dilution factor. Absorbance (A) was measured using UV-Vis spectrophotometer at 520 and 700 nm after 15 min incubation at room temperature, and the results were calculated as follows:

$$ A_{520} = (A_{520} - A_{700}) \times \text{pH} 1.0 - (A_{520} - A_{700}) \times \text{pH} 4.5 $$

The monomeric anthocyanin (MA) pigment concentration in the samples was calculated as:

Monomeric anthocyanin pigment (mg/liter) = \((A \times MW \times DF \times 1000)/ (\varepsilon \times 1)\)

where M represents the molar mass of cyanidin-3-glycoside (449.2 g/M), DF is the dilution factor, ε is molar extinction coefficient (28,900 I/M cm, and 1 is the cuvette optical path length (10 mm). The final anthocyanin concentration is expressed as milligram per 1000 ml of sample of cyanidin-3-glycoside.

### Determination of antioxidant activity

#### DPPH radical scavenging assay

The radical scavenging activity of the extracts against 2,2´-diphenyl-1-picrylhydrazyl (DPPH) radical was determined by the slightly modified method of Brand-Williams et al. (1995) as described by Mihaylova et al. (2015). Freshly prepared 0.4 mM solution of DPPH was mixed with the samples in a ratio of 2:0.5 (v/v). The light absorption was measured at 517 nm after 30 min incubation at room temperature. The DPPH radical scavenging activity was presented as a function of the concentration of Trolox having equivalent antioxidant activity expressed as the μM TE/g dw.

#### ABTS⁺ radical scavenging assay

The radical scavenging activity of the extracts against 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS⁺) was estimated according to Re et al. (1999). Briefly, ABTS radical cation (ABTS⁺) was produced by reacting ABTS stock solution (7 mM) with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. Afterward, the ABTS⁺ solution was diluted with ethanol to an absorbance of 0.7 ± 0.2 at 734 nm and equilibrated at 30 °C. After the addition of 1.0 ml of diluted ABTS⁺ solution to 0.01 ml of samples, the absorbance reading was taken at 30 °C after 6 min. The results were expressed as TEAC value (μM TE/g dw).

#### Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to the procedure of Benzie and Strain (1999) with slight modification. The FRAP reagent was prepared fresh daily and was warmed to 37°C prior to use. One-hundred and fifty microliters of plant extracts were allowed to react with 2850 μl of the FRAP reagent for 4 min at 37°C. The absorbance was recorded at 593 nm and the results were expressed as µM TE/g dw.

### Cupric ion reducing antioxidant capacity (CUPRAC) assay

The CUPRAC assay was carried out according to the procedure of Apak et al. (2004). One ml of CuCl₂ solution (10 mM) was mixed with 1 ml of neocuproine methanolic solution (0.0075 mM), 1 ml NH₄Ac buffer solution (pH 7.0), and 0.1 ml of herbal extract (sample) followed by addition of 1 ml water (total volume = 4.1 ml) and mixed well. Absorbance against a reagent blank was measured at 450 nm after 30 min. Trolox was used as a standard and the results were expressed as µM TE/g dw.

### Identification and quantification of phenolic acids

Qualitative and quantitative determination of phenolic acids was performed by using Elite LaChrome (Hitachi) HPLC system equipped with DAD and ELITE LaChrome (Hitachi) software. Separation of the phenolic acids was performed on Supelco Discovery HS C18 column (5 μm, 25 cm × 4.6 mm), operated at 30 °C under gradient conditions with mobile phase consist of 2 % (v/v) acetic acid (solvent A) and acetonitrile (solvent B) as reported by Terzieva et al. (2017). The gradient program used was: 0.1 min – 95 % A and 5 % B; 1–40 min: 50 % A and 50 % B; 40–45 min: 100 % B; 46-50 min: 95 % A and 5 % B. The detection of phenolic acids was carried out at 280 nm for gallic, protocatechuic and cinnamic acids and at 320 nm for chlorogenic, caffeic, ferulic, p-coumaric, sinapic, rosmarinic and chioconic acids. The flow rate was 0.8 ml/min.

### RESULTS AND DISCUSSION

Table 1 is a visual presentation of the results in respect of the total phenolic content, total flavonoid content and total monomeric anthocyanins occurring in the investigated extracts. The established total phenolic content varied between 5.02 ± 0.07 and 17.44 ± 0.24 mg GAE/g DW. The maximum polyphenolic extraction yield (17.44 ± 0.24 mg GAE/g dw) was obtained in the tincture extract, The same tendency was observed regarding the total flavonoid content (3766.27 ± 118.25 - 6909.66 ± 66.03 μM QE/g DW) and total monomeric anthocyanins, where the presence of a high concentration of cyanidins in the infusion and microwave extracts was not even detected. In comparison, Nurain et al. (2006) reported TFC of water pepper water and ethanol extracts – 424.17 ± 36.67 and 589.33 ± 12.83 μg QE/g extract and Zulfiker et al. (2011) detected TPC of 964.230 mg GAE/g dw for 97 % methanol extract.

According to the results from the phytochemical profile of P. hydropiper the tincture possesses higher activity among all investigated extracts. This is possibly due to the use of ethanol as solvent. Polar solvents are frequently used for recovering polyphenolic compounds from plant matrices. The most suitable solvents are aqueous mixtures containing ethanol, methanol, acetone, and ethyl acetate. The higher the polarity, better the solubility of the compounds (Načk and Shahidi, 2006). Ethanol is known as a good polyphenol extraction solvent and safe for human consumption. The addition of water to the ethanol improves the extraction rate. Among the water extracts decoction appeared as the most suitable technique. Water is a polar solvent, with a density, viscosity, and activity, which can impair solubility, transfer or contact with the matrix. Furthermore, water has benefits as a green extraction solvent because it is not only inexpensive and environmentally benign; but is also non-flammable, nontoxic, providing opportunities for clean processing and pollution prevention. Finally yet importantly, water is easy accessible by consumers. A higher temperature of extraction can increase solvent-matrix interaction too.

### Table 1 Total phenolic content (mg GAE/g DW), total flavonoid content (µM QE/g DW) and total monomeric anthocyanins content (µg/ml) of P. hydropiper (water pepper) extracts

<table>
<thead>
<tr>
<th>Extraction technique</th>
<th>Total polyphenolic content</th>
<th>Total flavonoid content</th>
<th>Total monomeric anthocyanins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infusion</td>
<td>5.02 ± 0.07</td>
<td>3766.27 ± 118.25</td>
<td>-</td>
</tr>
<tr>
<td>Decoction</td>
<td>7.58 ± 0.21</td>
<td>6385.35 ± 728.05</td>
<td>0.26 ± 0.14</td>
</tr>
<tr>
<td>Microwave</td>
<td>5.84 ± 0.07</td>
<td>2050.58 ± 100.23</td>
<td>-</td>
</tr>
<tr>
<td>Tincture</td>
<td>17.44 ± 0.24</td>
<td>6909.66 ± 66.03</td>
<td>3.17 ± 0.44</td>
</tr>
</tbody>
</table>

**-** not established

The antioxidant potential of the studied P. hydropiper extracts was evaluated by four different in vitro assays (DPPH and ABTS antiradical activities; FRAP and CUPRAC methods) in order to venerate the many authors’ recommendation for using several methods for plant antioxidant activity assessment (Schlies et al., 2002). The results are presented in Table 2. The values ranged from 50.94 ± 0.28 to 282.81 ± 3.16 µM TE/g DW. The highest results in respect of the antiradical activity (DPPH and ABTS assays) were established in the decoction - 115.49 ± 0.37 and 229.73 ± 7.41 µM TE/g DW. These results were in contrary to the highest values for the tincture in respect of FRAP and CUPRAC assays (Fe³⁺ and Cu²⁺ reducing activity) revealing the higher ability of the decoction to scavenge...
Table 2 In vitro antioxidant activity of P. hydropiper (water pepper) extracts (μM TE/g DW)

<table>
<thead>
<tr>
<th>Extraction technique</th>
<th>ABTS</th>
<th>DPPH</th>
<th>FRAP</th>
<th>CUPRAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infusion</td>
<td>69.69 ± 1.50</td>
<td>50.94 ± 0.28</td>
<td>84.32 ± 0.44</td>
<td>86.16 ± 0.95</td>
</tr>
<tr>
<td>Decoction</td>
<td>229.73 ± 7.41</td>
<td>115.49 ± 0.37</td>
<td>147.43 ± 3.12</td>
<td>205.71 ± 0.01</td>
</tr>
<tr>
<td>Microwave</td>
<td>118.76 ± 1.81</td>
<td>91.33 ± 1.18</td>
<td>118.16 ± 0.79</td>
<td>135.69 ± 3.82</td>
</tr>
<tr>
<td>Tincture</td>
<td>111.67 ± 0.91</td>
<td>100.19 ± 1.11</td>
<td>162.73 ± 1.3</td>
<td>282.81 ± 3.16</td>
</tr>
</tbody>
</table>

Table 3 Phenolic acids composition of P. hydropiper (water pepper) extracts (μg/g dw)

<table>
<thead>
<tr>
<th>Extraction technique</th>
<th>gallic acid</th>
<th>protocatechuic acid</th>
<th>chlorogenic acid</th>
<th>caffeic acid</th>
<th>p-coumaric acid</th>
<th>sinapic acid</th>
<th>rosmarinic acid</th>
<th>cichoric acid</th>
<th>cinnamic acid</th>
<th>total phenolic acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infusion</td>
<td>1056.84</td>
<td>68.97</td>
<td>435.91</td>
<td>33.27</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1745.77</td>
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<tr>
<td>Decoction</td>
<td>688.89</td>
<td>145.32</td>
<td>477.91</td>
<td>38.12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1208.36</td>
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<tr>
<td>Microwave</td>
<td>907.13</td>
<td>62.94</td>
<td>412.68</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1201.53</td>
</tr>
<tr>
<td>Tincture</td>
<td>148.48</td>
<td>1478.26</td>
<td>1503.53</td>
<td>56.52</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1333.74</td>
</tr>
</tbody>
</table>

CONCLUSION

The present study aimed at evaluating different extraction techniques in order to draw biologically active substances of P. hydropiper (water pepper). In this regard, four procedures were conducted and the phytochemical results obtained revealed water pepper as a good source of polyphenolic compounds, especially flavonoids and phenolic acids. The investigated samples consisted of bioactive compounds in moderate amounts, which could be possible due to the influence of the extraction process itself. The reported data provide valuable information about the most suitable extraction approach of P. hydropiper as a source of beneficial health effects.

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