Chemical composition and antioxidant activity of ultrasound-assisted extract of the endemic plant *Haberlea rhodopensis* Friv.

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Abstract The antioxidant capacity of extracts from leaves of *Haberlea rhodopensis* Friv. obtained by a highly efficient and simple method of the ultrasound-assisted extraction was investigated. The total polyphenolic content was determined spectrophotometrically using the Folin–Ciocalteu’s phenol reagent and the polyphenols composition was analyzed by HPLC method. Antioxidant activity of the extract was evaluated as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, 2,2-azinobis-3 ethyl benzothiazoline-6-sulphonic acid (ABTS) cation decolorization activity assays and the ferric reducing/antioxidant power method (FRAP). The total phenolics of the extract were determined to be 148.71 ± 5.33 mg GAE on a dry weight basis. The HPLC analysis showed a large number of flavonoids and phenolic acids present in the ultrasound ethanol extract of *H. rhodopensis*. Results also revealed that the major flavonoids were luteolin ($2730.18 \mu\text{g/g DW}$), hesperidin ($928.56 \mu\text{g/g DW}$) and kaempferol ($578.52 \mu\text{g/g DW}$), whereas the most abundant phenolic acids identified in the extract were ferulic acid ($630.48 \mu\text{g/g DW}$) and sinapic acid ($580.80 \mu\text{g/g DW}$). DPPH[•] and ABTS^{•+} values were 0.803 ± 0.007 and 0.769 ± 0.040 mmol TE/g DW, respectively and FRAP values were 1.362 ± 0.05 mmol TE/g DW.

Keywords *Haberlea rhodopensis* · Radical scavenging activity · RP-HPLC · Ultrasound extraction

Introduction

Haberlea rhodopensis Friv. belongs to the family Gesneriaceae and it is a Balkan endemic relict widely distributed mainly in the Rhodope Mountains, some regions of the Sredna gora Mountains and the Balkan. The plant appertains to the so called “resurrection plants” due to its ability to fall in anabiosis for a long time when there are unfavorable conditions and to restore back to normal when the conditions become appropriate. Despite the fact that several reliable methods were applied to study the antioxidant activity (Berkov et al. 2011; Mihaylova et al. 2013) of different extracts, *Haberlea rhodopensis* is still less explored and the chemistry of the family Gesneriaceae is poorly known. Different phytochemical studies indicated that *H. rhodopensis* contains flavonoids, tannins and polysaccharides (Radev et al. 2009), in addition to lipids (Stefanov et al. 1992) and saccharides (Müller et al. 1997). Baloutzov et al. (2009) found carbohydrates, flavonoids, tannins, phytosterols, glycosides, saponins and slimes in all organs (leaf, flower and root) of *H. rhodopensis* Friv., whereas Berkov et al. (2011) identified five free phenolic acids by gas chromatography–mass spectrometry with syringic acid being most prevalent of all. In another study (Mihaylova et al. 2013), luteolin, hesperidin, sinapic acid and ferulic acid were found to be the most prevailing compounds in the heat reflux 70 % alcohol extract of *H. rhodopensis*.

There is a need of looking for new sources of substances, which, accessible from daily food, could also influence unfavorable aging processes in the human body. Therefore, it seems to be a good aim to conduct investigations in isolation and characterization of biologically active components of plant origin, safe for people and showing high antioxidant activity. Different methods have been used for the extraction of various plants, however, the general disadvantages of all these methods are the potential losses due to oxidation, hydrolysis, and ionization during extraction and long extraction

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times (Li et al. 2005). Many natural products are thermally unstable and may degrade during thermal extraction. The ideal extraction methods could obtain the maximum of the bioactive constituents in a shortest processing time with a low cost (Wang et al. 2011). Ultrasound-assisted extraction (UAE) allows the solvent to penetrate into the cell walls. In addition, this method also prevents the possible chemical degradation of targeted compounds (Wang and Weller 2006). As long as this method has been applied in the extraction of many chemical constituents from different plant materials (Li et al. 2005; Ma et al. 2008; Dong et al. 2010), it has been evaluated as an alternative to the conventional extraction techniques being highly efficient, solvent and time reducing method, requiring low energy (Wang and Weller 2006).

The objective of the present study was to obtain for the first time extracts from *H. rhodopensis* using alternative ultrasound-assisted technique and thus to extend knowledge of this less known endemic plant. The presented phenolic constituents were established and the potential antioxidant properties of the extracts were also evaluated.

Materials and methods

Plant material and chemicals

Haberlea rhodopensis Friv. was collected from its natural habitat at Plovdiv region, Bulgaria. The plant leaves were air-dried at room temperature (25–28 °C), roughly grounded and stored in air-tight dark containers until extracted.

6-Hydroxy-2,3,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin-Ciocalteu's phenol reagent, gallic acid, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,4,6-Tripyridyl-S-triazine (TPTZ) and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were purchased from Sigma-Aldrich (Steinheim, Germany). Rosemary extract (RE) was supplied from Danisco, Denmark. All other chemicals and solvents used were of analytical grade and obtained from local producers.

Methods

Preparation of plant extract

Grounded leaves of *Haberlea rhodopensis* (0.5 g) were suspended in 70 % ethanol (ratio of raw material to solvent 1:60) and the UAE was carried out in an ultrasonic cleaning bath (UST 5.7–150, Rated power 240 W, temperature 50 °C, Siel, Bulgaria) for 30 min. After filtering, the obtained extract was stored at 4 °C without adding any preservatives.

HPLC analysis

The HPLC analysis of phenolic acids and flavonoids were performed by a Waters HPLC system, (Milford, MA, USA) equipped with binary pump (Waters 11525), a UV-VIS detector (Waters 2487) and Breeze 3.30 SPA software. Detailed conditions of HPLC analyses are reported previously (Marchev et al. 2011). Concentration of each individual compound was calculated based on external standard method and was converted to µg/g DW.

Total phenolic content (TPC)

The total polyphenol content was analyzed using the Folin-Ciocalteu method of Kujala et al. (2000) with some modifications. Each sample (1 ml) was mixed with 5 ml of Folin-Ciocalteu phenol reagent and 4 ml of 7.5 % Na₂CO₃. The mixture was vortexed well and left for 5 min at 50 °C. After incubation, the absorbance was measured at 765 nm. The TPC in the extracts was expressed as mg gallic acid equivalent (GAE) per g dry weight (DW).

DPPH radical scavenging activity

The ability of the extracts to donate an electron and scavenge DPPH radical was determined by the slightly modified method of Brand-Williams et al. (1995). Freshly prepared 4 × 10⁻⁴ mol methanolic 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. The DPPH radical scavenging activity was presented as a function of the concentration of Trolox - Trolox equivalent antioxidant capacity (TEAC) and was defined as the concentration of Trolox having equivalent AOA expressed as the mmol Trolox per g DW.

ABTS radical scavenging assay

The radicals scavenging activity of the ultrasound extract against radical cation (ABTS^{•+}) was estimated according to a previously reported procedure with some modifications (Re et al. 1999). The results were expressed as TEAC value (mmol TE/g DW).

Ferric-reducing antioxidant power assay (FRAP)

The FRAP assay was carried out according to the procedure of Benzie and Strain (1999). FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe (II)-tripyridyltriazine compound from colorless oxidized Fe (III) form by the action of electron donating antioxidants. The results were expressed as mmol TE/g DW.

Statistical analysis

The presented results are average from two independent experiments carried out in triplicates. The results were expressed as mean±SD and statistically analyzed using MS-Excel software.

Results and discussion

HPLC analysis

The ultrasound-assisted extraction as a novel extraction method is a promising way for plant extracts obtaining. Therefore, the identification and the quantification of the phenolic constituents present in the studied extract were of interest, in order to establish their influence over the extract properties.

The conducted RP-HPLC analysis showed that a large number of flavonoids and phenolic acids were presented in the ultrasound ethanol extract of *H. rhodopensis* (Table 1). In total 11 phenolic acids and eight flavonoids were identified. The most abundant phenolic acids were ferulic (630.48 µg/g DW)

and sinapic (580.80 µg/g DW) acids as the main representatives. Different amounts of ferulic and sinapic acids were also identified in alcohol extract obtained by traditional reflux technique (Mihaylova et al. 2013). This confirmed that with 70 % ethanol as solvent and applying regardless UAE or traditional refluxing the obtained results are comparable and the extracted substances are the same. In comparison Berkov et al. (2011) identified in methanol extracts from in vitro *H. rhodopensis* by GC-MS syringic acid as dominant.

The predominant flavonoids in the investigated sample were luteolin (2730.18 µg/g DW) and hesperidin (928.56 µg/g DW), as they were the most abundant constituents of the studied extract of all (Table 1). Unidentified compounds with characteristic spectra of phenolic acids and were also established.

The extraction properties could be clearly demonstrated by the HPLC results. As a confirmation, Mihaylova et al. (2013) established luteolin and hesperidin as predominant constituent in the *H. rhodopensis* 70 % alcohol extract obtained by reflux extraction as well.

The obtained results show that these particular substances were extracted with the solvent applied according both ultrasound-assisted and heat reflux methods. Their presence confirmed their role in the plant and can contribute to the potential antioxidant properties of the examined plant extract.

Table 1 Polyphenols content in ultrasound extract from *H. rhodopensis*

Polyphenols	µg/g DW
Phenolic acids	
Gallic acid	33.06
3,4-Dihydroxybenzoic acid	17.52
2-Hydroxybenzoic acid	100.92
Chlorogenic acid	64.26
Vanillic acid	179.82
Caffeic acid	398.4
Syringic acid	87.6
p-Coumaric acid	238.08
Sinapic acid	580.8
Ferulic acid	630.48
Cinnamic acid	40.2
Flavonoids	
Flavonols	
Myricetin	338.64
Kaemferol	578.52
Quercetine	225.48
Quercetine glycosides	
Rutin	309.42
Hyperoside	138.06
Flavanone glycosides	
Hesperidin	928.56
Flavones	
Apigenin	42.0
Luteolin	2730.18

Total phenolic content

The total phenolic content in *H. rhodopensis* leaves extract was established to be 148.71±5.33 mg GAE/g DW. Compared to previously reported total phenolic content values (151.24±10.35 mg GAE/g DW) for 70 % ethanol extract obtained by reflux extraction the amounts were similar (Mihaylova et al. 2013). Phenols and polyphenolic compounds, such as flavonoids, are widely found in many food products derived from plant sources, and they have been shown to possess significant antioxidant activities (Van Acker et al. 1996). Furthermore, phenols have been determined to play an important role for the survival of the plants under extreme conditions (Müller et al. 1997). Consequently the presence of those compounds in the studied extracts suggests their important role in the plant and might contribute toward its antioxidant activities.

Table 2 Antioxidant activity of ultrasound extract from *H. rhodopensis* and RE, mmol TE/g DW

Sample	TEAC _{DPPH} [*]	TEAC _{ABTS} ⁺⁺	FRAP
<i>H. rhodopensis</i> ^a	0.80±0.01	0.77±0.04	1.36±0.05
Rosemary extract ^b	1.75±0.07	1.01±0.20	1.51±0.01

^a Ultrasound extract

^b Commercial product

The method has some limitations; namely, the Folin-Ciocalteu's reagent can react with other non-phenolic reducing compounds such as ascorbic acid, glutathione, and etc. and can lead to overvaluation of the phenolic compounds. In samples rich in phenolics this is generally low relevance interference. On the other hand these compounds participate to the antioxidant potential of the samples. Moreover, several studies have successfully correlated the phenolic content with antioxidant activity (Tepe et al. 2006; Mihaylova et al. 2013).

Scavenging of DPPH[•] and ABTS^{•+} radicals

The antioxidant properties of natural products, such as plant extracts, are due to the nature of the bioactive compounds and sometimes to synergistic effects between them. However, the establishment of the contribution of each individual component to the total antioxidant activity is difficult and time consuming. The common procedure is to measure the total antioxidant capacity of the whole sample.

Various methodologies are widely used for evaluation of the antioxidant potential and, in this study, we determined the free radical scavenging capacity of *H. rhodopensis* using ABTS and DPPH methods, and their reducing capacity by the FRAP assay. The use of several methods to measure antioxidant activity may seem a redundancy, but since different authors used various methods, comparison of properties becomes easier if a large set of data is available.

In order to investigate the antioxidant activity of the obtained plant extract, experiments with two stable radicals DPPH[•] and ABTS^{•+} were conducted. Higher TEAC value indicates that a sample has stronger antioxidant activity. The results represented in Table 2 were 0.80 ± 0.01 and 0.77 ± 0.04 mmol TE/g DW, respectively. In comparison with the traditional heat reflux extraction the established TEAC values were lower. The TEAC_{ABTS} value of extract obtained using heat reflux technique was 1.34 ± 0.15 mmol TE/g DW (Mihaylova et al. 2013). That could be due to the principle difference in the two extraction procedures. In order to make objective consideration of the results, RE was used as reference compound. The TEAC values were 1.75 ± 0.07 and 1.01 ± 0.20 mmol TE/g DW toward DPPH[•] and ABTS^{•+}, respectively.

Antioxidant activity measured by DPPH showed the same relationships as did ABTS method, but TEAC values were higher. This might be explained by the unique mechanism and the unequal sensitivity of each method applied. However, in the future the study on extracts of *H. rhodopensis* could be expanding in order to analyze a correlation of antioxidant activity and single constituents; to elucidate the unknown phenolic compounds and to optimize the extraction conditions.

FRAP assay

The FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of TPTZ, forming an intense blue Fe^{2+} -TPTZ complex with an absorption maximum at 593 nm. The absorbance decrease is proportional to the antioxidant content (Benzie and Strain 1996). The FRAP value for the ultrasound extract of *H. rhodopensis* was 1.36 ± 0.05 mmol TE/g DW (Table 2). In addition, by comparison of the ferric reducing power of the obtained plant extract with RE, the reducing power of the reference was higher - 1.51 ± 0.01 mmol TE/g DW.

Conclusions

In conclusion, our survey can be considered as the first report on the phenolic constituents profile and the antioxidant properties of ultrasound-assisted extract from *H. rhodopensis* leaves and contributes to more comprehensive study of this still less explored endemic plant. The results indicated that the method applied is an effective for the extraction of phenolic compounds and they contribute significantly to the in vitro antioxidant capacity of the extracts. Based on the established results the investigated extract from *H. rhodopensis* could be considered as an effective source of natural antioxidants but further analysis of the constituents is needed in order to discover full characteristic of this resurrection plant and thus to explore any possibility of its application.

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