View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Repository of the Academy's Library

*Acta Alimentaria, Vol. 44 (3), pp. 326–332 (2015)*
*DOI: 10.1556/AAlim.2014.0009*

# TOTAL PHENOLIC CONTENT, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF *HABERLEA RHODOPENSIS* EXTRACTS OBTAINED BY PRESSURIZED LIQUID EXTRACTION

D. Mihaylova[a]*, A. Lante[b] and A. Krastanov[a]

[a]Department of Biotechnology, University of Food Technologies, 26 Maritza Blvd., 4002, Plovdiv. Bulgaria
[b]Department of Agronomy, Food, Natural Resources, Animals and Environment, Università di Padova, Viale dell`Universitá 16, Agripolis, 35020 Legnaro, Padova. Italy

(Received: 8 April 2013; accepted: 26 July 2013)

The present study was designed to investigate the antioxidant and antimicrobial activities of pressurized liquid extracts from *Haberlea rhodopensis* Friv. The total phenolic content was performed using the Folin-Ciocalteu phenol reagent. To determine the antioxidant activities of the extracts, several complementary tests were used: ABTS and DPPH radical scavenging activities, oxygen radical absorbance capacity, and ferric-reducing antioxidant power assay. The phenolic concentration was 15.98±0.09 and 9.42±0.06 mg GAE $g^{-1}$ DW for 70 and 85% ethanol extracts, respectively. Of all the performed methods, the highest antioxidant activity values were measured by the ORAC assay – 224.6±6.6 and 154.0±9.9 µM TE $g^{-1}$ DW for 70 and 85% ethanol extracts, respectively. Results also showed that both extracts exhibited very weak antimicrobial activity against the examined microorganisms. However, the 70% ethanol extract possessed higher inhibition ability, which correlated with higher total phenolic content and antioxidant activity.

**Keywords:** pressurized liquid extract, *Haberlea rhodopensis*, antioxidant activity, antimicrobial activity

Natural antioxidants play a very important role in the prevention of different diseases, such as cancer, arteriosclerosis, and neurodegenerative diseases (Chu et al., 2012; Fernández-Mar et al., 2012). Therefore, there is an increasing interest in the antioxidant effects of natural compounds from medicinal plants and pharmaceutical products for health (Ramful et al., 2011; Ventuani et al., 2011).

Recovery of antioxidant compounds from plant materials is typically accomplished through different extraction techniques, taking into account their chemistry and uneven distribution in the plant matrix. Solvent extraction is the most frequently used technique for isolation of plant antioxidant compounds. However, the extract yields and resulting antioxidant activities of the plant materials are strongly dependent on the nature of the extracting solvent, due to the presence of different antioxidant compounds of varied chemical characteristics and polarities that may or may not be soluble in a particular solvent (Peschel et al., 2006).

Alternative novel extraction procedures are now being sought after that will reduce extraction time and solvent consumption, increase sample throughput and improve analyte recovery. Pressurized liquid extraction (PLE) operates at high pressures and temperature above the boiling point of the organic solvent. The use of higher pressure facilitates the extraction of the analytes from samples by improving the solvent accessibility to the analytes that are trapped in the matrix pores. The use of PLE decreases significantly the total time of

* To whom correspondence should be addressed.
Phone ++ 359 32 603 645; e-mail: dashamihaylova@yahoo.com

0139–3006/$ 20.00 © 2015 *Akadémiai Kiadó, Budapest*

treatment and in addition, this method can be more effective and selective by changing some parameters (Choi et al., 2003; Ong & Len, 2003).

*Haberlea rhodopensis* is a resurrection species and glacial relic endemic in the mountains of the Balkan Peninsula in southeastern Europe (Djilianov et al., 2011). It has been proven that it can survive long periods of desiccation (for up to 2 years) and quickly resume normal growth within hours of re-watering. Despite the fact that several reliable methods were applied to study the antioxidant (Berkov et al., 2011; Mihaylova et al., 2011) and enzyme activity (Yahubyan et al., 2005) of its different extracts, this plant is still less explored. Radev and co-workers (2009) and Mihaylova and co-workers (2011) studied different extracts of the plant and reported inhibition against *St. aureus* and *Ps. fluorescens.*

The aim of the present study was to obtain extracts from *H. rhodopensis* for the first time using alternative PLE technique and thus to extend knowledge of this less explored plant. The potential antioxidant properties of the extracts were studied as well and the antimicrobial activity was measured.

## 1. Materials and methods

### *1.1. Plant material*

*Haberlea rhodopensis* Friv. was collected from its natural habitat (Permission №201/07.05.2009-Bulgarian Ministry of Environment and Water) at Plovdiv region, Bulgaria. The plant leaves were air-dried at room temperature (25–28 ºC), roughly grounded, and stored in air-tight dark containers.

### *1.2. Extracts preparation*

About 67 and 69 g of *H. rhodopensis* were randomly sampled from 200 g dry plant material. The PLE was carried out for 1.45 h in an automatic equipment (NM LAB/M Deputex 88, Limena, Padova, Italy) with 580 and 600 ml of ethanol:water solutions (70:30 and 85:15 v/v acidified with 0.1 M HCl, pH 3), previously deoxygenated by flushing with nitrogen as reported by Rossetto and co-workers (2005). The volume of each sample was measured to calculate the extract concentration (g $l^{-1}$). The extraction rate was 0.140 and 0.142, respectively The extracts were subdivided in dark glass bottles without headspace and stored at –20 ºC.

### *1.3. Total phenolic content (TPC)*

The TPC was analyzed using the method of Kujala and co-workers (2000) with some modifications. Each extract was mixed with Folin-Ciocalteu reagent and 7.5% $Na_2CO_3$. The mixture was vortexed and left for 5 min at 50 ºC. After incubation, the absorbance was measured at 765 nm by room temperature. The TPC was expressed as mg gallic acid equivalents (GAE) per g dry weight (DW).

### *1.4. DPPH radical scavenging assay*

The ability of the extracts to donate an electron and scavenge 2,2-diphenil-1-picrylhydrazyl (DPPH) radical was determined by the slightly modified method of Brand-Williams and co-workers (1995). Freshly prepared $4\times10^{-4}$ M methanolic solution of DPPH was mixed with the samples in a ratio of 2:0.5 (v/v). After 30 min incubation at room temperature the light absorption was measured at 517 nm. The DPPH radical scavenging activity was presented as a function of the concentration of Trolox. The unit of Trolox equivalent antioxidant capacity

(TEAC) was defined the concentration of Trolox having equivalent antioxidant activity expressed as the μM per g DW (μM TE $g^{-1}$ DW).

*1.5. ABTS radical cation decolourization assay*

The radical scavenging activity of the extracts against 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) was estimated according to RE and co-workers (1999). The results were expressed as TEAC value (μM TE $g^{-1}$ DW).

*1.6. Ferric-reducing antioxidant power (FRAP) assay*

The FRAP assay was carried out according to the procedure of BENZIE & STRAIN (1996) with slight modification. The FRAP reagent was prepared fresh daily and was warmed to 37 °C prior to use. 150 µl of plant extracts were allowed to react with 2850 µl of the FRAP reagent for 4 min at 37 °C and the absorbance was recorded at 593 nm. The results were expressed as µM TE $g^{-1}$ DW.

*1.7. Oxygen radical absorbance capacity (ORAC) assay*

The ORAC assay measures the antioxidant scavenging function against peroxyl radical induced by AAPH at 37 °C. The loss of fluorescence of fluorescein is an indication of the extent of damage from its reaction with the peroxyl radical (OU et al., 2001; HUANG et al., 2002; GOMES et al., 2005). ORAC values were expressed as µM TE $g^{-1}$ DW.

*1.8. Determination of antimicrobial activity (AMA)*

*Escherichia coli* ATCC 25922, *Salmonella enterica* subsp. *enterica* ATCC BAA-2162, *Pseudomonas aeruginosa* ATCC 9027 and *Staphylococcus aureus* ATCC 25093 were purchased from the National Bank of Industrial Microorganisms and Cell Cultures (Sofia, Bulgaria). Strains *Bacillus subtilis, Saccharomyces cerevisiae, Aspergillus niger* and *Rhizopus sp.* were provided by the Culture collection at the Department of Microbiology, University of Food Technologies, *Klebsiella pneumoniae* and *Listeria monocytogenes* were isolated from clinical samples.

The AMA of the examined extracts was analyzed by the agar-well diffusion method. To prepare suspensions, the tested microorganisms (TM) were cultured on Luria-Bertani (Sigma-Aldrich) agar and incubated at 37 °C overnight. Appropriate dilutions of each microbial suspension were used to inoculate the molten plate count agar (PCA) (Sigma-Aldrich), equilibrated in water bath, to obtain the concentrations given in Table 2. *A. niger* was grown on Wort agar (Sigma-Aldrich) at 30 °C for 48 hours and a suspension was prepared to inoculate the molten medium. The wells (6 mm in diameter) were cut from the agar and 60 µl of the tested extracts were delivered into them. Equal volumes of the solvents were used as controls. PCA plates were incubated at 37 ºC for 24 hours and Wort agar plates at 30 °C for 48 hours. All plates were examined for any zones of growth inhibition, and the diameters were measured (mm).

*1.9. Statistical analysis*

All measurements (except AMA) were carried out in triplicates. The results were expressed as mean±SD and statistically analyzed using MS-Excel software.

## 2. Results and discussion

### *2.1. Determination of TPC*

The TPC in 70 and 85% ethanol extracts from *H. rhodopensis* were 15.98±0.09 and 9.42±0.06 mg GAE $g^{-1}$ DW, respectively. 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 AOAs (VAN ACKER et al., 1996). Furthermore, phenols have been determined to play an important role in the survival of the plants under extreme conditions (MŰLLER et al., 1997). Consequently the presence of those compounds in the extracts suggested their important role in the plant. Several studies have successfully correlated the phenolic content with AOA (TEPE et al., 2006; MIHAYLOVA et al., 2011), which provoked our interest.

### *2.2. Determination of antioxidant activity (AOA) of the extracts*

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

### *2.3. DPPH, ABTS, FRAP and ORAC assays*

In order to investigate the AOA, experiments with two stable radicals $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ were conducted, $TEAC_{DPPH}{}^{\bullet}$ values were 72.42±0.18 and 30.44±0.25 μM TE $g^{-1}$ DW and the $TEAC_{ABTS}{}^{\bullet+}$ values were 72.98±0.77 and 37.99±2.84 μM TE $g^{-1}$ DW for the 70 and 85% ethanol extracts, respectively (Table 1). Higher TEAC value indicates that a sample has stronger AOA. The FRAP values for the investigated extracts of *H. rhodopensis* were as follows: 120.54±3.57 and 58.57±1.42 μM TE $g^{-1}$ DW. Using the ORAC assay the established results were 224.6±6.60 and 154.0±9.90 μM TE $g^{-1}$ DW.

The results of the total antioxidant capacity assays (Table 1) showed that the investigated extracts possessed AOA, which for the 70% ethanol extract was approximately two times higher than the capacity of the 85% one. This confirmed the results obtained from the TPC assay. Interestingly, the highest AOA values were measured by the ORAC assay. A slight difference among the results obtained by the DPPH, ABTS, ORAC, and FRAP assays was observed. This might be explained by the unique mechanism and the unequal sensitivity of each method applied. The authors therefore strongly suggested that, when analyzing the AOA of samples, it is better to use at least two methods due to the differences between the test systems (OU et al., 2002). From the results obtained it can be concluded that the 70% ethanol is more efficient as solvent in order to obtain extract with higher content of biologically active substances in terms of AOA. This is in agreement with previously conducted studies that established the effectiveness of 70% ethanol as solvent (MIHAYLOVA et al., 2011).

### *2.4. Evaluation of antimicrobial activity of the extracts*

The AMA was evaluated by measuring the inhibition zone against the TMs after correction with the diameter of wells and the zones of appropriate controls. According to the results of

antimicrobial screening given in Table 2, neither extracts showed any significant activity against the TMs. However, higher antimicrobial activities were obtained for 70% ethanol extract. The 85% ethanol extract has been shown to possess antimicrobial effect only against *A. niger* among the studied TMs, which corresponds well with the results of TPC and AOA assays. The 70% ethanol extract showed weak activity against *Kl. pneumoniae, A. niger, B. subtilis,* and *Rh.* sp., while neither of the extracts showed inhibitory activity toward *L. monocytogenes.* In comparison Radev and co-workers (2009) reported effect of *H. rhodopensis* against *S. aureus*.

*Table 1.* Total phenol content and antioxidant activity of extracts from *H. rhodopensis*

| Sample /Assay | TPC, mg GAE $g^{-1}$ DW | $TEAC_{DPPH}^{\bullet}$, μM TE $g^{-1}$ DW | $TEAC_{ABTS}^{\bullet+}$, μM TE $g^{-1}$ DW | ORAC, μM TE $g^{-1}$ DW | FRAP, μM TE $g^{-1}$ DW |
|---|---|---|---|---|---|
| 70% ethanol | 15.98±0.09 | 72.42±0.18 | 72.98±0.77 | 224.6±6.60 | 120.54±3.57 |
| 85% ethanol | 9.42±0.06 | 30.44±0.25 | 37.99±2.84 | 154.0±9.90 | 58.57±1.42 |

*Table 2.* Antimicrobial activity of extracts from *H. rhodopensis*

| Test microorganism | Inhibition zone diameter, mm | | Concentration of TM, CFU[a] $ml^{-1}$ in media |
|---|---|---|---|
| | 70% ethanol | 85% ethanol | |
| *E. coli* | n.d.[b] | n.d.[b] | $1.0*10^{10}$ |
| *Kl. pneumoniae* | 1;0[c] | n.d.[b] | $1.9*10^{11}$ |
| *S. enterica* subsp. *enterica* | n.d.[b] | n.d.[b] | $1.7*10^{9}$ |
| *Ps. aeruginosa* | n.d.[b] | n.d.[b] | $1.5*10^{6}$ |
| *L. monocytogenes* | n.d.[b] | n.d.[b] | $2.5*10^{6}$ |
| *St. aureus* | n.d.[b] | n.d.[b] | $1.4*10^{11}$ |
| *B. subtilis* | 1;1 | n.d.[b] | $1.0*10^{5}$ |
| *S. cerevisiae* | n.d.[b] | n.d.[b] | $1.0*10^{5}$ |
| *A. niger* | 1;0[c] | 3;3[c] | $1.0*10^{5}$ |
| *Rhizopus sp.* | 1;1[c] | n.d.[b] | $1.0*10^{5}$ |

[a]:CFU: colony-forming units; [b]: n.d.: not detected; [c]: the results of two parallel determinations

## 3. Conclusions

In summary, due to the insufficient information on *H. rhodopensis,* we applied the pressurized liquid extraction method for the first time in order to obtain new extracts and to reveal the characteristics of this resurrection plant. The extraction technique was applied with two different solvents – 70 and 85% ethanol – and the antioxidant and the antimicrobial activities were evaluated. Based on the conducted experiments the 70% ethanol extract was found to possess higher inhibition, which correlated with the higher total phenolic content and antioxidant activity.

*

This is a collaborative project and has been partially supported by the „Science and Business" Program at the Ministry of Education and Science, Bulgaria, BG051PO001/3.3-05, co-financed by the European Social Fund.

## References

Benzie, I.F.F. & Strain, J.J. (1996): The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal. Biochem., 239,* 70–76.

Berkov, S.H., Nikolova, M.T., Hristozova, N.I., Momekov, G.Z., Ionkova, I.I. & Djilianov, D.L. (2011): GC-MS profiling of bioactive extracts from *Haberlea rhodopensis*: an endemic resurrection plant. *J. Serb. Chem. Soc., 76*(2), 211–220.

Brand-Williams, W., Cuvelier, M.E. & Berset, C. (1995): Use of a free radical method to evaluate antioxidant activity. *LWT-Food Sci. Technol., 28*, 25–30.

Choi, M.P.K., Chan, K.K.C., Leung, H.W. & Huie, C.W. (2003): Pressurized liquid extraction of active ingredients (ginsenosides) from medicinal plants using non-ionic surfactant solutions. *J. Chromatogr. A.*, *983*, 153–162.

Chu, Y.F., Chen, Y., Brown, P.H., Lyle, B.J., Black, R.M., Cheng, I.H., Ou, B. & Prior, R.L. (2012): Bioactivities of crude caffeine: Antioxidant activity, cyclooxygenase-2 inhibition, and enhanced glucose uptake. *Fd Chem., 131*, 564–568.

Djilianov, D., Ivanov, S., Moyankova, D., Miteva, L., Kirova, E., Alexieva, V., Joudi, M., Peshev, D. & Van Den Ende, W. (2011): Sugar ratios, glutathione redox status and phenols in the resurrection species *Haberlea rhodopensis* and the closely related nonresurrection species *Chirita eberhardtii*. *Plant Biol.*, *13*, 767–776.

Fernández-Mar, M.I., Mateos, R., García-Parrilla, M.C., Puertas, B. & Cantos-Villar, E. (2012): Bioactive compounds in wine: Resveratrol, hydroxytyrosol and melatonin: A review. *Fd Chem.*, *130*, 797–813.

Gomes, A., Fernandes, E. & Lima, J.L. (2005): Fluorescence probes used for detection of reactive oxygen species. *J. Biochem. Biophys. Meth.*, *65*(2–3), 45–80.

Huang, D.J., Ou, B.X., Hampsch-Woodill, M., Flanagan, J.A. & Deemer, E.K. (2002): Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated beta-cyclodextrin as the solubility enhancer. *J. Agric. Fd Chem.*, *50*, 1815–1821.

Kujala, T.S., Loponen, J.M., Klika, K.D. & Pihlaja, K. (2000): Phenolics and betacyanins in red beetroot (*Beta vulgaris*) root: Distribution and effect of cold storage on the content of total phenolics and three individual compounds. *J. Agric. Fd Chem., 48*, 5388–5342.

Mihaylova, D., Bahchevanska, S. & Toneva, V. (2011): Examination of the antioxidant activity of *Haberlea rhodopensis* leaf extracts and their phenolic constituents. *J. Fd Biochem.,* first published online, doi:10.1111/j.1745-4514.2011.00609.x.

Müller, J., Sprenger, N., Bortlik, K., Boller, T. & Wiemken, A. (1997): Desiccation increases sucrose levels in *Ramonda* and *Haberlea*, two genera of resurrection plants in the *Gesneriaceae*. *Physiol. Plant.*, *100*, 153–158.

Ong E. & Len, S.M. (2003): Pressurized hot water extraction of berberine, baicalein and glycyrrhizin in medicinal plants. *Anal. Chim. Acta*, *482*, 81–89.

Ou, B.X., Hampsch-Woodill, M. & Prior, R.L. (2001): Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescence probe*. J. Agric. Fd Chem.*, *49,* 4619–4626.

Ou, B.X., Hunag, D.J., Hampsch-Woodill, M., Flanagan, J.A. & Deemer, E.K. (2002): Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: A comparative study. *J. Agric. Fd Chem.*, *50,* 3122–3128.

Peschel, W., Sanchez-Rabaneda, F., Diekmann, W., Plescher, A., Gartizia, I., Jimenez, D., Lamuela-Raventos, R., Buxaderas, S. & Condina, C. (2006): An industrial approach in the search of natural antioxidants from vegetable and fruit wastes. *Fd Chem.*, *97*, 137–150.

Radev, R., Lazarova, G., Nedialkov, P., Sokolav, K., Rukanova, D. & Tsokeva, Zh. (2009): Study on antibacterial activity of *Haberlea rhodopensis. Trakia J. Sci.*, 34–36.

Ramful, D., Aumjaud, B., Neergheen, V.S., Soobrattee, M.A., Googoolye, K., Aruoma, O.I. & Bahorun, T. (2011): Polyphenolic content and antioxidant activity of *Eugenia pollicina* leaf extract *in vitro* and in model emulsion systems. *Fd Res. Internat.*, *44*, 1190–1196.

Re, R., Pellegrini, N., Proggente, A., Pannala, A., Yang, M. & Rice-Evans, C. (1999): Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med., 26*, 1231–1237.

Rossetto, M., Lante, A., Vanzani, P., Spettoli, P., Scarpa, M. & Rigo, A. (2005): Red chicories as potent scavengers of highly reactive radicals: a study on their phenolic composition and peroxyl radical trapping capacity and efficiency. *J. Agric. Fd Chem.*, *53*, 8169–8175.

Tepe, B., Sokmen, M., Akpulat, H.A. & Sokmen, A. (2006): Screening of the antioxidant potentials of six *Salvia* species from Turkey. *Fd Chem., 95*, 200–204.

Van Acker, S.A.B.E., Van Den Berg, D.J., Tromp, M.N.J.L., Griffioen, D.H., Van Bennekom, W.P., Van Der Vijgh, W.J.F. & Bast, A. (1996): Structural aspects of antioxidant activity of flavonoids. *Free Radic. Biol. Med., 20*, 331–342.

Ventuani, S., Beghelli, E., Scalambra, E., Malisardi, G., Copetti, S., Toso, R.D., Baldisserotto, A. & Manfredini, S. (2011): Activity and stability studies of verbascoside, a novel antioxidant, in dermo-cosmetic and pharmaceutical topical formulations. *Molecules*, *16*, 7068–7080.

Yahubyan, G., Denev, I. & Gozmanova, M. (2005): Determination of the multiple isoforms of some antioxidant enzymes in *Haberlea rhodopensis.* -in: Gruen, B., Nikolova, M. & Donev, A. (Eds): *Proceedings of the Balkan scientific conference of biology in Plovdiv (Bulgaria)***,** 19th–21st May, 2005, pp. 226–230.