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GC–MS profiling of bioactive extracts from *Haberlea rhodopensis*: an endemic resurrection plant

STRAHIL H. BERKOV[1], MILENA T. NIKOLOVA[2], NEVENA I. HRISTOZOVA[3], GEORGI Z. MOMEKOV[4], ILIANA I. IONKOVA[4] and DIMITAR L. DJILIANOV[1]*

[1]*AgroBio Institute, 8 Dragan Tzankov Blvd., 1164-Sofia,* [2]*Institute of Botany, Bulgarian Academy of Sciences, 23 Acad. G. Bonchev Str., 1113-Sofia,* [3]*Faculty of Biology, Sofia University, 8 Dragan Tzankov Blvd., 1164-Sofia and* [4]*Faculty of Pharmacy, Medical University of Sofia, 2 Dunav Str., 1000- Sofia, Bulgaria*

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Abstract: GC–MS metabolic profiling of the apolar and polar fractions from methanolic extracts of *Haberlea rhodopensis* revealed more than one hundred compounds (amino acids, fatty acids, phenolic acids, sterols, glycerides, saccharides, *etc*.). Bioactivity assays showed that the polar fractions possessed strong free radical scavenging activity (IC_{50} = 19.95±14.11 μg ml^{-1} for fresh leaves and 50.04±23.16 μg ml^{-1} for desiccated leaves), while both the polar and apolar fractions failed to provoke any significant cytotoxic effects against the tested cell lines. Five compounds possessing antiradical activity were identified – syringic, vanillic, caffeic, dihydrocaffeic and *p*-coumaric acids.

Keywords: *Haberlea rhodopensis*; metabolites; free radical scavenging activity.

INTRODUCTION

Haberlea rhodopensis Friv. is a very rare Balkan endemite belonging to the group of extremely desiccation-tolerant (ressurection) plants which are capable of withstanding long periods of almost full desiccation and to recover quickly on water availability.[1,2] Carbohydrates and phenols were found to play an important role in the survival of plants under extreme conditions.[3] Phenolic compounds, accumulated in high amounts in ressurection plants, are assumed to protect the membranes against desiccation and free radical-induced oxidation.[4,5]

Ethnobotanical data that *Haberlea* leaves were used for the treatment of wounds and diseases of stock in the Rhodope region of Bulgaria stimulated our interest in this plant species. Similarly, *Myrothamnus flabelifolia*, a desiccation--tolerant plant accumulating gallotannins, is used in traditional folklore and medicine in southern Africa due to its wound-healing properties.[5] Alcoholic extracts

* Corresponding author. E-mail: d_djilianov@abi.bg
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of *H. rhodopensis* were found to possess strong antioxidant and antimicrobial activities.[6,7] Preliminary phytochemical studies indicated that this plant contains flavonoids, tannins and polysaccharides,[7] in addition to previously reported lipids[8] and saccharides.[3]

The aim of the present study was to perform metabolic profiling of this resurrection plant in parallel with antioxidant and cytotoxicity activity assays in an attempt to make a preliminary evaluation of its potential for application in phytotherapy.

EXPERIMENTAL

Plant material

Micropropagated plants, obtained by an *in vitro* propagation system developed in our laboratory,[9] were used in this study to avoid possible damage of natural habitats and problems resulting from handling material of unknown age, size and stage of plant growth. The plants were maintained routinely in culture rooms under a 16/8 h light/dark photoregime, at 22 °C with a light intensity of 75 μmol m^{-2} s^{-1}. The micropropagated *Haberlea* plants possess the same resurrection behaviour as plants taken from natural habitats.[6] Leaves from well-developed plantlets (about 3 months in culture) were taken out from the culture vessels and left to dry to the full air-dried stage in a culture room under controlled conditions (22–25 °C and 60 % relative humidity in the dark) or lyophilized to obtain the desiccated and fresh leaf samples, respectively.

Sample preparation

For metabolite analysis, 50 mg (DW) of leaf samples were macerated in 500 μl of methanol in Eppendorf tubes. 20 μl of nonadecanoic acid (C19:0, 2 mg ml^{-1}) and ribitol (2 mg ml^{-1}) were added as internal standards and the material was extracted for 30 min at 70 °C. Subsequently, 500 μl of chloroform was added and the material was extracted for a further 5 min at room temperature with vortex. Then, 300 μl of distilled water was added and the extract was centrifuged at 13,000 rpm for 10 min to separate the apolar and polar fraction. 300 μl aliquots of the polar and apolar fractions were dried by lyophilisation. Dried polar and apolar fractions were dissolved in 50 μl of pyridine and derivatized with *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA, 50 μl) for 90 min at 40 °C. The derivatized extracts were dissolved in 100 μl chloroform and injected into the GC–MS system. BSTFA and pyridine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

For the bioactivity assays, about 420 mg of dry plant material was extracted in a similar manner to the above-described method, using 4 ml of both methanol and chloroform and 2.5 ml of water to obtain the polar and apolar fractions. No internal standards were added.

GC–MS analyses

The GC–MS analyses were performed on a Hewlett Packard 7890 instrument coupled with MSD 5975 equipment (Hewlett Packard, Palo Alto, CA, USA) operating in EI mode at 70 eV. An HP-5 MS column (30 m×0.25 mm×0.25 μm) was used. The temperature programme was: 100–180 °C at 15 °C min^{-1} and 180–300 °C at 5 °C min^{-1} with a 10 min hold at 300 °C. Injector temperature was 250 °C. The flow rate of the carrier gas (helium) was 0.8 ml min^{-1}. A split ratio of 1:20 was used for the injection of 1 μl of the solutions.

Metabolites identification

The compounds contained in the polar and apolar fractions were identified as TMS derivatives with the help of the NIST 05 database (NIST Mass Spectral Database, PC-Version 5.0 – 2005, National Institute of Standardization and Technology, Gaithersburg, MD, USA), and other plant-specific databases: the Golm Metabolome Database (http://csbdb.mpimp-golm.mpg.de/ /csbdb/gmd/home/gmd_sm.html) and the lipid library (http://www.lipidlibrary.co.uk/ms/ ms01/ /index.htm), as well as literature data[10] based on the matching of the mass spectra and the Kovats retention indexes (*RI*). A syringic acid standard, purchased from Sigma-Aldrich (St. Louis, MO, USA), was co-chromatographed for confirmation of the major phenolic acid in the polar fraction. The measured mass spectra were deconvoluted using AMDIS 2.64 software before comparison with the databases. The groups of unidentified compounds were determined based on the specific mass spectral fragmentation and in comparison with the mass spectra of the known metabolites. All unknown compounds, comprising more than 0.1 % of the TIC (total ion current), were used to calculate the relative contribution of each metabolite group. The response ratios were calculated for each analyte relative to the internal standard (ribitol for the polar and nonadecanoic acid for the apolar metabolites) using the calculated areas for both components. The *RI* values of the compounds were measured with a standard *n*-hydrocarbon calibration mixture (C9–C36) (Restek, Cat No. 31614, supplied by Teknokroma, Spain) using AMDIS 2.64 software.

Determination of the free radical scavenging activity

The stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used for the determination of the free radical scavenging activity of the extracts.[11] Different concentrations of the extracts (5, 10, 20, 50, 100 and 200 μg mL^{-1} in methanol) were added to an equal volume (2.5 mL) of a methanolic solution of DPPH$^{\bullet}$ (0.3 mM, 1 mL). After 30 min at room temperature, the *Ab* values were measured at 517 nm using a Jenway 6320D spectrophotometer and converted into the percentage antioxidant activity using the following equation: DPPH$^{\bullet}$ anti-radical scavenging capacity (%) = 100((*Ab*(sample) – *Ab*(blank)×100/*Ab*(control)). Methanol (1.0 mL) plus plant extract solution (2.5 mL) was used as the blank, while DPPH$^{\bullet}$ solution plus methanol was used as the control. The extracts were measured in triplicate on two different days. The results are presented as the mean±standard error of IC_{50}.

Cytotoxic activity

The cell lines used in this study, namely HL-60 (acute myelocyte leukaemia), its multi--drug resistant sub-line HL-60/Dox, SKW-3 (KE-37 derivative) (T-cell leukaemia), and MDA-MB-231 (breast cancer), were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany). They were cultured under standard conditions – RPMI-1640 liquid medium supplemented with 10 % foetal bovine serum (FBS) and 2 mM L-glutamine, in cell culture flasks, housed at 37 °C in an incubator "BB 16-Function Line" Heraeus (Kendro, Hanau, Germany) with a humidified atmosphere and 5 % CO_2. The cell cultures were maintained in the logarithmic growth phase by supplementation with fresh medium two or three times weekly. The mdr-phenotype of HL-60/Dox was maintained by culturing cells in the presence of 0.2 μM doxorubicin. In order to avoid synergistic interactions, HL-60/Dox were maintained in an anthracycline-free medium (90 % RPMI 1640, 10 % FCS) for at least 72 h prior to the cell viability experiments.

Cellular viability after exposure to the tested compounds was assessed using the standard MTT-dye reduction assay as described by Mosmann[12] with some modifications.[13] The method is based on the reduction of the yellow tetrazolium dye MTT to a violet formazan pro-

duct *via* mitochondrial succinate dehydrogenase in viable cells. The exponentially growing cells were seeded in 96-well flat-bottomed microplates (100 µl well^{-1}) at a density of 1×10^5 cells per ml and after 24 h incubation at 37 °C, they were exposed to various concentrations of the tested compounds for 72 h. At least 8 wells were used for each concentration. After the incubation with the test compounds, 10 µl MTT solution (10 mg ml^{-1} in PBS) aliquots were added to each well. The microplates were further incubated for 4 h at 37 °C, after which the formed MTT-formazan crystals were dissolved by adding 100 µl well^{-1} of 5 % HCOOH-acidified 2-propanol. The MTT-formazan absorption was determined at 580 nm using a microprocessor-controlled microplate reader (Labexim LMR-1). Cell survival fractions were calculated as percentage of the untreated control.

Data processing and statistics

The cell survival data were normalized as percentage of the untreated control (set as 100 % viability). The IC_{50} values (concentrations causing 50 % scavenge of the DPPH$^{\bullet}$) were calculated using non-linear regression analysis (GraphPad Prism Software). The statistical processing of the biological data included the Student's *t*-test, whereby values of $p \leq 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

The free radical scavenging activity assays (Table I) showed that the polar fractions of both desiccated and fresh samples possessed strong antioxidant activity; the fraction from the desiccated leaves being more active ($p < 0.05$). Mainly saccharides, as well as organic acids, phenolic acids, phosphate-containing compounds and amino acids, showing high levels of biological variability[14] were found by GC–MS (Table II). The total amounts of disaccharides and trisaccharides were about two-times more in the samples obtained from the desiccated as compared to those from the fresh samples (Table III). The amounts of phosphate-containing compounds tended to decrease in the desiccated leaves, while glycerides, mainly glycerol, increased. There were no detectable changes in the total amount of amino acids.

TABLE I. Free radical scavenging activity of *H. rhodopensis* fractions

Fractions of *H. rhodopensis*	$IC_{50}\pm SE$ / µg ml^{-1}
Polar fraction of desiccated leaves	19.95±3.42[b]
Polar fraction of fresh leaves	50.04±3.44[b]
Apolar fraction of desiccated leaves	>200
Apolar fraction of fresh leaves	>200
Quercetin[a]	3.23±0.39
Syringic acid[a]	4.40±0.37

[a]Reference compound; [b]significantly different at $p < 0.05$

The antiradical activity of plant extracts may be explained by the presence of phenolic compounds.[15,16] Five free phenolic acids were detected in these fractions, with syringic acid dominating (**46**, more than 86 % of all the free phenolic acids) (Tables II and III). This compound was reported to be a potent antioxidant,

effectively inhibiting linoleic acid (fatty acid) peroxidation.[15] The concentration of syringic acid and of the other free phenolic compounds were found to be about 3-times less ($p < 0.05$) in the desiccated (2.8 % of the total ion current, TIC) than in the fresh leaves (7.8 % of the TIC) of *H. rhodopensis.* Similar results concerning the total phenolic acids were reported for *Ramonda serbica*, another resurrection plant. The predominant phenolic compounds found in *R. serbica*, however, were protocatechuic and chlorogenic acids.[4]

TABLE II. Metabolites found in *H. rhodopensis* fractions; identification: Golm database, NIST05, the lipid library, Madeiros and Simoneit[10] and standard; results represent the means±*SE* of the response ratios of measurements on 3 different fractionations from different samples (50 mg). The response ratios represent peak area ratios using ribitol (40 μg) for polar and nonadecanoic acid (40 μg) for apolar metabolites as quantitative internal standards

Compound	M^+/base ion	R_t min	Apolar fraction		Polar fraction	
			Fresh leaves	Desiccated samples	Fresh leaves	Desiccated samples
Glycolic acid (**1**)	205[a]/147	3.69	–	–	0.1±0.1	tr
L-Valine (**2**)	174/72	3.77	–	–	tr	0.1±0.03
Lactic acid (**3**)	234[a]/147	4.35	–	–	tr	tr
Phosphoric acid methyl ester (**4**)	256/241	4.76	–	–	tr	tr
Malonic acid (**5**)	248/147	4.98	–	–	tr	
4-Hydroxybutanoic acid (**6**)	233[a]/147	5.29	–	–	tr	tr
L-Serine (**7**)	–/132	5.54	–	–	tr	tr
Hydrocarbon – branched (**8**)	–/57	4.96	0.2±0.2	0.1±0.02	–	–
Glycerol (**9**)	293[a]/147	5.71	1.5±0.6	1.0±0.3	0.6±0.1	4.1±4.2
Phosphoric acid (**10**)	314/299	5.75	9.4±3.5	4.9±2.5	8.2±3.2	3.4±0.5
Succinic acid (**11**)	262/147	6.07	0.1±0.1	tr	2.0±1.0	0.5±0.1
Hydrocarbon – branched (**12**)	–/71	6.16	0.6±0.4	0.2±0.1		
2,3-Dihydroxypropanoic acid (**13**)	307/147	6.28	–	–	0.5±0.4	tr
UC (**14**)	–/145	6.32	2.9±0.4	–	–	–
Fumaric acid (**15**)	–/245	6.39	–	–	0.1±0.1	tr
3,4-Dihydroxyl-2-furanone (**16**)	262/147	6.71	–	–	0.3±0.1	tr
Hydrocarbon – branched (**17**)	–/57	7.03	0.4±0.01	0.3±0.1	–	–
Hexadecane (**18**)	–/57	7.31	0.3±0.2	0.2±0.1	–	–
Hydrocarbon – branched (**19**)	–/57	7.39	0.5±0.3	0.2±0.2	–	–
Hydrocarbon – branched (**20**)	–/71	7.51	0.2±0.1	tr	–	–
Hydrocarbon – branched (**21**)	–/71	7.72	3.4±2.8	1.2±0.3	–	–
Malic acid (**22**)	335[a]/147	7.73	0.5±0.3	0.3±0.03	10.4±5.5	4.0±0.7
Erythritol (**23**)	–/217	7.97	–	–	0.7±0.5	0.2±0.1
UC[b] (**24**)	–/355	8.62	1.9±0.5	–	–	–
Glutamine (**25**)	363/246	9.13	0.1±0.1	–	–	–
UC (**26**)	–/220	9.2	–	–	18.1±9.2	6.8±1.6
Xylonic acid (**27**)	364/217	9.34	–	–	4.0±3.0	7.8±1.8
UC (**28**)	–/355	9.34	1.9±0.2	–	–	–
Dodecanoic acid (**29**)	272/257	9.4	0.2±0.2	0.2±0.03	–	–
UM (**30**)	–/220	10.09	–	–	2.4±2.0	0.4±0.1

TABLE II. Continued

Compound	M^+/base ion	R_t min	Apolar fraction		Polar fraction	
			Fresh leaves	Desiccated samples	Fresh leaves	Desiccated samples
Hydrocarbon – branched (**31**)	–/71	10.1	2.2±1.7	0.9±0.1	–	–
UM (**32**)	–/218	10.15	–	–	15.5±18.2	0.2±0.2
UM (**33**)	–/205	10.32	–	–	5.1±1.9	0.2±0.03
Vanillic acid (**34**)	312/297	10.97	–	–	1.0±0.5	0.3±0.3
UM (**35**)	–/292	11.01	–	–	26.5±10.1	25.0±13.3
Ribonic acid (**36**)	511/292	11.05	–	–	23.4±5.4	22.7±3.0
Glycerol phosphate (**37**)	445/357	11.12	13.7±1.0	4.4±0.6	tr	0.7±0.4
UM (**38**)	–/217	11.34	–	–	4.6±2.9	2.0±0.9
Glucofuranose (**39**)	–/217	11.64	–	–	5.9±2.6	0.8±0.3
Fructose I (**40**)	–/217	11.81	–	–	10.2±2.2	6.6±3.8
Phytol I (**41**)	–/95	11.88	7.5±4.4	2.8±0.8	–	–
Fructose II (**42**)	–/217	11.94	–	–	11.5±2.8	5. 5±2.5
Tetradecanoic acid (**43**)	300/285	12.12	0.9±0.4	0.3±0.1	–	–
Fructose III (**44**)	–/204	12.28	–	–	6.0±3.0	1.0±0.4
UM (**45**)	–/217	12.36	–	–	14.2±5.6	2.7±1.1
Syringic acid (**46**)	342/327	12.94	4.3+0.9	8.8±4.2	43.8±10.5	24.9±2.6
Hydrocarbon – branched (**47**)	–/71	13.14	1.2±1.2	0.6±0.1	–	–
Glucose (**48**)	–/204	13.28	–	–	6.7±3.3	3.6±1.9
Caffeic acid (**49**)	396/293	13.5	–	–	0.1±0.1	tr
Dihydrocaffeic acid (**50**)	398/179	13.7	–	–	5.4±2.1	2.1±1.5
Ascorbic acid (**51**)	464/332	13.94	–	–	0.4±0.4	0.1±0.2
9-Hexadecenoic acid (**52**)	326/117	14.64	0.1±0.1	0.1±0.03	–	–
Hexadecanoic acid (**53**)	328/313	15.04	16.0±6.2	10.8±1.3	–	–
UC (**54**)	397/218	15.18	0.8±0.4	–	–	–
Heptadecanoic acid (**55**)	342/327	16.18	0.3±0.1	0.3±0.1	–	–
p-Coumaric acid (**56**)	396/396	16.7	–	–	0.4±0.2	0.1±0.1
Phytol II (**57**)	–/143	17.15	1.5±0.2	0.8±0.1	–	–
9.12-Octadecadienoic acid (**58**)	352/337	17.68	2.5±1.5	1.6±0.9	–	–
9-Octadecenoic acid (**59**)	354/339	17.78	1.9±0.7	2.3±0.8	–	–
UC (glyceride) (**60**)	402/314	17.9	12.0±7.3	7.3±0.2	–	–
Octadecanoic acid (**61**)	356/341	18.19	5.7±0.6	6.2±1.3	–	–
UC (Polyolphosphate) (**62**)	602/587	18.31	25.1±2.0	7.0±2.9	–	–
Uridine (**63**)	445[a]/217	21.76	–	–	–	0.2±0.02
2-Hexadecanoylglycerol (**64**)	459[a]/218	23.16	0.3±0.2	0.1±0.1	–	–
UD (**65**)	–/217	23.32	–	–	7.1±1.3	tr
UD (**66**)	–/361	23.44	–	–	72.9±14.4	183.2±52.0
1-Hexadecanoylglycerol (**67**)	459[a]/371	23.66	2.7±1.0	3.2±0.5	–	–
?UD (**68**)	–/361	23.78	–	–	67.7±19.4	59.3±24.1
?UD (**69**)	–/217	24.1	–	–	8.4±5.1	tr
?UD (**70**)	–/217	24.38	–	–	59.9±16.0	168.3±60.3
?UD (**71**)	–/361	24.48	–	–	21.9±9.6	32.9±32.3
?UD (**72**)	–/217	24.55	–	–	9.6±5.9	2.4±0.5
?UD (**73**)	–/217	24.7	–	–	14.3±1.0	7.1±12.9
?UD (**74**)	–/217	24.85	–	–	0.8±0.6	tr

TABLE II. Continued

Compound	M^+/base ion	R_t min	Apolar fraction		Polar fraction	
			Fresh leaves	Desiccated samples	Fresh leaves	Desiccated samples
UD (**75**)	–/361	25.07	–	–	11.0±7.1	55.5±30.0
UD (**76**)	–/361	25.18	–	–		47.3±59.5
Sucrose (**77**)	–/361	25.26	–	–	40.1±15.7	160.3±61.4
UD (**78**)	–/217	25.74	–	–	0.8±0.5	tr
UD (**79**)	–/191	25.85	–	–	1.0±0.7	2.0±1.8
1-Octadecanoylglycerol (**80**)	487[a]/399	26.39	3.6±1.2	4.5±0.7	0.9±0.6	1.0±0.2
Squalene (**81**)	409/69	26.83	0.4±0.1	0.4±0.1	–	–
Tetracosanoic acid (**82**)	440/425	26.99	0.1±0.1	0.1±0.1	–	–
UC(glycerol) (**83**)	530/193	29.14	27.9±0.2	21.9±4.4	–	–
UT (**84**)	–/217	30.27			1.1±0.4	0.7±0.2
Tocopherol (**85**)	502/502	31.05	7.0±2.9	6.2±1.0	–	–
Cholesterol (**86**)	458/329	31.15	1.2±0.4	1.9±0.3	–	–
UC(glycerol) (**87**)	558/133	31.57	27.6±6.1	24.3±0.6	–	–
Campestrol (**88**)	472/382	32.45	9.2±1.1	10.1±1.4	–	–
Stigmasterol (**89**)	484/83	32.82	0.6±0.1	0.6±0.3	–	–
UT (**90**)	–/217	33.46	–	–	6.7±2.4	13.8±5.3
UT (**91**)	–/217	33.57	–	–	10.8±2.5	11.9±2.1
β-Sitosterol (**92**)	486/396	33.6	39.4±5.2	41.4±4.3	–	–
UC (**93**)	396/381	33.79	–	–	4.4±3.0	0.7±0.7
UT (**94**)	–/361	33.83	–	–	–	26.2±22.1
UT (**95**)	–/217	34.33	–	–	14.9±10.9	29.8±6.1
UT (**96**)	–/217	34.75	–	–	3.0±1.4	4.2±3.8
UT (**97**)	–/217	35.18	–	–	3.7±2.4	6.9±1.9
UT (**98**)	–/217	35.42	–	–	7.8±5.7	21.4±12.9
UT (**99**)	–/217	35.81	–	–	9.8±2.4	33.4±33.4
UT (**100**)	–/361	35.97	–	–	10.8±7.3	24.9±7.1
Raffinose (**101**)	–/361	36.21	–	–	22.7±6.1	15.0±3.2
UT (**102**)	–/217	36.61	–	–	5.0±2.9	5.3±3.3
UT (**103**)	–/217	37.04	–	–	3.0±0.8	–
UC-diglyceride (**104**)	–/129	38.36	16.0±1.6	9.6±7.7	–	–
Total	–	–	255.6± ±39.7	187.0± ±12.3	648.1± ±114.4	1034.8± ±305.15

[a][M-15]$^+$; [b]compounds with "U" are unknown

As compared to desiccated leaves, the higher concentration of free phenolic acids in the fresh leaves is not in correlation with their lower antiradical activity, which indicates that other unidentified compounds contribute to the antiradical activity of the polar fractions. The presence of flavonoids and tannins was reported for *H. rhodopensis*[6] but, due to the limitations of GC–MS, such compounds were not detected in the present study.

The apolar fraction of the methanolic extract showed relatively weak antioxidant activity (Table I). This fraction consisted of glycerides (29 and 33 % of TIC of the fresh and desiccated samples, respectively), sterols (20 and 29 %,

respectively), phosphate-containing compounds (19 and 9 %, respectively), free fatty acids (11 and 12 %, respectively), polyenes (6%), phenolic acids and hydrocarbons. The relatively weaker antioxidant activity could be explained by the presence of small amounts of α-tocopherol and free phenolic acids.

TABLE III. Main groups of compounds in the extracts of *H. rhodopensis*; the results represent the means±*SE* of the response ratios and % of TIC (total ion current) of the measurements on 3 different fractionations from different samples (50 mg). The response ratios represents peak area ratios using ribitol (40 μg) for polar and nonadecanoic acid (40 μg) for apolar metabolites as quantitative internal standards

Compound	Apolar fraction				Polar fraction			
	Fresh leaves		Desiccated leaves		Fresh leaves		Desiccated leaves	
	Response ratio	% of TIC	Response ratio	% of TIC	Response ratio	% of TIC	Response ratio	% of TIC
Hydrocarbons	8.8±6.9	3.2	3.7±1.0	2.0	–	–	–	–
Phosphates	48.1±6.4	18.9	16.3±6.0	8.6	8.3±3.2	1.3	4.0±0.8	0.4
Organic acids	0.7±0.3	0.3	0.3±0.1	0.2	40.9±15.9	6.21	35.3±5.8	3.6
Phenolic acids	4.3±0.9	1.7	8.8±4.2[a]	4.6	50.6±13.4	7.8	27.4±4.4[a]	2.8
Fatty acids								
Saturated	23.2±7.6	8.9	17.8±2.8	9.5	–	–	–	–
Unsaturated	4.5±2.2	1.7	4.0±1.7	2.1	–	–	–	–
Polyenes	16.4±7.7	6.48	10.3±2.0	5.5	–	–	–	–
Glycerides	74.1±16.0	29.3	61.2±6.5	32.7	1.5±0.7	0.2	5.0±4.4	0.6
Sterols	50.4±6.8	20.0	54.0±6.3	28.8	–	–	–	–
Amino acids	–	–	–	–	0.1±0.003	0.02	0.1±0.1	0.01
Saccharides								
Mono-	–	–	–	–	109.7±55.2	17.0	48.3±24.6	4.6
Di-	–	–	–	–	315.2±106.7	48.8	720.5±334.8	70.0
Tri-	–	–	–	–	99.2±45.0	15.4	184.8±101.4	17.2
Syringic acid	4.3±0.9	1.7	8.8±4.2	4.6	43.8±10.5	6.7	24.9±2.6[a]	2.5

[a]Significantly different at $p < 0.05$

The polar and apolar fractions of *H. rhodopensis* were screened for their cytotoxic activity against a panel of four human tumour cell lines, representative for some important types of neoplastic diseases, including a multi-drug resistant cell line (Table IV). Both tested fractions failed to evoke any significant cytotoxic effects against any of the cell lines.

TABLE IV. Cytotoxic effects of the polar and apolar fractions of *H. rhodopensis* after 72 h continuous exposure (MTT-dye reduction assay); each value represents the arithmetic mean± ±*SE* from 8 independent experiments

Concentration mg/ml	HL-60 cells		HL-60/Dox cells		SKW-3 cells		MDA-MB-231 cells	
	Hc 30[a]	*Hc* 70[b]	*Hc* 30	*Hc* 70	*Hc* 30	*Hc* 70	*Hc* 30	*Hc* 70
0.00	100±2	100±2	100±7	100±7	100±2	100±2	100±5	100±2
0.10	103±2	101±2	103±3	100±3	101±3	95±6	100±3	100±3

TABLE IV. Continued

Concentration	HL-60 cells		HL-60/Dox cells		SKW-3 cells		MDA-MB-231 cells	
mg/ml	*Hc* 30	*Hc* 70	*Hc* 30	*Hc* 70	*Hc* 30	*Hc* 70	*Hc* 30	*Hc* 70
0.20	101±1	100±3	99±3	95±7	100±2	97±4	97±3	99±4
0.25	102±2	104±2	114±6	104±3	106±6	99±3	111±7	102±3
0.40	95±4	99±3	94±6	94±4	93±7	96±4	96±6	94±7
0.50	102±2	107±4	109±6	102±5	107±4	97±4	103±2	102±5

[a]Polar fractions; [b]apolar fractions

CONCLUSIONS

In conclusion, the polar fractions of *H. rhodopensis* showed potent free radical scavenging activity. GC–MS metabolic profiling of the polar and apolar fractions resulted in the detection of more than one hundred compounds, including several phenolic acids. In depth quantitative analysis of the phenolic complex (free and conjugated phenolic acids, flavonoids and polyphenols), however, is needed to reveal the relationship between antiradical activity and metabolites in the extracts of *H. rhodopensis*. The lack of any cytotoxic activity of the extracts indicates that the plant may be used in phytotherapy for its antiradical properties. In this respect, the desiccated leaves of *H. rhodopensis* are more suitable due to their higher antiradical activity.

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ИЗВОД

ГАСНОМАСЕНА АНАЛИЗА БИОЛОШКИ АКТИВНИХ ЕКСТРАКАТА ЕНДЕМСКЕ БИЉКЕ *Haberlea rhodopensis*

STRAHIL H. BERKOV[1], MILENA T. NIKOLOVA[2], NEVENA I. HRISTOZOVA[3], GEORGI Z. MOMEKOV[4], ILIANA I. IONKOVA[4] и DIMITAR L. DJILIANOV[1]

[1]AgroBio Institute, 8 Dragan Tzankov Blvd., 1164-Sofia, [2]Institute of Botany, Bulgarian Academy of Sciences, 23 Acad. G. Bonchev Str., 1113-Sofia, [3]Faculty of Biology, Sofia University, 8 Dragan Tzankov Blvd., 1164-Sofia и [4]Faculty of Pharmacy, Medical University of Sofia, 2 Dunav Str., 1000-Sofia, Bulgaria

Гасномасена анализа поларних и неполарних метаболита биљке *Haberlea rhodopensis* изолованих екстракцијом метанолом детектовала је више од сто једињења (амино-киселина, масних киселина, фенолних киселина, стерола, глицерида, сахарида, итд.). Тестови за одређивање биолошке активности су показали да поларне фракције имају изражену активност према слободним радикалима (IC_{50} = 19,95±14,11 μg ml^{-1} за свеже листове и 50,04±23,16 μg ml^{-1} за осушене листове), док ни поларне ни неполарне фракције нису испољиле значајне цитотоксичне ефекте на тестираним ћелијским линијама. Пет једињења са антирадикалском активношћу је идентификовано: сирингинска, ванилинска, кофеинска, дихидрокофеинска и *p*-кумаринска киселина.

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REFERENCES

1. J. Markovska, T. Tsonev, G. Kimenov, A. Tutekova, *J. Plant Physiol.* **144** (1994) 100
2. K. Georgieva, S. Lenk, C. Buschmann, *Photosynthetica* **46** (2008) 208
3. J. Muller, N. Sprenger, K. Bortlik, T. Boiler, A. Wiemken, *Physiol. Plant.* **100** (1997) 153
4. C. Sgherri, B. Stevanovic, F. Navari-Izzo, *Physiol. Plant.* **122** (2004) 478
5. J. Moore, K. Westall, N. Ravenscroft, J. Farrant, G. Lindsey, W. Brandt, *Biochem. J.* **385** (2005) 301
6. I. Ionkova, S. Ninov, I. Antonova, D. Moyankova, T. Georgieva, D. Djilianov, *Pharmazija* **1–4** (2009) 22
7. R. Radev, G. Lazarova, P. Nedialkov, K. Sokolova, D. Rukanova, Z. Tsokeva, *Trakia J. Sci.* **7** (2009) 34
8. K. Stefanov, Y. Markovska, G. Kimenov, S. Popov, *New Chem.* **31 (**1992) 2309
9. D. Djilianov, G. Genova, D. Parvanova, N. Zapryanova, T. Konstantinova, A. Atanassov, *Plant Cell Tissue Organ Cult.* **80** (2005) 115
10. P. Medeiros, B. Simoneit, *J. Chromatogr. A* **1141** (2007) 271
11. C. Cimpoiu, *J. Liq. Chromatogr. Relat. Technol.* **29** (2006) 1125
12. T. Mosmann, *J. Immunol. Met.* **65** (1983) 55
13. S. Konstantinov, H. Eibl, M. Berger, *Br. J. Haematol.* **107** (1999) 365
14. U. Roessner, C. Wagner, J. Kopka, R. Trethewey, L. Willmitzer, *Plant J.* **23** (2000) 131
15. F. Liu, T. Ng, *Life Sci*. **66** (2000) 725
16. D. Szwajgier, J. Pielecki, Z. Targonski, *Acta Sci. Pol.* **4** (2005) 129.