Cytoprotective and antioxidant effects of phenolic compounds from *Haberlea rhodopensis* Friv. (Gesneriaceae)

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ABSTRACT

Background: *Haberlea rhodopensis* Friv. (Gesneriaceae) is a rare poikilohydric endemic and preglacial relict growing in Balkan Peninsula. Previous investigations demonstrated strong antioxidant, antimicrobial and antimutagenic potential of alcoholic extract from the plant. **Objective:** The isolation of known caffeoyl phenylethanoid glucoside – myconoside and flavone-*C*-glycosides hispidulin 8-*C*-(2-*O*-syringoyl-β-glucopyranoside), hispidulin 8-*C*-(6-*O*-acetyl-2-*O*-syringoyl-β-glucopyranoside), and hispidulin 8-*C*-(6-*O*-acetyl-β-glucopyranoside) from the leaves of *H. rhodopensis* was carried out. The aim of this study was to investigate cyto-protective and antioxidant effects of isolated compounds. **Materials and Methods:** Antioxidant activity of isolated substances was examined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radicals; ferric reducing antioxidant power (FRAP) assay and inhibition of lipid peroxidation (LPO) in linoleic acid system by ferric thyocianate method. The compounds were investigated for their possible protective and antioxidant effects against *tert*-butyl hydroperoxide-induced oxidative stress in isolated rat hepatocytes. The levels of thiobarbituric acid reactive substances were assayed as an index of LPO. Lactate dehydrogenase leakage, cell viability, and reduced glutathione depletion were used as signs of cytotoxicity. **Results:** Myconoside demonstrated the highest DPPH radical scavenging, ABTS, FRAP, and antioxidant activity in linoleic acid system as well as the highest and statistically most significant protection and antioxidant activity against the toxic agent. **Conclusion:** Phenolic compounds isolated from *H. rhodopensis* demonstrated significant cytoprotective, radical scavenging potential, and inhibit lipid peroxidation, moreover, myconoside was found to be a new powerful natural antioxidant.

Key words: Antioxidant activity flavone-C-glycosides, cytoprotection, *Haberlea rhodopensis*, myconoside

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INTRODUCTION

Haberlea rhodopensis Friv. (Gesneriaceae) is a rare endemic and preglacial relict growing in Balkan Peninsula. It occurs in Central and Southern Bulgaria mainly in the Rhodope Mountains and some regions of the Sredna gora Mountains and the Stara Planina Mountains.[1] *H. rhodopensis* is a poikilohydric species which is highly desiccation-tolerant and able to revive upon re-hydration of vegetative tissues even after prolonged periods of complete dehydration. Its behaviour under dehydration and re-hydration has been the subject of photosynthetic and metabolic studies.[2] Recently, the presence of the caffeoyl phenylethanoid glucosides myconoside and paucifloside[3] and flavone *C*-glycosides – hispidulin 8-*C*-(6-*O*-acetyl-*ß*-glucopyranoside), hispidulin 8-*C*-(2-*O*-syringoyl-*ß*-glucopyranoside), and hispidulin 8-*C*-(6-*O*-acetyl-2-*O*-syringoyl-*ß*-glucopyranoside)[4] – has been reported.

The leaves *H. rhodopensis* were used in folk medicine for treatment of wounds and diseases of stock in the Rhodope region of Bulgaria. Alcoholic extracts prepared from the titled species were found to possess strong antioxidant

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and antimicrobial activity, reduced the frequency of chromosome aberrations in gamma-irradiated rabbit lymphocytes and exerted *in vivo* antimutagenic potential against the carcinogen cyclophosphamide.[5-9]

In vitro studies offer quick and reliable way for pharmacological assessing of new chemical entities of natural origin. The pharmacologically active new compounds with predictable hepatic metabolism have to be examined for cyto- and hepato-toxicity. The isolated hepatocytes system resembles a well-controlled, biological *in vitro* model with high drug-metabolizing capacities, which is included in the battery of recommended tests from the European Centre for the Validation of Alternative Methods (ECVAM). The main goal of ECVAM is to promote the acceptance of alternative methods, which are important for reducing, refining, and replacing the use of laboratory animals.[10]

The experimental intoxication induced by *tert*-butyl hydroperoxide (*t*-BuOOH) is widely used as an *in vitro* model for oxidative stress. The injury is explained mainly by the formation of divers free radicals, that initiate the process of lipid peroxidation (LPO).[11]

In order to identify the antioxidant principles in the titled species 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as well as ferric reducing antioxidant power (FRAP) activity and inhibition of LPO in linoleic acid system have been employed. Furthermore, the protective effect of these compounds against *t*-BuOOH-induced oxidative stress on isolated rat hepatocytes model has been elucidated, as well. To the best of our knowledge, no study on the cyto-protective and antioxidant activity of these compounds has appeared, so far.

MATERIALS AND METHODS

Chemicals and reagents

Column chromatography (CC) was conducted using Diaion™ HP-20 and MCI gel® CHP20P (Supelco, USA); dry column vacuum chromatography (DCVC) was carried out on Silica Gel 60 (15-40 μm) (Merck, Germany); gel filtration (GF) on Sephadex® LH-20 (Sigma, USA). Thin-layer chromatography was performed on Silica Gel 60 F_{254} or RP-18 F_{254s} (Merck, Germany). DPPH, linoleic acid, ferrous chloride, ABTS, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), BHT, potassium persulfate and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™) were from Sigma-Aldrich USA. All the other chemicals used including the solvents, were of analytical grade. All solvents were of high performance liquid chromatography grade and were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO, USA). The following chemical for isolation and incubation of hepatocytes were used: Pentobarbital sodium (Sanofi, France), N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) (Sigma-Aldrich, Germany), NaCl (Merck, Germany), KCl (Merck), D-glucose (Merck), $NaHCO_3$ (Merck), KH_2PO_4 (Scharlau Chemie SA, Spain), $CaCl_2 \times 2H_2O$ (Merck), $MgSO_4 \times 7H_2O$ (Fluka AG, Germany), collagenase from *Clostridium histolyticum* type IV (Sigma-Aldrich), albumin, bovine serum fraction V, minimum 98% (Sigma-Aldrich), Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (Sigma-Aldrich), 2-thiobarbituric acid (TBA) (4,6-dihydroxypyrimidine-2-thiol) (Sigma-Aldrich), trichloroacetic acid (TCA) (Valerus, Bulgaria), 6-hydroxydopamine (Merck), 2,2'-dinitro-5,5'-dithiodibenzoic acid (DTNB) (Merck), lactate dehydrogenase (LDH) kit (Randox, UK), D(+)sucrose (Fluka, Germany), NaH_2PO_4 (Merck), $MgCl_2 \cdot 6H_2O$, Percoll (Sigma-Aldrich), (3-[4,5-dimethylthiazol-2-yl]-2,5diphenyl-tetrazolium bromide) (Sigma-Aldrich), Dimethyl sulfoxide (DMSO) (Valerus, Bulgaria).

Plant material

The leaves of *H. rhodopensis* Friv. were collected in June 2009 from wild populations near Bachkovo, Rodope Mountains. A voucher specimen (No. 20090600) is kept at the Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Sofia.

Extraction and isolation

Air-dried powdered leaves (126.1 g) were extracted with dichloromethane (DCM) (3 l) and then with 95% EtOH (6 l) using percolation procedure. The resultant extract (57.3 g) was suspended in water and treated with *n*-BuOH (10×200 ml). The BuOH extract (21.0 g) was subjected to a GF on Sephadex LH-20 using MeOH as eluent and then to a CC on Diaion HP-20 (40 × 450 mm) with mobile phase MeOH–H_2O (80:20). Subsequent series of flash-chromatography on MCI gel (eluent H_2O–MeOH, 40:60), a DCVC (50×20 mm) on silica gel (eluent toluene–EtOAc–MeOH–HCOOH, 7:20:1.5:0.5) and GF on Sephadex LH-20 (eluent MeOH) carried out in order to obtain pure compounds **1-4**. The identification of all compounds was achieved by ultra-violet (UV), infrared, high-resolusion mass spectrometry (HR-MS), ^1H and ^{13}C nuclear magnetic resonance (NMR) and 2D NMR experiments. The structures were also confirmed by comparing with the previously reported spectral data.[3-4] The compounds (**1-4**) [Figure 1] were identified as myconoside (**1**), hispidulin 8-*C*-(2-*O*-syringoyl-*β*-glucopyranoside) (**2**), hispidulin 8-*C*-(6-*O*-acetyl-2-*O*-syringoyl-*β*-glucopyranoside) (**3**), and hispidulin 8-*C*-(6-*O*-acetyl-*β*-glucopyranoside) (**4**). All compounds **1-4** have been previously reported for the titled species[4].

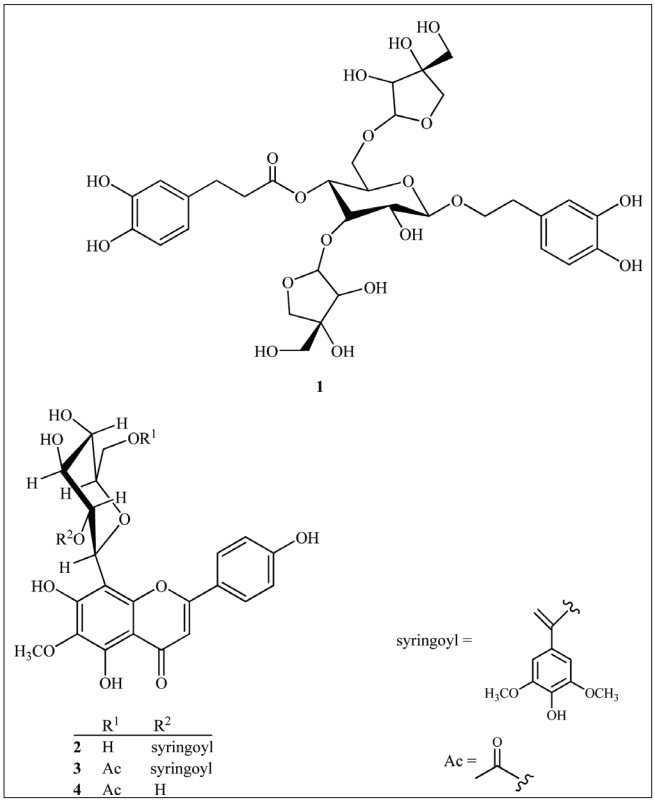

Figure 1: Structures of the compounds **1-4**

Determination of antioxidant activity

1,1-Diphenyl-2-picrylhydrazyl radical-scavenging activity

Scavenging activity of the phenolic compounds against DPPH radical was assessed according to the method previously described.[12] Briefly, 2 ml of each compound in MeOH (0.1 mM) was mixed with 2 ml of DPPH methanol solution (0.1 mM). The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm. Ascorbic acid in MeOH (0.1 mM) was used as reference. The ability to scavenge DPPH radical was calculated by the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \cdot 100,$$

where $\text{Abs}_{\text{control}}$ is the absorbance of the DPPH radical in methanol; $\text{Abs}_{\text{sample}}$ is the absorbance of the DPPH radical solution mixed with sample. All determinations were performed in triplicate ($n = 3$).

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical scavenging assay

For ABTS assay, the procedure followed the method previously described[12] with some modifications. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium per-sulphate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 14 h at room temperature in the dark. The solution was then diluted by mixing 2 ml ABTS solution with 60 ml methanol to obtain an absorbance of 0.71 ± 0.01 units at 734 nm using a spectrophotometer. A fresh ABTS solution was prepared for each assay. One ml of compound in MeOH (0.1 mM) was allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min. The ABTS scavenging capacity of the compound was compared with that of ascorbic acid and the percentage inhibition was calculated as by the following equation:

$$\text{ABTS radical scavenging activity (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \cdot 100,$$

where $\text{Abs}_{\text{control}}$ is the absorbance of ABTS radical in methanol; $\text{Abs}_{\text{sample}}$ is the absorbance of an ABTS radical solution mixed with sample. All determinations were performed in triplicate ($n = 3$).

Total antioxidant activity ferric reducing antioxidant power

The FRAP assay was done according to Zheleva-Dimitrova *et al.*,[12] with some modifications. The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \times 3H_2O$ and 16 ml $C_2H_4O_2$), pH 3.6, 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3 \times 6H_2O$ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $FeCl_3 \times 6H_2O$ solution and then warmed at 37°C before using. 0.1 ml of compound in MeOH (0.1 mM) was allowed to react with 2 ml of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were then taken at 593 nm. The standard curve was linear between 0.03 and 1 mM Trolox™. Results are expressed in trolox equivalent (TE). Ascorbic acid in MeOH (0.1 mM) was used as reference. All determinations were performed in triplicate ($n = 3$).

Determination of antioxidant activity in linoleic acid system by the ammonium thiocyanate ferrous tripyridyltriazine complex method

The antioxidant activity of the studied compounds against LPO was measured using ferrous tripyridyltriazine complex (FTC) assay, as previously described,[12] with some modifications. The reaction solution, containing 0.2 ml of 0.1 mM compound in MeOH, 0.2 ml of linoleic acid emulsions (25 mg/ml in 99% ethanol) and 0.4 ml of 50 mM phosphate buffer (pH 7.4), was incubated in the dark at 40°C. A 0.1 ml aliquot of the reaction solution was then added to 3 ml of 70% (v/v) ethanol and 0.1 ml of 30% (w/v) ammonium thiocyanate. Precisely 3 min after the addition of 0.1 ml of 20 mM ferrous chloride

in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance of the resulting red color was measured at 500 nm. Aliquots were assayed every 24 h until the day after the absorbance of the control solution (without compound) reached maximum value. Ascorbic acid and BHT in MeOH (0.1 mM) were used as positive controls. All determinations were performed in triplicate ($n = 3$).

Animals

Male Wistar rats (body weight, 200-250 g) were used. Rats were housed in plexiglass cages (3 per cage) in a 12/12 light/dark cycle, temperature 20 ± 2°C. Food and water were provided *ad libitum*. Animals were purchased from the National Breeding Centre, Sofia, Bulgaria. All experiments were performed after at least 1 week of adaptation to this environment. The experimental procedures were approved by the Institutional Animal Care and Use Committee at the Medical University-Sofia, Bulgaria. The principles stated in the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes were followed strictly throughout the experiment.

Isolation and incubation of rat hepatocytes

Rats were anesthetized with sodium pentobarbital (0.2 ml/100 g). *In situ* liver perfusion and cell isolation were performed as described by Fau *et al.*[13] with modifications.[14] After portal catheterization, the liver was perfused with 100 ml HEPES buffer (pH = 7.85), containing 10 mM HEPES, 142 mM NaCl, 7 mM KCl, 5 mM glucose and 0.6 mM EDTA (pH = 7.85), followed by 200 ml HEPES buffer (pH = 7.85), without any addition and finally 200 ml HEPES buffer, containing collagenase type IV (50 mg/ 200 ml) and 7 mM $CaCl_2$ (pH = 7.85). The liver was excised, minced into small pieces and the hepatocytes were dispersed in 60 ml Krebs-Ringer-bicarbonate (KRB) buffer (pH = 7.35), containing 1.2 mM KH_2PO_4, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5 mM KCl, 5 mM $NaHCO_3$, 4.5 mM glucose, and 1% bovine serum albumin. After filtration, the hepatocytes were centrifuged at 500*g* for 1 min and washed three times with KRB buffer. Cells were counted under the microscope and the viability was assessed by Trypan blue exclusion (0.05%).[15] Initial viability averaged 89%. Cells were diluted with KRB, to make a suspension of about 3×10^6 hepatocytes/ml. Incubations were carried out in 25 ml Erlenmeyer flasks. Each flask contained 3 ml of the cell suspension (i.e., 9×10^6 hepatocytes). Incubations were performed in a 5% CO_2 + 95% O_2 atmosphere.[13]

Biochemical determinations in isolated rat hepatocytes

The biochemical parameters were determined by spectrophotometric methods using a Spectro UV–visible spectroscopy split spectrophotometer.

Lactate dehydrogenase release

LDH release in isolated rat hepatocytes was measured as described by Fau *et al.*[15]

Glutathione stimulating harmone depletion

At the end of the incubation, isolated rat hepatocytes were recovered by centrifugation at 4°C, and used to measure intracellular reduced glutathione stimulating harmone (GSH), which was assessed by measuring non-protein sulfhydryls after precipitation of proteins with TCA, followed by measurement of thiols in the supernatant with DTNB. The absorbance was measured at 412 nm.[13]

Lipid peroxidation

Hepatocyte suspension (1 ml) was taken and added to 0.67 ml of 20% (w/v) TCA. After centrifugation, 1 ml of the supernatant was added to 0.33 ml of 0.67% (w/v) 2-TBA and heated at 100°C for 30 min. The absorbance was measured at 535 nm, and the amount of TBA-reactants was calculated using a molar extinction coefficient of malondialdehyde (MDA) 1.56×10^5/M/cm.[13]

Statistical analysis

Statistical analysis of the results that were produced by isolated rat hepatocytes model was performed by applying the Student's *t*-test, with $P < 0.05$ considered statistically significant. All results ($n = 12$) are expressed as mean ± SD.

RESULTS AND DISCUSSION

The radical scavenging and FRAP activity of compounds (0.1 mM in MeOH) were compared with those of ascorbic acid at the same concentration (0.1 mM in MeOH) and expressed as % of inhibition against DPPH, ABTS, and TE, respectively [Table 1]. Antioxidant capacities measured by three different methods appeared in the following order: ABTS assay > DPPH assay > FRAP assay.

Myconoside **1** demonstrated the highest DPPH (89.9% ± 0.3%), ABTS (99.6% ± 0.1%) and FRAP (2.49 TE ±

Table 1: 1,1-Diphenyl-2-picrylhydrazyl, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt-radical scavenging and ferric reducing antioxidant power-activity of studied compounds (0.1 mM)

Compound	DPPH %	ABTS %	FRAP TE
1	89.9±0.3	99.6±0.1	2.49±0.01
2	10.9±0.1	91.9±0.2	-
3	3.9±0.3	88.9±0.1	-
4	5.5±0.4	87.7±0.4	-
Ascorbic acid	96.2±0.2	96.2±0.4	0.69±0.50

DPPH: 1,1-Diphenyl-2-picrylhydrazyl; ABTS: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; FRAP: Ferric reducing antioxidant power; TE: Trolox equivalent

0.01 TE) activity comparable to these of ascorbic acid. Phenylethanoid glycosides are well known for a wide range of biological properties including antioxidant activity.[7,8] Among flavonoids compound **2** showed the highest DPPH (24.3% ± 0.1%) and ABTS (92.0% ± 0.2%) radical scavenging activity.

Numerous studies revealed radical scavenging activity of flavonoids and extracts from different medicinal and nutrition plants as *Abelmosus esculentus*,[16] *Sambucus ebulus*,[17] *Crocus sativus*,[18] *Thymus vulgaris*[19] as well as extracts of *H. rhodopensis*.[9] However, no detailed evaluation of antioxidant capacity of pure compounds (myconoside and C-flavone glycosides) from *H. rhodopensis* was undertaken so far.

In this study, the inhibition of LPO of compounds (0.1 mM in MeOH) was determined in linoleic acid system using the FTC method. This method measures the amount of peroxide produced during the initial stages of oxidation, which is the primary product of oxidation. Myconoside **1** was found to be the most active and hindered the oxidation of linoleic acid for all 5 days [Figure 2]. Flavonoids did not manifest any ability to inhibit LPO compare to the control.

Hepatocyte incubation with *t*-BuOOH (0.075 mM) resulted in statistically significant reduction of cell viability by 73% ($P < 0.001$), increased LDH leakage by 553% ($P < 0.001$), depletion of cell GSH by 83% ($P < 0.001$) and increased thiobarbituric acid reactive substances (TBARS) by 1006% ($P < 0.001$) compared to the control [Tables 2-5].

The compounds **1-4**, administered alone, revealed toxic effects on isolated rat hepatocytes model [Figure 3], that were manifested as statistically significant decreasing of cell viability (compound **1** – by 32% [$P < 0.001$]; **2** – by 15% [$P < 0.001$]; **3** – by 23% [$P < 0.001$], and **4** – by 13% [$P < 0.01$]), GSH level (compound **1** – by 39% [$P < 0.01$]; **2** – by 39% [$P < 0.01$]; **3** – by 33% [$P < 0.01$], and **4** – by 33% [$P < 0.01$]) as well as increasing of LDH leakage (compound **1** – by 424% [$P < 0.001$]; **2** – by 474% [$P < 0.001$]; **3** – by 314% [$P < 0.001$], and **4** – by 417% [$P < 0.001$]), compared to the control. The tested substances had no statistically significant effect on TBARS level [Figure 3]. The toxicity of silymarin at 0.1 mM was higher compared to tested compounds (**1**-**4**). It significantly decreased cell viability by 46% ($P < 0.001$); GSH level – by 44% ($P < 0.001$) and increased LDH leakage by 472% ($P < 0.001$); TBARS level – by 188% ($P < 0.001$).

In combination with *t*-BuOOH, all tested compounds showed statistically significant reducing of the damage caused by the hepatotoxic agent and preserving of cell viability [Table 2], decreasing of LDH leakage [Table 3], preserving of GSH level [Table 4] and reducing of lipid damage [Table 5]. The effects were similar to those of silymarin in combination with *t*-BuOOH. Applied together with *t*-BuOOH, myconoside (**1**) showed better hepatoprotective and antioxidant effects compared to compounds **2-4** and silymarin. The results were statistically significant versus control as well as versus *t*-BuOOH treatment.

In experimental toxicology the *in vitro* systems play an important role for the investigation of xenobiotic biotransformation and reveal the possible mechanisms of toxic stress and its protection.

The main structural features of flavonoids required for efficient radical scavenging could be summarized as follows:[20]

An *ortho*-dihydroxy (catechol) structure in the B ring, for electron delocalization;

2,3-double bond in conjugation with a 4-oxo function in the C ring provides electron delocalization from the B ring;

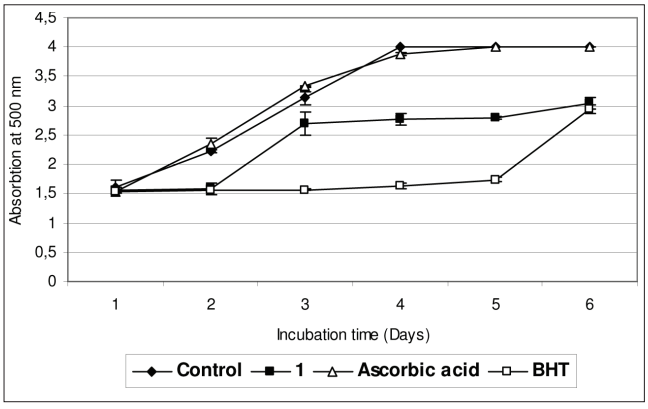

Figure 2: Antioxidant activity of myconoside 1 (0.1 mM) compared with ascorbic acid (vitamin C) and BHT on linoleic acid system

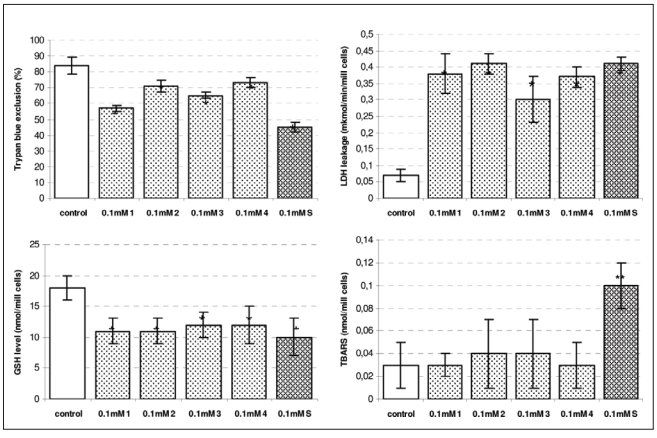

Figure 3: The effects of phenolic compounds (**1**-**4**) from *Haberlea rhodopensis* and silymarin (S) (0.1 mM, ***P*<0.01, ****P*<0.001) versus control on isolated rat hepatocytes model, administered alone: (a) On Trypan blue exclusion; (b) on lactate dehydrogenase leakage; (c) on glutathione stimulating harmone depletion; (d) on lipid peroxidation

Table 2: Effect of phenolic compounds (1-4) from *Haberlea rhodopensis* and silymarin (0.1 mM) on Trypan blue exclusion and lactate dehydrogenase leakage in isolated rat hepatocytes

Group	Trypan blue exclusion (%)	Effect versus control (%)	LDH leakage (μmol/min/106 cells)	Effect versus control (%)
Control	84±5	100	0.07±0.02	100
1	57±2***	↓ 32	0.38±0.06***	↑424
2	71±4***	↓ 15	0.41±0.03***	↑ 474
3	65±2***	↓ 23	0.30±0.07***	↑ 314
4	73±3**	↓ 13	0.37±0.03***	↑ 417
Silymarin	45±3***	↓ 46	0.41±0.02***	↑ 472

**P<0.01; ***P<0.001 versus control; LDH: Lactate dehydrogenase

Table 3: Effect of phenolic compounds (1-4) from *Haberlea rhodopensis* and silymarin (0.1 mM) on glutathione depletion and lipid peroxidation in isolated rat hepatocytes

Group	Glutathione stimulating harmone level (nmol/106 cells)	Effect versus control (%)	TBARS (nmol/106 cells)	Effect versus control (%)
Control	18±2	100	0.03±0.02	100
1	11±2**	↓ 39	0.03±0.01	0
2	11±2***	↓ 39	0.04±0.03	↑ 21
3	12±2**	↓ 33	0.04±0.03	↑ 24
4	12±3**	↓ 33	0.03±0.03	↑ 3
Silymarin	10±3***	↓ 44	0.10±0.02***	↑ 188

GSH: Glutathione stimulating harmone; TBARS: Thiobarbituric acid reactive substances; **P<0.01, ***P<0.001 versus control

Table 4: Effect of phenolic compounds (1-4) from *Haberlea rhodopensis* and silymarin (0.1 mM) in combination with *tert*-butyl hydroperoxide (0.075 mM) on Trypan blue exclusion in isolated rat hepatocytes

Group	Trypan blue exclusion (%)	Effect versus control (%)	Effect versus *t*-BuOOH (%)
Control	84±5	100	
t-BuOOH	23±2***	↓ 73	100
t-BuOOH+**1**	77±5*,+++	↓ 8	↑ 235
t-BuOOH+**2**	49±2***,+++	↓ 42	↑ 113
t-BuOOH+**3**	47±3***,+++	↓ 44	↑ 104
t-BuOOH+**4**	44±3***,+++	↓ 48	↑ 91
t-BuOOH+Silymarin	62±2***,+++	↓ 26	↑ 170

*P<0.05; ***P<0.001 versus control; +++P<0.001 versus *t*-BuOOH; *t*-BuOOH: *tert*-butyl hydroperoxide

hydroxyl groups at positions 3 and 5 provide hydrogen bonding to the oxo group.

According to the previously stated criteria, all flavones are less effective DPPH radical scavengers and this has been confirmed experimentally. However, in ABTS assays all flavonoids display lower antioxidant activities compared to ascorbic acid (96.2% ± 0.4%). The acetylation of the glucose moiety in the structure of flavonoids **3** and **4** always results in decreasing of antioxidant activity.

All isolated flavonoids are *C*-glycosides and do not present FRAP activity. It is assumed that the binding sites for trace metals in the molecule of flavonoids are the catechol moiety in the ring B. The presence of a 3-hydroxyl group in the heterocyclic ring also increases the radical-scavenging activity, while additional hydroxyl or methoxyl groups at positions 3, 5 and 7 of ring A and C seem to be less important.[20]

Perfused rat hepatocytes seem to be a convenient *in vitro* system for investigating xenobiotic biotransformation and the possible mechanisms of toxic stress and its protection. It is a suitable model for evaluation of the cyto-protective effects of some prospective biologically active compounds, both synthetical and of plant origin. Isolated hepatocytes provide the opportunity to evaluate the effects by direct interactions of the studied compounds with endogenous factors. For measuring cell viability, the Trypan blue test was employed. LDH is one of most commonly used enzyme markers, as its increased release is an indicator of membrane damage.[21] In addition, the increased LDH leakage corresponds to decreased cell viability. It is known that reduced GSH plays an important role in cell detoxification and protection.[11] Assessment of the quantity of GSH indicates the possible toxic hepatic metabolism of xenobiotics.[11] The level of TBARS was measured as a biomarker of LPO.

In present study the effects of phenolic compounds (**1-4**) isolated from *H. rhodopensis* were assessed in a model of *t*-BuOOH-induced oxidative stress on isolated rat hepatocytes.

The effects of compounds **1-4** on rat hepatocytes, administered alone at a concentration of 0.1 mM were studied as well. The results showed that compounds **1-4** exerted toxic effects [Tables 2 and 3], manifested by a decrease of cell viability, GSH level and by an increase of LDH leakage and TBARS level. The toxic effects of compounds **1-4**, compared to the toxic effect of silymarin were weaker.

It is known that metabolism of *t*-BuOOH to free radicals undergoes through several steps. In microsomal suspension, in the absence of NADPH, t-BuOOH undergo one-electron oxidation to a peroxyl radical

Table 5: Effect of phenolic compounds (1-4) from *Haberlea rhodopensis* and silymarin (0.1 mM) in combination with *tert*-butyl hydroperoxide (0.075 mM) on lactate dehydrogenase leakage in isolated rat hepatocytes

Group	LDH leakage (μmol/(min·106 cells)	Effect versus control (%)	Effect versus t-BuOOH (%)
Control	0.07±0.02	100	
t-BuOOH	0.47±0.01***	↑ 553	100
t-BuOOH+**1**	0.19±0.03***,+++	↑ 167	↓ 59
t-BuOOH+**2**	0.39±0.03***,++	↑ 438	↓ 18
t-BuOOH+**3**	0.38±0.02***,++	↑ 431	↓ 19
t-BuOOH+**4**	0.43±0.02***,+	↑ 490	↓ 10
t-BuOOH+silymarin	0.26±0.03***,+++	↑ 263	↓ 44

***P<0.001 versus control; +P<0.05; ++P<0.01; +++P<0.001 versus; *t*-BuOOH; *t*-BuOOH: *tert*-butyl hydroperoxide; LDH: Lactate dehydrogenase

Table 6: Effect of phenolic compounds (1-4) from *Haberlea rhodopensis* and silymarin (0.1 mM) in combination with *tert*-butyl hydroperoxide (0.075 mM) on glutathione depletion in isolated rat hepatocytes

Group	Glutathione stimulating harmone level (nmol/106 cells)	Effect versus control (%)	Effect versus t-BuOOH (%)
Control	18±2	100	
t-BuOOH	3±2***	↓ 83	100
t-BuOOH+**1**	11±2***, +++	↓ 39	↑ 267
t-BuOOH+**2**	10±2***, +++	↓ 44	↑ 233
t-BuOOH+**3**	8±3***, +++	↓ 56	↑ 167
t-BuOOH+**4**	9±3***, +++	↓ 50	↑ 200
t-BuOOH+silymarin	11±2***, +++	↓ 39	↑ 267

***P<0.001 versus control; +++P<0.001 versus *t*-BuOOH; GSH: Glutathione stimulating harmone; *t*-BuOOH: *tert*-butyl hydroperoxide

Table 7: Effect of phenolic compounds (1-4) from *Haberlea rhodopensis* and silymarin (0.1 mM) in combination with *tert*-butyl hydroperoxide (0.075 mM) on lipid peroxidation in isolated rat hepatocytes

Group	TBARS (nmol/106 cells)	Effect versus control (%)	Effect versus t-BuOOH (%)
Control	0.03±0.02	100	
t-BuOOH	0.37±0.03***	↑ 1006	100
t-BuOOH+**1**	0.12±0.02***, +++	↑ 264	↓ 67
t-BuOOH+**2**	0.21±0.03***, +++	↑ 548	↓ 41
t-BuOOH+**3**	0.22±0.02***, +++	↑ 570	↓ 39
t-BuOOH+**4**	0.23±0.02***, +++	↑ 597	↓ 37
t-BuOOH+silymarin	0.21±0.03***, +++	↑ 527	↓ 43

***P<0.001 versus control; +++P<0.001 versus *t*-BuOOH; *t*-BuOOH: *tert*-butyl hydroperoxide; TBARS: Thiobarbituric acid reactive substances

[Reaction 1]. Whereas in the presence of NADPH this hepatotoxic chemical undergo one-electron reduction to an alkoxyl radical [Reaction 2]. Furthermore, in isolated mitochondria and intact cells, *t*-BuOOH has been shown to undergo β-scission to the methyl radical [Reaction 3]. All these radicals cause LPO processes.[11]

$(CH_3)_3COOH \rightarrow (CH_3)_3COO\bullet + e^- + H^+$ (Reaction 1)

$(CH_3)_3COOH + e^- \rightarrow (CH_3)_3CO\bullet + OH^-$ (Reaction 2)

$(CH_3)_3CO\bullet \rightarrow (CH_3)_2CO + \bullet CH_3$ (Reaction 3)

Pre-incubation of the hepatocytes with compounds **1-4** significantly protected against *t*-BuOOH toxicity [Tables 4-7]. Compounds **1-4**, during *t*-BuOOH-induced hepatotoxicity, preserved the cell viability and significantly decreased LDH leakage in the medium, compared to *t*-BuOOH. On cellular GSH, compounds **1-4** had protective effects in combination with *t*-BuOOH. *t*-BuOOH caused an elevation of the LPO marker TBARS. In combination with the toxic agent, compounds **1-4** significantly decreased the level of TBARS.

The effects of flavone-C-glycosides **2-4** on the examined parameters were smaller than or similar to the effects of silymarin. The compound **1** (myconoside) had stronger effect on these parameters than the flavolignane mixture silymarin – the classical hepatoprotector and antioxidant.

Our results were supported by literature data about an antioxidant activity on the total extract of endemic plant *H. rhodopensis*. The results of those experiments showed higher SOD-like activity compared to a reference compound Trolox™ (a water-soluble vitamin E analog). The authors explained these results with the probable existence of some phytochemicals as flavonoides and antocianines (cianidine and quercetine) into the total extract of *H. rhodopensis* which were known as strong scavenging and antioxidant agents.[5]

Yahubyan *et al*.[22] in their experiments found multiple forms of several antioxidant enzymes in leaves of the resurrection plant *H. rhodopensis*. Native Polyacrylamide gel electrophoresis (PAGE) showed the presence of six multiple superoxide dismutase isoforms in the protein extract from fresh leaves, and the differential visualization revealed that four of them belonged to Cu, Zn-SOD isoforms, one belonged to Mn-SOD and one belonged to Fe-SOD. The same method showed one form of nonspecific Guaiacol peroxidase and two multiple isoforms of ascorbate peroxidise.[22]

Based on the information available in literature as well as the results from our investigations, we can suggest that the

cyto-protective effects of phenolic compounds (**1-4**) from *H. rhodopensis* on rat hepatocytes might be due to their free radical scavenging and antioxidant activity.

CONCLUSIONS

Compound **1** (myconoside) demonstrated the highest DPPH radical scavenging, ABTS, FRAP and antioxidant activity in linoleic acid system. In isolated rat hepatocytes, the examined compounds **1-4**, administered alone, showed hepatotoxic effects – weaker than effects of silymarin itself. On model of *t*-BuOOH-induced oxidative stress in rat hepatocytes, compound **1** (myconoside) showed the highest statistically significant protection and antioxidant activity against the toxic agent, then compounds **2-4** and silymarin – the classical hepatoprotector and antioxidant. A good correlation between the cyto-protective effects on rat hepatocytes and *in vitro* free radical scavenging and antioxidant activity of the phenolic compounds from *H. rhodopensis* was observed.

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