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Research Article

Simultaneous quantification of the major flavonoids from wild spinach by UHPLC-HRMS and their neuroprotective effects in a model of H_2O_2-induced oxidative stress on SH-SY5Y cells

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Abstract

A modified UHPLC-HRMS method for simultaneous quantification of eight flavonoids from the aerial parts of the wild spinach (*Chenopodium bonus-henricus* L.) was re-validated for specificity, the limit of detection and quantitation limit, linearity, accuracy, and precision. The glycosides of spinacetin (**Chbhnf-04, Chbhnf-06,** and **Chbhnf-08**) and patuletin (**Chbhnf-01**) were the predominant compounds. The total amount of assayed flavonoids from the aerial parts of a title plant was estimated to be 1.82% and 1.4% in two different populations from Vitosha Mountain (Bulgaria). The neuroprotective properties of compounds **Chbhnf-02**, **Chbhnf-04**, **Chbhnf-06**, **Chbhnf-07**, **Chbhnf-08** were further assessed using a model of H_2O_2-induced oxidative stress on human neuroblastoma SH-SY5Y cells. All tested flavonoids demonstrated statistically significant neuroprotective activity close to that of silibinin. Patuletin (**Chbhnf-07**) and spinacetin **(Chbhnf-08)** triglycosides showed the most protective effects at the lowest concentration of 50 μM.

Keywords

Chenopodium bonus-henricus, flavonoids, neuroprotection, oxidative stress, quantification

Introduction

The genus *Chenopodium* (Amaranthaceae) numbers a wide range of species (more than 200) and is native to all the continents with exception of Antarctica as well as in some distant archipelagoes (such as Juan Fernandez, New Zealand, and Hawaii) (Nedialkov and Kokanova-Nedialkova 2021). *Chenopodium bonus-henricus* L. (Amaranthaceae) is a perennial herbaceous plant and is spread in the mountainous regions of Bulgaria (Grozeva 2011). The leaves and flowering tops of Good King Henry (*C. bonus-henricus* L.) are used as a vegetable similar to spinach in some European traditional cuisines. In Italy, Spain, and England it is used in soups or stews and roughly in salads. In Turkey, it is known as "yabanı ispanak" (wild spinach). Canadians have also cultivated the plant as a daily

vegetable. The shoots and flower clusters are eaten like asparagus and broccoli, respectively (Kokanova-Nedialkova et al. 2017). Nine flavonol glycosides of patuletin, 6-methoxykaempferol, and spinacetin were isolated from the aerial part of *C. bonus-henricus*. All flavonoids (100 μM), compared to silibinin (100 μM), significantly reduced the cellular damage caused by CCl_4 in rat hepatocytes, preserved cell viability and GSH level, decreased LDH leakage, and reduced lipid damage. The compounds showed marginal or no cytotoxicity on the HepG2 cell line, even used in high concentrations (Kokanova-Nedialkova et al. 2017). Besides, these flavonoids possessed DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) radical-scavenging activity as well as significantly inhibited the lipid peroxidation in a linoleic acid system by the ferric thiocyanate method (Kokanova-Nedialkova and Nedialkov 2017). Additionally, the glycosides of patuletin, 6-methoxykaempferol, and spinacetin from *C. bonus-henricus* L. were investigated for neuroprotective, anti-α-glucosidase, and lipase activities. All tested flavonoids (100 μM) showed statistically significant neuroprotective activities on isolated rat brain synaptosomes using a 6-hydroxydopamine *in vitro* model. They preserved synaptosome viability as well as the reduced glutathione level. Anti-α-glucosidase and lipase activities of the tested compounds were established by measuring the levels of the released 4-nitrophenol using LC-MS. Patuletin glycosides possessed similar activity to acarbose. All flavonoids exhibited prolipase activity and could be used in the treatment of cachexia. The most active were flavonoids, which contain esterified ferulic acid (Kokanova-Nedialkova et al. 2020).

As a continuation to our studies on phytochemistry and pharmacology of the aerial parts of *C. bonus-henricus* L., in the present study, we reported a re-validation of a modified UHPLC-HRMS method for simultaneous quantification of eight major flavonoids from two populations in Vitosha Mountain (Bulgaria). Besides, their neuroprotective effects in a model of H_2O_2-induced oxidative stress on human neuroblastoma SH-SY5Y cells were established, as well.

Material and methods

Apparatus, materials, and chemicals

UHPLC-HRMS analysis was performed using a Thermo Scientific Dionex Ultimate 3000 RSLC (Germering, Germany) consisting of 6-channel degasser SRD-3600, high-pressure gradient pump HPG-3400RS, autosampler WPS-3000TRS, and column compartment TCC-3000RS coupled to Thermo Scientific Q Exactive Plus (Bremen, Germany) mass spectrometer. All the reagents used were of analytical grade. The main flavonoids (purity 95–96%), patuletin-3-O-[β-apiofuranosyl(1→2)]-β-glucopyranosyl(1→6)-β-glucopyranoside (**Chbhnf-01**), patuletin-3-O-gentiobioside (**Chbhnf-02**), 6-methoxykaempferol-3-O-[β-apiofuranosyl(1→2)]-β-glucopyranosyl(1→6)-β-glucopyranoside (**Chbhnf-03**), spinacetin-3-O-[β-apiofuranosyl (1→2)]-β-glucopyranosyl(1→6)-β-glucopyranoside (**Chbhnf-04**), spinacetin-3-O-gentiobioside (**Chbhnf-06**), patuletin-3-O-(5″′-O-E-feruloyl)-β-D-apiofuranosyl(1→2)[β-D-glucopyranosyl (1→6)]-β-D-glucopyranoside (**Chbhnf-07**), spinacetin-3-O-(5″′-O-E-feruloyl)-β-D-apiofuranosyl (1→2)[β-D-glucopyranosyl(1→6)]-β-D-glucopyranoside (**Chbhnf-08**), 6-methoxykaempferol-3-O-(5″′-O-E-feruloyl)-β-D-apiofuranosyl(1→2)[β-D-glucopyranosyl (1→6)]-β-D-glucopyranoside (**Chbhnf-09**) were previously isolated from the aerial parts of *C. bonus-henricus* L. (Kokanova-Nedialkova et al. 2017). The cell culture medium Roswell Park Memorial Institute (RPMI) 1640 Medium, Fetal Bovine Serum 10%, L-glutamine, trypsin, 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), hydrogen peroxide solution 30% (w/w) were obtained from Sigma-Aldrich (Taufkirchen, Germany).

Plant material

The aerial parts of *Chenopodium bonus-henricus* L. were collected in a flowering stage at altitudes of 1200 m (Zheleznitsa village) and 1734 m (Kumata hut), from Vitosha mountain, Bulgaria in May-June 2020. The plants were identified by P. Nedialkov and the voucher specimens from the plant populations (No. SOM-177438 and No. SOM-177439) were deposited at the National Herbarium, Bulgarian Academy of Sciences, Sofia, Bulgaria.

Preparation of MeOH extracts

The aerial parts of *C. bonus-henricus* L. were dried in a shade, and the powdered plant materials from Zheleznitsa village (200.24 mg) and Kumata hut (200.22 mg) were extracted with 80 vol. % MeOH (60 mL) by ultrasonic-assisted extraction for 30 min. The MeOH extracts were diluted to 100 mL 80 vol. % MeOH. The resulting solutions were filtered and the first 10 mL were removed. An aliquot (10 mL) of each solution was evaporated to dryness, then dissolved in water, and further purified by solid-phase extraction over RP_{18}. The sorbents were first washed with H_2O, then eluted with 80 vol. % MeOH (12 × 500 μL) in 10.0 mL volumetric flasks and diluted to the nominal volume with the same solvent (solution A1 and A2). Subsequently, 2 mL of solutions A1 and A2 were diluted to 25 mL 80 vol. % MeOH (solutions B1 and B2). Further, 1 mL of solutions B1 and B2 were diluted to 5 mL 80 vol. % MeOH (solution C1 and C2). Solutions C1 and C2 were used for LC-MS quantification of flavonoids in the aerial parts of *C. bonus-henricus* L.

UHPLC-HRMS conditions

UHPLC-HRMS conditions were published previously (Kokanova-Nedialkova and Nedialkov 2021). The product ions at *m/z* 317.0656 (for **Chbhnf-03** and **Chbhnf-09**),

333.0605 (for **Chbhnf-01**, **Chbhnf-02**, and **Chbhnf-07**) and 347.0761 (for **Chbhnf-04**, **Chbhnf-06**, and **Chbhnf-08**) with 5.0 ppm isolation window were used as quantifiers. Xcalibur software ver. 4.0 was used for data acquisition and processing.

Method validation

The quantification of flavonoids was carried out using the external standard method. Each of the flavonoids was dissolved in 25 mL 80 vol. % MeOH (primary solutions). The stock standard solution of eight flavonoids was prepared by combining the aliquots (1 mL) of each primary solution and diluting it to 10 mL with 80 vol. % MeOH. It was stored in the refrigerator at 4 °C. The working standard solutions of appropriate concentration were prepared by diluting the stock standard solution with 80 vol. % MeOH.

External standard calibrations were established on six data points covering the concentration range of 12.875–412 ng/mL for (**Chbhnf-01**) and (**Chbhnf-02**), 13.125–420 ng/mL for (**Chbhnf-03**), (**Chbhnf-06**) and (**Chbhnf-08**), 13–416 ng/mL for (**Chbhnf-04**), 12.750–408 ng/mL for (**Chbhnf-07**), and 13.250–424 ng/mL for (**Chbhnf-09**).

The limit of detection (LOD) of an analytical procedure is the lowest analytical concentration at which an analyte(s) could be detected qualitatively. Typically, peak heights are two or three times the noise level. The quantitation limit (LOQ) is also the lowest concentration at that level analyte can be quantitated with acceptable precision, requiring peak heights 10 to 20 times higher than the baseline noise. This signal-to-noise ratio is a good rule of thumb. Limits of detection (LODs) were calculated according to the expression 3.3σ/S, where σ was the standard deviation of the response and S the slope of the calibration curve. Limits of quantification (LOQs) were established from the expression 10σ/S (ICH 2005; Kazusaki et al. 2012).

Accuracy is the closeness of the analytical results obtained by the analyses to the true values and is usually presented as a percent of nominal (ICH 2005; Kazusaki et al. 2012). The accuracy of analytes was evaluated by applying the entire extraction procedure to a control plant matrix that had been spiked with a standard solution of analytes at three concentrations close to that expected in the real plant samples. The accuracy data was recorded as percent recovery of the spiked concentration with relative standard deviations. Each solution was tested in triplicate.

The precision of an analytical method is the amount of variation in the results obtained from multiple analyses of the homogeneous samples. Intra-day precision (repeatability), defines the precision obtained using the same operating conditions over a designated short period (typically ≤1 day). Inter-day precision (intermediate precision), defines the precision obtained using the same operating conditions, typically within the same laboratory, over a designated period (typically ≥1 day) (ICH 2005; Borman and Elder 2017). The intra-day and inter-day precision were determined by analyzing the calibration samples during a single day and on three different days, respectively. The intra-day variation was determined by analyzing the nine replicates on the same day and the inter-day variation was determined on three consecutive days. The retention times (RT) and recovery were obtained for the assayed compounds. The relative standard deviation (RSD) was taken as a measure of precision.

Human neuroblastoma cell line SH-SY5Y

Cultivation

Human neuroblastoma cell line SH-SY5Y was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). SH-SY5Y cells were maintained in 75 ml flasks at 37 °C in a humidified atmosphere with 5% CO_2. The cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum and 2 mM L-glutamine. At 95% confluence, they were plated in 96-well plates for the next experiments.

In vitro cell viability assay

The SH-SY5Y cells were seeded in 96-well microplates at a density of 2×10^4 cells/well and allowed to attach to the well surface for 24 h at 37 °C in a humidified atmosphere with 5% CO_2 (24 h). Five different concentrations of flavonoids (50, 100, 200, 400, and 800 μM) were added to cells and incubated for 24 h. For each concentration, a set of at least 8 wells were used. The cell viability was estimated by MTT-dye reduction assay (Mosmann 1983).

In vitro model of H_2O_2-induced oxidative stress

The model of oxidative stress damage on neuroblastoma SH-SY5Y cell line was achieved by H_2O_2 treatment of cells. SH-SY5Y cells were seeded at a density of 3.5×10^4 cells/well in 96-well plates and allowed to attach at the bottom of the wells for 24 h. Furthermore, the cell medium was aspirated, and the cells were treated with solutions of flavonoids (50, 100, 200, 400, and 800 μM) in RPMI for 60 min before H_2O_2 exposure. Afterward, the SH-SY5Y cells were washed with phosphate-buffered saline (PBS) to remove the extracellular amount of the tested compounds. Subsequently, the treatment with a solution of hydrogen peroxide (H_2O_2, 1 mM) in PBS for 15 min accomplished the SH-SY5Y damage. Silibinin was used as a reference compound because of its protective activity (Kokanova-Nedialkova et al. 2020). After 24 h, the amount of attached viable cells was evaluated by MTT assay. Negative controls (cells without H_2O_2 treatment) were considered as 100% protection and hydrogen peroxide-treated cells as 0% protection.

Statistical analysis

Statistical analysis was performed by one-way analysis of variance with Dunnett's post hoc test. Differences were accepted to be significant when $P < 0.05$. All statistical analysis was carried out on Graph Pad 6 software (GraphPad Software, Inc., La Jolla, CA, USA).

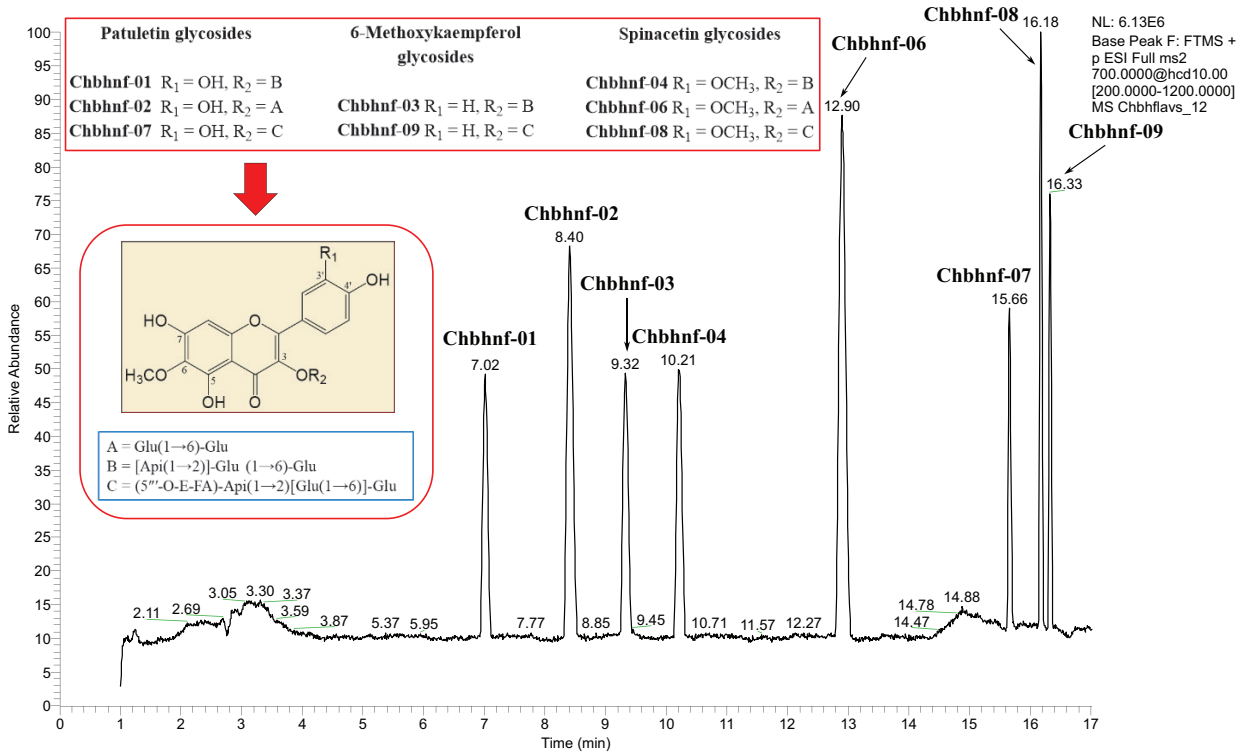

Figure 1. A chromatogram of the standard mixture of flavonoids.

Results and discussion

A modified ultra-high performance liquid chromatography – high-resolution mass spectrometry (UHPLC-HRMS) was used to establish the quantity of the main flavonoids in the aerial parts of *C. bonus-henricus* L. from two populations in Vitosha mountain (Bulgaria) in this work.

The efficiency of the extraction procedure and chromatographic conditions were given in a previous report (Kokanova-Nedialkova and Nedialkov 2021).

Quantitative determination of the main flavonoids in the aerial parts of *C. bonus-henricus* L. was performed by the method of the external standard. Eight previously isolated flavonoids from a title plant were used as external standards (Kokanova-Nedialkova et al. 2017) (Figure 1). Extracted ion chromatograms (EIC) were employed for the calculation of the selected analytes. The calculated monoisotopic mass of the product ions due to the aglycone part of the analytes was used for the construction of EIC with an isolation window of 5 ppm. The quantifier ions of 6-methoxykampferol (**Chbhnf-03** and **Chbhnf-09**), patuletin (**Chbhnf-01, Chbhnf-02** and **Chbhnf-07**), and spinacetin (**Chbhnf-04, Chbhnf-06** and **Chbhnf-08**) glycosides were with *m/z* 317.0656, 333.0605 and 347.0761, respectively.

The calibration curves were linear over the concentration range of 12.875–412 ng/mL for (**Chbhnf-01**) and (**Chbhnf-02**), 13.125–420 ng/mL for (**Chbhnf-03**), (**Chbhnf-06**) and (**Chbhnf-08**), 13–416 ng/mL for (**Chbhnf-04**), 12.750–408 ng/mL for (**Chbhnf-07**), and 13.250–424 ng/mL for (**Chbhnf-09**). All calibration curves showed very good linear regressions and the correlation coefficients were $R^2 > 0.998$ (Table 1).

The method showed that LODs and LOQs were 1.59 ng/mL and 4.81 ng/mL (**Chbhnf-01**), 1.14 ng/mL and 3.44 ng/mL (**Chbhnf-02**), 1.99 ng/mL and 6.05 ng/mL (**Chbhnf-03**), 1.31 ng/mL and 3.96 ng/mL (**Chbhnf-04**), 1.16 ng/mL and 3.52 ng/mL (**Chbhnf-06**), 1.56 ng/mL and 4.71 ng/mL (**Chbhnf-07**), 1.97 ng/mL and 5.98 ng/mL (**Chbhnf-08**) and 0.97 ng/mL and 2.95 ng/mL (**Chbhnf-09**), respectively (Table 1).

Table 1. Linearity of calibration curve for the eight flavonoids.

Marker compound	Linear range (ng/mL)	Regression equations	R^2	LOD (ng/mL)	LOQ (ng/mL)
Chbhnf-01	12.875 ÷ 412	y = 53052x – 440933	0.9987	1.59	4.81
Chbhnf-02	12.875 ÷ 412	y = 81917x – 686762	0.9987	1.14	3.44
Chbhnf-03	13.125 ÷ 420	y = 53868x – 467219	0.9994	1.99	6.05
Chbhnf-04	13.000 ÷ 416	y = 56272x – 384872	0.9995	1.31	3.96
Chbhnf-06	13.125 ÷ 420	y = 112020x – 847350	0.9994	1.16	3.52
Chbhnf-07	12.750 ÷ 408	y = 30118x – 248618	0.9990	1.56	4.71
Chbhnf-08	13.125 ÷ 420	y = 40092x – 281735	0.9998	1.97	5.98
Chbhnf-09	13.250 ÷ 424	y = 31702x – 259428	0.9997	0.97	2.95

The accuracy of the analytes was checked by addition of a standard solution mixture at three concentrations (32.96, 65.92, and 98.88 ng/mL for **Chbhnf-01** and **Chbhnf-02**; 33.60, 67.20, and 100.80 ng/mL for **Chbhnf-03**, **Chbhnf-06,** and **Chbhnf-08**; 33.28, 66.56 and 99.84 ng/mL for **Chbhnf-04**; 32.64, 65.28 and 97.92 ng/mL for **Chbhnf-07**; 33.92, 67.84 and 101.76 ng/mL for **Chbhnf-09**) close to that expected in the real plant samples.

Blank samples from the same unspiked plant material were analyzed at the same time as the spiked samples and the measured values were subtracted. Besides, the related compounds showed overall recoveries ranging from

95.33% to 99.77% with RSD ranging from 0.65% to 2.99%. A good agreement between the spiked and determined concentrations indicated acceptable accuracy (Table 2).

Table 2. Accuracy of the UHPLC-HRMS method.

Flavonoids	Added (ng/mL)	Found[a] (ng/mL)	Recovery[a] (%)	RSD (%)
Chbhnf-01	32.96	32.29 ± 0.87	97.98 ± 2.64	2.69
	65.92	64.79 ± 0.69	98.28 ± 1.05	1.07
	98.88	97.95 ± 0.95	99.06 ± 0.96	0.97
Chbhnf-02	32.96	31.94 ± 0.39	96.89 ± 1.18	1.22
	65.92	64.72 ± 1.08	98.17 ± 1.64	1.67
	98.88	97.58 ± 0.92	98.68 ± 0.93	0.94
Chbhnf-03	33.60	32.35 ± 0.48	96.29 ± 1.44	1.49
	67.20	65.76 ± 1.03	97.85 ± 1.53	1.56
	100.80	98.60 ± 2.11	97.82 ± 2.09	2.14
Chbhnf-04	33.28	32.21 ± 0.96	96.78 ± 2.89	2.99
	66.56	64.17 ± 1.73	96.41 ± 2.60	2.69
	99.84	99.61 ± 2.22	99.77 ± 2.22	2.23
Chbhnf-6	33.60	32.42 ± 0.70	96.50 ± 2.08	2.16
	67.20	64.20 ± 1.17	95.54 ± 1.75	1.83
	100.80	98.03 ± 2.03	97.26 ± 2.01	2.07
Chbhnf-07	32.64	31.12 ± 0.20	95.33 ± 0.62	0.65
	65.28	62.90 ± 1.11	96.36 ± 1.69	1.76
	97.92	95.56 ± 1.79	97.59 ± 1.82	1.87
Chbhnf-08	33.60	32.11 ± 0.68	95.56 ± 2.02	2.11
	67.20	64.25 ± 0.85	95.61 ± 1.26	1.32
	100.80	99.08 ± 2.43	98.29 ± 2.41	2.45
Chbhnf-09	33.92	32.57 ± 0.26	96.01 ± 0.76	0.79
	67.84	65.88 ± 1.04	97.11 ± 1.53	1.58
	101.76	98.67 ± 0.69	96.97 ± 0.67	0.69

[a] Values are the mean ± SD (n = 3).

The precision of the retention times was determined by analyzing the calibration samples during a single day and on three different days, respectively. The RSDs of retention times of the analytes were ≤ 0.15 for intra-day and ≤ 0.14 for inter-day precision assays, respectively. Also, the related compounds showed overall recoveries ranging from 96.83% to 102.52% (for intra-day and inter-day precision assays) with RSDs from 0.35% to 2.34%. (Tables 3 and 4).

Table 3. Evaluation of intra-day precision (repeatability) of the UHPLC-HRMS method.

Compds.	Intra-day precision (repeatability)			
	RT ± SD (min)	RSD	Recovery ± SD (%)	RSD (%)
Chbhnf-01	7.01 ± 0.010	0.15	101.00 ± 2.16	2.14
Chbhnf-02	8.40 ± 0.007	0.08	100.76 ± 1.98	1.96
Chbhnf-03	9.33 ± 0.010	0.11	101.68 ± 1.25	1.23
Chbhnf-04	10.20 ± 0.013	0.13	100.23 ± 1.89	1.88
Chbhnf-06	12.90 ± 0.009	0.07	98.18 ± 1.48	1.50
Chbhnf-07	15.66 ± 0.006	0.04	99.30 ± 2.05	2.06
Chbhnf-08	16.18 ± 0.006	0.04	97.53 ± 1.82	1.87
Chbhnf-09	16.33 ± 0.005	0.03	97.03 ± 0.75	0.77

Table 4. Evaluation of inter-day precision of the UHPLC-HRMS method.

Compds.	Inter-day precision (intermediate precision)			
	RT±SD (min)	RSD	Recovery ± SD (%)	RSD (%)
Chbhnf-01	7.01 ± 0.010	0.14	100.13 ± 2.17	2.16
Chbhnf-02	8.40 ± 0.010	0.12	100.43 ± 2.35	2.34
Chbhnf-03	9.33 ± 0.009	0.09	102.52 ± 0.73	0.72
Chbhnf-04	10.21 ± 0.009	0.09	98.79 ± 0.88	0.89
Chbhnf-06	12.91 ± 0.006	0.05	99.66 ± 0.64	0.64
Chbhnf-07	15.66 ± 0.007	0.04	97.53 ± 0.34	0.35
Chbhnf-08	16.18 ± 0.006	0.04	97.83 ± 0.93	0.95
Chbhnf-09	16.33 ± 0.005	0.03	96.83 ± 0.77	0.80

The modified UHPLC-HRMS method was applied for quantification of eight major flavonoids in the aerial parts of *C. bonus-henricus* L. from two different populations (Zheleznitsa and Kumata hut) in Vitosha mountain (Bulgaria). The results show that the flavonoids of spinacetin (**Chbhnf-04, Chbhnf-06** and **Chbhnf-08**) and patuletin (**Chbhnf-01**) were the predominant compounds and reached 0.53%, 0.25%, 0.29%, 0.26% in the aerial parts of *C. bonus-henricus* from Kumata hut (KH) and 0.32%, 0.19%, 0.21%, 0.20% in the aerial parts collected from Zheleznitsa (ZH), respectively (Table 5). The glycosides of patuletin (**Chbhnf-02** and **Chbhnf-07**) and 6-methoxykaempferol (**Chbhnf-03**) were found in smaller quantities ranging from 0.09 to 0.17%. The flavonoid (**Chbh-**

Table 5. Content of flavonoids in the aerial parts of *C. bonus-henricus* L. from Kumata hut (KH) and Zheleznitsa (ZH).

Compds.	Flavonoids	Content of flavonoids (%)	
		KH	ZH
Chbhnf-01	patuletin-3-O-[β-apiofuranosyl(1→2)]-β-glucopyranosyl(1→6)-β-glucopyranoside	0.26 ± 0.0036	0.20 ± 0.0025
Chbhnf-02	patuletin-3-O-gentiobioside	0.10 ± 0.0012	0.09 ± 0.0004
Chbhnf-03	6-methoxykaempferol-3-O-[β-apiofuranosyl(1→2)]-β-glucopyranosyl(1→6)-β-glucopyranoside	0.17 ± 0.0035	0.15 ± 0.0015
Chbhnf-04	spinacetin-3-O-[β-apiofuranosyl(1→2)]-β-glucopyranosyl(1→6)-β-glucopyranoside	0.53 ± 0.0137	0.32 ± 0.0024
Chbhnf-06	spinacetin-3-O-gentiobioside	0.25 ± 0.0031	0.19 ± 0.0011
Chbhnf-07	patuletin-3-O-(5″′-O-E-feruloyl)-β-D-apiofuranosyl (1→2)[β-D-glucopyranosyl (1→6)]-β-D-glucopyranoside	0.14 ± 0.0012	0.15 ± 0.0012
Chbhnf-08	spinacetin-3-O-(5″′-O-E-feruloyl)-β-D-apiofuranosyl (1→2)[β-D-glucopyranosyl(1→6)]-β-D-glucopyranoside	0.29 ± 0.0058	0.21 ± 0.0020
Chbhnf-09	6-methoxykaempferol-3-O-(5″′-O-E-feruloyl)-β-D-apiofuranosyl(1→2)[β-D-glucopyranosyl (1→6)]-β-D-glucopyranoside	0.08 ± 0.0019	0.09 ± 0.0009
The total content of flavonoids (%)		1.82	1.4

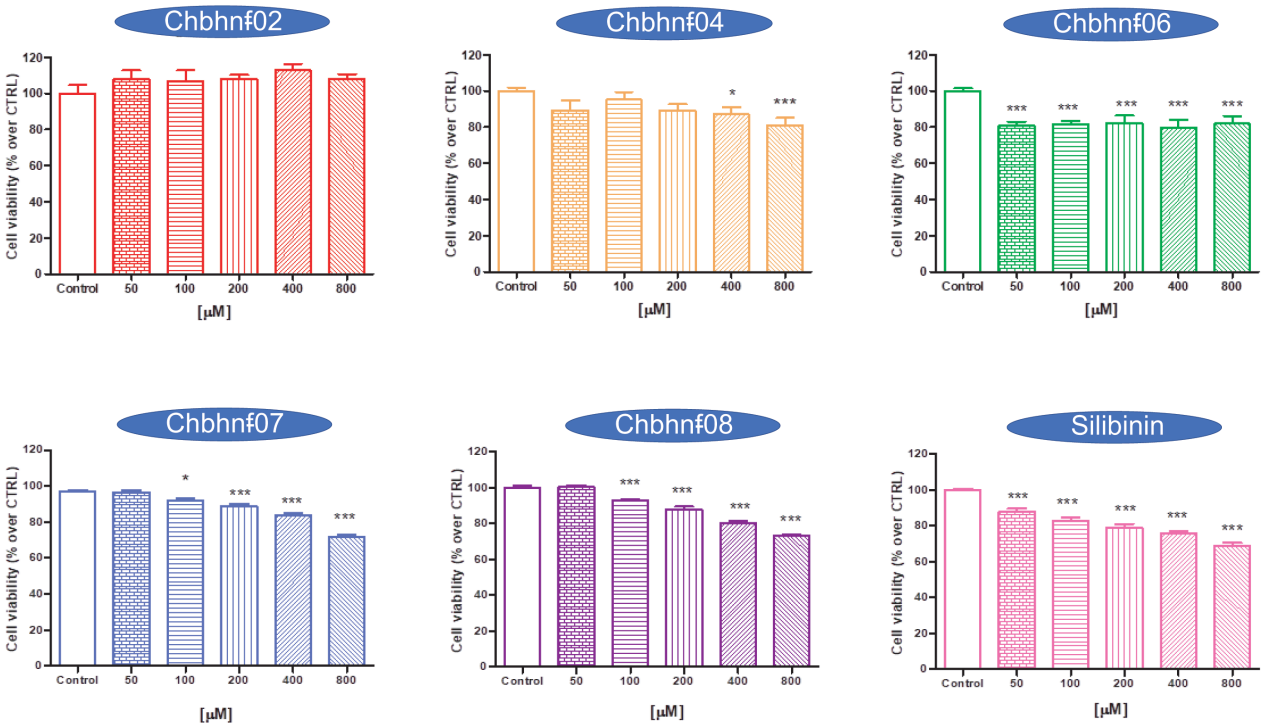

Figure 2. Effect of flavonoids and silibinin on the viability of neuroblastoma SH-SY5Y cells. Data are presented as means from three independent experiments ± SD (n = 8). *P < 0.05, ***P < 0.001, vs. untreated control (one-way analysis of variance with Dunnet's post hoc test).

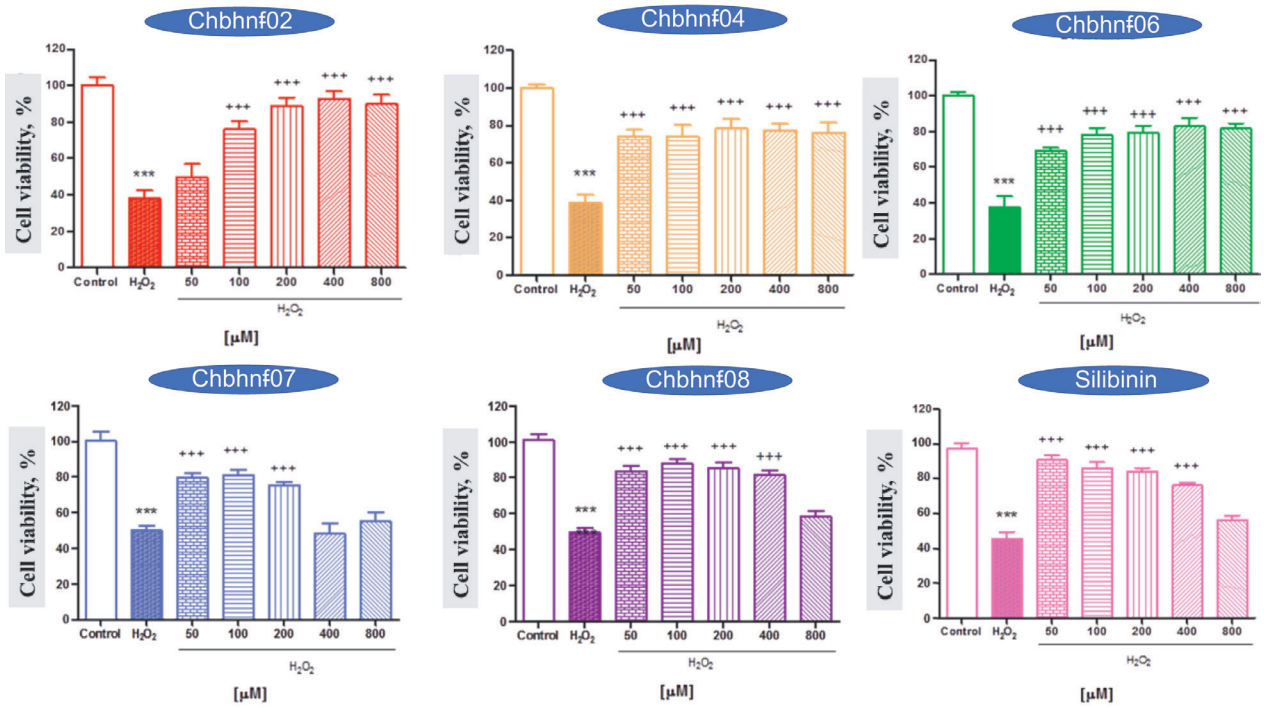

Figure 3. Effect of flavonoids and silibinin on the viability of SH-SY5Y cells in a model of H_2O_2-induced toxicity. Data are presented as means from three independent experiments ± SD (n = 8). ***P < 0.001, vs. untreated control; $^{+++}$P < 0.001, vs. H_2O_2 group. (one-way analysis of variance with Dunnet's post hoc test).

nf-09) was found in the smallest amount (0.08% in KH and 0.09% in ZH). The total amount of assayed flavonoids was estimated to be 1.82% in the aerial parts from KH and 1.4% in ZH (Table 5).

The neuroprotective properties of compounds **Chbhnf-02**, **Chbhnf-04**, **Chbhnf-06**, **Chbhnf-07,** and **Chbhnf-08** were further assessed using a model of H_2O_2-induced oxidative stress on SH-SY5Y cells. SH-SY5Y cells possess

morphological and biochemical characteristics of human neurons and represent a suitable *in vitro* model for studying the mechanisms of damage and neuroprotection in different neurodegenerative diseases as Parkinson's, Alzheimer's diseases, etc. (Agholme et al. 2010; Xicoy et al. 2017).

The study started with the determination of the potential cytotoxic effects of five tested flavonoids on SH-SY5Y cells after 24 h incubation. The cell viability was determined by MTT assay as a marker for mitochondrial function. The cytotoxicity was classified according to ISO 10993-5 standard: cell viability above 80% was considered as non-cytotoxic; within 80%–60% as weak; 60%–40% as moderate and below 40% as strong cytotoxicity (ISO 2009). The results showed a lack or low cytotoxicity of all tested compounds in concentrations up to 400 μM (<20%) (Figure 2). **Chbhnf-07** and **Chbhnf-08** showed increased cytotoxicity at the highest concentration (800 μM) similar to those of silibinin (***P < 0.001).

Furthermore, the neuroprotective effects of flavonoids (**Chbhnf-02**, **Chbhnf-04**, **Chbhnf-06**, **Chbhnf-07,** and **Chbhnf-08**) were explored in a model of H_2O_2-induced oxidative stress in SH-SY5Y cells. H_2O_2 is commonly used as an inducer of oxidative stress *in vitro* models. The mechanism of H_2O_2-induced cell damages includes the production of reactive hydroxyl radicals and by the products of Fenton's reaction that further interact directly with cellular components to damage proteins, lipids and DNA.

Patuletin diglycoside (**Chbhnf-02**) showed protection against H_2O_2-oxidative stress damage in the high concentration range from 200 to 800 μM. Triglycosides of patuletin (**Chbhnf-07**) and spinacetin (**Chbhnf-08**) showed a high protective effect at concentrations up to 400 μM. Spinacetin glycosides **Chbhnf-04** and **Chbhnf-06** possessed protective effects in the entire studied concentration range (50–800 μM). The most prominent neuroprotection *in vitro* was observed with triglycosides of patuletin and spinacetin, respectively compounds **Chbhnf-07** and **Chbhnf-08**. Their protective cell viability effects were seen at the lowest concentration of 50 μM (Figure 3). The ability to preserve SH-SY5Y cell viability was probably due to the presence of esterified ferulic acid in their moiety, which is well known for its antioxidant properties and ability to scavenge reactive oxygen species (ROS) (Itagaki et al. 2009).

The results from the present study showed that in the model of H_2O_2-induced oxidative stress in SH-SY5Y cells, the flavonoids **Chbhnf-07** and **Chbhnf-08** possessed the comparable effect as those of silibinin, while the effects of the other flavonoids were close to silibinin to less extent. Additionally, we suggested that the neuroprotective effects of tested patuletin, 6-methoxykaempferol, and spinacetin glycosides are attributed to their antioxidant activity and ability to scavenge ROS.

The results of this study were in good agreement with previously published research on neuroprotective activities of these flavonoids on isolated rat brain synaptosomes using a 6-hydroxydopamine *in vitro* model (Kokanova-Nedialkova et al. 2020).

Conclusions

A modified UHPLC-HRMS method for simultaneous quantification of eight flavonoids from wild spinach (*Chenopodium bonus-henricus* L.) was re-validated for specificity, the limit of detection, and quantitation limit, linearity, accuracy, and precision. The flavonoids of spinacetin and patuletin (**Chbhnf-01**) were the predominant compounds. The total amount of assayed flavonoids was estimated to be 1.82% in the aerial parts from Kumata hut and 1.4% in Zheleznitsa. All tested flavonoids demonstrated statistically significant neuroprotective activity in a model of H_2O_2-induced oxidative stress *in vitro*, which resembles those of natural antioxidant silibinin. Patuletin (**Chbhnf-07**) and spinacetin **(Chbhnf-08)** triglycosides possessed the most protective effects at the lowest concentration of 50 μM.

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