

Saponins from the roots of *Chenopodium bonus-henricus* L. with neuroprotective and anti- α -glucosidase activities

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Received 14 February 2021 ♦ Accepted 19 March 2021 ♦ Published 27 April 2021

Citation: Kokanova-Nedialkova Z, Kondeva-Burdina M, Nedialkov P (2021) Saponins from the roots of *Chenopodium bonus-henricus* L. with neuroprotective and anti- α -glucosidase activities. Pharmacia 68(2): 387–392. <https://doi.org/10.3897/pharmacia.68.e64425>

Abstract

Six saponins of phytolaccagenin, bayogenin, medicagenic acid, 2 β -hydroxygypsogenin, and 2 β -hydroxyoleanoic acid from the roots of *Chenopodium bonus-henricus* L. were investigated for neuroprotective and anti- α -glucosidase activities. All tested saponins (10 μ M) 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. The bayogenin glycoside (**Chbhs-05**) possessed the most prominent neuroprotective effect. The anti- α -glucosidase activity of the tested saponins was established by measuring the levels of the released 4-nitrophenol using LC-MS. Bonushenricoside B (**Chbhs-07**) showed the highest inhibitory effect against α -glucosidase (44.1%) compared to the positive control acarbose (36.3%) at a concentration of 625 μ M.

Keywords

Anti- α -glucosidase activity, *Chenopodium bonus-henricus*, neuroprotection, saponins, synaptosomes

Introduction

The species from the genus *Chenopodium* have been used as leafy vegetables and important subsidiary grain crops for human and animal foodstuff. Diverse species of this genus have been employed as medicinal agents in traditional systems of medicine (Kokanova-Nedialkova et al. 2009). *Chenopodium bonus-henricus* L. (Amaranthaceae) is widespread in Europe, Western Asia, and North America. The leaves and flowering tops of Good King Henry are used as a vegetable in the same manner as spinach in some European countries (Kokanova-Nedialkova et al.

2017). *C. bonus-henricus* is a perennial herbaceous plant and is spread in the mountainous regions of Bulgaria (Grozeva 2011). In Bulgarian folk medicine, the extracts of the roots have been used for the treatment of bronchitis, laryngitis, rheumatism, gout, constipation, dermatitis, and eczema. A decoction of the roots of *C. bonus-henricus* (also known as “chuyen”) is used in the food industry to produce “tahin” and “white halva” (Kokanova-Nedialkova et al. 2019a). Recently phytochemical investigation of the roots of *C. bonus-henricus* led to the isolation of six saponins of phytolaccagenin, 2 β -hydroxyoleanoic acid, bayogenin, 2 β -hydroxygypsogenin, and medicagenic acid. The

MeOH extract and saponins showed moderate or marginal cytotoxicity on five leukemic cell lines (HL-60, SKW-3, Jurkat E6-1, BV-173, and K-562) and stimulatory effects on interleukin-2 production in PHA/PMA stimulated Jurkat E6-1 cells (Kokanova-Nedialkova et al. 2019a).

Furthermore, UHPLC-HRMS based profiling of a purified MeOH extract from the roots of *C. bonus-henricus* tentatively identified 15 saponins of six saponin classes. Additionally, the MeOH extract and the saponins have exerted hepatoprotective and antioxidant activities comparable to those of silymarin on *in vivo* and *in vitro* models of CCl₄-induced liver damage, respectively (Kokanova-Nedialkova et al. 2019b).

In the present study, we reported a determination of the anti- α -glucosidase activity of six saponins from the roots of *C. bonus-henricus* by measuring the 4-nitrophenol levels with LC-MS as well as their neuroprotective activities on *in vitro* conditions of 6-hydroxydopamine-induced oxidative stress.

Material and methods

Materials, chemicals, and apparatus

The saponins, 3-O- β -D-glucuronopyranosyl-medicagenic acid-28-O- β -D-xylopyranosyl(1 \rightarrow 4)- α -L-rhamnopyranosyl(1 \rightarrow 2)- α -L-arabinopyranosyl ester (**Chbhs-01**), Bonushenricoside A (3-O- α -L-arabinopyranosyl-phytolaccagenin-28-O- β -D-glucopyranosyl ester) (**Chbhs-03**), 3-O- β -D-glucuronopyranosyl-bayogenin-28-O- β -D-glucopyranosyl ester (**Chbhs-04**), 3-O- α -L-arabinopyranosyl-bayogenin-28-O- β -D-glucopyranosyl ester (**Chbhs-05**), 3-O- β -D-glucuronopyranosyl-2 β -hydroxygypsogenin-28-O- β -D-glucopyranosyl ester (**Chbhs-06**), Bonushenricoside B (3-O- β -D-glucuronopyranosyl-2 β -hydroxyoleanic acid-28-O- β -D-glucopyranosyl ester) (**Chbhs-07**) were previously isolated from the roots of *C. bonus-henricus* L. (Kokanova-Nedialkova et al. 2019a).

HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) as well as other chemicals, trichloroacetic acid (TCA), Percoll, 6-hydroxydopamine (6-OHDA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), α -glucosidase from *Saccharomyces cerevisiae* were obtained from Sigma Aldrich. NaCl, KCl, D-glucose, CaCl₂·2H₂O, 2,2'-dinitro-5,5'-dithiodibenzoic acid (DTNB), D-sucrose, MgCl₂·6H₂O, NaH₂PO₄ were purchased from Merck. Acarbose and *p*-nitrophenyl- α -D-glucopyranoside were obtained from Alfa Aesar.

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.

Experimental animals

Male Wistar rats (bodyweight 200–250 g) were used. The rats were housed in plexiglass cages (three per cage) in a 12/12 light/dark cycle, under standard laboratory conditions (ambient temperature 20 \pm 2 °C and humidity 72 \pm 4%) with free access to water and standard pelleted rat food 53-3, produced according to ISO 9001:2008. The animals were purchased from the National Breeding Center, Sofia, Bulgaria. Seven days of acclimatization was allowed before the commencement of the study and a veterinary physician monitored the health of the animals regularly. The vivarium (certificate of registration of farm No. 0072/01.08.2007) was inspected by the Bulgarian Drug Agency to check the husbandry conditions (No. A-11-1081/03.11.2011). All performed procedures were approved by the Institutional Animal Care Committee and the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) (Council of Europe 1991) were strictly followed throughout the experiment.

Isolation and incubation of synaptosomes

Synaptosomes were prepared from brains of old male Wistar rats, as previously described by Taupin et al. (1994), using Percoll gradient.

Two types of buffers were prepared: Buffer A: HEPES 5 mM and sucrose 0.32 M; Buffer B: NaCl 290 mM, MgCl₂·2H₂O 0.95 mM, KCl 10 mM, CaCl₂·2H₂O 2.4 mM, NaH₂PO₄ 2.1 mM, HEPES 44 mM, D-glucose 13 mM. Brain homogenates were prepared with buffer A and centrifuged at 1000 rpm for 10 min at 4 °C. After centrifugation, the supernatants were removed and centrifuged again under the above-mentioned conditions. Supernatants were taken and subjected to centrifugation three times at 10 000 rpm for 20 min at 4 °C. The last two centrifugations were for the purification of the synaptosomes.

Isolation of synaptosomes was performed with the help of a colloidal silicon solution (Percoll). A 90% stock solution of Percoll was used. Percoll solutions of two concentrations, 16%, and 10%, were prepared. Then 4 mL of Percoll 16% and 10% were added in test-tubes and 90% Percoll (7.5% Percoll) was added to the sediment from the last centrifugation. The tubes were centrifuged for 20 min at 15 000 rpm at 4 °C. After centrifugation, three layers were formed in the tubes. The lower layer contained mitochondria, the upper layer – lipids, and the middle layer (between 16% and 10% Percoll) – synaptosomes. The middle layer of each tube was harvested and buffer B with glucose was added. The mixture was centrifuged at 10 000 rpm for 20 min at 4 °C. After centrifugation, the sediment with the synaptosomes was mixed with buffer B with glucose.

The synaptosomes were incubated with 10 μ M of the saponins. After the incubation, MTT-test was performed to determine synaptosomal vitality by a method described by Mungarro-Menchaca et al. (2002).

Determination of reduced glutathione (GSH)

The level of reduced glutathione was determined by measuring the non-protein SH-groups after precipitation of the proteins with trichloroacetic acid. After the incubation, synaptosomes were centrifuged at 400 rpm for 3 min. The sediment was treated with 5% trichloroacetic acid and left for 10 min on ice. Samples were centrifuged at 8000 rpm for 10 min (2 °C). The supernatant was removed to determine the level of GSH and was stored at -20 °C. Immediately before the measurement, the samples were neutralized with 5 M NaOH. The presence of thiols in the supernatant was determined using Ellman reagent. The resulting yellow color was measured spectrophotometrically ($\lambda = 412 \text{ nm}$) (Robyt et al. 1971).

Model of 6-hydroxydopamine (6-OH-DA)-induced neurotoxicity

This *in vitro* model resembles the neurodegenerative processes occurring in Parkinson's disease. Dopamine metabolism and oxidation lead to the formation of reactive oxygen species (ROS) and reactive quinones. They induce dopamine neurotoxicity and neurodegeneration (Stokes et al. 2002). The synaptosomes were incubated with 150 μM 6-OHDA and the saponins (10 μM) for 1 hour.

Statistical analysis

Statistical analysis was performed using the statistical program MEDCALC. Results are expressed as mean values with a standard error of the mean (\pm SEM) for six experiments. The significance of the data was assessed using the non-parametric Mann-Whitney test. Values of $p < 0.05$; $p < 0.01$ were considered statistically significant. Three parallel samples were used.

α -Glucosidase inhibitory activity assay

The assay mixture was prepared according to the method of Kang et al. (2011) with some modifications (Kokanova-Nedialkova et al. 2020). Briefly, the mixture (160 μL) contained 20 μL of a sample in phosphate buffer (pH 6.8) containing 10% DMSO (or phosphate buffer containing 10% DMSO as a control), 100 μL phosphate buffer (pH 6.8), and 20 μL enzyme solution (0.2 U/mL α -glucosidase in phosphate buffer) were mixed and incubated at 37 °C for 15 min, and then, 20 μL substrate solution (2.5 mM *p*-nitrophenyl- α -D-glucopyranoside prepared in the same buffer) were added. The reaction was processed at 37 °C for 15 min and stopped by adding 840 μL AcCN. Then the supernatant was used for LC-MS analysis after centrifugation at 12 000 rpm for 10 min. The amount of the *p*-nitrophenol released from *p*-nitrophenyl- α -D-glucopyranoside was quantified using LC-MS. The inhibitory rates (%) were calculated according to the formula:

$$\% \alpha - \text{Glucosidase inhibitory activity} = \frac{A_k - A_{\text{sample}}}{A_k} \times 100,$$

where A_k is the area of the *p*-nitrophenol peak in the control and A_{sample} is the area of the *p*-nitrophenol peak in the sample.

Acarbose was taken as a standard α -glucosidase inhibitor. All determinations were performed in triplicate ($n = 3$).

Statistical analysis

The statistical program "MEDCALC" was used for the analysis of the data. The results were expressed as mean (\pm standard deviation, SD) of three independent experiments, each performed in triplicate.

LC-MS method for measuring 4-nitrophenol levels in the α -glucosidase inhibitory activity assay

One μL of all samples were injected on a column for LC-MS measuring 4-nitrophenol levels in α -glucosidase inhibitory activity assay (Kokanova-Nedialkova et al. 2020). UHPLC separations were performed on Thermo Scientific Hypersil GOLD column (USA) (2.1 \times 50 mm, 1.9 μm) at 40 °C. Each chromatographic run was carried out with a binary mobile phase consisting of water containing 0.1%(v/v) formic acid (A) and acetonitrile also with 0.1% (v/v) formic acid (B). A gradient program was used as follows: 0–3 min, 20% B; 3–4 min, 20–95% B; 4–6 min, 95% B. The flow rate was 0.3 mL min⁻¹ and the sample injection volume was 1 μL . Operating conditions for the HESI source used in a negative ionization mode were: -2.5 kV spray voltage, the capillary and the probe heater temperature were 320 °C, sheath gas flow rate 38 a.u., auxiliary gas flow 12 a.u. (a.u. refer to arbitrary values set by the Exactive Tune software) and S-Lens RF level 50.00. Nitrogen was used for sample nebulization and collision gas in HCD cell. FullMS-SIM was used as an MS experiment where the resolution, AGC target, max. IT and mass range were 70000 (at m/z 200), 3e6, 200 ms, and m/z 133–143, respectively. XIC chromatograms at m/z 138.0186 ($[\text{M}-\text{H}]^-$ of 4-nitrophenol) with 5 ppm isolation were used to take peak area. Xcalibur software ver. 4.0 was used for data acquisition and processing.

Results and discussion

In the present study, six saponins of phytolaccagenin, bayogenin, medicagenic acid, 2 β -hydroxygypsogenin, and 2 β -hydroxyoleanoic acid isolated from the roots of *Chenopodium bonus-henricus* L. (Figure 1) (Kokanova-Nedialkova et al. 2019a) were tested for their neuroprotective and anti- α -glucosidase activities.

Along with the other brain models (brain slices, primary neuronal cultures), the isolated nerve terminals –

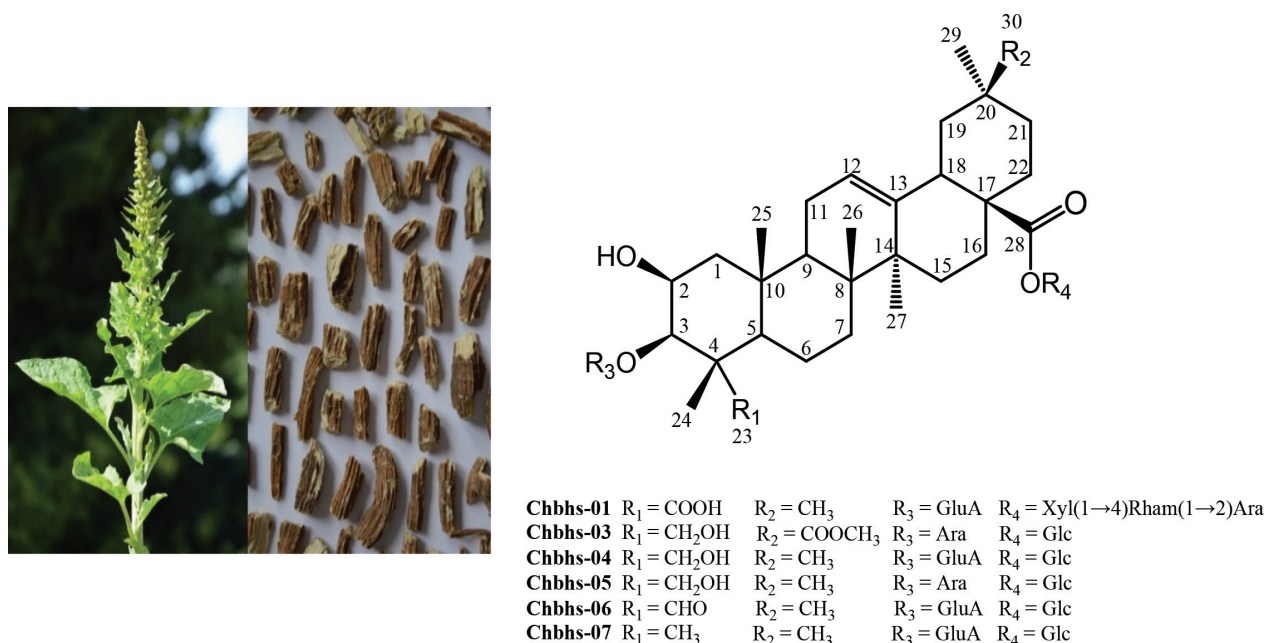


Figure 1. Saponins from the roots of *C. bonus-henricus* L.

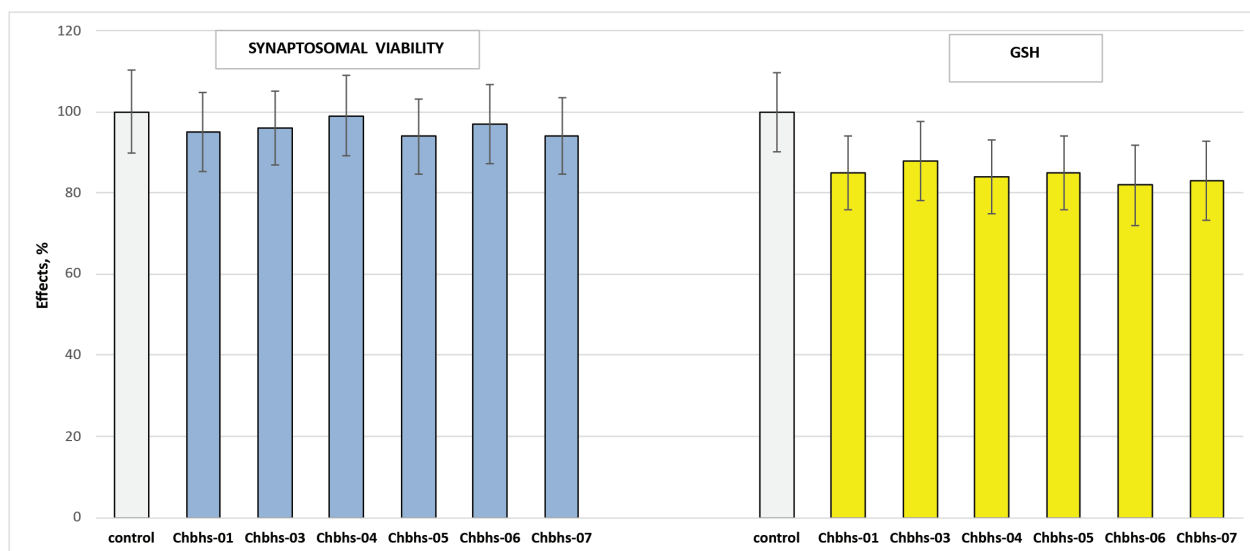


Figure 2. Effects of the saponins, isolated from the roots of *Chenopodium bonus-henricus* (at concentration 10 μ M), administered alone on isolated rat brain synaptosomes.

synaptosomes, are an important tool for the investigation of the synaptic function in the brain on a molecular level. Using this useful model system for assessment of the neuroprotective properties of the saponins we evaluated their effect on isolated rat brain synaptosomes. The necessary rat brain synaptosomes were prepared by using Percoll reagent.

Administered alone, the saponins did not reveal statistically significant neurotoxic effects on isolated rat brain synaptosomes. They did not change the synaptosomal viability and the level of reduced glutathione (GSH), compared to the control (non-treated synaptosomes) (Figure 2).

The treatment of isolated rat brain synaptosomes with 6-OHDA is a reliable and commonly used *in vitro* model for the investigation of processes, which play role in neurodegenerative disease, including Parkinson's and

Alzheimer's disease. The mechanism of 6-OHDA neurotoxicity includes the formation of ROS and reactive metabolites, as a result of its metabolism in mitochondria of the neuronal cells (Stokes et al. 2002). The mechanism of destruction of the nerve terminals is thought – involvement of oxidation of 6-OHDA to a *p*-quinone and production of a free radical or of superoxide anion. The species intermediate reacts covalently with the nerve terminal and permanently inactivates it. When administered alone, 6-OHDA (150 μ M) decreased synaptosomal viability and GSH level by 50%, compared to the non-treated synaptosomes (Figure 3).

In a model of 6-OHDA-induced neurotoxicity, all saponins (at concentration 10 μ M) revealed statistically significant neuroprotective effects, compared to 6-OHDA, by preserving the synaptosomal viability and level of GSH (Figure 3).

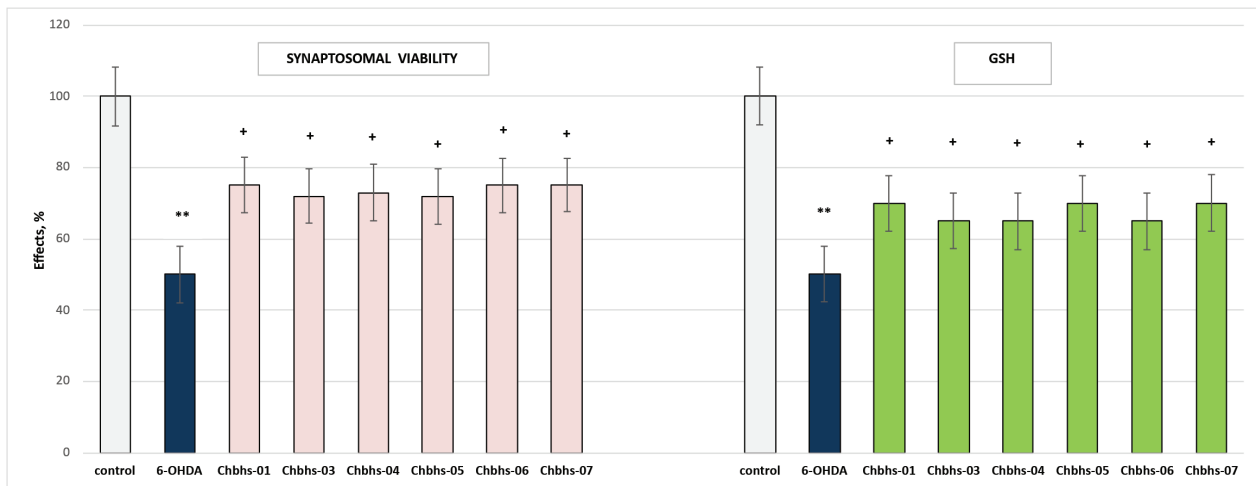


Figure 3. Effects of the saponins, isolated from the roots of *Chenopodium bonus-henricus* (at concentration 10 μ M), with combination with 6-OHDA, on isolated rat brain synaptosomes.

* $p < 0.01$ vs control (non-treated synaptosomes); + $p < 0.05$ vs toxic agent (6-OHDA).

Compared to the toxic agent, the saponins preserved the synaptosomal viability between 25% and 28%. Glycosides of phytolaccagenin (**Chbhs-03**) and bayogenin (**Chbhs-04** and **Chbhs-05**) prevented a 6-OHDA-induced decrease of the synaptosomal viability by 28%, 27%, and 28%, respectively. The results also showed that glycosides of medicagenic acid (**Chbhs-01**), 2 β -hydroxygypsogenin (**Chbhs-06**), and 2 β -hydroxyoleanoic acid (**Chbhs-07**) preserved the synaptosomal viability by 25%. The most active were glycosides of phytolaccagenin (**Chbhs-03**) and bayogenin (**Chbhs-05**) which preserved cell viability by 28%, respectively (Figure 3).

The saponins **Chbhs-03**, **Chbhs-04**, and **Chbhs-06** prevented 6-OHDA-induced GSH depletion by 15%. The most active were glycosides of medicagenic acid, bayogenin and 2 β -hydroxyoleanoic acid, saponins **Chbhs-01**, **Chbhs-05**, and **Chbhs-07** that preserved the GSH levels by 20% compared to the 6-OHDA group, respectively (Figure 3).

The results from the present study showed that in the model of 6-OHDA-induced oxidative stress on isolated rat synaptosomes the most active saponin was a glycoside of bayogenin (**Chbhs-05**), which preserved synaptosomal viability and level of GSH by 28% and 20%, respectively.

We suggested that these neuroprotective effects of the saponins might be due to possible antioxidant activity and preservation of reduced glutathione – the main cell protector, which reacted and scavenged the reactive metabolites, produced by 6-OHDA.

Saponins, an important group of bioactive plant natural products, are glycosides of triterpenoid or steroidal aglycones. Their diverse biological activities are ascribed to their different structures. Saponins have long been recognized as key ingredients in traditional Chinese medicine. Accumulated evidence suggests that saponins have significant neuroprotective effects on the attenuation of central nervous system disorders, such as stroke, Alzheimer's disease, Parkinson's disease, and Huntington's disease. However, our understanding of the mechanisms underlying the observed effects remains incomplete. Based on

recently reported data from basic and clinical studies, Sun et al. (2015) highlights the proposed mechanisms of their neuroprotective function including antioxidant, modulation of neurotransmitters, anti-apoptosis, anti-inflammation, attenuating Ca^{2+} influx, modulating neurotrophic factors, inhibiting Tau phosphorylation, and regeneration of neural networks.

The anti- α -glucosidase activity of the tested saponins was established by measuring the amount of released *p*-nitrophenol from *p*-nitrophenyl- α -D-glucopyranoside using LC-MS. The inhibitory effect of saponins against α -glucosidase activity was shown in Table 1.

Table 1. α -Glucosidase inhibitory activity of saponins and acarbose at a concentration of 625 μ M.

Samples	α -Glucosidase inhibitory activity (%)
Chbhs-01	ND
Chbhs-03	ND
Chbhs-04	ND
Chbhs-05	ND
Chbhs-06	30.0 \pm 1.5
Chbhs-07	44.1 \pm 3.4
Acarbose	36.3 \pm 2.2

ND – not detected.

The glycoside of 2 β -hydroxyoleanoic acid (**Chbhs-07**) showed the highest inhibitory effect against α -glucosidase (44.1%) which was better than that of the positive control acarbose (36.3%). Furthermore, the glycoside of 2 β -hydroxygypsogenin (**Chbhs-06**) possessed a lower anti- α -glucosidase activity (30.0%) compared to the saponin (**Chbhs-07**)(44.1%). Both saponins (**Chbhs-06** and **Chbhs-07**) contained glucuronic acid (GluA) at C-3 and glucose (Glu) at C-28 of the aglycone. The only difference between the two aglycones was a substituent at C-23, which was the -CHO group for the glycoside of 2 β -hydroxygypsogenin (**Chbhs-06**) and -CH₃ group for 2 β -hydroxyoleanoic acid glycoside (**Chbhs-07**). The saponins **Chbhs-01**, **Chbhs-03**, **Chbhs-04**, and **Chbhs-05** did not show any inhibitory effect against α -glucosidase. The re-

sults of this study were in good agreement with previously published research on the structure-activity relationship of saponins against α -glucosidase activity (Dou et al 2013).

Conclusions

Six saponins of phytolaccagenin, bayogenin, medicagenic acid, 2 β -hydroxygypsogenin, and 2 β -hydroxyoleanoic acid from the roots of *Chenopodium bonus-henricus* L. were investigated for neuroprotective and anti- α -glucosidase activities. All tested saponins showed statistically significant neuroprotective activities on isolated rat brain synaptosomes using a 6-hydroxydopamine *in vitro* model.

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The bayogenin glycoside (**Chbhs-05**) possessed the most prominent neuroprotective effect. The anti- α -glucosidase activity of the tested saponins was established by measuring the levels of released 4-nitrophenol using LC-MS. Bonushenricoside B (**Chbhs-07**) and 2 β -hydroxygypsogenin glycoside (**Chbhs-06**) possessed significantly high anti- α -glucosidase activity (44.1% and 30.0%) compared to acarbose (36.3%), respectively.

Acknowledgements

This work was supported by the Medicinal Science Council at Medical University of Sofia (grant number D-75/2019).