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Cholinesterase inhibitors isolated from bilberry fruit

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ABSTRACT

Cholinesterases (ChEs) are key enzymes in the pathogenesis of Alzheimer's disease (AD). A growing body of evidence suggests that plants deliver compounds able to inhibit ChEs (e.g., huperzine A, galanthamine, and physostigmine), thus playing a beneficial therapeutic role in the treatment of AD. Screening for cholinesterase inhibitors (ChEIs) in selected fruits and vegetables showed that extract prepared from bilberry fruit effectively inhibited the activity of acetyl- and butyrylcholinesterase. The purification of ChEIs from bilberry fruit followed by HPLC-UV, FT-IR, NMR, and LC-MS demonstrated that the studied compounds were derivatives of chlorogenic and benzoic acids. These results confirm that bilberry fruit may serve as a useful source of ChEIs, leading to the attenuation of memory deficit caused by AD.

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1. Introduction

Cholinesterases (ChEs) are key enzymes participating in the pathogenesis of Alzheimer's disease (AD). This neurodegenerative disorder can be characterized by pathological lesions of the central nervous system (CNS), such as extracellular senile plaques (SPs) formed by amyloid- β (A β), as well as intracellular neurofibrillary tangles (NFTs), which are aggregates of hyperphosphorylated tau (τ) protein (Castellani, Rolston, & Smith, 2010). Oxidative stress is also reported to

cause devastating changes in brain tissue (Butterfield, Reed, Newman, & Sultana, 2007). Elevated acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities (Rao, Sridhar, & Das, 2007) contribute to acetylcholine (ACh) deficits (Schliebs & Arendt, 2011). As a result, memory loss is observed. Additionally, there is evidence that ChEs contribute to the formation of SP and NFT as well as to an increment of the toxicity of A β deposits (Ciro, Park, Burkhard, Yan, & Geula, 2012).

Currently, cholinesterase inhibitors (ChEIs) are used for the elevation of ACh levels at cerebral cortex synapses.

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According to the US Food and Drug Administration, ChEIs rivastigmine, tacrine, donepezil, and galanthamine are approved for use at mild-to-moderate stages of AD (Contestabile, 2011). Natural ChEIs are rivastigmine (a derivative of physostigmine purified from the seeds of Calabar bean *Physostigma venenosum*), galanthamine (isolated from *Galanthus nivalis* bulbs), and huperzine A (purified from *Huperzia serrata*; Mukherjee, Kumar, Mal, & Houghton, 2007). However, ChEIs cause a number of side effects after long-term administration to patients, as has been discussed earlier (Alva & Cummings, 2008). Therefore, research on new ChEIs and new inhibitor-containing food is still necessary.

Anti-ChE activity has previously been detected in extracts from a considerable number of edible plants: e.g., *Malus domestica*, *Musa paradisiaca*, *Prunus persica*, *Anethum graveolens*, *Apium graveolens*, and *Solanum tuberosum* (Szwajgier & Borowiec, 2012b). In the past, AChE inhibitory activity was detected in other *Vaccinium* species: lowbush blueberry (*V. angustifolium*, wild blueberry; Papandreou et al., 2009), Caucasian whortleberry (*V. arctostaphylos*; Gholamhoseinian, Moradi, & Sharifi-Far, 2009), and *V. oldhamii* (also known as *V. ciliatum*; Lee, Lee, Yang, Baek, & Kim, 2004).

Given the role of oxidative stress in AD (Giasson, Ischiropoulos, Lee, & Trojanowski, 2002) as well as the results of our preliminary research, bilberry fruit was chosen for this study due to its antioxidant (Milivojevic et al., 2012) and anti-ChE (Szwajgier & Borowiec, 2012b) properties. The isolation of ChEIs from bilberry fruit followed by the characterization of the chemical structures of isolated compounds was performed.

2. Material and methods

2.1. Chemicals

AChE (from *Electrophorus electricus*), BChE (from equine serum), acetylthiocholine iodide (ATCh), *s*-butyrylthiocholine chloride (BTCh), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), eserine, methanol (CHROMASOLV, ≥99.9%), deuterium oxide (D_2O , 99.99 atom% D), methanol- d_4 (99.96 atom% D), methanol with 0.1% v/v formic acid (LC-MS CHROMASOLV), water with 0.1% v/v formic acid (LC-MS CHROMASOLV), and Tris-HCl buffer were purchased from Sigma-Aldrich (Poznań, Poland). Nitrogen was obtained from Linde (Lublin, Poland). Other reagents were of analytical grade and were purchased from P.O.Ch. (Gliwice, Poland).

2.2. Plant material

Test samples of fresh bilberry fruit (*Vaccinium myrtillus* L.) in harvest maturity were purchased at the local market and were immediately frozen (−20 °C) until use. Fruits were harvested in forests in Lublin province (51° 14' 29.3604" N, 22° 29' 47.9544" E). The identity of fruit samples was authenticated by Prof. Kazimierz Głowniak (Department of Pharmacognosy with Medicinal Plants Laboratory, Medical University of Lublin, Chodźki 1, Lublin, Poland).

2.3. Preparation of juice

Bilberry juice was obtained using a juice extractor (15 min, Thermomix TM31, Vorwerk, Germany) followed by centrifugation (30 min, 4 °C, $13,131 \times g$). The supernatant solution was ultrafiltered (4 °C, Vivaflow 50, 5 kDa PES membrane, Masterflex L/S Economy Drive pump, Cole-Parmer Instrument Co., Vernon Hills, IL, USA). The ultrafiltrate solution was freeze dried (−50 °C, Labconco FreeZone 2.5, Labconco, Fort Scott, KS, USA) and frozen (−20 °C) until use.

2.4. Determination of ChE inhibitory activities

Inhibition of ChE was evaluated in each fraction obtained at every stage of purification, based on the modified method of Ellman, Courtney, Andres, and Featherstone (1961). Daily prepared solutions of reagents were dissolved in Tris-HCl buffer (100 mmol, pH 8.0). The reaction mixture was composed of 0.035 mL of studied sample, 0.035 mL of ATCh or BTCh (1.5 mmol), 0.194 mL of DTNB (0.3 mmol DTNB containing 10 mmol NaCl and 2 mmol $MgCl_2 \cdot 6H_2O$), 0.020 mL of AChE or BChE solution (0.28 units/mL), and 0.086 mL of Tris-HCl buffer (100 mmol, pH 8.0). The absorbance (405 nm, 22 °C) was read after 15 min (BChE) or 30 min (AChE) using a 96-well microplate absorbance reader (microplate reader Sunrise, Tecan, Grödig, Austria). Blank samples containing either eserine (181.5 μmol) or Tris-HCl buffer instead of a studied sample were simultaneously examined as a positive and a negative control, respectively. A blank sample containing DTNB and ATCh (or BTCh) and completed to the final reaction volume with Tris-HCl buffer was used to control the spontaneous hydrolysis of the substrate.

The inhibitory activity of the studied sample was calculated using the calibration curves of eserine (0.00–5.34 mmol). The inhibitory activity was expressed in eserine equivalents (μmol Es), as proposed earlier (Szwajgier & Borowiec, 2012a, 2012b). The samples were analyzed in two or eight repeats.

2.5. Preparative HPLC

A BioLogic DuoFlow system (Bio-Rad, Hercules, CA, USA) consisting of two BioLogic pumps, a QuadTec UV-Vis detector, and a BioFrac fraction collector. A concentrated solution prepared from freeze-dried ultrafiltrate (5 mL, 0.5 g dry mass/mL) was injected into the column (Eurospher 100-5 C18, 250×20 mm, Knauer, Berlin, Germany). Various optimized gradients were tested in this study (for details, personal communication). The final gradient (10 mL/min) was composed of deionized water (A) and HPLC-grade methanol (B): 0–10 min 100% A, 10–14 min 20 → 30% B, 14–30 min 30% B, 30–33 min 30 → 90% B, 33–43 min 90% B, 43–51 min 90 → 0% B, 51–59 min 100% A. Detection was performed at 245, 280, 365, and 530 nm. All signals were analyzed using the BioLogic DuoFlow V.5.10 Build 2 program (Bio-Rad).

The AChE inhibitory activity was measured in each fraction (7 mL), as described earlier. This separation was repeated 87 times. Fractions containing inhibitors were combined and concentrated under a vacuum (approximately 200-fold to ~50 mL, 40 °C, −0.09 MPa), and frozen (−80 °C).

2.6. Acid hydrolysis

The acid hydrolysis of a combined fraction (obtained as described in Section 2.5., 50 mL) was performed. The reaction mixture consisted of 50 mL of the fraction, 200 mg of ascorbic acid, and 1.525 mL of 35–38% HCl (final HCl concentration: 1 mol). After incubation (1 h, 85 °C, water bath), the mixture was cooled down and frozen (24 h, –20 °C). Immediately before analysis, the thawed solution was filtered (syringe filters 0.45 µm, Merck Millipore, Darmstadt, Germany) followed by separations using analytical HPLC.

2.7. Analytical HPLC

A Knauer HPLC system consisted of two Smartline 100 pumps, a dynamic mixer, a 0.1 mL loop and Retriever 500 fraction collector. Detection (at 245 or 300 nm) was performed using a UV-Vis detector (Linear Instruments, Reno, NV, USA) coupled with an IF2 interface. The absorption spectra of the tested sample in UV-Vis (NanoDrop 2000c UV-VIS Spectrophotometer, Thermo Scientific, Waltham, MA, USA) were investigated in order to determine the optimal wavelength. For separations, a Hypersil ODS-2 column (250 mm × 4.6 mm, 5 µm, Thermo Scientific) was coupled with a LiChospher 100 RP18 column (250 mm × 4.0 mm, 5 µm, Macherey-Nagel, Düren, Germany) and corresponding precolumns (max. pressure 30 MPa). The optimized gradient (1 mL/min) was formed by deionized water (A) and 90% HPLC-grade methanol in deionized water (B): 0–10 min 100% A, 10–70 min 0 → 100% B, 70–76 min 100% B, 76–80 min 100 → 0% B, 80–95 min 100% A. Signals were analyzed using the Eurochrom 3.05 P5 program (Knauer). Fractions (typically from 0.005 to ~0.03 mL) corresponding to single peaks of compounds were collected manually during each separation, wherein an individual sample was injected into the system (approximately 140 times). The AChE inhibitory activity was evaluated in every fraction (peak), as described earlier. Fractions containing inhibitors (with the best purified fraction Bi) were freeze dried (–50 °C, Labconco FreeZone 2.5, Labconco) followed by FT-IR, NMR, and LC-MS.

2.8. Ion exchange

Ionic compounds were removed from fraction Bi using an AG 501-X8 mixed bed resin (Bio-Rad) following the producer's instructions. One gram of the resin was added to 20 mL of fraction Bi dissolved in deionized water (0.15 mg dry mass/mL) followed by stirring (24 h, 4 °C, magnetic mixer), decantation, filtration (syringe filters 0.45 µm, Merck Millipore), and drying under nitrogen.

2.9. FT-IR spectroscopy

FT-IR spectra were collected using Nicolet 6700 FT-IR Spectrometer (Thermo Scientific). The Smart iTR ATR sampling accessory was used. Fraction Bi was applied on ATR as freeze-dried powder. The spectra were collected over the range 4000–650 cm^{–1}. The sample was examined twice. Two hundred scans were averaged with a spectral resolution of 4 cm^{–1}. The final average spectrum was calculated and normalized for the sample. The baseline correction was obtained using Omnic Soft-

ware (Thermo Scientific) and the OriginPro 8.5 program (OriginLab Corp., Northampton, MA, USA).

2.10. NMR spectroscopy

NMR analyses were performed using a Bruker Avance 500 Spectrometer (Bruker, Rheinstetten, Germany) in D₂O and methanol-d₄ solutions. Due to the complex composition and low concentration of the Bi fraction (about 0.4 mg dry mass/mL) and even after a long acquisition time, only the most intensive signals were observed in ¹H-NMR, ¹³C-NMR, and DEPT-135 spectra. The recorded spectra were obtained using frequency 500.13 MHz, acquisition time 3.17 s, sweep width 10,330.26 Hz, receiver gain 228.00 in ¹H-NMR spectra; frequency 125.77 MHz, acquisition time 1.10 s, sweep width 29,761.00 Hz, receiver gain 2050.00 in ¹³C-NMR spectra; frequency 125.77 MHz, acquisition time 1.63 s, sweep width 20,160.68 Hz, receiver gain 2050.00 in DEPT-135 spectra.

2.11. LC-MS

LC-MS analysis of the Bi fraction was performed using a Shimadzu LC-MS-IT-TOF instrument with ES ionization (heat block and CDL temperature: 200 °C, argon gas flow: 1500 mL/min), connected to a Shimadzu Prominence chromatograph (Shimadzu, Kyoto, Japan) consisting of two LC-20AD XR pumps and an SPD-20A UV detector (254 and 354 nm). LC-MS grade solvents (containing 0.1% v/v formic acid) were deionized water (A) and methanol (B). The separations were performed for 15 min using isocratic elution with A:B 9:1 v/v (0.15 mL/min) and Kinetex C18 100A (100 mm × 2.1 mm, 2.6 µm, Phenomenex, Torrance, CA, USA). Signals were analyzed using Lab-Solutions LC-MS software (Shimadzu). The main components of the isolated fractions were characterized after the isolation of the precursor ions (pseudomolecular ions) in an ion trap and then collided with argon to induce the fragmentation. Also, the elemental composition of the isolated compounds was studied. Formula Predictor software (Shimadzu) or the ChemBioDraw program (PerkinElmer, Waltham, MA, USA) was used for chemical structure drawing and analysis.

2.12. Statistical analysis

The routine statistical tests were used (mean values, standard deviations; STATISTICA 8.0, StatSoft, Cracow, Poland).

3. Results

At first, compounds present in the freeze-dried ultrafiltrate from bilberry juice (molecular weight cutoff <5 kDa) were separated using the preparative HPLC (e.g., Fig. S1). Forty-eight fractions were obtained, among which 1–20 exhibited anti-AChE activity (0.43 ± 0.04–1.16 ± 0.04 µmol Es). After 87 repeats of the separation presented in Fig. S1, fractions 1–20 were combined and concentrated to obtain an inhibitory activity equal to 1.98 ± 0.04 µmol Es. The UV-Vis absorption of this sample revealed the presence of nonprotonated dienes (245 nm) or

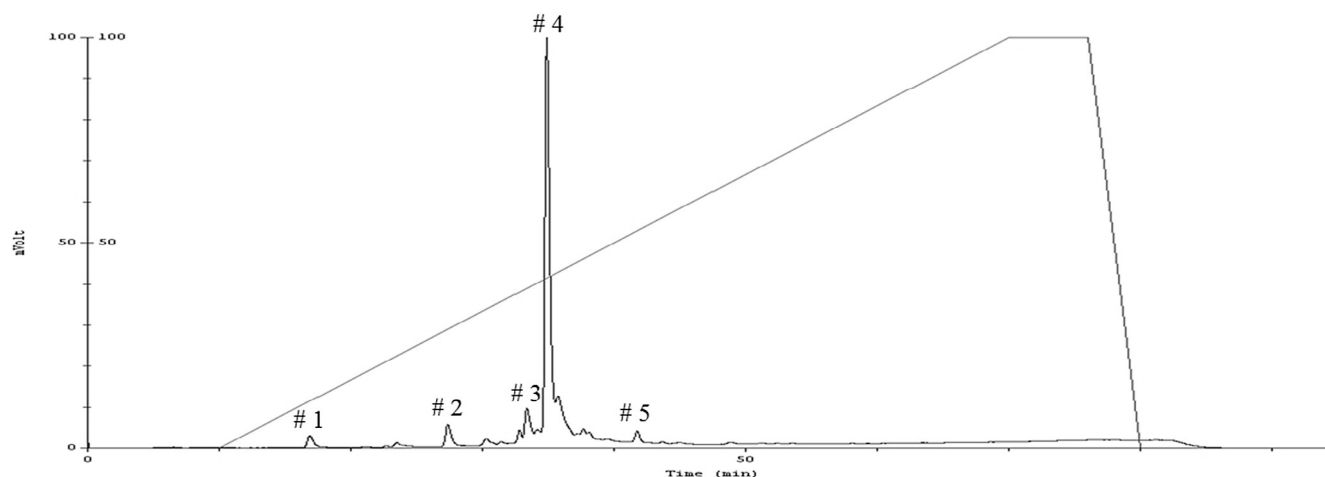


Fig. 1 – Analytical HPLC of Bi fraction (UV detection at 300 nm). Retention times of peaks: #1 (16.97 min), #2 (27.37 min), #3 (33.37 min), #4 (34.88 min), and #5 (41.75 min).

aromatics and polyalkylaromatics (280 nm; Jiang, Huang, Weitkamp, & Hunger, 2007).

The sample obtained after the combination of fractions 1–20 was very complex (Fig. S2a). For this reason, acid hydrolysis of the fraction was carried out reflecting the phenomenon occurring in the human digestive tract where glycosides undergo hydrolysis to aglycones (Stahl et al., 2002). After the hydrolysis, the separation of compounds was substantially improved (Fig. S2b and S2c). The anti-AChE activity was detected in the fraction representing a peak marked as Bi ($0.88 \pm 0.02 \mu\text{mol Es}$).

After 140 repeats of the separation presented in Fig. S2c, Bi fractions were combined and freeze dried to obtain 1 mg dry mass. It was evaluated that 1.5 kg of bilberry fruit was used in the study, and the approximate yield of fraction Bi was 0.066 mg/100 g of fresh fruit. The final Bi fraction (standardized to obtain 1 mg dry mass/mL) exhibited a high anti-AChE and anti-BChE activity (2.00 ± 0.08 and $1.50 \pm 0.02 \mu\text{mol Es}$, respectively). After the isolation, the Bi fraction was once more chromatographed in the same way (Fig. 1). The main peak (#4) as well as other minor peaks can be seen in this chromatogram.

The FT-IR spectrum of the Bi fraction (Fig. S3) revealed the presence of single (O—H, C—H, C—C, C—O) and double (C=O, C=C) bonds. The chemical band assignment suggests that com-

pounds in the Bi fraction may contain aromatic phenol rings and functional groups typical for alkanes, alcohols, or carbonyls (Table 1).

The NMR spectra were registered; however, despite the long acquisition time, only the most intensive signals were recorded. The ^{13}C -NMR signals obtained using methanol- d_4 were at 113.1, 115.9, 117.8, 124.0, and 146.9 ppm. The DEPT-135 spectrum obtained using D_2O revealed signals at 115.6, 117.2, 123.8, 149.4, 161.6, and 169.9 ppm. These results suggest the presence of —CH groups in the Bi fraction. Moreover, multiplets were registered in the ^1H -NMR spectrum with the use of methanol- d_4 (Fig. S4), suggesting the presence of sugars as well as vinyl, aliphatic, and aromatic compounds. The ^1H -NMR spectrum obtained in D_2O (Fig. S5) revealed the following multiplets, which were partially identified: 3.83 ppm (singlet), 6.33 ppm (doublet, $J = 16.1 \text{ Hz}$), 6.87 ppm (doublet, $J = 8.2 \text{ Hz}$), 7.43 ppm (doublet, $J = 11.7 \text{ Hz}$), and 7.55 ppm (doublet, $J = 16.1 \text{ Hz}$). The singlet at 3.83 ppm was assigned to the —COCH₃ group. Doublets at 6.33 and 7.55 ppm, as well as at 6.87 and 7.43 ppm, were assigned to two —CH=CH— groups. In both cases, vinyl or heteroaromatic protons were identified. Due to the multiplets in the aromatic region, it was suggested that a number of structurally similar chemical compounds containing sugars or aromatic fragments were in the Bi fraction.

Table 1 – Summary of FT-IR wavenumbers and characteristic band assignments recorded for the Bi fraction.

Wavenumber [cm^{-1}]	Group	Characteristics	
3307	O—H	Alcohol, phenol	H-bonded (broad and strong)
2916	C—H	Alkane	CH_2 (asymmetrical stretching)
1675	C=O	Ester and carbonyl	
1606	C=C	Aromatic	
1538	C—C aromatic conjuncted with C=C		
1443	C—H	Alkane	CH_3 (asymmetrical stretching), ester CH_3
1342			
1270	CO (stretching), OCH (scissoring), COOH		
1194	C—O	Alcohol, ester	
1066			C=O free acid group
1024			CO (stretching), CC (stretching), OCH (scissoring), ring
939	Ring	CCH (scissoring), COH (scissoring)	

After LC–MS analysis of sample Bi in isocratic mode, the HPLC (Fig. S6a) and the total ion chromatograms (TIC, Fig. S6b) were recorded. It was assumed that the benzene ring caused UV absorption at 254 nm (retention times 7.1–8.1 and 10.0 min; Kumar, 2006). The UV absorption at 354 nm (retention time 10.0 min) was probably caused by compounds containing a chromen fragment (Tsimogiannis, Samiotaki, Panayotou, & Oreopoulou, 2007). Taking into consideration both chromatograms (Fig. S6) and mass spectra of the Bi fraction, an attempt was made to identify the structure of potential ChE inhibitors.

Compound A. Retention times 6.6 and 7.9 min. After the insightful analysis of pseudomolecular ions (m/z): 267 $[M + H]^+$ and 289.1261 $[M + Na]^+$, the only possible formula $C_{11}H_{22}O_7$ (accuracy 1.04 ppm) was proposed. The fragmentation spectrum of this compound was not recorded. Nevertheless, it was assumed that the observed mass and formula could match the simple glycoside presented in Fig. 2a.

Compound B. Retention time 6.8 min. The following ions (m/z): 367.0990 $[M + Na]^+$ and 343.1020 $[M - H]^-$ were detected. The fragmentation spectrum of the anion at m/z 343.1020 $[M - H]^-$ yielded the 163.0451 Da ion. The proposed formula is $C_9H_7O_3$ (Fig. S7). The hydroxycinnamic anion can be proposed, with the molecular formula $C_{15}H_{20}O_9$ (accuracy: 3.27 and 4.37 ppm, respectively; Fig. 2b). This compound is most likely a glycoside of protonated caffeic acid. Unfortunately, further fragmentation was impossible.

Compound C. Retention time 7.1 min. The following ions (m/z): 339 $[M + Na]^+$, 317 $[M + H]^+$ (15%), and 315 $[M - H]^-$ were observed. The ions: 227 Da (10%), 153 Da (100%), and 109 Da (10%) were observed in the fragmentation pattern of ion m/z 315 $[M - H]^-$. The positive ion 155 Da was obtained after the fragmentation of ion m/z 317 $[M + H]^+$ (Fig. S8). Due to these results, the molecular formula $C_{13}H_{16}O_9$ was proposed. On the basis of the high intensity of fragmentation ions 153, 109, and 155 Da, the dihydroxybenzoate derivative (3-(β -D-glucopyranosyloxy)-4-hydroxybenzoic acid) was proposed (Fig. 2c).

Compound D. Retention time 7.5 min, detected ions at m/z : 404.1530 $[M + Na]^+$ (100%), 382.1694 $[M + H]^+$ (80%) and 380.1549 $[M - H]^-$. The proposed formula is $C_{15}H_{27}NO_{10}$ (accuracy: 5.44, 2.09, and 0.00 ppm, respectively). After the fragmentation of ion m/z 382.1694 $[M + H]^+$, ion 220.1198 Da ($C_9H_{18}NO_5^+$) was recorded. The fragmentation of the anion at m/z 380.1549 $[M - H]^-$ yielded ions: 362.1410 Da ($C_{15}H_{24}NO_9^-$) and 146.0858 Da ($C_6H_{12}NO_3^-$; Fig. S9). The proposed structure can be an isomer of methylacetamide-deoxy-O-fucopyranosyl-glucopyranoside (Fig. 2d).

Compound E. Retention time 7.5 min. The compound was characterized by ions at m/z : 337.0897 $[M + Na]^+$ and 313.0933 $[M - H]^-$. The proposed molecular formula was $C_{14}H_{18}O_8$ (with accuracy 0.89 and 1.28 ppm, respectively, in the case of both ions). The fragmentation of the anion at m/z 313.0933 $[M - H]^-$ yielded ion 151 Da. The possible compound could be glucovanillin or an isomer of cyanoneside (Fig. 2e). The observed fragmentation pattern matched both compounds closely (Fig. S10). Glucovanillin is also known as vanillin (or 4-hydroxy-3-methoxybenzaldehyde) glucoside. None of these compounds have been found in bilberry fruit yet. Nevertheless, it is known that bilberry fruit is a source of vanillic acid (Díaz-García, Obón, Castellar, Collado, & Alacid, 2013).

Compound F. Retention time 7.8 min. This compound was characterized by the following ions (low intensity): 397.1095 and 373.1149 Da. The nature of the concomitant inorganic positive ion was not determined. After the fragmentation, the positive ion 367 Da was obtained. The proposed formula is $C_{16}H_{22}O_{10}$ (accuracy: 2.52 and 2.41 ppm in the range of positive and negative ions, respectively), and the proposed compounds are geniposidic acid or 3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl 3,4,5-trimethoxybenzoate (Fig. 2f). Geniposidic acid is the main constituent in *Eucommia ulmoides* and can efficiently protect PC-12 cells against the cytotoxicity caused by $A\beta_{25-35}$ (Zhou et al., 2009). However, the ChE inhibitory activity of geniposidic acid has not yet been tested. Also, the presence of this compound in bilberry or any other plant belonging to the *Vaccinium* genus has not yet been reported.

Compound G. Retention time 8.1 min. The following pseudomolecular ions were observed (m/z): 331 $[M + H]^+$ (10%), 353 $[M + Na]^+$ (100%), 369 $[M + K]^+$ (40%) and 329 $[M - H]^-$ (100%), 375 $[M + HCO_2]^-$ (80%), 443 (30%), and 659 $[2M - H]^-$ (25%). Based on a precise assessment of the masses of ions (353.0845 and 329.0877 Da), the molecular formula $C_{14}H_{18}O_9$ was proposed (accuracy: 1.13 and 0.3 ppm for positive and negative ions, respectively). Since the fragmentation of the sodium adduct ion was uncertain, the fragmentation path of anion 329.0877 Da leading to ions 167.0387 Da (100%) and 152.0173 Da (15%) was analyzed. In the case of anion 167.0387 Da, a methoxy derivative of hydroxybenzoic acid $C_8H_7O_4$ (Fig. S11) can be proposed. Therefore, the tested compound may be an ester or ether of a methoxy-hydroxybenzoic acid bound to hexose ($C_8H_7O_3-O-C_6H_{11}O_5$). In contrast, it could also be the well-known 4-(β -D-glucopyranosyloxy)-3-methoxybenzoic acid (Fig. 2g), previously isolated from *Carthamus oxyacantha* (Johansen, Wubshet, Nyberg, & Jaroszewski, 2011). It is important to note that ion 353 Da was also eluted at 7.5 min. Therefore, it was suggested that more than one isomer of this compound was present in the Bi fraction.

Compound H. Retention time 10.0 min, m/z 355.1024 $[M + H]^+$. The molecular formula $C_{16}H_{18}O_9$ was proposed. After the fragmentation of the precursor ion, the following fragmentation ions were detected: 193.0671 Da (80%), 175.0600 Da (10%), 163.0390 Da (100%), 147.0593 Da (50%), 145.0300 Da (20%), and 135.0395 Da (trace). The molecular formula $C_{16}H_{18}O_9$ was confirmed by the precise measurement of the ion mass equal to 353.0888 Da $[M - H]^-$ followed by the fragmentation path: 191.0584 Da (100%; $C_7H_{11}O_6$, an anion of quinic acid), 179.0378 Da (30%; $C_9H_7O_4$, an anion of caffeic acid), 135.0501 Da (10%). Therefore, it was suggested that compound $C_{16}H_{18}O_9$ could be an isomer of chlorogenic acid (e.g., 4-O-caffeoylquinic acid or 5-O-caffeoylquinic acid; Fig. 2h). This result was supported by the NMR spectra (Figs. S5 and S12), which showed signals derived from $-CH$ groups of caffeic acid. The result of the fragmentation of the real HPLC standard of chlorogenic acid was similar to that observed after the fragmentation of the studied ion 355 Da. Indeed, the following fragmentation ions were obtained: 163, 145, and 135 Da (Fig. S13). However, during the presented comparison of the unknown compound with the standard of chlorogenic acid, different retention times were recorded. For this reason, we assume that the isolated compound was not chlorogenic acid.

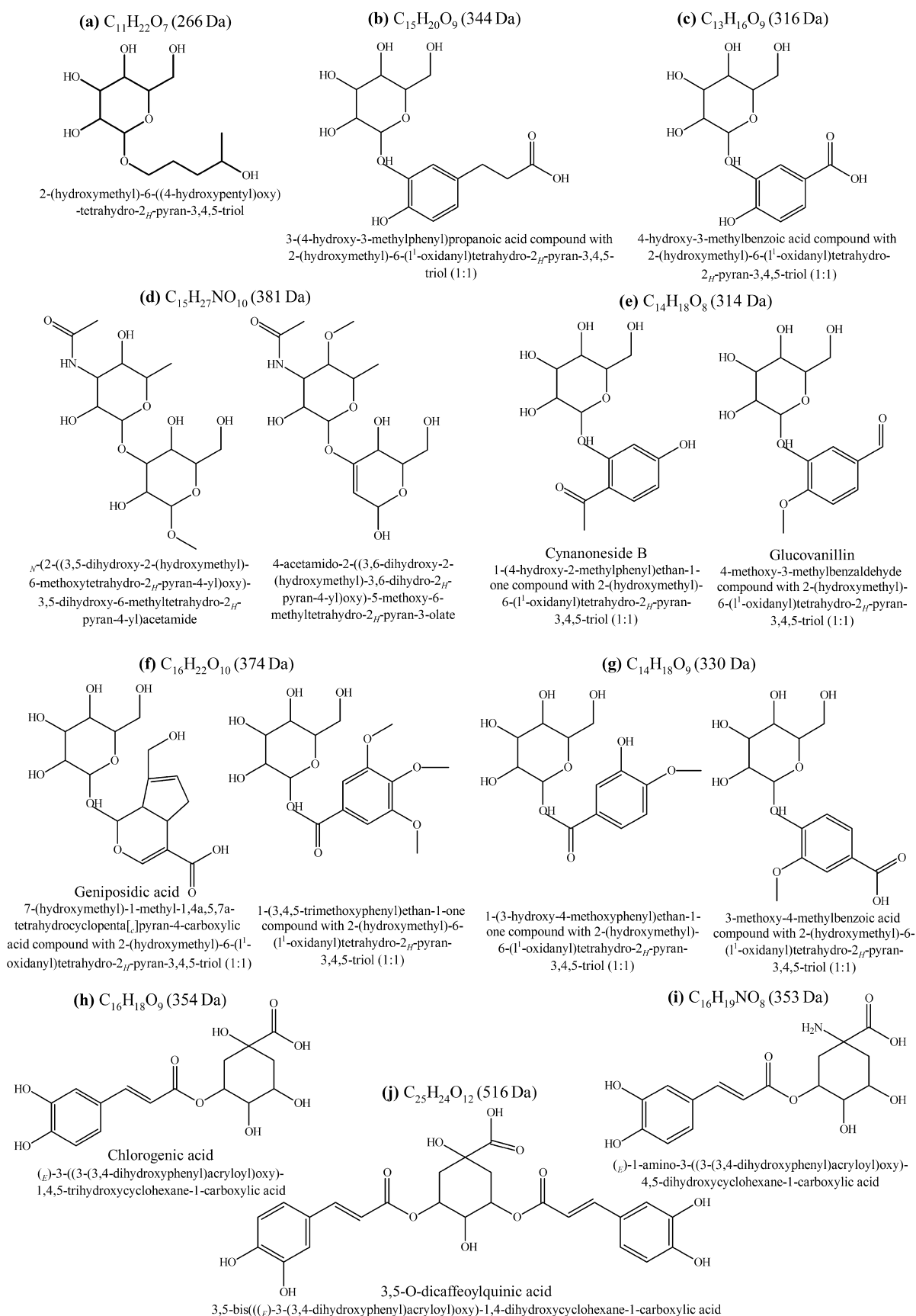


Fig. 2 – The structural formulas of $C_{11}H_{22}O_7$ (a), $C_{15}H_{20}O_9$ (b), $C_{13}H_{16}O_9$ (c), $C_{15}H_{27}NO_{10}$ (d), $C_{14}H_{18}O_8$ (e), $C_{16}H_{22}O_{10}$ (f), $C_{14}H_{18}O_9$ (g), $C_{16}H_{18}O_9$ (h), $C_{16}H_{19}NO_8$ (i), and $C_{25}H_{24}O_{12}$ (j).

Compounds I and J. The following pseudomolecular ions of the compounds eluted at 10.2 and 14.7 min were found (m/z): 354.1187 $[M + H]^+$, 376 $[M + Na]^+$, and 352 $[M - H]^-$. Therefore, the molecular formula $C_{16}H_{19}NO_8$ (accuracy 1.13 ppm) was proposed (Fig. 2i). Additionally, compounds eluted at 11.1 and 12.6 min were characterized by the pseudomolecular ion pairs, 354 and 516 Da (positive ions) and 353 and 514 Da (negative ions), and ion 538 Da $[M + Na]^+$ (low intensity). Therefore, the molecular formula $C_{22}H_{29}NO_{13}$ was proposed. The presence of hexose could reflect the difference in the masses of the pairs of ions (354 versus 516 Da, 353 versus 514 Da). Thus, it was assumed that compound $C_{22}H_{29}NO_{13}$ could be a glycoside of compound $C_{16}H_{19}NO_8$. After the further fragmentation of anion 514 Da and positive ion 354 Da, the following fragmentation ions were obtained: 470 Da ($-CO_2$), 230 Da, 218 Da, and 179 Da and ions 192 and 146 Da, respectively. The studied compound(s) could be an (the) amine derivative(s) of chlorogenic acid in its glycosidic form. The fragmentation ions could be decarboxylated glucoside (470 Da), a dihydroxycinnamate anion (179 Da), a protonated derivative of decarboxylated aminoquinic acid (146 Da), and a protonated derivative of aminoquinic acid (192 Da; Fig. S14). Compound 515 Da could be a derivative of dicaffeoylquinic acid (e.g., 3,5-O-dicaffeoylquinic acid, MW = 516.45 g/mol; Fig. 2j), which has already been detected in bilberry fruit (Rieger, Müller, Guttenberger, & Bucar, 2008).

4. Discussion

The presented results confirm that bilberry fruit is an interesting source of ChEIs, including derivatives of chlorogenic and benzoic acids, purified within the framework of this study. The presence of phenolic acids in bilberry fruit is well documented (e.g., Ochmian, Oszmiański, & Skupień, 2009). The exact determination of the structural formulas of some studied compounds was not possible, but functional groups were well characterized. The presence of at least one $-OH$ group substituted in the phenol ring in *meta*- or *para*- position was considered. The presence of a $-NH_2$ group (at position C1 in quinic acid) and/or a hexose (*meta*- substitution in the phenol ring) was confirmed in the chlorogenic acid derivatives. A hexose as well as a $-OCH_3$ group (in the *meta*- or *para*- position of the phenol ring) was probably present in the derivatives of benzoic acid. Due to the different retention times of chlorogenic acid and its isomer found in the Bi fraction, it seems that the position of functional groups in a molecule is essential for ChE inhibitory activity.

The inhibition of ChE by phenolic acids (including chlorogenic acid as well as 3- or 4-hydroxybenzoic acids) has already been reported (Szwajgier, 2013; Szwajgier & Borowiec, 2012a). It was demonstrated that the presence of a $-OH$ group and/or a $-OCH_3$ group in the phenol ring increased the anti-ChE activity. Moreover, the methyl or ethyl esters of phenolic acids were more effective ChEIs than the corresponding free phenolic acids. Additionally, it was shown that chlorogenic acid exhibited higher anti-ChE activity (especially anti-BChE) than caffeic acid (Szwajgier, 2013). Kwon et al. (2010) demonstrated that the administration of chlorogenic acid to mice (3,

6, or 9 mg/kg, per os) inhibited, ex vivo, the AChE activity in the hippocampus and the frontal cortex as well as scopolamine-induced memory impairment (examined using a few behavioral tests). On the other hand, Orhan, Kartal, Tosun, and Sener (2007) reported on the inhibition of BChE by chlorogenic acid, but free caffeic and quinic acids were inefficient in this context. Akhtar et al. (2011) showed that anti-AChE activity could be due to the presence of a $-OH$ group in *ortho*- position in the phenol ring, but the methylation was not efficient. Katalinić et al. (2010) indicated that BChE inhibitory activity increased proportionally with an increasing number of $-OH$ groups in the phenol ring of flavonoids, wherein the position of $-OH$ groups was important in this context. Also, the methylation of the phenol ring in *para*- position contributed to increased anti-AChE activity as well as exerting a general neuroprotective action (Uriarte-Pueyo & Calvo, 2010).

The preparative and analytical HPLC used in this study led to sufficient purification of the Bi fraction for structure elucidation. Previously, the LC-MS method coupled with biochemical detection was applied for the rapid online analysis of the anti-AChE activity of complex biological matrices (de Jong, Derks, Bruneel, Niessen, & Irth, 2006). Rhee et al. (2004) separated ChEI from *Nerine bowdenii* using two-step HPLC (preparative scale with μ -Bondapak C18 and analytical scale with Lichrospher 60 RP).

In the present study, acid hydrolysis was carried out in order to remove nonphenolic parts. However, the structure elucidation indicated that glycosidic bonds were still in the Bi fraction. It is worth mentioning that the ChE inhibitory activity of phenolic glycosides has previously been reported. Hillhouse, Ming, French, and Towers (2004) showed that the flavonoid glycosides isolated from *Rhodiola rosea* (gossypetin-7-O-L-rhamnopyranoside and rhodioflavonoside) were effective AChE inhibitors. Rollinger, Hornick, Langer, Stuppner, and Prast (2004) reported that scopoletin (coumarin), as well as scopolin (glucoside), could be used as potential AChE inhibitors that increase the extracellular ACh concentration in rat brains. On the other hand, the fitting of a flavonoid molecule to the active site of BChE was limited by a sugar moiety. As a result, the inhibitory activity of flavonoid glycosides was lower in comparison with that of aglycones (Katalinić et al., 2010). In another work (Uriarte-Pueyo & Calvo, 2010), it was demonstrated that the mono-acetylation of the sugar moiety in flavones from *Galeopsis ladanum* L. (Lamiaceae) contributed to the increase of the anti-AChE activity of these compounds.

It should be stressed that phenolic glycosides are effectively hydrolyzed in the human organism, but phenolic glucosides can also be absorbed from the small intestine without hydrolysis (Hollman, 2004). Additionally, the hydrolysis of phenolic acid esters (e.g., chlorogenic acid) is observed in the digestive tract as a result of the activity of bacterial esterases (Couteau, McCartney, Gibson, Williamson, & Faulds, 2001).

Although phenolic acids (e.g., 5-O-caffeoylquinic acid or 4-hydroxybenzoic acid) have been shown to have effects on CNS in mice after ingestion (Ohnishi et al., 2006), their permeation through the blood-brain barrier has never been proven (Diniz et al., 2007). Lardeau and Poquet (2013) suggested that 5-O-caffeoylquinic acid cannot be considered promising candidate for an entry in the brain and for a direct effect on CNS.

However, an indirect protective effect of these compounds on the brain cannot be excluded (Lee et al., 2012). Therefore, further studies will be required to investigate this issue or to develop other methods of administration of active compounds.

5. Conclusions

Our results confirm that bilberry fruit is an interesting source of AChE and BChE inhibitors. Selected compounds possessing the inhibitory activity were purified followed by structure elucidation, namely the derivatives of chlorogenic acid (e.g., 4-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, or 3,5-O-dicaffeoylquinic acid) and benzoic acid (e.g., 3-(β -D-glucopyranosyloxy)-4-hydroxybenzoic acid or 4-(β -D-glucopyranosyloxy)-3-methoxybenzoic acid). Our study is the first to characterize ChE inhibitors from bilberry fruit in detail. However, further studies are required to confirm the established molecular formulas and structures of compounds. In our opinion, both preparative and analytical-scale HPLC should be improved as critical stages of the purification procedure.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2014.10.008.

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