ANTICHOLINESTERASE AND ANTIOXIDANT ACTIVITIES OF COMMERCIAL PREPARATIONS FROM *Ginkgo biloba* LEAVES

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Abstract. *Ginkgo biloba* preparations from leaves are widely used for the treatment of mild cognitive dysfunctions. This work compared thirteen commercial dietary supplements with fresh *G. biloba* leaves. Anticholinesterase activities and the levels of total phenolics were studied using corresponding spectrophotometric methods. Antioxidant activities were tested using ABTS and DPPH free radicals. Phenolic acids and quercetin contents were determined using HPLC-DAD. *G. biloba* preparations more effectively inhibited the activity of butyrylcholinesterase than acetylcholinesterase with significant (p < 0.05) differences between preparations. Selected preparations had both the highest content of total phenolics and the antioxidant activity (with ABTS and/or DPPH) whereas in the case of other samples, adverse results were obtained. Significant (p < 0.05) differences in the quercetin content were seen between individual preparations. Gallic, protocatechuic, syringic, 4-OH-benzoic, chlorogenic, caffeic, sinapic, ferulic, 4-OH-cinnamic and o-coumaric acids were detected in studied samples. The preliminary characterization of acetyl- and butyrylcholinesterase inhibitors from *G. biloba* with Sep-Pak C18 and polyvinylpolypyrrolidone revealed that these compounds are phenolics, although non-phenolics exhibiting the inhibitory activity were present in the leaves. The study aiming the purification of cholinesterase inhibitors from *G. biloba* is in progress.

Key words: antioxidant activity, phenolics, acetylcholinesterase, butyrylcholinesterase, inhibitors

INTRODUCTION

The efficiency of *Ginkgo biloba* preparations for the treatment of mild cases of memory impairment has been appreciated worldwide. This herb is usually used in the form of ethanol or ethanol/water extracts or in a solid form (tablets, capsules). There is

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a great number of *in vitro* studies (also those involving cell lines) that confirm the positive activity of *Ginkgo biloba* constituents. However, the definitive significance in this matter have the studies involving groups of healthy or impaired patients who received *G. biloba* preparations for a longer period of time. Indeed, scientific databases provide a sufficient number of decent papers in this field. Standardized extract Egb 761 is one of the most recognized *G. biloba* preparation in the market. As it was shown in the past, this preparation was very effective during the treatment of dementia of Alzheimer's type (DAT) in the double-blind, randomized, placebo controlled parallel experiment involving a group of 20 patients. The memory improvement in the studied group was observed after 3 months of the trial while the deterioration was seen in the placebo group [Maurer et al. 1997]. In another study, the delay in the loss of capacities needed to cope with the demands of daily living (measured using Geriatric Evaluation of Relative's Rating Instrument) by the patients with dementia was observed. After 26 and 52 weeks of the treatment with Egb 761, this delay was considerable and ranged from 10 to 21 months, respectively. In DAT patients, the estimated delay was 16 and 25 months, respectively [Haan and Hörr 2004]. Numerous positive effects of Egb 761 on patients with probable Alzheimer's disease (AD) as well as possible AD combined with cerebrovascular disease or vascular dementia were pointed out in some other randomised, double-blind works, as pointed out e.g. by Scripnikov et al. [2007]. Egb 761 effectively improved numerous measures of multiple sclerosis (fatique, symptom severity, functionality) in humans [Johnson et al. 2006]. The same preparation also abolished cognitive deficits (caused by the chronic stress) in rats leading to normal levels [Walesiuk et al. 2006] and improved learning deficits, cognition and compensated deficits in the cerebral metabolism in these animals [Hoyer et al. 1999]. In another study (double-blind, randomized, placebo-controlled, multi-dose, balanced, crossover design), 20 healthy young volunteers consumed standardized *G. biloba* extract GK501. The improvement of the dose-dependent "speed of attention" factor, the quality of memory factor as well as the speed of memory factor after the consumption of variable doses of GK501 were observed [Kennedy et al. 2000].

The charactersitic feature of AD is the loss of cholinergic neurons responsible for memory and cognition. Also, the imbalance in the butyrylcholinesterase (BChE) and acetylcholinesterase (AChE) activity was reported as early as in 1981 by Whitehouse et al. and, thereafter, by many other authors. The application of cholinesterase inhibitors for the slowing-down of the development of AD (by the decrement of AChE and BChE activity) has been investigated since the 1980s. Until today, this is the only method approved for the treatment of AD in the world. However, numerous authors, as thoroughly reviewed by Sabbagh [2006], have reported a number of side effects after the long term administration of synthetic cholinesterase inhibitors to patients (donepezil, rivastigmine and galantamine). Therefore, the search for new inhibitors as well as new inhibitor-rich food raw materials is a priority. *Ginkgo biloba* preparations are leading over-the counter herbal preparations in the world and there is a considrable number of *G. biloba* preparations available on the market. However, the quality of these preparations is not always verified. The aim of this work was the comparison of anticholinesterase and antioxidant activities of the preparations available in Poland. The preliminary attempt to identify main groups of cholinesterase inhibitors in the selected *G. biloba* preparation was made.

MATERIALS AND METHODS

Collection of samples. Thirteen commercial products containing extract or dry extract of *G. biloba* leaves, registered in Poland as pharmaceuticals or dietary supplements (tab. 1) were tested in this study. All products were available without prescription and were obtained on the local market (pharmacies). *G. biloba* leaves studied in this survey were collected in three locations: at the Maria Curie-Skłodowska University botanical garden (Lublin, N 51.26216°, E 22.51613°), near Kopernika street (Bełżyce, N 51.17608°, E 22.26770°) and Skrzetuskiego street (Lublin, N 51.23912°, E 22.51622°). The identity of plant samples was authenticated by prof. dr hab. Kazimierz Głowniak from the Department of Pharmacognosy with Medicinal Plants Laboratory, Medical University of Lublin, Chodzki 1, Lublin, Poland. The voucher specimens are deposited in the Department of Biotechnology, Human Nutrition and Science of Food Commodities, University of Natural Sciences, Skromna street 8, 20-704 Lublin, Poland. Leaves were frozen at -20ºC until use.

Table 1. *Ginkgo biloba* preparations from leaves studied in this work

Preparation[a]	Market form	*G. biloba* content in the tablet (capsule) (mg)	Dose proposed by the producer (mg)
A	capsules	40	120
B	capsules	80	160–240
C	capsules	60	60 –120
D	tablets	50	150–300
E	tablets	60	60–120
F	tablets	40	120
G	tablets	80	160–240
H	capsules	80	80–240
I	tablets	40	40–80
J	tablets	60	120–80
K	capsules	60	60–120
L	tablets	35	70
M	tincture	–	15–30 ml·day⁻¹

[a] names and detailed compositions of preparations are available from authors

Reagents and chromatographic media. AChE, BChE, acetylthiocholine iodide (ATChI), S-butyrylthiocholine chloride (BTCh), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), eserine, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), potassium persulphate, 6-hydroxy-2,5,7,8-tetrametylchroman-2-carboxylic acid (Trolox®), HPLC standards of phenolic acids and quercetin, PVPP and Sephadex® LH-20 were purchased from Sigma-Aldrich (USA). 2,2-diphenyl-1-picrylhydrazyl

(DPPH) was purchased from ICN Biomedicals Inc., Aurora (USA). Sep-Pak C18 cartridges were purchased from Waters (Ireland). Chromatography-grade eluents, Folin-Ciocalteu reagent and other chemicals were purchased from P.O.Ch. (Gliwice, Poland).

Determination of dry mass (d.m.). The accurately weighted portion of 0.5–1 g of the sample (in the case of preparation M approx. 1 ml) was finely ground in the mortar and dried at 103 ±2°C for 16 h. The sample was then weighted and dried again until the loss of weight was less than 0.005 g (typically 17–18 h). Weighting of the samples was performed with the precision of 0.001 g. The analysis was performed in triplicate.

Inhibition of AChE and BChE. The portion equal to 0.75 g of the d.m. of the sample was finely ground with 5 ml of Tris-HCl buffer (50 mM $\cdot$ L^{-1}, pH 8.0). The sample was then transferred to Eppendorf tubes and centrifuged (9300g, 20 min, room temperature). In this way, each studied sample was standardized in order to obtain 150 mg of d.m. $\cdot$ ml^{-1}. The preparation M (Tinctura) was dried in order to remove ethanol and the solids were dissolved in Tris-HCl buffer. Enzyme activities were measured using Ellman's method [Ellman et al. 1961] with some modifications described in detail previously [Szwajgier and Borowiec 2012a]. Daily prepared solutions of reagents were dissolved in Tris-HCl buffer (50 mmol$\cdot$$L^{-1}$, pH 8.0). The reaction mixture consisted of: 1 ml of 0.3 mmol $\cdot$ L^{-1} DTNB (containing 10 mmol $\cdot$ L^{-1} NaCl and 2 mmol $\cdot$ L^{-1} $MgCl_2$ $\cdot$ 6 H_2O), 0.2 ml of ATChI or BTCh (1.5 mmol $\cdot$ L^{-1}), 0.575 ml of Tris-HCl buffer (50 mmol $\cdot$ L^{-1}, pH 8.0), 0.025 ml of AChE or BChE solution (0.28 units $\cdot$ ml^{-1}) and 0.2 ml of the studied sample. The temperature during the measurement (405 nm) was 22°C. The absorbance was read after 20 min (BChE) or 60 min (AChE). Blank samples containing either eserine (90.7 μmol $\cdot$ L^{-1}) or Tris-HCl buffer instead of the studied sample were also run. The increase of the absorbance due to the spontaneous hydrolysis of the substrate was monitored using blanks containing DTNB and ATChI (BTCh) completed to the final reaction volume with Tris-HCl buffer. The inhibitory activity was calculated using the calibration curves prepared using eserine at 0.09 μmol $\cdot$ L^{-1}– 6.10 μmol $\cdot$ L^{-1} (AChE) and 0.09 μmol $\cdot$ L^{-1}– 8.57 μmol $\cdot$ L^{-1} (BChE). Each sample was analyzed in eight repeats.

Preparation of water and ethanol extracts for antioxidant activity determination. *Water extract:* the sample (100 mg of d.m.) was flushed with 5 ml of double deionized (DDI) water (85°C) and incubated in water bath (85°C) for 5 min followed by cooling to 20°C. The preparation M (Tinctura) was vacuum concentrated (35°C, -0.09 MPa) and freeze-dried (FreeZone 2.5 system, Labconco, USA) in order to remove ethanol followed by dissolving in DDI water. Ethanol (EtOH) extract: the sample (100 mg of d.m.) was extracted using 5 ml of EtOH : water solution (96:4 v/v) and left overnight at 4°C. Then, the sample was centrifuged (13 400 g, 10 min, 4°C) and the supernatant was used for studies. Where applicable, preparation M (Tinctura) was vacuum concentrated (35°C, -0.09 MPa) in order to increase the d.m. content prior analyses.

Antioxidant activity (ABTS). The analysis was performed as described by Miller et al. [1993] with some modifications. A solution containing ABTS (7 mmol $\cdot$ L^{-1}) and potassium persulphate (2.45 mmol $\cdot$ L^{-1}) in DDI water was prepared and left at ambient temperature for 24 h. Directly prior to analysis, the absorbance of this solution (734 nm) was adjusted to 0.70 ±0.02. The volume of reagents in the spectrophotometric cuvette was: 1.5 ml of ABTS solution and 0.15 ml of the sample. Changes in the absorbance (20°C) at 734 nm were measured every 10 s for 2 min. For each sample, measurements

were repeated at least four times. The total antiradical activity was calculated using the calibration curve obtained with a series of Trolox solutions (0.1–1 mmol · L^{-1}) and expressed as TEAC (Trolox Equivalent Antioxidant Capacity).

Antiradical activity (DPPH). The method of Brand-Williams et al. [1995] was used with some minor modifications. In short, a volume of 0.05 ml of the studied extract was mixed with 1.95 ml of DPPH solution in 96% (v/v) methanol (0.06 mmol · L^{-1}). Absorbance was read every 10 s for 2 min at 515 nm. The antioxidant activity was calculated using Trolox as essentially described above. Blanks were run followed by the subtraction of background absorbances from studied samples. All samples were run in four repeats.

Total phenolic content. The sample (500 mg of d.m.) was left overnight at 4°C with 5 ml of EtOH : water solution (7:3 v/v). The sample was then centrifuged (13 400 g, 30 min, 4°C) and proteins were removed from the supernatant by mixing with the same volume of acetonitrile (ACN), centrifugation (15 700 g, 30 min, 4°C), followed by another mixing with ACN and centrifugation in the same manner. The sample M (Tinctura) was studied without any previous preparation. The analysis was performed using a modified method of Folin and Ciocalteu [1927]. After pH adjustment to 5.2, 20 μL of the sample was mixed with 500 μL of the Folin–Ciocalteu reagent (0.2 mol · L^{-1}) and 1.080 ml of DDI water. After 1 min (at room temperature), 1.5 ml of a 20% (v/v) sodium carbonate water solution was added. After vortexing, the mixture was left for 2 h in darkness and the absorbance was measured at 760 nm against the blank sample. The calibration curve was prepared using standard solutions of gallic acid in the range of 0.1–2.0 mg · ml^{-1}.

HPLC analysis of quercetin. The sample (100 mg of d.m.) was mixed with 5 ml of EtOH : water solution (70 : 30 v/v) and left overnight at 4°C (optimization of the quercetin extraction not presented, personal communucation). Samples were then transferred to Eppendorf tubes and centrifuged (14 000 g, 20 min, 4°C). The sample M (Tinctura) was studied without any previous preparation. Directly prior HPLC, every sample was filtered using Millipore filters (0.45 μm). The HPLC system consisted of two Gilson 306 Separation Module piston pumps, Gilson PhotoDiode Array Detector 170, Gilson loop (20 μl), manometric module Gilson 805 and dynamic mixer 811C. Waters Symmetry C18 column (USA, 250 mm, 4.6 mm i.d., 5 μm), coupled with Waters Symmetry C18 precolumn (5 μm, 8 × 20 mm) was used for separations. Eluents were as follows: eluent A – 1% (v/v) acetic acid in DDI water, eluent B – 50% HPLC-grade ACN in DDI water. Signals were monitored at 370 nm (the main wavelength) in the range of 200–600 nm. The time-eluent program was elaborated in our laboratory and was as follows: 0–10 min 92% A, 8% B; 10–55 min 92%→0% A, 8→100% B; 55–70 min 0→92% A, 100→8% B. The eluent flow was 0.8 ml · min^{-1} (2050 p.s.i.).

Mild alkaline hydrolysis and extraction of total phenolic acids. The content of total phenolic acids was determined after the alkaline hydrolysis. Hydrolysis was performed according to Nardini and Ghiselli [2003] with slight modifications. The sample (0.2 g of d.m. or 0.5 ml of sample M) was sonicated (Sonics Vibra Cell, at 70% amplitude/50 sec pulses and 5 sec off, total time 20 min) with NaOH solution (0.8 mol · L^{-1}, 2 ml). The sample was then purged for 5 min using CO_2 and left in the darkness for 24 h at the ambient temperature. After hydrolysis, the pH of the samples was adjusted to 1.0

using HCL solution (2 mol · L^{-1}) and the sample was extracted three times, each time using a fresh portion of ethyl acetate (5 ml). The organic part was evaporated to dryness (40°C, -0.09 MPa) and the residue was dissolved in methanol (1 ml). The sample was filtered using Millipore filters (0.45 μm) and directly analyzed by HPLC-DAD. Every sample was extracted twice. The within-one-day repeatability of HPLC method for HPLC standards of phenolic acids (gallic, protocatechuic, syringic, 4-OH-benzoic, chlorogenic, caffeic, sinapic, ferulic, 4-OH-cinnamic and 2-OH-cinnamic acid) tests were below 6% and were satisfactory. Also, in the reproducibility of the HPLC method, the coefficients of variation were below 8% and 7% (tested using ferulic and p-coumaric acid, respectively). The recoveries of phenolic acids subjected to the alkaline hydrolysis were performed in our laboratory and they were in the range of 87–104%. These recoveries were taken under consideration during the recalculation of results. In results, the sum of free phenolic acids (free + released due to the alkaline hydrolysis) was presented.

HPLC analysis of free phenolic acids. The quantitative analysis of free phenolic acids was performed in extracts prepared using the following extraction solutions: EtOH : DDI water (98:2 v/v), EtOH : DDI water (70:30 v/v) or DDI water. Each time, the finely ground sample (100 mg of d.m.) was flushed using 5 ml of the extraction solution and left overnight at 4°C. Then, the sample was centrifuged (13 400 g, 10 min, 4°C) and the supernatant was used for studies. Prior the injection, the sample was filtered using Millipore filters (0.45 μm). Gilson HPLC system was used (as described above) and the method of Kim et al. [2006] was adopted for this study. Eluents were as follows: eluent A – 1% (w/v) acetic acid in DDI water, eluent B – 50% HPLC-grade ACN in DDI water. The eluent program was as follows: START 92% A, 8% B 0–10 min; 70% A, 30% B 10–40 min; 60% A, 40% B 40–55 min; 92% A, 8% B 55–70 min. Signals were monitored at 320 nm, 280 nm, 260 nm and 360 nm and in the scanning mode (200–600 nm). In results, the sum of individual free phenolic acids in samples was presented.

Fractionation using Sep-Pak C18. Sep-Pak C18 cartridge was flushed with 1 ml of acidified methanol (pH 2.0–3.0) followed by 5 ml of acidified DDI water (pH 2.0–3.0) which was then removed from the cartridge. The similar water extract from *G. biloba* preparation H, as used for the determination of the anticholinesterase activity, was purified in Sep-Pak (see above). The volume of 0.5 ml of the extract was loaded onto the column followed by flushing with acidified DDI water (5 ml) and methanol 98% (v/v) (10 ml). Both fractions were collected separately. The water fraction was filled up to 10 ml using Tris-HCl buffer (pH 8.0). In methanol fraction, the solvent was completely evaporated (30°C, -0.09 MPa) and solids were dissolved in 10 ml of Tris-HCl buffer (pH 8.0). Simultaneously, the dilution of the raw (non-treated) extract in DDI water, equal to the final dilution after Sep-Pak C18 purifications, was made. Therefore, the efficiency of the purification procedure could be evaluated. The three samples were analyzed for the anti-ChE activities in eight repeats. The whole separation procedure was performed twice.

Fractionation using PVPP. The similar water extract from *G. biloba* preparation H, as used for the determination of the anticholinesterase activity, was used in this analysis. PVPP (150 mg) was added to the extract (5 ml) followed by chilling (4°C, 30 min) and centrifugation (9300 g, 10 min, 4°C). The anti-AChE and anti-BChE activity of the

supernatant was tested in eight repeats as described earlier. The whole fractionation procedure was performed twice.

Statistical analysis. Routine statistical tests were used and all presented results are means with standard deviations. Differences between results were calculated using Tukey's HSD test (STATISTICA 8.0, StatSoft, Poland) and were considered significant at $p < 0.05$. Different superscript letters denote a significant difference at $p < 0.05$.

RESULTS AND DISCUSSION

Anticholinesterase activities of *G. biloba* preparations are presented in fig. 1 and 2. It can be noticed that preparations much more effectively inhibited BChE than AChE. Both enzymes exhibit 65% amino-acid homology and they are characterized by the similar molecular form as well as the active site [Allderdice et al. 1991]. However, the characteristic features of AChE are the presence of serine (Ser 200), glutamate (Glu 327) and histidine (His 440) in the active site as well as the presence of anionic residues (Asp 443 i Glu 199) in the direct neighbourhood leading to the active site. Therefore, the differences in the inhibition of both enzymes can be observed in herein presented as well in other works [e.g. Giacobini 2003]. More importantly, significant ($p < 0.05$) differences of the inhibitory activity of individual enzymes could be observed. The anti-AChE activities were equal to 0.38 ± 0.05–1.66 ± 0.22 µg of eserine · ml^{-1} whereas anti-BChE activities were equal to 0.87 ± 0.38–8.01 ± 1.26 µg of eserine · ml^{-1}. It was pointed out in the past that different types of dementia and brain dysfunctions

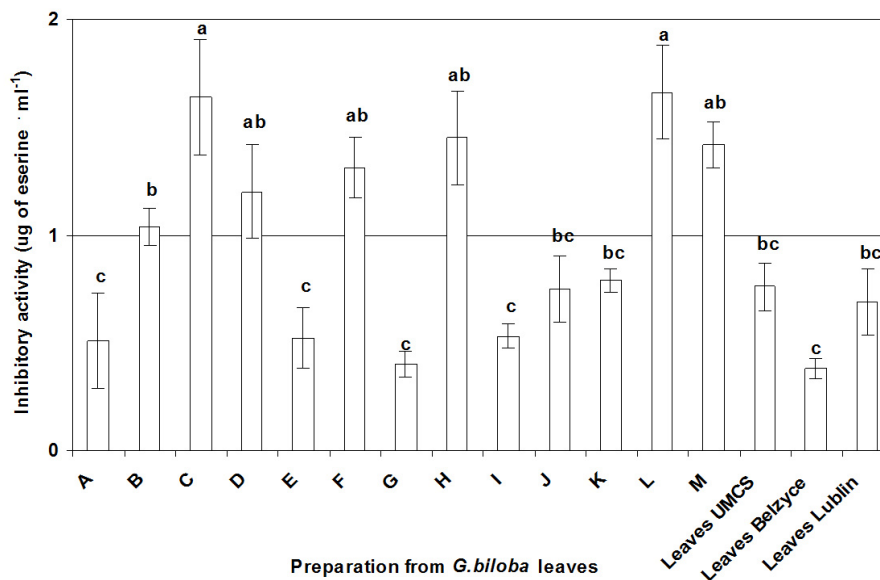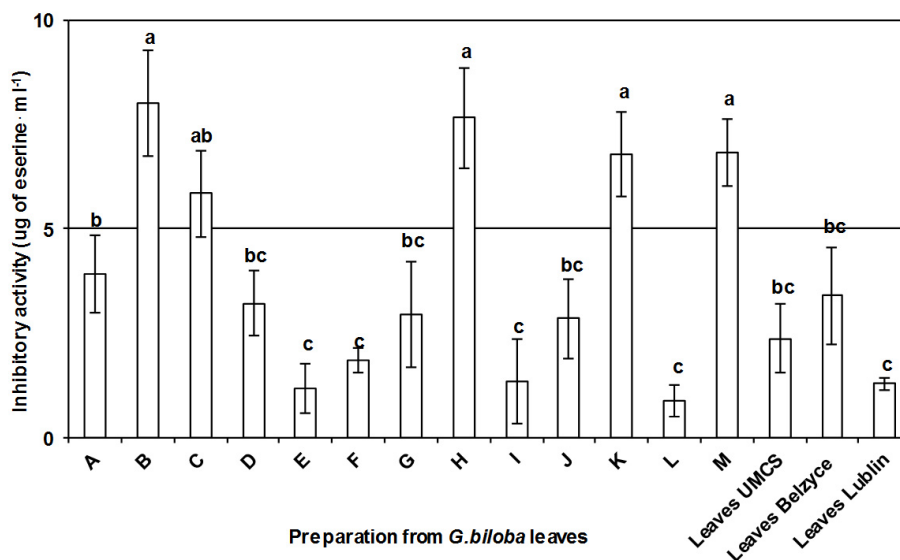Fig. 1. Anti-AChE activity of *G. biloba* leaves preparations, n = 8

Fig. 2. Anti-BChE activity of *G. biloba* leaves preparations, n = 8

lead to the imbalance in the acetylcholine: AChE + BChE system. For example, in the case of AD, the significant decrease of the AChE activity can be seen whereas the BChE activity in the brain is elevated [Giacobini 2000]. Therefore, the type of the preparation applied in the particular dysfunction should be considered taking under consideration differences in the inhibition of AChE and BChE.

Phenolic acids and flavonoids were previously repeatedly pointed out as very effective antioxidants [e.g.: Young et al. 2008, Szwajgier 2009]. Also, it was previously shown [Szwajgier 2012, Szwajgier and Borowiec 2012b] that phenolic compounds are efficient inhibitors of AChE and BChE. The comparative analysis of phenolic acids in *G. biloba* preparations was previously not performed. In the case of *G. biloba* preparations herein studied, the differences in the total phenolic content were significant and ranged from 1.56 ±0.13 (preparation I) to 24.57 ±0.67% in d.m. (*G. biloba* UMCS leaves). The difference in the total phenolic content corresponded to differences in the antioxidative activitiy measured using both ABTS and DPPH free radicals (fig. 4 and 5). Extracts prepared from samples B, H, M and from UMCS leaves exhibited both the highest total phenolic content and the antioxidant activity with ABTS and DPPH free radicals (EtOH extracts or/and water extracts). On the other hand, both types of extracts from preparations I, J, K and L were poor sources of phenolic compounds and exhibited low or very low ability to "scavenge" free ABTS and DPPH radicals. The comparison of *G. biloba* preparations I–L with fresh leaves leads to the conclusion that, during the production of these preparations, leaves were improperly stored or processed, leading to the loss of phenolic compounds.
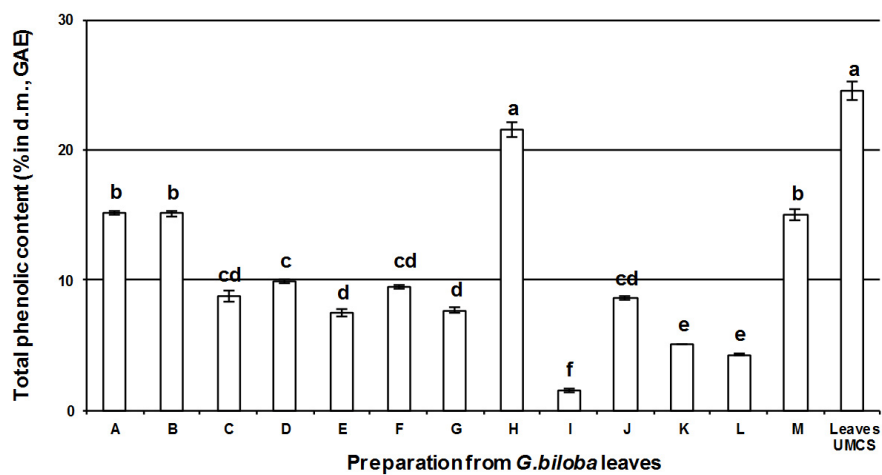
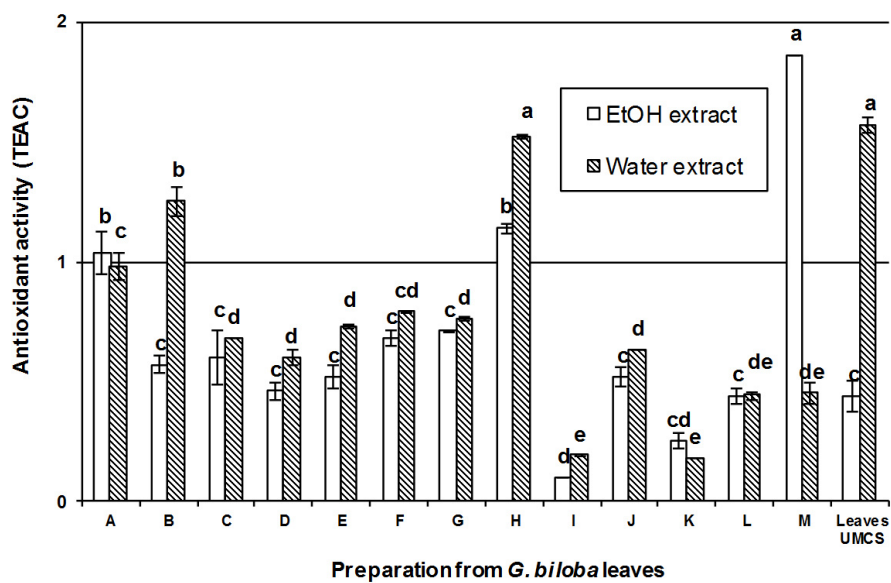Fig. 3. Total content of polyphenols in preparations produced from *G. biloba* leaves, n = 3

Fig. 4. Antioxidant activity of *G. biloba* preparations with ABTS, n = 4

Quercetin is a well-known flavonoid (flavonol) repeatedly detected in *G. biloba* leaves [e.g.: Kwon et al. 2009, Kang et al. 2010]. Extracts studied in our work contained differentiated levels of quercetin in the dry mass (fig. 6). It is interesting to notice that preparation M (commercial Tinctura produced using EtOH) as well as our ethanol:water extract from fresh *G. biloba* leaves contained very low levels of quercetin (similarly as preparations B, F and G). This result can be explained by the possible difference in the method of the production of particular commercial preparations. Unfortunately, producers usually provide a limited information about the composition of the preparation. Selected commercial preparations had no information concerning the content of active compounds (e.g. the total content of phenolic compounds, ginkgo–flavone glycosides, terpenoids, flavonols etc.). Gawron-Gzella et al. [2010] compared eleven preparations containing dry extract of *G. biloba* leaves (three pharmaceuticals, eight dietary supplements and one dry extract of leaves). The authors tested the contents of the main active groups of constituents of G. *biloba* leaves (individual flavonoids, total flavonoids, flavonoids after hydrolysis, total terpene lactones) as well as the levels of toxic ginkgolic acids. It was stated that majority of the dietary supplements had reduced content of bioactive compounds and elevated concentrations of ginkgolic acids, whereas pharmaceutical preparations met relevant quality criteria.

Phenolic acids are often omitted during the determination of the levels of active compounds present in *G. biloba*. However, these molecules are minimally degraded during the ingestion and absorption in the gastrointestinal tract in comparison to other polyphenols [e.g.: Nardini et al. 2006]. Moreover, phenolic acids are products of the degradation of more complex phenolic compounds consumed daily. In this way, free
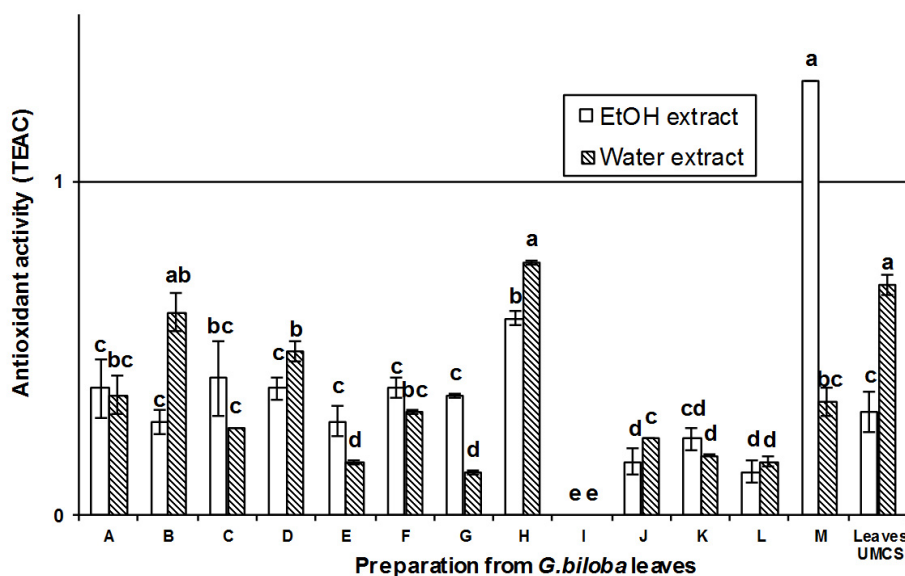

Fig. 5. Antioxidant activity of *G. biloba* preparations with DPPH, n = 4
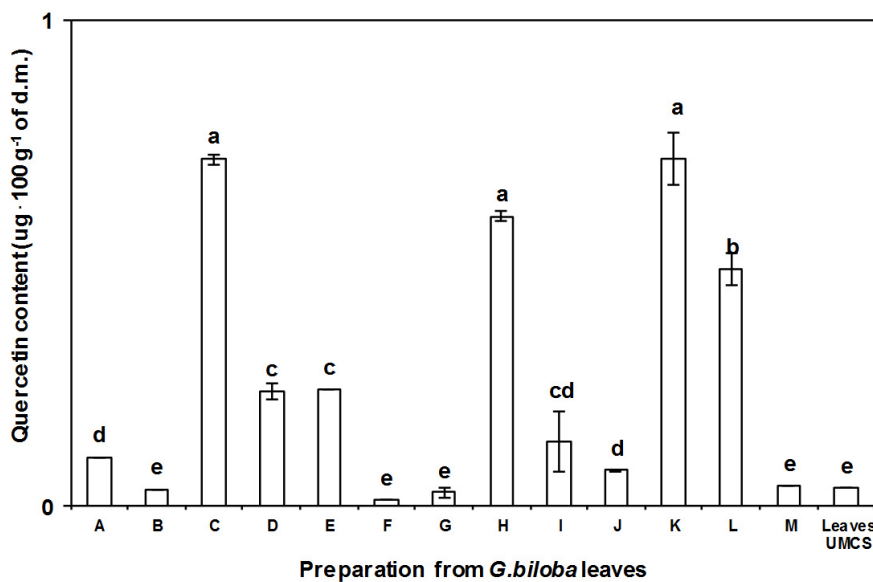
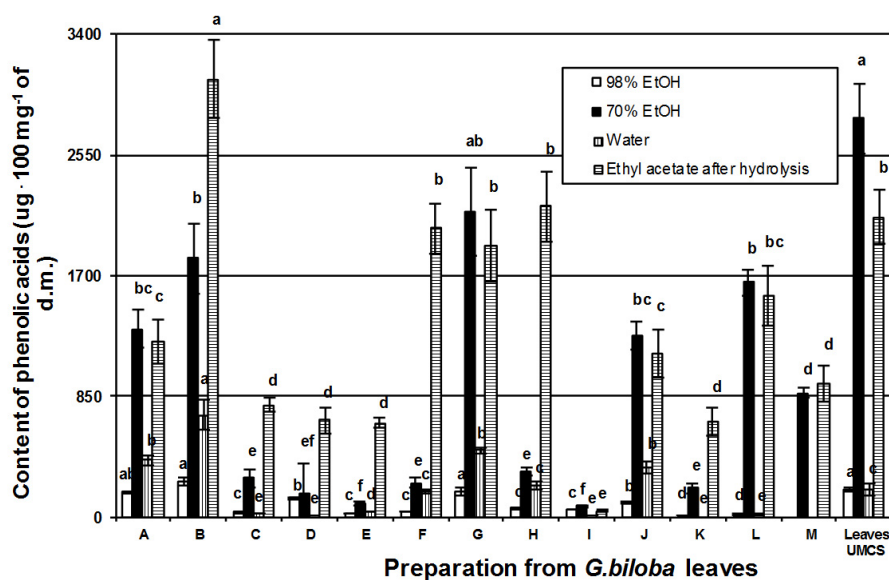Fig. 6. Quercetin content in *G. biloba* preparations, n = 4

Fig. 7. The sum of phenolic acids extracted from *G. biloba* preparations, n = 2

phenolic acids can easily exert the antioxidant activity *in vivo* [Deprez et al. 2000]. In this study, the following phenolic acids were detected using HPLC-DAD: gallic, proto-catechuic, syringic, 4-OH-benzoic, chlorogenic, caffeic, sinapic, ferulic, 4-OH-cinnamic and o-coumaric acid. The content of individual phenolic acids in preparations is not presented due to the volume of results (personal communication). However, the aim of this work was the comparison of *G. biloba* preparations rather than the detailed characterization of the content of individual phenolic acids. As it can be seen in fig.7, the highest total content of phenolic acids was obtained after the alkaline hydrolysis followed by extraction with ethyl acetate. However, 7:3 EtOH : water (v/v) was also an efficient extraction solution whereas DDI water and 89:2 (v/v) EtOH : water were ineffective for this purpose. Once again, it can be noticed that some preparations (B, F, G, L) were a rich source of phenolic acids whereas in other preparations (C, D, E, I, K), phenolic acids were at low concentrations.
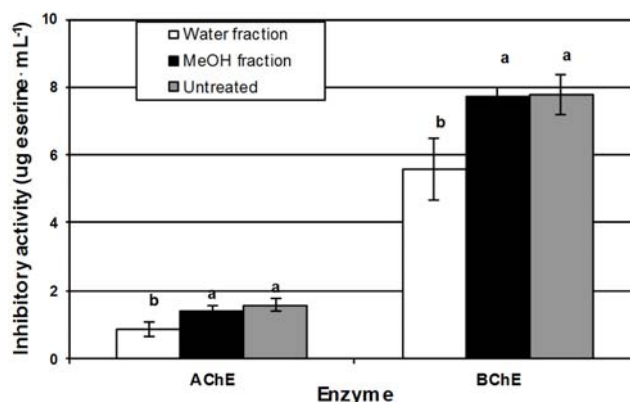

Fig. 8. The influence of fractions obtained using Sep-Pak C18 on the activity of cholinesterases, n = 3
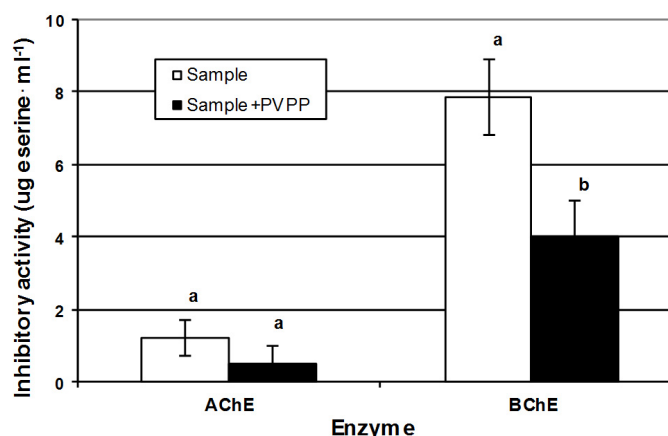

Fig. 9. The influence of fractions obtained using PVPP on the activity of cholinesterases, n = 3

In the next part, the more detailed characterization of *G. biloba* preparation H was performed due to the high anticholinestrase and antioxidant activity. Also, this preparation was a rich source of total phenolics and quercetin. The aim of this part of the study was to test if acetyl- and butyrylcholinesterase inhibitors were phenolic compounds. The preliminary characterization of AChE and BChE inhibitors was performed using Sep-Pak C18 (fig. 8). The anti-BChE activity after the Sep-Pak C18 separation was significantly lower in the water fraction. There was no statistical difference between the methanol fraction and the untreated sample. However, such an univocal result was not obtained in the case of the anti-AChE activity. Therefore, polivinylpolypirrolidone (PVPP) was used in order to separate phenolic compounds from other components of *G. biloba* preparation H (fig. 9). The removal of phenolic compounds from *G. biloba* preparation H by PVPP caused the decrease of both anti-AChE and anti-BChE activity (although the statistically significant difference was seen only in the case of the anti-BChE activity). Results obtained using Sep-Pak C18 and PVPP give the assumption that cholinesterase inhibitors from *G. biloba* can be phenolic compounds, although cholinesterase inhibitors belonging to other groups can be also present in *G. biloba* leaves. The similar result was previously obtained in our laboratory in the case of wild strawberry (*Fragaria vesca*) [Szwajgier and Borowiec 2012a]. The strong evidence exists that polyphenols containing the C_6-C_3-C_6 group [Szwajgier 2012] as well as simple phenolic acids [Szwajgier and Borowiec 2012b] are efficient cholinesterase inhibitors. Therefore, next studies aiming the purification of cholinesterase inhibitors from *G. biloba* are now in progress.

CONCLUSIONS

Ginkgo biloba preparations are widely used for the treatment of mild cases of memory impairment as well as for the increment of the antioxidant status of different body compartments (including the brain tissue). However, commercial enzyme preparations studied in this work exhibited very differentiated anticholinesterase and antioxidant activities. Some preparations were very poor sources of cholinesterase inhibitors and phenolic antioxidants. Differences in results can be (at least partly) caused by the various content of phenolic compounds in studied preparations. The identification of main groups (main constituents) responsible for the anticholinesterase activity of preparations produced from *G. biloba* leaves is a priority.

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ZDOLNOŚĆ DO HAMOWANIA AKTYWNOŚCI CHOLINOESTERAZ I AKTYWNOŚCI PRZECIWUTLENIAJĄCE HANDLOWYCH PREPARATÓW Z LIŚCI *Ginkgo biloba*

Streszczenie. Preparaty wytworzone z liści *G. biloba* są szeroko wykorzystywane do leczenia łagodnych zaburzeń pamięci. Praca miała na celu porównanie trzynastu preparatów, jak również świeżych liści *G. biloba*. Hamowanie acetylo- i butyrylocholinoesterazy i zawartość związków fenolowych określono odpowiednimi metodami spektrofotometrycznymi. Aktywności przeciwutleniające zbadano przy użyciu wolnych rodników ABTS i DPPH. Zawartości kwasów fenolowych i kwercetyny określono za pomocą HPLC-DAD. Preparaty z liści *G. biloba* efektywniej hamowały aktywność acetylo- i butyrylocholinoesterazy przy istotnych statystycznie (p < 0,05) różnicach między badanymi próbami. Niektóre preparaty wykazywały największą całkowitą zawartość związków fenolowych i jednocześnie największą aktywność przeciwutleniającą (z ABTS i/lub DPPH), podczas gdy w przypadku innych preparatów zaobserwowano wyniki odwrotne. Wykryto znaczące (p < 0,05) różnice w zawartości kwercetyny w preparatach. Badane próby zawierały szereg kwasów fenolowych: galusowy, protokatechowy, syryngowy, 4-OH-benzoesowy, chlorogenowy, kawowy, synapinowy, ferulowy, 4-OH-cynamonowy i o-kumarowy. Wstępna charakterystyka inhibitorów acetylo- i butyrylocholinoesterazy przeprowadzona przy użyciu złoża Sep-Pak C18 i przy użyciu poliwinylopolipirolidonu wskazuje, że inhibitorami cholinoesteraz mogą być związki fenolowe, jak i składniki nie należące do fenoli, obecne w liściach *G. biloba*. Obecnie trwają badania mające na celu wyizolowanie inhibitorów cholinoesteraz z liści *G. biloba*.

Słowa kluczowe: aktywność przeciwutleniająca, związki fenolowe, acetylocholinoesteraza, butyrylocholinoesteraza, inhibitory