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SCREENING FOR CHOLINESTERASE INHIBITORS IN SELECTED FRUITS AND VEGETABLES

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ABSTRACT

Plants deliver a great number of compounds among which some of them can exert a beneficial therapeutic role in the treatment of Alzheimer's disease (e.g. huperzine A, galanthamine, physostigmine). In the present study it was shown that 17 edible fruits or vegetables effectively inhibited AChE and/or BChE. The highest anti-ChE activity was demonstrated in the case of the juice produced from peach (*Prunus persica* L.), water extracts prepared from dill leaves, wild strawberry fruit, potato tubers and juices produced using apples var. *Idared* and *Champion*. Extracts from parsley (leaves) and celery exhibited significantly higher activity towards AChE than BChE. AChE activity was not diminished by extracts prepared from strawberry, bean, broccoli, parsley (root) and tomato. These results confirm that selected fruits and vegetables can be an interesting source of ChE inhibitors with the possible potential in the context of the restoring of cognitive function and improving the memory. Therefore, there is a possibility to compose the diet containing fruits and vegetables aiming the protection against memory deficits caused by AD.

Key words: acetylcholinesterase**,** butyrylcholinesterase**,** inhibitor, Alzheimer's disease

INTRODUCTION

Cholinesterases (ChE) are responsible for the termination of the nerve impulse transmission at the cholinergic synapses by fast hydrolysis of acetylcholine (ACh) [49]. The enzymes are the products of two different genes located on human chromosome 7 and 3 (acetylcholinesterase- AChE and butyrylcholinesterase- BChE, respectively) with the 52% amino acid sequence homology [4]. In the rat brain, AChE is responsible for 80% of ChE activity with the rest of the activity attributed to BChE [42]. The activity of human brain AChE is 1.5-fold (temporal and parietal cortex) up to 60-fold (caudate nucleus) higher than that of BChE [23], whereas the BChE : AChE ratio in the cortex of patients suffering from Alzheimer's disease (AD) is elevated from 0.6 to 11 [25]. It was shown that BChE activity in the brains altered by AD was increased by 120% while AChE activity was 10-15% lower than in the healthy brain [24]. The increase of BChE activity in brains suffering from AD was pointed out in a considerable number of recent reports [4, 14, 38, 63]. Additionally, both AChE and BChE form neuritic plaques and

neurofibrillary tangles in a human AD brain [3, 73, 81]. Neuritic plaques are extracellular neurotoxic deposits primarily composed of amyloid beta peptides. Neurofibrillary tangles (NFTs) are aggregates of hyperphosphorylated tau protein. The role of cholinesterases in the progress of AD as well as the damage of the cholinergic system in the brain was elucidated [67, 70] what resulted in the cholinergic therapy which was defined in the early 1990s. Nowadays, the most popular and efficient treatment of AD is the use of AChE and BChE inhibitors [10, 47], although a recent report suggests that ChE inhibitors cause only symptomatic benefits [74]. ChE inhibitors agonize the muscarinic and nicotinic receptors as well as modulate nicotinic receptors [11, 12, 78]. Anticholinesterase (anti-ChE) activity can also be useful in the treatment of other severe disorders like Parkinson's disease, vascular dementia and dementia with Lewy bodies [3, 13], Down's Syndrome [40], traumatic brain injury [82], Wernicke-Korsakoff disease [2, 32], delirium [72], migraine [54], ataxia and an autoimmune disorder like *myasthenia gravis* [52]. Moreover, Friedman [20] reviewed reports concerning the use of selected ChE inhibitors for the treatment of schizophrenia and suggested that ChE inhibitors can cooperate with muscarinic agonists and therefore can be used as cognitive enhancers. In the USA and Europe, only four ChE inhibitors are accepted for the treatment of mild to moderate phases of AD: donepezil (Aricept®), rivastigmine (Exelon®), galanthamine (Razadyne, formerly Reminyl®) and tacrine (Cognex®) [10, 41] (Fig. 1). Tacrine and donepezil are synthetic compounds whereas rivastigmine is a derivative of physostigmine previously purified from the seeds of *Physostigma venenosum* (Calabar bean). Another natural inhibitors isolated from plants are galanthamine and huperzine A [52]. Galanthamine is present in the bulbs of a spring flower called common snowdrop (*Galanthus nivalis*). Galanthamine was used in selected countries for the reversal of the neuromuscular blockade and treatment of *myasthenia gravis* [27]. Huperzine A is an alkaloid from the club moss *Huperzia serrata*, which was broadly used for ages as the Chinese medicinal herb. Huperzina A improves the memory of seniors and patients with AD without any side effects [80]. Among these ChE inhibitors, some can be recognized as selective AChE inhibitors (donepezil, galanthamine, physostigmine and eptastigmine) whereas rivastigmine exhibits the inhibitory activity towards both AChE and BChE [27, 55]. Tacrine is more effective towards BChE than AChE [41]. Other potent ChE inhibitors tested at different stages of clinical trials are, among others, metrifonate, phenserine, huperzine, velnacrine [12], Amridin, eptastigmine, KA-672, P-11012, P-11149, TAK147 and Zifrosilone [19]. Mohamed and Rao [51] reported on the class of 2,4-disubstituted pyrimidine derivatives possessing the potent anti-ChE activity. The role of BChE should be taken under consideration as the BChE-positive glia in the brains of patients with AD were seen [50]. Moreover, it was shown that BChE (next to AChE) was an important component of the neuritic plaques. These plaques exhibited the 87% affinity to BChE reactivity (K-variant of BChE). It was previously proved that K-variant of BChE caused the increased susceptibility of brains to develop certain forms of AD [3]. Furthermore, some premises exist that BChE in plaques can elevate the neurotoxicity of neuritic plaques [81]. In this context, the parallel inhibition of both BChE and AChE can be considered during the treatment of AD. This strategy can probably lead to the increased efficiency of the therapy [41].

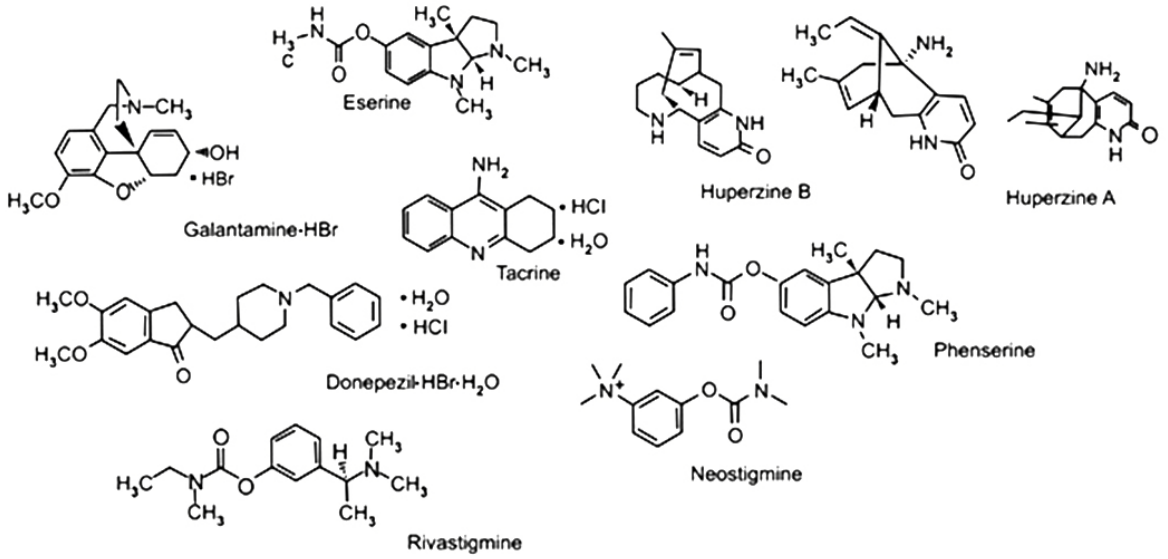


Fig. 1. Selected AChE and BChE inhibitors [76].

It is well known that plant extracts are used in traditional medicine for the treatment of variety disorders including cognitive impairments. For instance, *Withania somnifera* (known as 'ashwagandha') was mentioned as early as in the indian ancient writings as a compound of diet that improved learning and memory processes [22]. The root has been used for almost 4,000 years [31]. Also, different fruits were also used for the medical purposes [35]. The

mechanisms responsible for the anti-dementia effects of extracts from many plants are not yet fully understood. Some plants or plant extracts exhibit anti-cholinesterase activity [7, 34, 52, 57, 68] but other mode of actions include: facilitation of ACh synthesis [65], potentiation of ACh receptor functioning [88], antioxidant activity [6, 89], neuroprotection [35, 45], elevation of the levels of neurotrophic factors [46, 69] and, last but not least, modulation of neurotransmitter-receptor systems [59, 85]. Therefore, the aim of this study was to identify the anti-ChE activity of selected fruits and vegetables commonly consumed worldwide as it can be supposed that natural plants can be a rich source of new ChE inhibitors.

MATERIALS AND METHODS

Plant materials

Edible parts of selected fruits, vegetables and herbal spices commonly consumed in Poland were studied: garden strawberry (*Fragaria* x *ananassa),* wild strawberry (*Fragaria vesca*), apple (*Malus domestica*)*,* banana (*Musa paradisiaca*), plum (*Prunus domestica*), peach (*Prunus persica*), pomegranate (*Punica granatum*)*,* grapes (*Vitis vinifera*), dill (*Anethum graveolens*), celery (*Apium graveolens*), broccoli (*Brassica oleracea* var. *italica*), caraway (*Carum carvi*), tomato (*Lycopersicon esculentum*), parsley (*Petroselinum crispum*), potato (*Solanum tuberosum*) and bean (*Vicia faba*). Test samples were purchased on the local market in the fresh or frozen form and were directly frozen (-20 ^{o}C) until use.

Reagents

AChE (from electric eel *Electrophorus electricus,* cat. no C3389), BChE (from equine serum, cat. no C7512), acetylthiocholine iodide (ATChI cat. no 01480), S-butyrylthiocholine chloride (BTCh, cat. no B3128), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, cat. no D8130), eserine (physostigmine, cat. no E8375) and Tris-HCl buffer were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were from P.O.Ch. (Gliwice, Poland).

Determination of dry matter

Dry mass of each studied sample was determined after the drying until the constant mass (105 ± 5 ^{o}C, 28 h). These dry masses were taken under consideration in order to obtain similar concentrations of tested samples in the reaction mixtures during the testing of the anticholinesterase activities.

Extraction

The thawed plant samples (except juices of apples and peach) were macerated with buffer in a mortar (3 min) followed by centrifugation (4 °C, 10 min, 9300 x g). Juices from apples and peaches were obtained by using of extractor for juice (Thermomix, type TM31, Vorwerk, Germany, 3min) followed by centrifugation (4 °C, 10 min, 9300 x g).

Assay for anticholinesterase activity

The method of Ellman et al [16] in the modification described by Ingkaninan et al. [33] was used for the measurement of the anti-ChE activity. The daily prepared solutions of reagents were dissolved in Tris-HCl buffer (50 mmol $\cdot dm^{-3}$, pH 8.0). The reaction mixture consisted of: 1 cm^3 of 0.3 mmol $\cdot dm^{-3}$ DTNB (containing 10 mmol $\cdot dm^{-3}$ NaCl and 2 mmol $\cdot dm^{-3}$ $MgCl_2 \bullet 6\ H_2O$), 0.200 cm^3 of ATChI or BTCh (1.5 mmol $\cdot dm^{-3}$), 0.575 cm^3 of Tris-HCl buffer (50 mmol $\cdot dm^{-3}$, pH 8.0), 0.025 cm^3 of AChE or BChE solution (0.28 units $\cdot cm^{-3}$) and 0.2 cm^3 of the studied sample. Prior the analyses, each studied sample was standardized in order to obtain 115 mg of dry matter $\cdot cm^{-3}$. The temperature during the measurement (405 nm, Spekol 11, Carl Zeiss Jena) was approx. 22 ^{o}C. The absorbance was read after 30 min (BChE) or 60 min (AChE). Blank samples containing either eserine (90.7 µmol $\cdot dm^{-3}$) or Tris-HCl buffer instead of the studied sample were simultaneously studied. The increase of the absorbance due to the spontaneous hydrolysis of the substrate was monitored using the blanks containing DTNB and ATChI (BTCh) solutions 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 $\cdot dm^{-3}$ – 6.10 µmol $\cdot dm^{-3}$ (AChE) and 0.09 µmol $\cdot dm^{-3}$ – 8.57 µmol $\cdot dm^{-3}$ (BChE). All samples were analyzed in eight repeats.

Purification of the wild strawberry extract using Sep-Pak C18 cartridge

Sep-Pak C18 cartridge (Waters, Ireland) was flushed using acidified methanol (pH 2.0 – 3.0) and acidified deionized (DDI) water (pH 2.0 – 3.0). Then, the wild strawberry buffer extract (0.5 cm^3) was injected into cartridge followed by flushing with acidified DDI water (5 cm^3) and methanol (10 cm^3, both fractions were collected separately). The water fraction was then combined with the strawberry buffer extract and filled up to 10 cm^3 using Tris-HCl buffer (pH 8.0). Methanol was completely evaporated (Büchi, type SB, Glassapparatefabrik Flawil, 30 ^{o}C, -0.09 MPa) and the sample was diluted in 10 cm^3 of Tris-HCl buffer (pH 8.0). The purification was repeated twice using new Sep-Pak C18 cartridges at a time. Simultaneously, the dilution of the raw extract equal to that obtained after Sep-Pak

C18 purifications was prepared in DDI water ($1:19_{v:v}$) in order to evaluate the efficiency of the purification procedure. All samples were analyzed for the anti-ChE activity in eight repeats.

Purification of the wild strawberry extract using PVPP

PVPP (150 mg) was added to the wild strawberry extract (8 cm^3) followed by chilling (4 ^oC, 30 min) and centrifugation (4 ^oC, 10 min, 9300 x g). The anti-ChE activity was analyzed in eight repeats as described earlier.

Sephadex LH-20 chromatography

The thawed wild strawberry (200 g) was macerated with Tris-HCl buffer to the final volume of 200 cm^3 in a mortar (10 min) followed by centrifugation (4 °C, 10 min, 9300 x g), filtration using filter paper (Munktell, 84 g $\cdot m^{-2}$) and ultrafiltration (4 °C, 12 h, 10 kDa). The sample was then concentrated to 6 cm^3 (35 °C, 2.5 h, -0.09 MPa). The components of the wild strawberry sample (5 cm^3) were separated using the Biologic LP Fast Protein Liquid Chromatography system (FPLC, Bio-Rad, USA): LP peristaltic pump, fraction collector type 2110 and Biorad UV detector (280 nm). Separations were carried out in Sephadex LH-20 gel placed in Economy glass column (Bio-Rad, 1.5 i.d. x 100 cm). The eluent was DDI water (0.35 $cm^3 \cdot min^{-1}$, 30 h). Seventy nine fractions (8 cm^3) were gathered and analyzed for the presence of ChE inhibitors (n=4). The selected fractions exhibiting the highest activities were further purified using the HPLC system.

HPLC-DAD

The HPLC system (Gilson, USA) consisted of two model 306 pumps, model 170 diode array detector, model 805 manometric module, model 811C dynamic mixer, 0.020 cm^3 loop and UniPoint 3.01 software. The analytical column was used (Symmetry C18, 4.6 mm x 250 mm, particle size 5 µm Waters, Ireland) with Symmetry C18 (8 x 20 mm, 5 µm) precolumn. The eluent (0.8 $cm^3 \cdot min^{-1}$) was formed by 1% (w/v) acetic acid solution in DDI water (A) and acetonitrile in DDI water ($1:1_{v:v}$) (B) using the following program: 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. The detection was carried out at 260 nm, 280 nm, 320 nm and 365 nm. All separated chromatogram peaks were freeze-dried, diluted in buffer (1 cm^3) and analyzed for the anti-ChE activity (n=3).

Statistical analysis

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

RESULTS AND DISCUSSION

The inhibiton of ChEs by the test extracts are presented in Table 1.

Table 1. The inhibiton of ChEs by the test extracts or juices.

Family	Plants	Part used	Inhibitory activity (eserine µmol $\cdot dm^{-3}$)	
			anti-AChE	anti-BChE
Apiaceae	*Anethum graveolens* L. (dill)	leaf→extract	6.10 ± 1.09	8.57 ± 1.05
	Apium graveolens L. (celery)	root→extract	6.10 ± 1.23	1.82 ± 1.05
	Carum carvi L. (caraway)	fruit→extract	4.47 ± 1.23	6.65 ± 0.87
	Petroselinum crispum Mill. (parsley)	leaf→extract	6.10 ± 1.02	0.47 ± 1.05
		root→extract	0.07 ± 0.36	0.76 ± 0.51
Brassicaceae	*Brassica oleracea* L. var. *italica* Plenck (broccoli)	flower head →extract	0.07 ± 0.04	2.87 ± 0.69
Ericaceae	*Vaccinium myrtillus* L. (bilberry)	fruit→extract	4.32 ± 0.68	3.72 ± 0.75
Fabaceae	*Vicia faba* L. (bean)	seed→extract	0.07 ± 0.04	2.25 ± 0.36

Lythraceae	*Punica granatum* L. (pomegranate)	fruit→extract	1.53 ± 1.02	1.38 ± 1.02
Musaceae	*Musa paradisiaca* L. (banana)	immature fruit →extract	3.60 ± 0.65	4.14 ± 0.91
		mellow fruit →extract	1.78 ± 1.16	1.20 ± 0.80
Rosaceae	*Fragaria* × *ananassa* Duch. (garden strawberry)	fruit→extract	0.07 ± 0.07	0.29 ± 0.29
	Fragaria vesca L. (wild strawberry)	fruit→extract	6.10 ± 1.13	8.46 ± 1.13
	Malus domestica Borkh. var. Champion (apple)	fruit→juice	5.48 ± 0.76	6.57 ± 0.80
	Malus domestica Borkh. var. Gloster (apple)	fruit→juice	3.34 ± 0.58	2.47 ± 0.73
	Malus domestica Borkh. var. Golden delicious (apple)	fruit→juice	3.16 ± 0.58	1.53 ± 0.80
	Malus domestica Borkh. var. Idared (apple)	fruit→juice	6.10 ± 0.62	7.63 ± 0.80
	Malus domestica Borkh. var. Jonagold (apple)	fruit→juice	3.01 ± 0.54	2.00 ± 0.47
	Malus domestica Borkh. var. Red delicious (apple)	fruit→juice	2.91 ± 0.58	1.45 ± 0.73
	Prunus domestica L. (plum)	fruit→extract	3.09 ± 1.02	2.22 ± 1.02
	Prunus persica L. (peach)	fruit→juice	6.10 ± 0.58	8.57 ± 0.84
Solanaceae	*Lycopersicon esculentum* Mill. (tomato)	fruit→extract	0.07 ± 0.18	1.89 ± 0.62
	Solanum tuberosum L. (potato)	tuber→extract	6.10 ± 1.16	7.66 ± 0.51
Vitaceae	*Vitis vinifera* L. (grapes)	fruit→extract	0.98 ± 0.91	0.98 ± 0.94

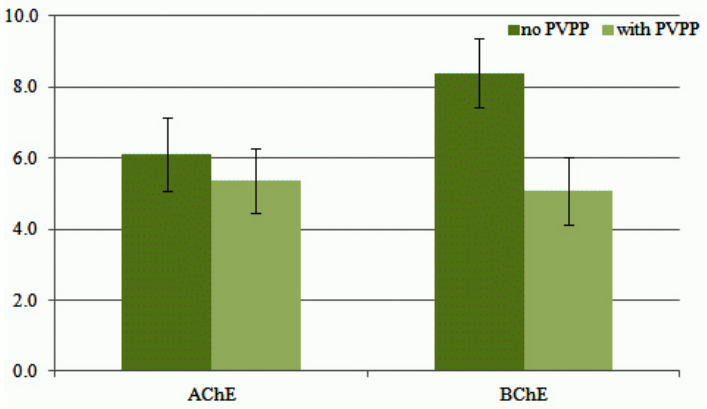
Our preliminary studies showed that the juice from peach (*Prunus persica* L.) more effectively inhibited both ChEs than the buffer extract obtained from the whole fruit (data not shown). Indeed, the inhibition of both AChE and BChE by peach juice was complete (100%). Previous studies showed that *Prunus persica* L. Batsch water extract (PPE prepared from sliced seeds) very effectively inhibited AChE and increased the extracellular ATCh concentration in the synaptic cleft of the hippocampus in rats [39, 75]. Thus, this extract can be recognized as the potential source of ChE inhibitors with long-lasting effect on the central cholinergic system. In our study, wild strawberry very effectively inhibited AChE (6.10 ± 1.13 Es µmol $\cdot dm^{-3}$- approx.100%) and BChE (8.46 ± 1.13 Es µmol $\cdot dm^{-3}$- approx. 98%). Also, *Prunus domestica* inhibited both AChE (3.09 ± 1.02 Es µmol $\cdot dm^{-3}$, 50%) and BChE (2.22 ± 1.02 Es µmol $\cdot dm^{-3}$, approx. 25%). Six varieties of apples were studied within this work. All extracts from apples were effective ChE inhibitors, especially Idared (AChE: 6.10 ± 0.62 Es µmol $\cdot dm^{-3}$, 100%; BChE: 7.63 ± 0.80 Es µmol $\cdot dm^{-3}$, approx. 89%) and Champion (AChE: 5.48 ± 0.76 Es µmol $\cdot dm^{-3}$, approx. 90%, BChE: 6.57 ± 0.80 Es µmol $\cdot dm^{-3}$, approx. 76%). Previously, diminished age-related oxidative damage was seen after the regular administration of apple juice to mice [9]. Supplementation of the diet with the apple juice concentrate restored the antioxidant potential and attenuated the overexpression of presenilin-1 (PS-1) [8] and the generation of amyloid beta fibrils [58]. Among samples from *Apiaceae* family, the extract from dill exhibited the maximum inhibition of ChEs (Table 1, equal to approx. 100% inhibition of both ChEs). These results as well as other recent reports [60] prove that dill plant can be a potential source of inhibitors for the treatment of AD. Park et al. [60] showed that methanol extracts from dill fruit (but not leaves) protected cell line derived from a pheochromocytoma of the rat adrenal medulla (PC12 cells) from amyloid beta-induced toxicity with ED50 equal to 18.8 µg $\cdot cm^{-3}$. The extract from parsley leaves effectively inhibited AChE (6.10 ± 1.02 Es µmol $\cdot dm^{-3}$, 100% inhibition) but was the weak BChE inhibitor (0.47 ± 1.05 Es µmol $\cdot dm^{-3}$, approx. 5% inhibition). Similar results were obtained in the case

of celery (AChE: 6.10 ± 1.23 Es µmol ·dm^{-3}, 100%; BChE: 1.82 ± 1.05 Es µmol·dm^{-3}, approx. 20%). Gholamhoseinian et al. [21] detected the low ability of the methanolic extract from celery leaves to inhibit AChE by 4.7% versus approx. 100% in our studies. Additionally, the extract from Chinese celery seeds contains L-3-*n*-Butylphthalide (L-NBP), the compound that effectively attenuated learning deficits and improved long-term spatial memory, reduced total cerebral amyloid beta plaque deposition, lowered amyloid beta levels in brain homogenates by removing the diffuse amyloid beta deposits in a triple-transgenic AD mouse model (3xTg-AD). In addition, L-NBP regulated amyloid precursor protein (APP) processing towards the nonamyloidogenic pathway [62]. Taking under consideration these results it can be concluded that celery can be a promising multitarget drug for AD. The extract from potato tubers very effectively inhibited both AChE (6.10 ± 1.16 Es µmol ·dm^{-3}, 100%) and BChE (7.66 ± 0.51 Es µmol ·dm^{-3}, approx. 89%). These results were different from former findings proving that glycoalkaloids from *Solanaceae* family (mainly alfa-solanine and alfa-chaconine) more effectively inhibited BChE (91.5 ± 1.6% by solanine and 92.8 ± 2.1% by chaconine) than AChE (76.8 ± 2.7% and 67.3 ± 0.4% respectively) [48]. The significant inhibitory activity was detected in the present study in the buffer extract of caraway (AChE: 4.47 ± 1.23 Es µmol ·dm^{-3}, approx. 73%; BChE: 6.65 ± 0.87 Es µmol ·dm^{-3}, 77%). Adsersen et al. [1] detected a very low inhibitory activity towards AChE of methanolic extract prepared from caraway root. However, in our study the seeds of caraway were used and the tested extract was at higher concentration (115.0 mg dry mass ·cm^{-3}) than in the above cited work (0.1 mg ·cm^{-3}). The extract from banana fruit exhibited the significant inhibiton of AChE (3.60 ± 0.65 Es µmol ·dm^{-3}, approx. 58%) and BChE (4.14 ± 0.91 Es µmol ·dm^{-3}, approx. 48%). Gupta and Gupta [28] detected no anti-AChE activity in the leaves of banana *Musa paradisiaca*. However, Ingkaninan et al. [34] detected the inhibition of AChE (29% ± 4.7%) by the methanol extract from banana (*Musa sapientum)* fruit (0.1 mg ·cm^{-3}). The difference between the results obtained in the herein presented and cited work was probably caused by the plant origin, different concentration of dry mass, extraction methods etc. We also noticed that the ability to inhibit the enzymes by *Musa sapientum* fruit extract was reduced by approx. 50% (AChE) and 70% (BChE) after the ripening of the fruit. The observations concerning the decrease of the inhibitory activity after the ripening were similar to those obtained by Fletcher et al. [18] for tomato fruit. The bilberry extract considerably inhibited AChE (4.32 ± 0.68 Es µmol ·dm^{-3}) as well as BChE (3.72 ± 0.75 Es µmol ·dm^{-3}). Bilberry can be not only a great source of ChE inhibitors but also it is considered the rich source of anthocyanins [56]. The potential antioxidant property of anthocyanins is well known [64, 90]. Giasson et al. [26] connected the oxidative stress with the pathology of AD. Ramirez et al. [66] tested the effect of the diet containing the lyophilised *Vaccinium* berries on the memory improving. Results showed that the dietary intake of the bilberry anthocyanins enhanced the memory, especially working and short-term memory. These findings suggest that *Vaccinium* berries may prevent from the memory deficiency which is the major symptom of AD. The anti-ChE activity of the extract from bean seeds was lower than expected (inhibition of BChE at 2.25 ± 0.36 Es µmol ·dm^{-3}, approx. 25%) with no activity towards AChE. On the contrary to our results, a report of approx. 50% inhibiton of both ChE by the extract obtained from the whole plant was previously published by Orhan [57]. Orhan et al. [57] studied the chloroform-methanol (1:1$_{v:v}$) extract (1 mg ·cm^{-3}) whereas the concentration of dry mass in our buffer extract was 115-fold higher. Therefore, the difference between these results was probably caused by the selection of different plant parts or by the solvents used. In our study, water was used as the solvent because it is applied during the preparation of beans (and other fruits and vegetables) at home and in the industrial scale. Another reason for the different results can be the temperature of storage. We tested the samples that were stored at -20 ^{o}C. In consequence, phenolic antioxidant content could be significantly decreased followed by the evolution in the anti-ChE activity [84]. The low anti-BChE activity was seen in the case of extracts from broccoli (2.87 ± 0.69 Es µmol ·dm^{-3}, approx. 33%) and tomato (1.89 ± 0.62 Es µmol ·dm^{-3}, approx. 21%). It was also seen that these extracts were insufficient AChE inhibitors. The broccoli extract was tested because of the high content of polyphenols, especially isothiocyanate derivatives [5] which may reduce inflammation and thereupon cause the increasing resistance to many diseases. A noted interest in the research of anti-ChE activity of tomato was seen after the isolation of a new inhibitor alfa-tomatin and the detection of AChE in the tomato plant [18]. Indeed, many species of plants are sources of both AChE and ACh [29, 53, 77] and at the same time they could contain ChE inhibitors as well as compounds with ability to decolorize the Ellman's reaction products [28]. In addition, it was shown that the AChE activity in tomato seedlings depended on the location in particular organs and tissues, conditions of plant growth (especially light treatment), growth phase, plant species [83]. It is of note that tomato plant is used as a system for the production of human amyloid beta- a potential vaccine against AD [86]. The slight ability to inhibit BChE by the extract prepared from ripe tomatoes can be the confirmation of the previous studies [18] that the activity of ChE inhibitors evolves during the ripening. In presented study we showed that fruit extract from pomegranate inhibited both AChE and BChE (1.53 ± 1.02 Es µmol ·dm^{-3}, approx. 24% and 1.38 ± 1.02 Es µmol ·dm^{-3}, approx. 15%, respectively). Also, the leaves of pomegranate and grapes exhibited considerable anti-AChE activities [28]. Gholamhoseinian et al. [21] detected the inhibition of AChE (11.5%) by methanol extract from pomegranate fruit hulls (50 µg crude extract ·cm^{-3}).

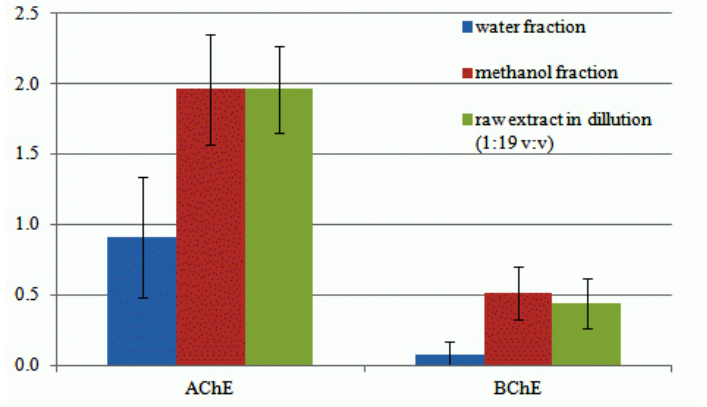
Extract from grapes inhibitied AChE (0.98 ± 0.91 Es µmol ·dm^{-3}, approx. 15%) as well as BChE (0.98 ± 0.94 Es µmol ·dm^{-3}, approx. 11%). It was stated that the pomegranate seed extract is the potential substance useful for the treatment of cognitive dysfunction because it relieves cognitive deficit in mice in the passive avoidance and elevated plus maze tasks [43]. The husk obtained from pomegranate is a good source of beta-secretase inhibitors and

therefore it can be used for the inhibition of the formation of toxic and reactive fragments of amyloid beta [44]. The similar properties were seen in the case of the extract from grapevine stems. Especially, stilbenes (mainly resveratrol and (+)-ampelopsin) inhibited the aggregation of amyloid beta and therefore *in vivo* protected against brain cell dysfunction in AD [87]. The extract prepared from parsley roots exhibited the low ability to inhibit BChE (0.76 ± 0.51 Es µmol ·dm^{-3}, approx. 8%) and the lack of anti-AChE activity was seen. However, Adsersen et al. [1] reported the significant (21%) inhibition of AChE by methanol extract from underground parts of parsley (at a concentration of 0.1 mg dry mass ·cm^{-3}). The possible cause of these discrepancies between herein presented and the cited results could be the use of different extraction solvents. It also confirms Adsersen's assumption that the compounds responsible for the inhibition of ChE in the extract from parsley roots are essential oils containing terpenes [1]. The low inhibition of ChEs was obtained in the case of the garden strawberry extract (0.29 ± 0.29 Es µmol ·dm^{-3}, below 3%) as well as the lack of AChE inhibition by this fruit extract. We have examined strawberry because the high total antioxidant activity of this fruit was reported [30, 37]. Shukitt-Hale et al. [71] also proved that the dietary supplementation with strawberry extract effectively attenuated brain and behavior deficits in aged rats. On the other hand, Joseph et al. [36] showed that strawberry extract didn't influence the interaction between dopamine and amyloid beta 25-35 on the Ca^{2+} regulation in the M1-transfected COS-7 cells.

The wild strawberry extract exhibited one of the highest anti-ChE activity among all tested plant samples. It was seen that this extract inhibited AChE (5.37 ± 0.91 Es µmol ·dm^{-3}) and BChE (5.08 ± 0.94 Es µmol ·dm^{-3}) after the removal of phenolic compounds by PVPP but the inhibitory activity was lower than in the case of raw extract (AChE: 6.10 ± 1.03 Es µmol ·dm^{-3}; BChE: 8.39 ± 0.98 Es µmol ·dm^{-3}) (Graph 1.). Also, the inhibition of both ChEs was seen in the methanol fraction (AChE: 1.96 ± 0.39 Es µmol ·dm^{-3}; BChE: 0.51 ± 0.19 Es µmol ·dm^{-3}) obtained after the purification in Sep-Pak C18 cartridges. This result was similar to that obtained in the case of raw extract (AChE: 1.96 ± 0.31 Es µmol ·dm^{-3}; BChE: 0.44 ± 0.18 Es µmol ·dm^{-3}) exhibited by the raw extract from the fruit (Graph 2.). This procedure was used for the characterization of ChE inhibitors from wild strawberry. The results suggest that ChE inhibitors from this fruit can be both phenolic as well as non-phenolic compounds. Peñarrieta et al. [61] pointed out that the wild strawberry fruit exhibits high total antioxidant capacity due to remarkable content of phenolic compounds, mainly ellagic and gallic acid, cyanidin, pelargonidin, quercetin, kaempferol, catechin and their derivatives.



Graph 1. The inhibition of ChEs (Es µmol ·dm^{-3}) by the wild strawberry extract purified using PVPP, n=8.



Graph 2. The inhibition of ChEs (Es µmol ·dm^{-3}) by the wild strawberry extract fractionated using Sep-Pak C18, n=8.

Therefore, next parts of this study aimed the purification of potential ChE inhibitors from the wild strawberry extract. The compounds present in the buffer extract from the wild strawberry were separated using gel filtration/adsorption chromatography in Sephadex LH-20 (Fig. 2.). Seventy nine fractions were obtained with fractions 14 – 24 exhibiting the anti-ChE activity. The highest overall inhibitory activity was present in fractions 23

(anti-AChE: 3.74 ± 0.64 Es µmol ·dm^{-3}, anti-BChE: 3.78± 0.49 Es µmol ·dm^{-3}) and 15 (anti-AChE: 2.29 ± 0.31 Es µmol ·dm^{-3}, anti-BChE: 3.30 ± 0.26 Es µmol ·dm^{-3}). Most probably, wild strawberry fruit extract can be the source of at least two effective ChE inhibitors and the difference in the chemical structure and/or molecular weight as well as the lipophilicity can be the reason of the difference in the retention time of these compounds.

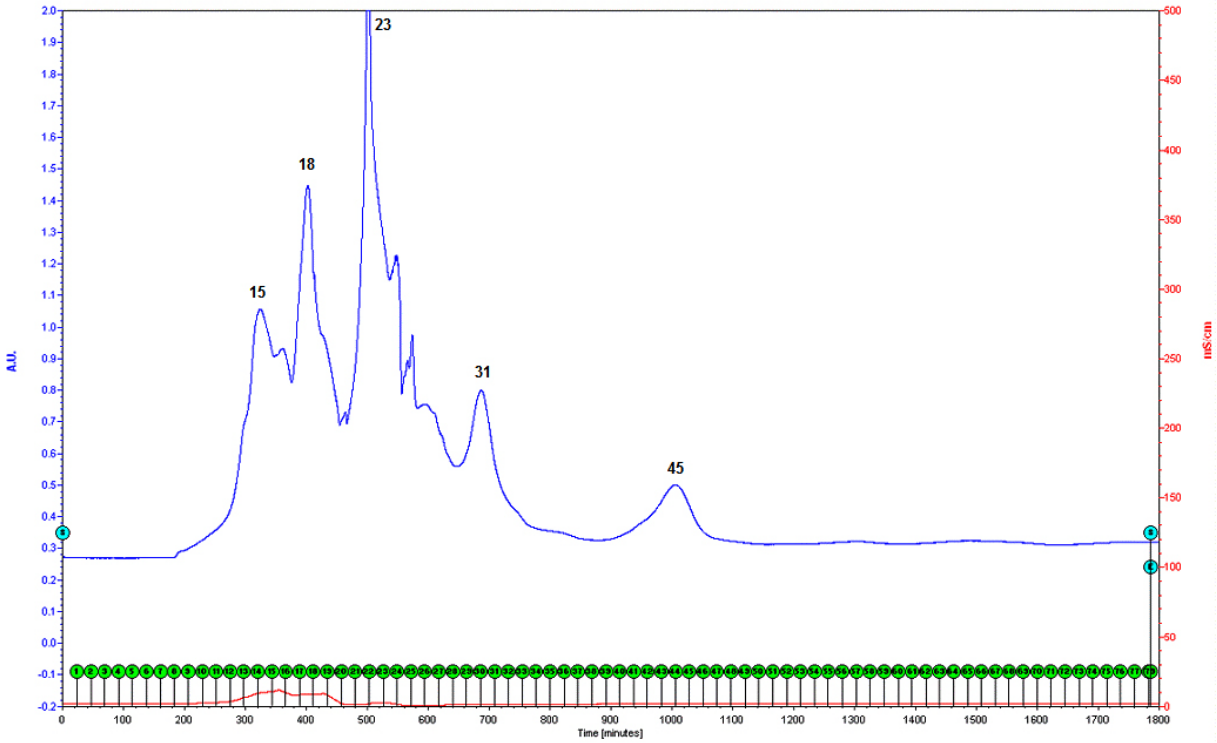


Fig. 2. FPLC chromatogram after the separation of compounds from wild strawberry buffer extract (280 nm).

Raw buffer extract from wild strawberry was very complex (Fig. 3.). However, fractions 15 and 23 obtained in FPLC system were significantly purified and these fractions were further separated using analytical HPLC system (Fig. 4. and 5.). No activity was observed in eluted fractions obtained from sample 15 separated in HPLC-UV system. The separation of fraction 23 revealed the presence of a chromatogram peak with anti-BChE activity (2.91 ± 0.62 Es µmol ·dm^{-3}). Previously, HPLC analysis was applied for the on-line anti-ChE activity testing by Ingkaninan et al. [33] and de Jong et al. [15]. These assays were used for the rapid detection and identification of cholinesterase inhibitors using the on-line coupled UV-MS-biochemical detection.

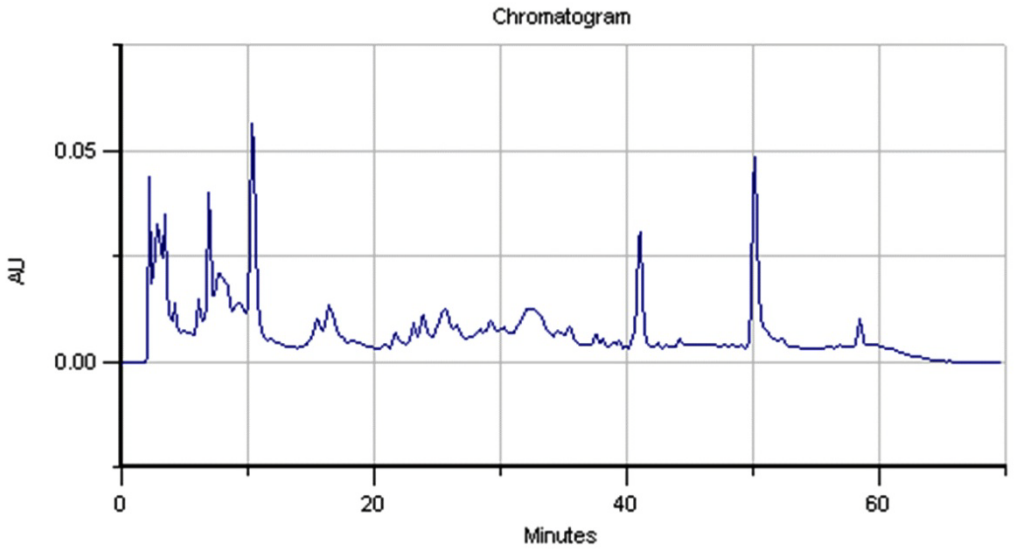


Fig. 3. HPLC chromatogram of the wild strawberry buffer extract with UV detection (280 nm).

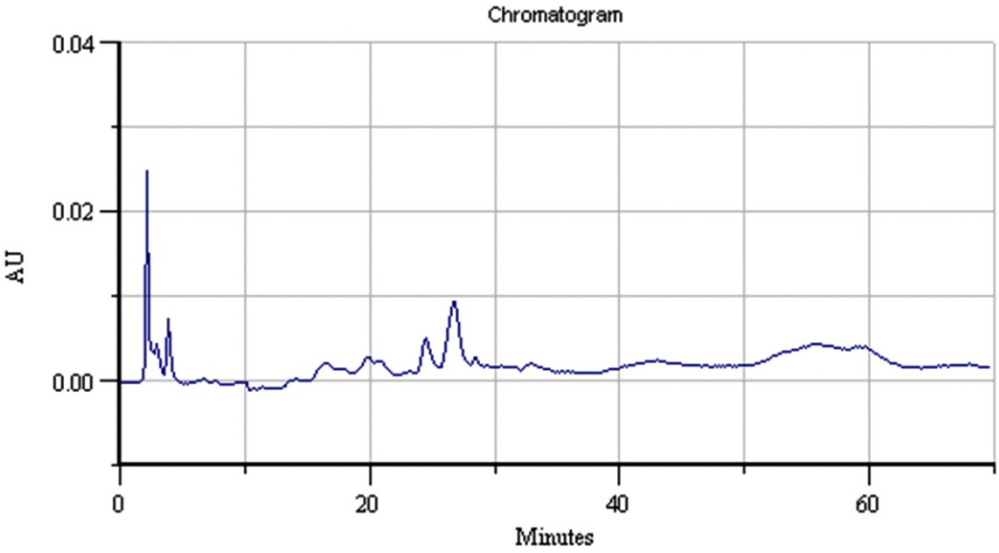


Fig. 4. HPLC chromatogram of FPLC fraction number 15 with UV detection (280 nm).

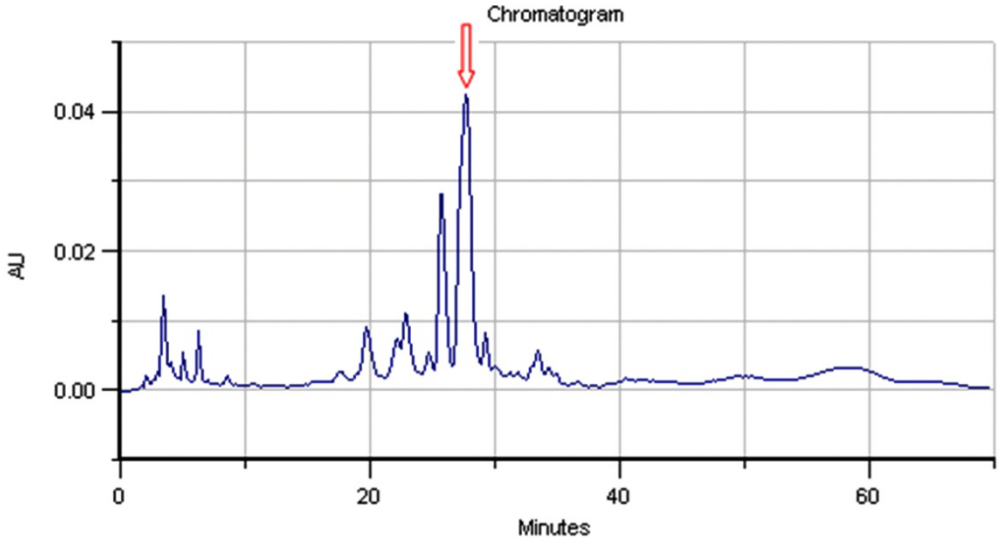


Fig. 5. HPLC separation of compounds present in fraction 23 (280 nm). The active fraction was marked.

Previously, it is well documented that edible fruits, vegetables and herbs were sources of anti-ChE compounds: *Punica granatum*, *Musa paradisiaca*, *Vitis vinifera* [28, 34], *Vicia faba* [57], *Prunus persica* [75], *Salvia officinalis, Melissa officinalis*, *Laurus nobilis*, *Mentha suaveolens, Lavandula angustifolia*, *Lavandula pedunculata* [17], *Carum carvi, Petroselinum crispum* [1], *Bacopa monniera*, *Ginkgo biloba* [52] and *Withania somnifera* [79]. The presented paper reports on the AChE and/or BChE inhibitory activities of many other edible plants which have never previously been mentioned. However, further works should be undertaken in order to thoroughly characterize the chemical structure of main cholinesterase inhibitors from these sources.

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