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ANALYSIS OF THE ACTIVITY OF HYDROXYCINNAMIC ACIDS FROM GREEN AND ROASTED COFFEE EXTRACTS AS ACETYLCHOLINESTERASE INHIBITORS USING AN ISOTHERMAL METHOD OF TITRATION CALORIMETRY

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Abstract. Acetylcholinesterase (EC 3.1.1.7) is a major enzyme responsible for the pathogenesis of Alzheimer's disease (AD). This enzyme regulates the nerve impulse conduction by hydrolyzing the excess of acetylcholine in the synaptic cleft, decreasing its level in the brain. A low level of acetylcholine observed in neurodegenerative diseases contributes to the formation of amyloid plaques and neurofibrillary degeneration, which together with the development of AD spread in the cerebral cortex. That is why natural, non-toxic AChE inhibitors are sought for. The aim of the study was to assess the degree of inhibition of AChE activity by single hydroxycinnamic acids and coffee extracts depending on the type of coffee and its roasting. The study was carried out by means of isothermal titration calorimetry (ITC), determining thermodynamic interactions and parameters. ITC analysis showed that the most stable bonds and strong interactions were characterized by 3-caffeoylquinic acid with AChE, and also was the most effective AChE inhibitor. Analysis of coffee extracts showed that the highest activity to AChE was characterized by Robusta green coffee.

Key words: acetylcholinesterase, green and roasted coffee, Alzheimer's disease, ITC.

INTRODUCTION

Alzheimer's Disease (AD) is chronic and slowly progressing neurodegenerative disease, characterized by accumulation of amyloid plaques, which cause toxic changes in the brain leading neuronal cell death (Muñoz-Moreno et al. 2018). In the first stage AD is symptom--free, therefore the most cases were diagnosed in older people in advanced stage of the disease, with symptom, such as motor and memory function disturbances, which impair their quality of life (Nelson and Tabet 2015; Dao et al. 2017; Connors et al. 2018). The pathophysiology of AD is not yet fully understood, however according to many scientific studies the main reason of disease development is impaired cholinergic system, resulting in lowered level of neurotransmitters acetylcholine (ACh) (Liu et al. 2016). The most important roles in the disease play enzymes: acetylcholinesterase (AChE) and butyryl cholinesterase (BChE), which catalyzes the hydrolysis of ACh (Wang et al. 2017).

AChE is an enzyme located in the central and peripheral nervous system and is characterized by active site, called catalytic triad. The active catalytic site consisting of anionic site and serine are mainly responsible for the hydrolysis of choline esters, including ACh. Reduced level of ACh contributes to formation of β-amyloid and its deposition as fibrils leading to inflammation (Beste et al. 2018; Dizdar et al. 2018; Moradi et al. 2018).

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AChE inhibitors cause the delay in the development of degenerative changes by protection against expression of phosphorylated tau protein which is a β-amyloid precursor. Available drugs inhibiting human AChE include substances such as: tacrine, rivastigmine, that with long-term using show adverse side effects. That is why, we are looking for natural, not toxic inhibitors of AChE. Many scientific studies confirm the beneficial activity of coffee extract as a safe and effective therapeutic agent against AD (Bogdanov 2018; Ulus et al. 2018). Coffee is one of the most consumed product in the word, it is a valuable source of caffeine, which is a stimulant of the central nervous system. Bioactive components found in coffee are also chlorogenic acids (CGAs), characterized by potential anti-inflammatory and antioxidant properties (Riberio et al. 2014).

The aim of our studies was to determine the inhibition of AChE activity and protection of ACh by specific interaction with GGAs and different coffee extract, depending of the degree of roasting.

MATERIAL AND METHODS

Roasting and preparing coffee extract

The objects of our study were the green Arabica (*Coffea arabica* L.) Brasil Cerrado and Robusta (*Coffea canephora* L.) India Parchment coffee beans, purchased from Bero Polska (Gdynia, Poland). The coffee beans (200 g) were roasted in a convective automatic CBR-101 (Gene Café, Gyeonggi-Do, Korea) at 230ºC for 9 min to achieve light roasted beans and 12 min for dark roasting. Green, light and dark roasted beans were ground and seeds size ranging from 480 to 680 µm was achieved by sieving. The preparation of coffee extracts included mixing of 400 g of ground coffee beans with water at 1 : 5.75 (w/w) ratio to water; in next step these suspensions were boiled in a pressure vessel at 110ºC for 10 min (PS-5682 Vienna, Austria). The suspensions were chilled in a water bath at 40ºC for 20 min. In order to clarify the coffee extracts, they were filtered under vacuum pump (KNF 18035.3 N, Neuberger, NJ, USA), according to (Budryn et al. 2018). The extracts were freeze-dried and store at –25ºC for calorimetric research.

Isothermal titration calorimetry

Evaluation of interaction between AChE (EC 3.1.1.7 from electric eel, lyophilized powder 200-1.000 units/mg protein; from Sigma Aldrich, St. Louis, MO, USA) and hydroxycinnamic acid (from PhytoLab, Vestenbergsgreuth, Germany) or coffee extracts, and inhibition of acetylcholinesterase was carried out using the isothermal titration calorimetry (ITC), MicroCal PEAQ-ITC200 (Malven, Worcestershire, UK). The measuring cell (0.2 mL) was filled with degassed 10 µmol/L AChE solution in ultrapure water, the syringe (2 µL) was supplemented with titrant of degassed 10 mmol/L aqueous solutions: CGAs or green or light/dark roasted coffee extracts and acetylcholine. Measurements were carried out at 36.6ºC with continuous string (307 rpm), total number of injections was 19, time of all analysis was 45 min. If binding occurs, heat is either absorbed or released and was recorded over time and showed as the raw data of heat in kcal/s against time. The plot showed the heat of interactions between AChE and GCAs or coffee extracts with ACh (Fig. 1, 2).

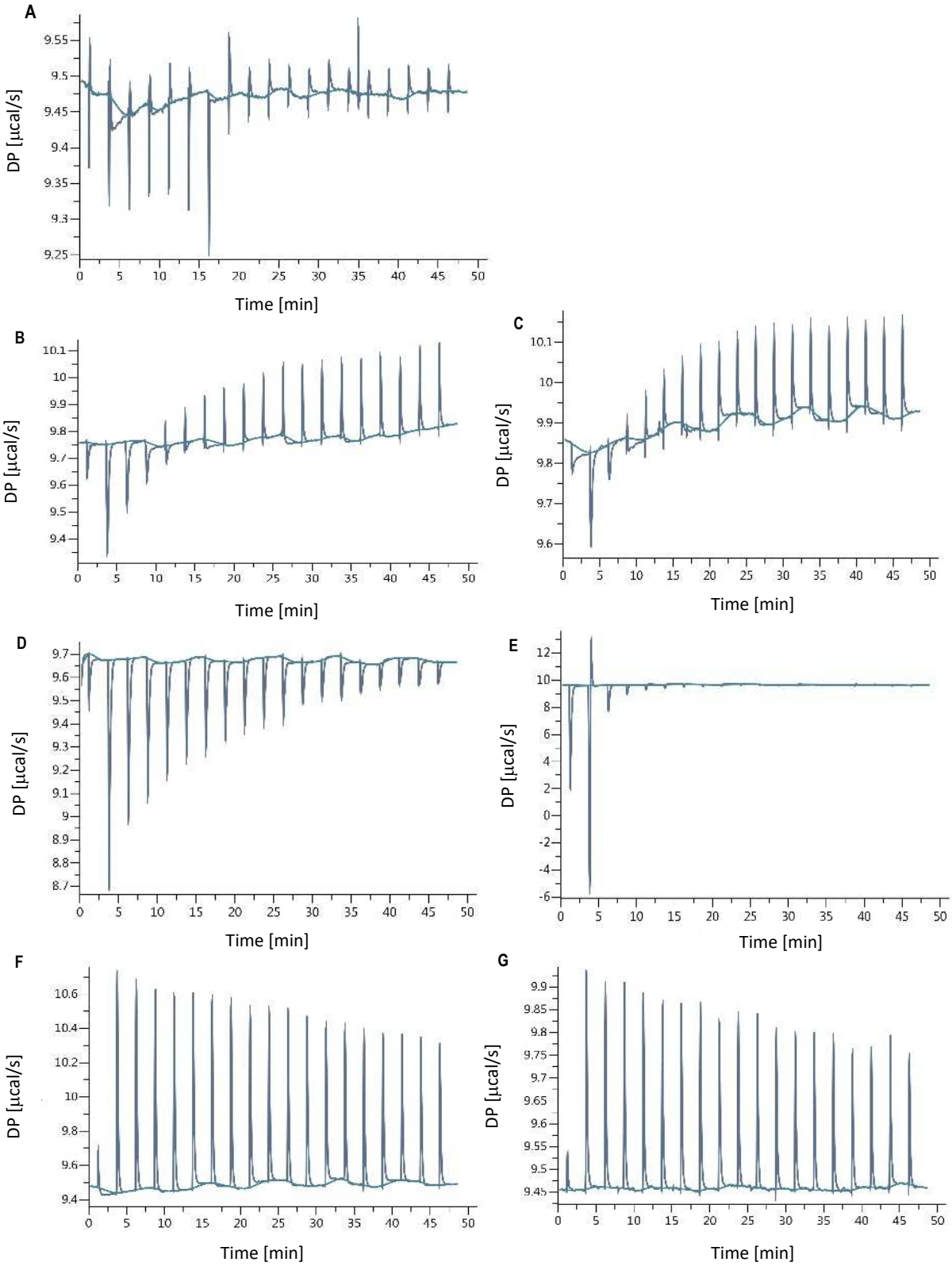

Fig. 1. ITC raw data: A – hydrolysis of ACh by AChE; B – reduced hydrolysis of Ach by AChE with 3-caffeoylquinic acid; C – effect of interactions of AChE with 3-O-caffeoylquinic acid; D – reduced hydrolysis of ACh by AChE with dihydrocaffeic acid; E – effect of interactions of AChE with dihydrocaffeic acid; F – reduced hydrolysis of ACh by AChE with caffeine; G – effect of interactions of AChE with caffeine

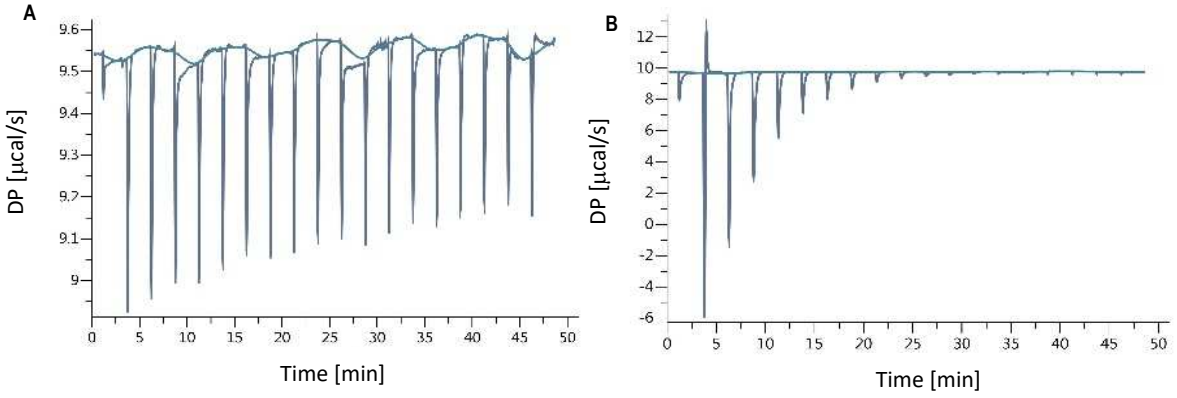

Fig. 2. ITC raw data: A – reduced hydrolysis of ACh by AChE with coffee extract of green Robusta; B – effect of interactions of AChE with extract of green Robusta

During the experiments dissociation constant (K_D), Michaelis constant (K_m), binding constants (K_A), Gibbs free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) has been determined. Based on the energetic effects IC_{50} of AChE inhibition was also determined, which was the concentration of an inhibitor causing the decrease of AChE activity by 50% and Ki was calculated.

Statistical analysis

Statistical analysis for ITC measurements was performed using Statistica 10.0 software; uniform groups were determined of the average values of six measurements and their standard deviation, as well as one-way ANOVA (analysis of variation) at the significance level $P < 0.05$.

RESULTS AND DISCUSSION

Interactions between acetylcholinesterase and chlorogenic acids or caffeine and coffee extracts

Analysis was carried out in order to determine interaction of CGAs, caffeine or coffee infusions two different species Arabica and Robusta with AChE and determining the best conditions of coffee processing to achieve high activity of AChE inhibition. The research was carried out using isothermal titrimetric calorimetry method, specifying thermodynamic parameters of interaction showed in Table 1, 2. ITC is a simple and thorough analysis, which allows you to study specific ligand binding reactions with protein, evaluate binding mechanisms and inhibitor design (Draczkowski, Matosiuk, Jozwiak 2014). During intermolecular bond is formed, the energy is either released or absorbed in the form of heat showed as raw data in Fig. 1, 2.

The raw data in Fig. 1 and 2 have shown that the direct titration of CGAs into AChE was mostly an exothermic process, which was confirmed by changes of reaction enthalpy ΔH. The enthalpy of interactions ΔH between AChE and chlorogenic acids and caffeine, was negative, which have range from –0.503 kJ/mol for caffeic acid to –30.26 kJ/mol for dihydrocaffeic acid (Table 1, Fig. 1 D, E).

Table 1. Parameters of interactions between acetylcholinesterase and chlorogenic acids or caffeine

Compound	K_D [µmol/L]	$K_A \cdot 10^3$ [L/mol]	ΔH [kJ/mol]	ΔG [kJ/mol]	ΔS [J/mol · K]	Inhibitory activity at 10 µmol/L inhibitor +10 µmol/L ACh: µmol/L AChE [%]	IC_{50} [µmol/L inhibitor: µmol/L AChE]	K_i [µmol/L] K_m for ACh 48.5
Caffeic acid	186.0 ± 9.40	0.537 ± 0.04	–0.503 ± 0.045	–16.22 ± 1.27	50.71 ± 1.95	68.59 ± 2.45	7.29 ± 0.39	0.112 ± 0.009
Ferulic acid	29.0 ± 2.04	34.48 ± 1.25	–0.682 ± 0.052	–26.94 ± 2.07	84.75 ± 3.13	48.70 ± 1.18	10.27 ± 0.45	0.123 ± 0.002
3-O-Caffeoylquinic acid	7.27 ± 0.226	137.50 ± 15.2	–28.13 ± 1.19	–30.50 ± 2.45[e]	7.67 ± 0.87	93.00 ± 2.22[b]	5.37 ± 0.17[a,b]	0.421 ± 0.012[a]
5-O-Caffeoylquinic acid	16.7 ± 1.45	59.88 ± 6.8	–11.72 ± 0.86	–28.37 ± 1.33[c]	53.71 ± 1.33	80.00 ± 1.09	6.25 ± 0.40	0.420 ± 0.029[a]
4-O-Caffeoylquinic acid	11.5 ± 1.15	86.95 ± 9.1	–14.79 ± 2.30[a]	–28.33 ± 1.94[c]	43.69 ± 1.47	90.53 ± 1.80	5.52 ± 0.24[a]	0.955 ± 0.043
3,5-Di-O-Caffeoylquinic acid	10.2 ± 0.836	98.0 3± 8.3	–14.03 ± 2.95[a,b]	–29.50 ± 1.83[c]	49.94 ± 1.05	93.15 ± 2.95[a,b]	5.36 ± 0.33 [a,b]	0.251 ± 0.013
4,5-Di-O-Caffeoylquinic acid	9.11 ± 0.675	109.87 ± 9.0	60.19 ± 5.27	–29.92 ± 1.64[d,e]	290.42 ± 11.37	38.93 ± 0.54	12.84 ± 0.64	0.604 ± 0.036
Dihydrocaffeic acid	5.41 ± 0.115	184.84 ± 16.4	–30.26 ± 3.12	–31.30 ± 1.05[d]	3.36 ± 0.13	19.28 ± 0.15	25.93 ± 2.29	0.843 ± 0.001
Caffeine	127.0 ± 7.36	1.27 ± 0.39	0.446 ± 0.052	–16.78 ± 2.34	55.58 ± 2.12	73.81 ± 1.12	6.70 ± 0.26	0.748 ± 0.008

Values are expressed as mean value ± SD; different letters (a–e) in the same row indicate that values are significantly different ($P < 0.05$).

Table 2. Parameters of interactions between acetylcholinesterase and coffee extracts

Coffee extract	K_D [µmol/L]	$K_A \cdot 10^3$ [L/mol]	ΔH [kJ/mol]	ΔG [kJ/mol]	ΔS [J/mol · K]	Inhibitory activity at 0.25 µL/µM AChE and 10 µM ACh [%]	IC_{50} [µmol/L inhibitor: µmol/L AChE]	K_i [µmol/L] K_m for ACh 48.5
Robusta green	0.008 ± 0.002	11.50 ± 0.49	–8.17 ± 0.12	–35.87 ± 1.49	89.42 ± 0.28	8.64 ± 0.12	12.73 ± 0.16	0.687 ± 0.001
Robusta light roasted	1.35 ± 0.25	0.74 ± 0.09	–1.63 ± 0.05	–17.00 ± 0.39	49.60 ± 0.35	6.59 ± 0.01	16.69 ± 0.19	0.531 ± 0.009
Robusta dark roasted	0.776 ± 0.013	1.29 ± 0.46	–2.90 ± 0.25	–18.48 ± 0.38	50.28 ± 0.18	13.71 ± 0.33	8.02 ± 0.03	0.749 ± 0.004
Arabica green	0.149 ± 0.004	6.71 ± 0.32	–3.26 ± 0.14	–22.75 ± 1.12	62.90 ± 0.33	10.56 ± 0.48	10.41 ± 0.09	0.824 ± 0.007
Arabica light roasted	1.77 ± 0.011	0.56 ± 0.01	–1.24 ± 0.09	–16.34 ± 0.49	48.73 ± 0.12	5.70 ± 0.08	19.3 ± 0.23	0.911 ± 0.011
Arabica dark roasted	1.66 ± 0.006	0.86 ± 0.03	–2.30 ± 0.18	–27.43 ± 0.86	48.83 ± 0.16	6.15 ± 0.01	17.89 ± 0.39	0.833 ± 0.012

The endothermic effect was recorded at interactions of ligands such as 4,5-di-O--caffeoylquinic acid and caffeine (Fig. 1 F, G), were ΔH was 0.446 kJ/mol and 60.19 kJ/mol, respectively. The value of ΔH showed that AChE is most strongly bind with dihydrocaffeic acid and 3-O-caffeoylquinic acid where it amounted –30.26 kJ/mol and –28.13 kJ/mol respectively. It was also observed that interactions with these ligands have been characterized by highly negative values of ΔG of −31.30 and −30.50 kJ/mol, respectively, suggesting van der Waals interaction and hydrogen bonds formation with catalytic site of AChE. The lowest value of ΔG was observed similar for caffeic acid and caffeine at about −16 kJ/mol. All tested compounds showed positive entropy changes ΔS indicating hydrophobic interactions as well (Table 1). The positive ΔS and negative ΔG additionally show conformational changes after binding in the catalytic pocket of the enzyme (Leavitt and Freire 2001). The binding constant K_A of the above interactions ranged from 0.537 to 184.84×10^3 L/mol for caffeic acid and dihydrocaffeic acid, respectively. The high value of K_A was showed for dihydrocaffeic acid 84.84×10^3 L/mol and next for 3-O-caffeoylquinic acid $137{,}50\times10^3$ L/mol.

ITC analysis of coffee extracts showed strong effects of interaction with the enzyme of green Robusta, were was the highest affinity determined ΔG −35.87 kJ/mol and binding constant 11.50×10^3 L/mol and is was due to the highest concentration of polyphenols contained in green Robusta coffee (Table 2, Fig. 2 A, B). The concentration of CHAs according to Budryn in green Robusta coffee is 4 time greater then roasted coffee, was 54.35 − 13.06% (Budryn et al. 2017). Analyzing the research we can observe that the process of roasting coffee caused a reduction of affinity K_A, ΔH and ΔG. However, the increase levels on affinity were characterized by light roasted brewed coffee. This may suggest that the early Maillard reaction products, besides chlorogenic acids, may also have an effect on interactions with AChE.

Inhibition of acetylcholinesterase

We determined the ability to inhibit the activity of the enzyme by subtracting the energetic effect of interaction of the AChE with CGAs or coffee extracts from energetic effect of interaction of the AChE with ACh in the presence of coffee compounds or extracts. The analysis of bioactive compounds showed the highest AChE inhibitory activity of 3-O-caffeoylquinic and 3,5-di-O-caffeoylquinic acid, which amounted about 93%, the lowest levels of inhibition was showed by dihydrocaffeic, 4,5-di-O-caffeoylquinic and ferulic acid under 50% (Table 1). We calculated also IC_{50} to show the ability of ligands to inhibit the activity of AChE in terms of molar ratio. The highest values of IC_{50} were observed both for 3-O-caffeoylquinic and 3,5-di-O-caffeoylquinic acid at about 5.3 μmol/L, the lowest IC_{50} was calculated for dihydrocaffeic acid 25.93 μmol/L. These results suggest that although dihydrocaffeic acid showed high affinity for AChE manifested as high ΔG value, its inhibitory properties are at a low level. The 3-O-caffeoylquinic acid shows the most stable complex and strong interaction with AChE and also the highest inhibitory activity. K_M of ACh hydrolysis by AChE according to Draczkowskiego amounts 73.9 μmol/L, and in our study it was evaluated at 48.5 μmol/L (Draczkowski et al. 2016) and the differences might depend from the

temperature of the experiment. The high value of K_i was showed by 4-O-caffeoylquinic acid 0.955 μmol/L, and its isomers 3-O-caffeoylquinic and 5-O-caffeoylqunic acids, the most abundant chlorogenic acids in coffee brews both exhibited K_i value at about 0.42 μmol/L.

The study of coffee extracts showed the inhibitory activity for AChE in the range from 5.70 to 13.71 %. The green Arabica and Robusta coffee extracts showed the highest affinity and stability of interactions with AChE. The IC_{50} values of these extracts were 10.41 μmol/L and 12.73 μmol/L, respectively, and the K_i amounted 0.531 μmol/L, 0.901 μmol/L. The analysis conducted with chlorogenic acids and their metabolite showed their high potential to form stable adducts with AChE (Table 1), which suggests that thanks to high concentrations of chlorogenic acids in green Arabica and Robusta comparing to roasted beans, this group of compounds affects high binding affinity of coffee extracts for AChE (Budryn et al. 2018). Besides is potential anti-neurodegenerative activity green coffee does not contain acrylamide, which is formed during roasting coffee beans. Acrylamide is a by-product of the Maillard reaction, which in high concentration is a neurotoxic and carcinogenic agent (Budryn et al. 2015). For this reason above of all, green coffee beans should be considered as potential natural AChE inhibitor.

CONCLUSIONS

The conducted studies on the interactions of coffee extracts and their bioactive compounds with acetylcholinesterase and on inhibition of the enzyme activity showed that chlorogenic acids might have the most beneficial properties. The coffee extracts, thanks to the high content of polyphenols and caffeine are potential products limiting hydrolysis of acetylcholine, which can be used to prevention and therapy of Alzheimer's disease. Chlorogenic acids are mainly responsible for inhibiting the enzyme activity, which concentration is the highest in green coffee extract.

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ANALIZA AKTYWNOŚCI KWASÓW HYDROKSYCYNAMONOWYCH Z EKSTRAKTÓW ZIELONEJ I PRAŻONEJ KAWY JAKO INHIBITORÓW ACETYLOCHOLINOESTERAZY, Z WYKORZYSTANIEM METODY IZOTERMICZNEJ KALORYMETRII MIARECZKOWEJ

Streszczenie. Acetylocholinoesteraza (EC 3.1.1.7) jest głównym enzymem odpowiedzialnym za patogenezę choroby Alzheimera (AD). Enzym ten reguluje przewodnictwo impulsów nerwowych poprzez hydrolizę acetylocholiny w szczelinie synaptycznej, obniżając jej stężenie w mózgu. Niskie stężenie acetylocholiny obserwowane w chorobach neurodegeneracyjnych przyczynia się do powstania blaszek amyloidowych oraz zwyrodnień neurofibrylarnych, które wraz z rozwojem AD rozprzestrzeniają się w korze mózgowej. Dlatego poszukuje się naturalnych nietoksycznych inhibitorów AChE. Celem przeprowadzonych badań była ocena stopnia hamowania aktywności AChE przez pojedyncze kwasy hydroksycynamonowe oraz ekstrakty z kawy, w zależności od gatunku kawy oraz jej stopnia prażenia. Badanie przeprowadzono metodą izotermicznej kalorymetrii miareczkowej (ITC), określając interakcje i parametry termodynamiczne. Analiza ITC wykazała, że najbardziej stabilnymi wiązaniami i silnym

oddziaływaniem z AChE charakteryzował się kwas 3-kawoilochinowy, który był także najskuteczniejszym inhibitorem AChE. Analiza ekstraktów kawy wykazała, że najwyższą aktywnością w odniesieniu do AChE charakteryzowała się kawa zielona robusta.

Słowa kluczowe: acetylocholinoesteraza, zielona i prażona kawa, choroba Alzheimera, ITC.

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