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Research article

In vitro screening for acetylcholinesterase and butyrylcholinesterase inhibition and antimicrobial activity of chia seeds (*Salvia hispanica*)

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ABSTRACT

Background: Chia seeds are gaining increasing interest among food producers and consumers because of their prohealth properties.
Results: The aim of this work was to evaluate the potential of chia seeds to act as acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitors. The highest inhibitory activity against AChE and BChE was observed for colored seed ethanol extracts. A positive correlation was found between the presence of quercetin and isoquercetin as well as protocatechuic, hydroxybenzoic, and coumaric acids and the activity of extracts as AChE and BChE inhibitors. It has also been shown that grain fragmentation affects the increase in the activity of seeds against cholinesterases (ChE). Furthermore, seeds have been shown to be a source of substances that inhibit microbial growth.
Conclusions: It was found that the chia seed extracts are rich in polyphenols and inhibit the activity of ChEs; therefore, their use can be considered in further research in the field of treatment and prevention of neurodegenerative diseases.
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1. Introduction

Salvia hispanica L., commonly known as chia, is an annual plant of the Lamiaceae family. It grows in dry and semi-dry climate, and it is native to the present-day Mexico and Guatemala. Chia fruits are schizocarps, which contain large numbers of white, gray, brown, or black seeds of size 1–2 mm. The largest colored or white seeds can be consumed as food because they are large enough to be mechanically separated from the mixture [1,2]. Recent research has shown that chia seeds have high potential to be used in the food industry because their ingredients can be beneficial to health. Many bioactive compounds with high antioxidative potential have been identified in chia seeds, *e.g.,* phenolic acids (gallic acid, caffeic acid, chlorogenic acid, cinnamic acid, ferulic acid, and p-coumaric acid), flavonoids, (quercetin, kaempferol, epicatechin, rutin, and apigenin), antioxidants (tocopherols), and sterols [1,3]. Apart from that, chia seeds have high content of polyunsaturated fatty acids (80.4%), with linolenic acid and linoleic acid being predominant. Chia seeds also contain monounsaturated fatty acids (mostly oleic acid). The content of saturated fatty acids in chia seeds amounts to 8.65%, and it is similar to the content of these acids in flax seeds (7.87%). The ratio between omega-6 and omega-3 fatty acids in chia seeds is 0.35 [1,4]. Because of the antioxidative properties of chia seeds, they may significantly prevent type 2 diabetes because they have a hypotensive effect, which changes the blood rheological parameters and reduces obesity [2,5,6]. Research on the physical properties of chia seeds proves that chia gel can replace up to 25% of oil or eggs, and this replacement does not affect the texture, color, or taste of the dish [7,8,9,10]. Chia seeds are said to have a wide range of health beneficial effects. Many *in vitro* and *in vivo* studies indicate their high bioactivity. Chia seeds are thought to play a significant role in the regulation of carbohydrate and lipid metabolism [11]. Hydrolysates of chia seed proteins have been proved to inhibit the angiotensin-converting enzyme (ACE) [5]. As chia seeds have antioxidative properties, they play a significant role in the prevention of type 2 diabetes because they reduce blood pressure, coagulation factors, and obesity. The effect of chia in a biosystem has

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been described in numerous studies [2,3,4,5,6]. However, there are no unequivocal data to show that chia seeds and its compounds prevent neurodegenerative diseases. Researchers observed considerably reduced activity of choline acetyltransferase in different parts of the brain of patients suffering from Alzheimer's disease. They also noted neuronal degeneration in the nucleus basalis of Meynert, reduced choline uptake, and reduced release of acetylcholine. All these observations led to the cholinergic hypothesis of the development of Alzheimer's disease [12,13]. It became more important because the contemporary therapy is based on cholinesterase (ChE) inhibitors only [14], which provide symptomatic treatment [15]. The available data describing the role of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) show that the inhibition of these enzymes in the central nervous system increases the concentration of acetylcholine in the forebrain. This improves the cognitive functions and delays neurodegenerative lesions such as those in Alzheimer's disease. Because of rather unfavorable pharmacokinetic parameters of registered compounds, researchers worldwide search for new AChE and BChE inhibitors. New compounds might be found in plants containing the following groups of compounds: alkaloids (indoles, steroids, piperidines, and amaryllidaceae), phenylpropanoids (furanocoumarins, xanthones, and flavonoids), and terpenoids (diterpenes) [12,16]. Recently, the antimicrobial effect of plant materials has also been considered. This group of plant materials also includes those containing mucus, *e.g.*, polysaccharides (galactosyl oligosaccharides). Their presence in the digestive system positively influences its functioning by promoting the growth of beneficial bacteria and inhibiting the growth of pathogenic microorganisms in the human gastrointestinal tract, which may cause the recognition of seeds as a raw material of prebiotic importance. They protect the upper respiratory tract mucosa from irritation in the oral cavity and pharynx, and they prevent coughing. The protective layer of plant mucus on the surface of the upper respiratory tract mucosa may inhibit the development of microorganisms and reduce inflammation. Apart from that, the antimicrobial effect of plant components may improve the microbial quality of food made from these plants [7,8].

No toxic effects of polyphenols present in food on nerve cells have been observed. However, there are reports in literature that flavonoids, especially those used at high, nonphysiological concentrations, may have pro-oxidative effects. It is suggested that flavonoids having a pyrogallol or a catechol moiety, under the presence of copper (II) ions, undergo autoxidation, which in turn leads to the formation of copper (I) ions and the semiquiinone radical. The reaction of copper (I) ions with oxygen produces superoxide anion radicals, followed by hydrogen peroxide. The resulting copper (I) ions bind to the DNA, and then, because of interaction with H_2O_2, reactive oxygen species forming copper hydroperoxide (Cu (I) OOH) connections are produced. The Cu (I) OOH complex can be treated as a hydroxyl radical-binding system, which upon release causes oxidative modification of DNA, mainly the thymine residue. In contrast, the oxidized form of the flavonoid, *e.g.,* in the form of the semiquinone or benzoquinone radical, undergoes a nonenzymatic reduction with the participation of NADH, which consequently initiates a cycle of redox reactions; as a result, large amounts of reactive oxygen species are produced. Excess polyphenols can be harmful because chelates with iron significantly reduce the level of this element in the body. Tannins have the ability to bind iron ions. In addition, a high content of polyphenols in food inactivates digestive enzymes [14,17,18,19].

Further, comprehensive research on plant polyphenols is necessary, considering the interaction between many components found in the extracts next to each other. An important aspect that should be considered is the dependence of phytochemicals on the concentration and presence of additional factors (*e.g.,* microflora and metal ions), which may result in the compound, instead of the expected therapeutic effect, causing serious damage to the body, thus acting inversely than expected (anti- and pro-oxidative effects). A better understanding of the mechanisms of action of the described phytochemicals is therefore necessary if polyphenols are to become effective therapeutics not only in theory but also in practice.

In view of the aforementioned facts, the aim of this study was to assess the potential of chia seeds to act as AChE and BChE inhibitors and as a source of substances with antimicrobial effect. Commercially available whole and fine-ground white and colored chia seeds were used in this study.

2. Materials and methods

2.1. Material

Commercially available white and colored (gray, brown, and black) chia seeds from Salta, Argentina, were used (24°45′06.4″S 65°29′33.9″W) in the research. The seeds were hermetically packed in plastic containers and stored at 20°C until use. The basic chemical composition was identified (34.8 g/100 g lipids, 4.5 g/100 g ash, 19.4 g/100 g protein, 23.4 g/100 g dietary fiber, and 17.4 g/100 g other carbohydrates). Whole and fine-ground seeds were investigated. They were ground in a Retsch Grindomix GM 200 (Haan, Germany) at 360-second intervals, at a speed of 4000 × g and temperature of 21°C.

2.2. Extraction

Whole and ground white and colored seeds underwent aqueous and aqueous-ethanol extraction. Triple extraction was used to obtain an aqueous extract. A sample weight of 50 g of chia flour made from white or colored seeds was flooded with a total amount of 1000 ml of water (at consecutive amounts of 400, 300, and 300 ml) at a temperature of 85°C, and the extraction was performed for 10 min. The extraction procedure was repeated three times. Each time, the extract was filtered, centrifuged (2697 × g, 15 min), and decanted. Each fraction was filtered (Whatman 1:11 μm), and the supernatants were combined and lyophilized. An aqueous-ethanol extract (40%) was obtained by initial flooding of 50 g of chia flour with 400 ml of the solvent. Each time, the samples were shaken in a water bath for 15 min at a temperature of 21°C and stable amplitude, and the supernatant was decanted. At the second and third stages of extraction, the sample of chia seeds was flooded with 300 ml of the solvent. The solutions from the three stages were combined and filtered (Whatman 1:11 μm); thus, the final extract was obtained. The total extraction time was 45 min. The supernatants were combined, and the solvent was evaporated in a vacuum rotary evaporator. The aqueous remnant was lyophilized.

2.3. Content of phenolic acids and flavonols

Phenolic acids and flavonols were analyzed in the extracts obtained in accordance with the extraction methods described above. Qualitative and quantitative analyses of polyphenols were carried out using an Agilent Infinity high-performance liquid chromatograph (Agilent Technologies), according to the methodology described by Kobus et al. [20] (Fig. 2). The following chromatographic parameters were applied for individual compounds:

The flavonols and phenolic acids were isolated by extraction into the solid phase. A Chromabond System (Macherey Nagel, Germany) coupled to Discovery® DSC-18, 3 ml, 500 mg columns (Sigma-Aldrich, Supelco) containing modified silica gel was used. The columns were conditioned with 5 ml of methanol. The trials were conducted using the column with a sample amount of 5 ml, and the compounds were eluted using 5 ml of methanol. The last fraction was collected in a flask up to 10 ml and injected into the HPLC system. Phenolic acids were separated using a NovaPack C18 column (5 mm, 150 × 3.9 mm). The separation took place at a mobile phase flow gradient and temperature of 20°C. Two solvents were used in the mobile phase:

water (pH 2.6) treated with orthophosphoric acid (phase A) and acetonitrile/water (50:50 *V/V*, (phase B)). The flow rate of the mobile phase was 1 ml/min. The gradient program started with 100% solution A and ended with 50% solution B after 50 min of separation. Analyses were detected with a UV detector at a wavelength of 250 nm for p-hydroxybenzoic acid, protocatechuic acid, gallic acid, and vanillic acid and at a wavelength of 320 nm for caffeic acid, chlorogenic acid, p-coumaric acid, and ferulic acid. The same column was used to analyze the content of flavonoids. The separation took place in a gradient, at a temperature of 40°C. Two solvents were used in the mobile phase: 0.3% aqueous solution of formic acid (A) and acetonitrile (B). The gradient program started with 85% solution A and ended with 25% solution B after 40 min of separation. The flow rate of the mobile phase was 1 ml/min. The detection took place at a wavelength of 370 nm. The following compounds were measured in the extracts: myricetin, quercetin, rutin, hyperoside, isoquercetin, kaempferol, and astragalin. The identification and measurement of the quantities of phenolic acids and flavonoids in the extracts were on the basis of calibration curves.

2.4. Cholinesterase inhibition (ChE)

The modified spectrometric method developed by Ellman et al. [21] was used to measure the activity of the extracts as AChE and BChE inhibitors. A POLARstar Omega – BMG LABTECH plate reader was used for taking measurements of 96-well plates of a maximum volume of 300 μl. The hydrolysis of acetylthiocholine/butyrylthiocholine caused a change in color. The absorbance of the enzymes was measured at a wavelength of 412 nm, 10 min after pipetting on a microplate.

Fresh solutions of reagents were prepared in Tris–HCl buffer (50 mmol/dm^3, pH 8). The enzyme solutions were prepared by solving 2 U/ml in 2.0 ml of phosphate buffer. The reaction mixture was composed of 0.035 ml of the sample under analysis, 0.086 cm^3 of Tris–HCl buffer (50 mmol/dm^3, pH 8), 0.035 cm^3 ATChI or BTCh (1.5 mmol/dm^3), 0.194 cm^3 DTNB (0.3 mmol/dm^3 with 10 mmol/dm^3 NaCl and 2 mmol/dm^3 $MgCl_2 \times 6\ H_2O$), and AChE or BChE solution. During measurements, the temperature was approximately 22°C. Absorbance was measured after 15 min (BChE) or 30 min (AChE). Eserine was chosen as the reference compound. It is a highly toxic indole alkaloid, which can be found in Calabar bean seeds. It is a very effective ChE inhibitor. Simultaneously, a positive control sample containing eserine (90.7 μmol × dm^3), a well-known ChE inhibitor, and a negative control sample without the ChE inhibitor were analyzed. The background of the samples was considered. The anti-ChE activity was calculated from serine calibration curves for concentrations of 0.08 μmol/dm^3 to 6.50 μmol/dm^3 (AChE) and 0.08 μmol/dm^3 to 8.30 μmol/dm^3 (BChE). All the samples were investigated eight times. The results were expressed as inhibition percentage.

2.5. Antimicrobial activity

Indicator microorganisms, gram positive: *Clostridium difficile* ATCC 9689, *Clostridium butyricum* ATTC 860, *Listeria monocytogenes* ATCC 7644, *Enterococcus faecalis* ATTC 29212, *Staphylococcus aureus* ATCC 25923, *Listeria ivanovii* ATTC 19119, *Listeria innocua* ATTC 33090 and *Lactococcus lactis*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus paracasei*, *Streptococcus thermophilus*, *Lactobacillus reuteri* DSM 12246, *Bifidobacterium animalis*, *Bifidobacterium longum*, *Bifidobacterium lactis*, and *Bifidobacterium infantis*; gram negative: *Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 12453, *Klebsiella pneumoniae* ATCC 31488, *Enterobacter hormaechei* ATCC 700323, *Enterobacter aerogenes* ATCC 13048, *Rhodococcus equi* ATCC 6939, *Alcaligenes faecalis* ATCC 35655, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 14028, and *Salmonella enteritidis* ATCC 13076; and yeast: *Candida krusei* ATCC 14243, *Candida albicans* ATTC 10231, were transferred to test tubes containing Mueller-Hinton medium. They were cultured at 37°C for 24 h. Next, the liquefied agar medium was inoculated with 10% (*v*/*v*) 24 h indicator culture and poured into Petri dishes to obtain a distinct confluent layer. After solidification of the broth medium inoculated with the indicator microorganisms, wells were made with a cork borer. Each well was supplemented with 150 μL of liquid chia seed medium. Next, the diameters of the indicator bacteria growth inhibition or reduction were measured. The inhibition of the growth of the indicator microorganisms was manifested by complete lightening around the place where the liquid culture/supernatant was transferred. It indicated bactericidal activity of the bacterial strain. Bacteriostatic properties were determined by measuring the diameter of the growth inhibition zone (indicator strain growth limitation).

2.6. Statistical analysis

The data were analyzed statistically using STATISTICA™ PL 13.1 (StatSoft, Poland). Individual parameters were described statistically. The results presented in the article are the arithmetic mean of at least two series replicated three times.

Table 1
Content of flavonols and phenolic acids in *chia (Salvia hispanica L.)* water and ethanol-water extracts.

Sample/chemical	WWW	WWG	WEW	WEG	CWW	CWG	CEW	CEG
Rutin	3.54^{a} ± 0.09	10.68^{c} ± 1.17	5.14 ^{a} ± 2.14	14.10^{d} ± 2.31	7.68^{b} ± 0.22	22.54^{e} ± 1.77	13.97^{d} ± 2.32	29.61^{f} ± 1.77
Astragalin	0.01^{a} ± 0.00	0.01^{a} ± 0.02	0.02 ^{a} ± 0.02	0.03^{a} ± 0.00	1.24 ^{b} ± 0.11	0.94^{b} ± 0.06	0.35^{b} ± 0.32	0.46^{b} ± 0.00
Hyperoside	1.46 ^{b} ± 0.02	1.41 ^{b} ± 0.02	0.00^{a} ± 0.00	0.03 ^{a} ± 0.00	3.39 ^{b} ± 0.16	3.33 ^{b} ± 0.04	3.03 ^{b} ± 0.22	3.06^{b} ± 0.03
Quercetin	3.20 ^{b} ± 0.02	3.15 ^{b} ± 0.05	0.71 ^{a} ± 0.03	0.97 ^{a} ± 0.03	1.20 ^{a} ± 0.01	0.91 ^{a} ± 0.02	0.80 ^{a} ± 0.02	0.94 ^{a} ± 0.02
Isoquercetin	1.73 ^{a} ± 0.02	1.36 ^{a} ± 0.02	1.30 ^{a} ± 0.04	1.19 ^{a} ± 0.02	3.11 ^{b} ± 0.01	2.36 ^{a} ± 0.01	2.04 ^{a} ± 0.01	2.40 ^{a} ± 0.01
Myricetin	0.01 ^{a} ± 0.00	0.01 ^{a} ± 0.00	0.43 ^{a} ± 0.02	0.94 ^{a} ± 0.03	0.61 ^{a} ± 0.01	0.57 ^{a} ± 0.01	0.57 ^{a} ± 0.02	0.64 ^{a} ± 0.02
Kaempferol	2.63 ^{b} ± 0.02	2.83 ^{b} ± 0.02	0.15 ^{a} ± 0.00	0.97 ^{a} ± 0.01	0.75 ^{a} ± 0.01	0.57 ^{a} ± 0.03	0.24 ^{a} ± 0.00	0.86 ^{a} ± 0.01
Isorhamnetin	0.01 ^{a} ± 0.02	0.01 ^{a} ± 0.02	0.05 ^{a} ± 0.02	0.46 ^{a} ± 0.01	0.32 ^{a} ± 0.01	0.24 ^{a} ± 0.04	0.34 ^{a} ± 0.03	0.32 ^{a} ± 0.01
Protocatechuic acid	1.33 ^{a} ± 0.02	1.34 ^{a} ± 0.02	1.87 ^{a} ± 0.02	3.92 ^{b} ± 0.02	4.64 ^{b} ± 0.12	3.51 ^{b} ± 0.12	1.61 ^{a} ± 0.02	1.60 ^{a} ± 0.02
p-Hydroxybenzoic acid	1.52 ^{b} ± 0.03	1.57 ^{b} ± 0.01	2.94 ^{b} ± 0.02	4.08 ^{b} ± 0.02	2.67 ^{b} ± 0.06	2.35 ^{b} ± 0.07	0.32 ^{a} ± 0.03	0.39 ^{a} ± 0.01
Vanillic acid	2.74 ^{a} ± 0.02	3.05 ^{a} ± 0.05	15.45 ^{b} ± 0.02	20.17 ^{b} ± 0.07	0.84 ^{a} ± 0.22	0.63 ^{a} ± 0.02	0.48 ^{a} ± 0.03	0.59 ^{a} ± 0.02
Caffeic acid	5.62 ^{b} ± 0.04	5.05 ^{b} ± 0.02	8.42 ^{c} ± 0.07	9.32 ^{c} ± 0.02	0.88 ^{a} ± 0.01	0.62 ^{a} ± 0.04	0.49 ^{a} ± 0.02	0.67 ^{a} ± 0.03
Gallic acid	5.50 ^{b} ± 0.02	5.05 ^{b} ± 0.04	5.61 ^{b} ± 0.02	6.63 ^{b} ± 0.02	0.01 ^{a} ± 0.00	0.01 ^{a} ± 0.03	0.01 ^{a} ± 0.04	0.01 ^{a} ± 0.00
Chlorogenic acid	15.39 ^{c} ± 0.02	14.67 ^{c} ± 0.13	21.37 ^{d} ± 0.09	31.64 ^{e} ± 0.23	5.45 ^{b} ± 0.02	7.74 ^{b} ± 0.09	2.60 ^{a} ± 0.02	5.87 ^{b} ± 0.04
p-Coumaric acid	0.54 ^{a} ± 0.02	0.78 ^{a} ± 0.02	5.12 ^{b} ± 0.06	8.64 ^{b} ± 0.11	0.81 ^{a} ± 0.02	0.61 ^{a} ± 0.02	0.48 ^{a} ± 0.04	0.58 ^{a} ± 0.02
Ferulic acid	3.48 ^{a} ± 0.02	2.56 ^{a} ± 0.05	5.62 ^{b} ± 0.02	7.55 ^{b,c} ± 0.06	9.99 ^{c} ± 0.32	6.80 ^{b} ± 0.03	6.34 ^{b} ± 0.30	6.65 ^{b} ± 0.06

The mean values in the line marked with different small letters indicate significance of differences ($P \leq 0.05$). Abbreviation: WWW: white whole chia seed water extract; WWG: white ground water chia seed extract; WEW: white whole ethanol chia seed extract; WEG: white ground ethanol chia seed extract; CWW: color whole chia seed water extract; CWG: color ground water chia seed extract; CEW: color whole ethanol chia seed extract; CEG: color ground ethanol chia seed extract.

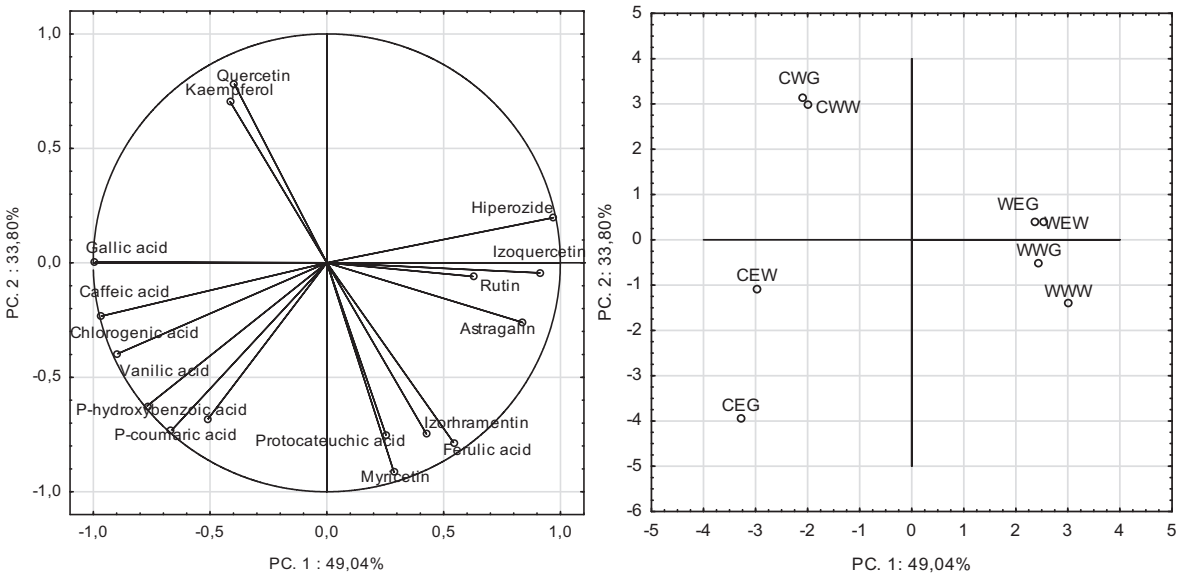

Fig. 1. PC analysis for quality indexes in the profile of flavonol and phenolic acid analysis in chia seed extracts.

The mean values of the traits under study were compared by analysis of variance for factor systems with a diversified number of observations. Differences between groups were assessed by Tukey's test or Spjotvoll's test (extended Tukey's test for samples of unequal sizes). The assumptions of the analysis were also checked. Pearson correlation coefficients were calculated to assess the strength of relation between the samples under analysis. The significance of the correlation coefficient was checked by means of Student's t-test. The significance of statistical inference was $\alpha = 0.05$. Nonlinear (exponential) regression equations were used to determine the character and trend of dependences between the activities under analysis. Apart from that, principle component analysis (PCA) was also used.

3. Results and discussion

Cui et al. [22], Derrien et al. [23], Gramza-Michałowska et al. [24], and Hempel et al. [25] proved that when different solvents were used for extraction, the extracts were characterized by different composition, which resulted in different bioactivity, including the antioxidative, antimicrobial, and enzyme-inhibitory effects.

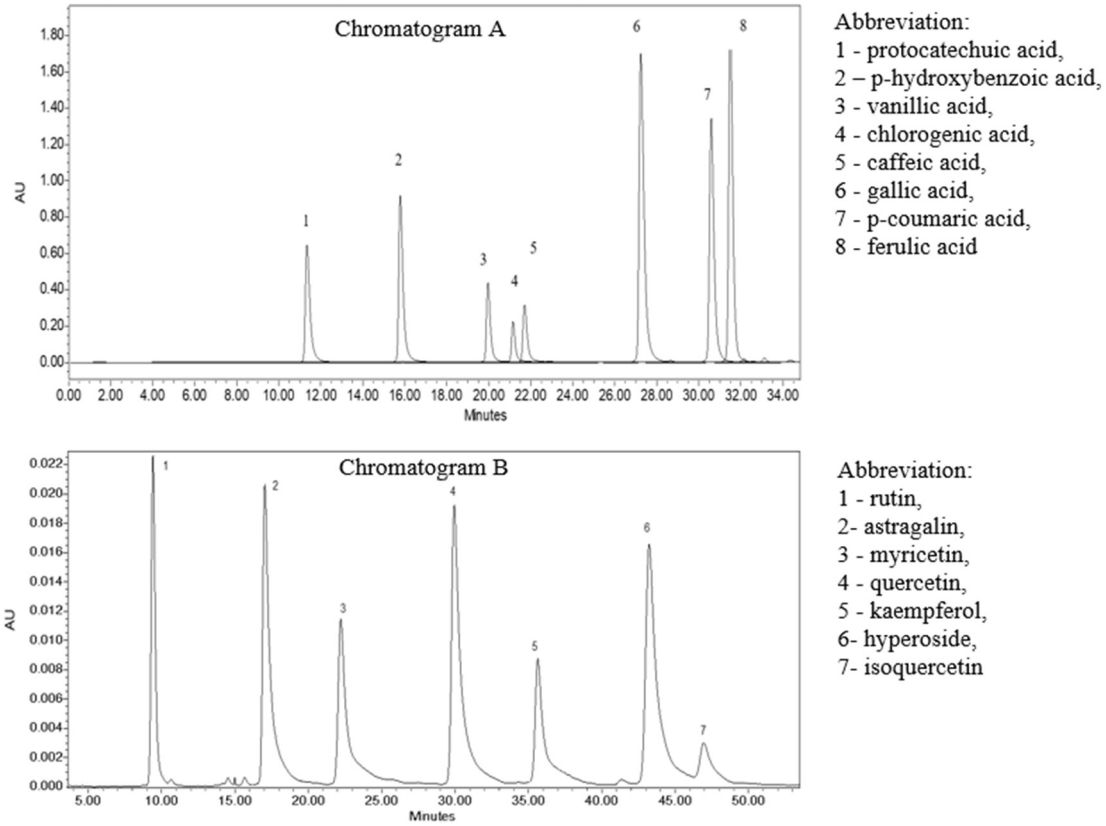

Fig. 2. Chromatogram of the pattern distribution of phenolic acids (Chromatogram A) and flavonols (Chromatogram B).

Monosaccharides, oligosaccharides, organic acids, pigments, tannins, polyphenols, *etc.* can be found in extracts. Their proportions differ according to the solvent used for extraction [17,26]. Numerous reports indicate that sugars as well as anthocyanins and tannins are transferred to the solvent as a result of aqueous extraction. The much lower content of polyphenols affects their lower antiradical potential [26]. Polyphenols are components of plant ingredients used for nutrition. They affect their bioactivity and are responsible for antioxidative, antimicrobial, and health-promoting effects. This activity is caused by the specific structure of polyphenols, which includes aromatic compounds and hydroxyl groups [2]. There have been numerous analyses of the composition of polyphenols in chia seeds. The results of these analyses coincide with the results of our study. The extracts were analyzed for the content of phenolic acids and flavonols. The results of measurements are shown in Table 1 and Fig. 1. The analysis revealed significant differences in the composition of phenolic acids in the chia seed extracts. Chlorogenic acid, ferulic acid, and protocatechuic acid predominated in the white seed extracts (Fig. 3). They amounted to 6–31% of the total content of phenolic acids. Vanillic acid, caffeic acid, gallic acid, and chlorogenic acid predominated in the colored seed extracts. Aqueous-ethanol extracts from colored seeds had statistically the highest total content of phenolic acids. The research also revealed that the grinding of seeds increased the amount of phenolic acids extracted by means of the aqueous-ethanol mixture. There were no differences between fine-ground and whole-seed aqueous extracts in the content of phenolic acids. Next, the content of flavonols in the chia seed extracts was analyzed qualitatively and quantitatively. The content of the following compounds was measured: rutin, astragalin, hyperoside, quercetin, isoquercetin, myricetin, kaempferol, and isorhamnetin. The analyses revealed similarities in the qualitative composition of flavonols from the chia seed extracts, but there were quantitative differences. Rutin was the predominant flavonol in both extracts made from whole and ground seeds. Its content ranged from 3.54 mg/g in WWW to 29.61 mg/g in CEG. The chia seeds also contained hyperoside, quercetin, and isoquercetin. The extracts contained minimal amounts

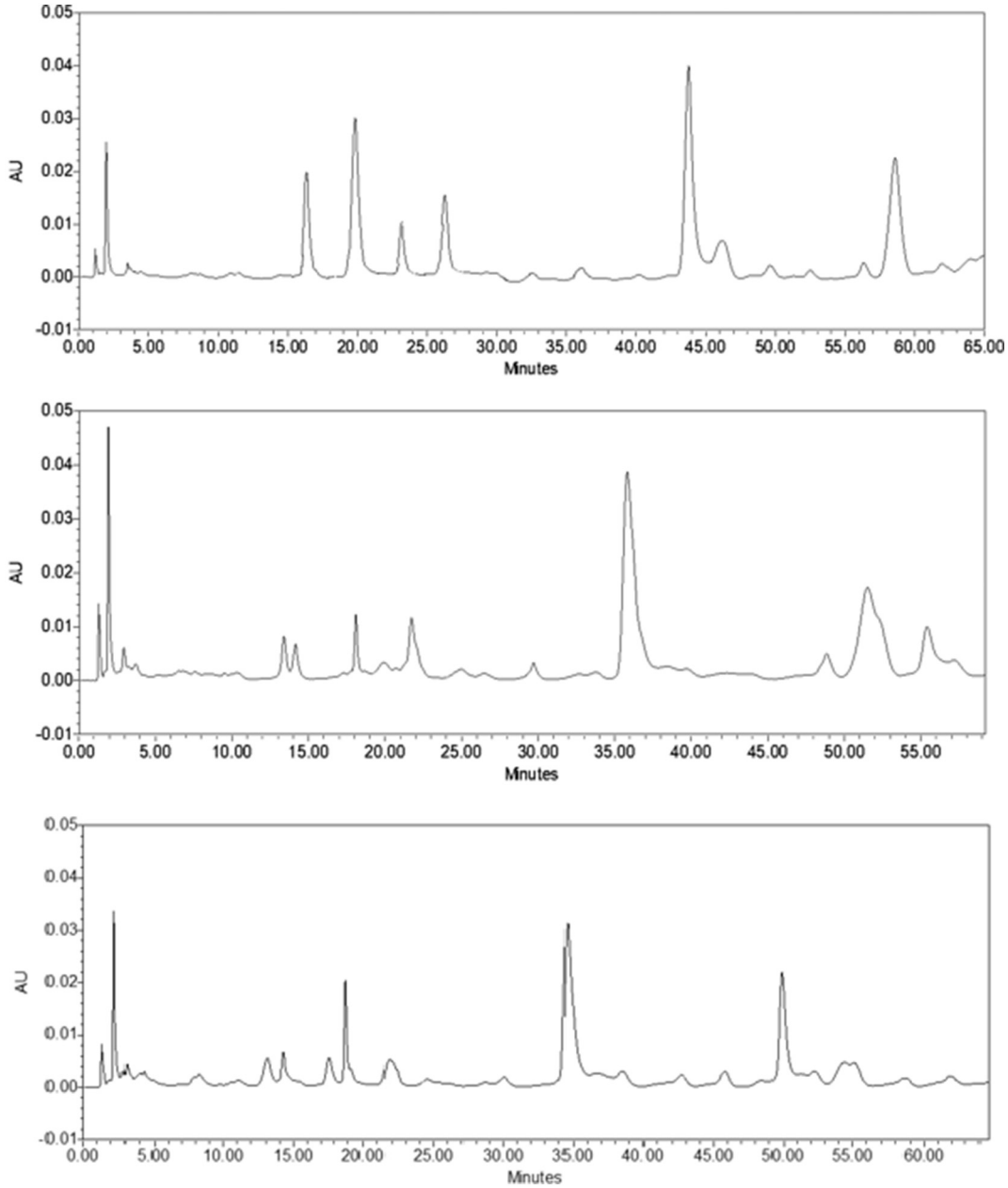

Fig. 3. Chromatograms of flavonols in chia seed water and ethanol–water extracts–examples.

of astragalin, myricetin, and kaempferol. The total content of flavonols in ethanol-aqueous and aqueous extracts from ground seeds was 23–51% greater than that in the extracts from whole seeds. The total content of flavonols in the extracts from white seeds was 20–43% lower than that in the extracts from colored seeds. The PCA (biplot) projection of the results of qualitative analysis of the extracts from white and colored seeds in the arrangement of two first components (PC1 and PC2), which were responsible for more than 80% of variability in the composition, showed that the samples differed in their content of active components and the influence of individual compounds on the extract characteristics. The close position of phenolic acid vectors showed that these indicators were positively correlated. On the other hand, the opposite position of flavonol vectors and acids showed negative correlation. The grouping of white seed extracts on the right side of the diagram and a group of samples of ethanol extracts from colored seeds and aqueous extracts from colored seeds indicate that the samples were more diversified in their content of the compounds under analysis. The results of statistical analysis proved that the type of seeds, *i.e.*, white or colored seeds, and the solvent type (for colored seeds) influenced the profile of polyphenols contained in the extract. Oliveira-Alves et al. [3] indicated caffeic acid, ferulic acid, and protocatechuic acid as the main acids found in seed husks and whole seeds. Similar to the findings of our study, Marineli et al. [27] pointed to the high content of myricetin, quercetin, kaempferol, and chlorogenic acid in chia seeds. These compounds have multidirectional effects in a biosystem, which result in a health-promoting effect. According to reports in literature, phenolic acids and flavonols are ChE inhibitors, which can be used for the prevention and treatment of neurodegenerative diseases such as Alzheimer's disease [28]. As mentioned above, there are two types of ChEs, *i.e.*, AChE and BChE, which differ in their susceptibility to inhibitors, reaction to substrate excess, and substrate binding specificity. Our study proved the difference between BChE and AChE in their susceptibility to chia seed inhibitors. The research did not reveal clear dependences between the activities of aqueous or ethanol extracts from whole or ground chia seeds. In contrast to other esterases, both enzymes hydrolyze choline esters at a very high, although different, rate [29]. Apart from that, they exhibit peptidase and aryl acylamidase activity [30]. The inhibition of ChE activity is of key importance in symptomatic treatment of Alzheimer's disease. ChE inhibitors block enzymes, and thus, they increase the content of acetylcholine in cholinergic synapses and improve neuronal transmission. The leaf and fruit extracts were characterized in terms of their capacity to inhibit the activity of ChEs, *i.e.*, AChE and BChE. The results of the analyses are shown in Fig. 2 and Fig. 3. The inhibition capacity was expressed as eserine equivalents. All the chia extracts exhibited ChE inhibition capacity in a concentration-dependent manner. The chia seed extracts inhibited AChE more than BChE, on average between 33% and 58% of their activity. Apart from that, ethanol extracts from white and colored seeds inhibited ChE more than aqueous extracts. All the ethanol extracts exhibited greater AChE inhibition activity. Their activity decreased in the following order: CEG > CEW > WEG > WEW. There was a different dependence observed in the assessment of the activity of the extracts against BChE. The extracts from colored seeds exhibited the highest inhibitory activity. It was characterized in the following order: CEG > CEW > CWG > CWW. Ethanol extracts from colored seeds exhibited the greatest inhibitory activity against both AChE and BChE. Apart from ethanol extracts from white seeds, all the samples from ground seeds exhibited greater activity against ChEs. The known CChE inhibitors belong to different groups of compounds such as carbamates, acridines, piperidines, and polyphenols. It is thought that a vast majority of ChE inhibitors are alkaloids including indole and steroid alkaloids. There are an increasing number of scientific reports on the capacity of numerous phenolic acids and flavonols to inhibit ChE activity [30]. The same observation was made in our study. The statistical analysis of research results confirmed the positive correlation between the presence of quercetin, isoquercetin, protocatechuic acid, hydroxybenzoic acid, and coumaric acid and the inhibitory activity of chia seed extracts against AChE and BChE (Table 2).The capacity to inhibit ChE activity by chlorogenic acid as well as 3- and 4-hydroxybenzoic acid was also documented in the research described by Szwajgier et al. [12] and Szwajgier and Borówiec [31]. The research showed that the inhibitory activity was influenced by the distribution and number of –OH and/or OCH3 groups substituted in the phenyl ring. Apart from that, the research also proved that the methyl or ethyl esters of phenolic acids inhibited ChEs more effectively than the corresponding free acids. Similarly, Akhtar et al. [13] proved that the activity of a particular compound against AChE was significantly influenced by the presence of the —OH group in the ortho position in the phenyl ring and the presence of the acidic group. With regard to the methylation of the acidic and hydroxyl groups, the authors observed lesser enzyme-inactivating capacity. Kwon et al. [14] made similar observations about the ChE inhibitory activity. Orhan et al. [16] proved high activity of

Table 2
Correlation coefficients between AChE i BChE and estimated components in chia seed extracts.

Compound	AChE activity	BChE activity
Rutin	0,333 ^{NS}	0,432 ^{NS}
Astragalin	0,316^{NS}	0,412 ^{NS}
Hiperozide	0,514^{NS}	0,423 ^{NS}
Quercetin	0,790*	0,723*
Izoquercetin	0,442 ^{NS}	0,812*
Myricetin	0,336^{NS}	0,177^{NS}
Kaempferol	0,265 ^{NS}	0,309 ^{NS}
Izorhramentin	0,514 ^{NS}	0,452 ^{NS}
Total flavonols	0,399^{NS}	0,531 ^{NS}
Protocateuchic acid	0,812*	0,687*
P-hydroxybenzoic acid	0,243 ^{NS}	0,755*
Vanilic acid	0,611 ^{NS}	0,611 ^{NS}
Caffeic acid	0,811*	0,144^{NS}
Gallic acid	0,423 ^{NS}	0,087^{NS}
Chlorogenic acid	0,744*	0,721*
p-coumaric acid	0,782*	0,556 ^{NS}
Ferulic acid	0,442^{NS}	0,445 ^{NS}
Total acids	0,154^{NS}	0,269 ^{NS}

* $p \leq 0,05$, NS – statistically insignificant.

Table 3
The antimicrobial activity tests on potential pathogenic bacteria.

LP	Microorganism	Seeds not incubated		Seeds incubated 24 h	
		Not ground	Ground	Not ground	Ground
		Inhibition zone diameter (mm)			
1	*Clostridium difficile* ATCC 9689	25	28	20	20
2	*Clostridium butyricum* ATTC 860	15	18	17	19
3	*Listeria monocytogenes* ATCC 7644	24	28	25	27
4	*Pseudomonas aereuginosa* ATCC 27853	20	27	15	17
5	*Salmonella typhimurium* ATCC 14028	14	18	18	18
6	*Salmonella enteritidis* ATCC 13076	11	15	14	15
7	*Proteus mirabilis* ATCC 12453	14	17	15	17
8	*Escherichia coli* ATCC 25922	5	8	7	8
9	*Klebsiella pneumoniae* ATCC 31488	–	–	–	–
10	*Enterobacter hormaechei* ATCC 700323	–	–	–	–
11	*Enterobacter aerogenes* ATCC 13048	–	–	–	–
12	*Enterococcus faecalis* ATTC 29212	–	–	–	–
13	*Staphylococcus aureus* ATCC 25923	–	–	–	–
14	*Listeria ivanovii* ATTC 19119	–	–	–	–
15	*Listeria innocua* ATTC 33090	–	–	–	–
16	*Acinetobacter baumanii* ATCC 19606	–	–	–	–
17	*Rhodococcus equi* ATCC 6939	–	–	–	–
18	*Alcaligenes faecalis* ATCC 35655	–	–	–	–
19	*Candida krusei* ATCC 14243	–	–	–	–
20	*Candida albicans* ATTC 10231	–	–	–	–

Table 4
The antimicrobial activity tests on potential probiotic bacteria.

LP	Microorganism	Seeds not incubated		Seeds incubated for 24 h	
		Not ground	Ground	Not ground	Ground
		Inhibition zone diameter (mm)			
1	*Lactococcus lactis*	8	11	6	10
2	*Lactobacillus acidophilus*	6	8	3	8
3	*Lactobacillus bulgaricus*	6	5	3	4
4	*Lactobacillus paracasei*	5	5	4	4
5	*Streptococcus thermophilus*	2	4	3	4
6	*Lactobacillus reuteri* DSM 12246	–	2	1	2
7	*Bifidobacterium animalis*	–	–	–	–
8	*Bifidobacterium longum*	–	–	–	–
9	*Bifidobacterium lactis*	–	–	–	–
10	*Bifidobacterium infantis*	–	–	–	–

chlorogenic acid against BChE. The authors also proved that caffeic acid, which was formed as a product of chlorogenic acid decomposition, did not exhibit the inhibitory activity. The findings of the research conducted by Balkis et al. [15] were similar to the results of our study. The researchers confirmed the inhibition of ChE activity by quercetin, and they proved the enzyme inactivity caused by rutin and hydroxybenzoic acid. The research showed that the grinding of seeds affected the activity against BChE, but it was impossible to prove whether it increased the inhibitory activity against AChE. However, the research showed that ethanol extraction increased the ChE inhibitory activity of the extracts from white and colored seeds. The statistical analysis of the results proved the positive correlation between the presence of quercetin, isoquercetin, protocatechuic acid, hydroxybenzoic acid, and coumaric acid and the inhibitory activity of chia seed extracts against AChE and BChE (Table 2).

The research included analyses of antimicrobial properties of whole and ground chia seeds. Apart from that, the antimicrobial activity was tested for each fraction suspended in saline solution immediately after preparation and 24 h after incubation at room temperature. The antimicrobial activity was tested on model microorganisms with pathogenic potential and on probiotic bacteria (Table 3 and Table 4). Whole and ground chia seeds exhibited antimicrobial potential against pathogens of the genera *Salmonella* and *Clostridium* as well as *L. monocytogenes*, *P. aeruginosa, E. coli,* and *P. mirabilis*. The ground (nonincubated) seeds exhibited the highest antimicrobial activity against *C. difficile* species (25 mm). The same values were noted for *L. monocytogenes*, where a 25-mm clear zone was observed for whole incubated seeds. Ground (nonincubated) seeds exhibited the highest antimicrobial activity against *L. monocytogenes* (28 mm) and *P. aeruginosa* (27 mm) (Table 3, Fig. 4A). All the species of microorganisms against which chia seeds exhibited their inhibitory activity are classified as pathogens harmful to humans and animals. Their presence in food causes serious poisoning, which is usually fatal (Fig. 5).

The next stage of the research involved assessment of the influence of white and colored chia seeds on probiotic microorganisms. The seeds (especially ground ones) exhibited antimicrobial activity against six out of ten species under investigation. The ground seeds exhibited the strongest effect against *L. lactis* (11 mm), *L. acidophilus* (8 mm), *L. bulgaricus* (5 mm), *L. paracasei* (5 mm), *L. rhamnosus* (5 mm), and *S. thermophilus* (5 mm) (Table 4, Fig. 4B). However, it is noteworthy that the bacteriostatic effect of the seeds on the potentially probiotic species was much lesser than their effect on the microorganisms with pathogenic potential. The results showed that ground chia seeds had greater antimicrobial potential than whole seeds. Polyphenols in chia seeds also exhibit antimicrobial activity, which depends on their concentration in the environment. At low concentrations, polyphenols react with lipids and proteins in the cell membrane. Consequently, the membrane is no longer semipermeable. Ions and other components leak from the cell. As a result of the short duration of contact with these compounds, microorganisms become resistant to them. Exposure to polyphenols or their solutions for long duration at higher concentrations denatures proteins including enzymes and disorders the metabolism in bacterial cells, thereby causing their death [32]. At present, there is an increasing number of studies that attempt to determine the effectiveness of polyphenols as natural substances, thus limiting the development of microorganisms in food [33,34]. These studies have also attempted to determine whether polyphenols can be used for the production of packages from natural polymers with antimicrobial properties. However, the use of these compounds in food and packages might be limited by the fact that they alter the sensory properties of food products. Their antimicrobial activity consists in direct killing or reduction of the phagocytic effect of microorganisms, inhibition of biofilm formation, reduced adhesion and internalization into the host cells, and neutralization of toxins

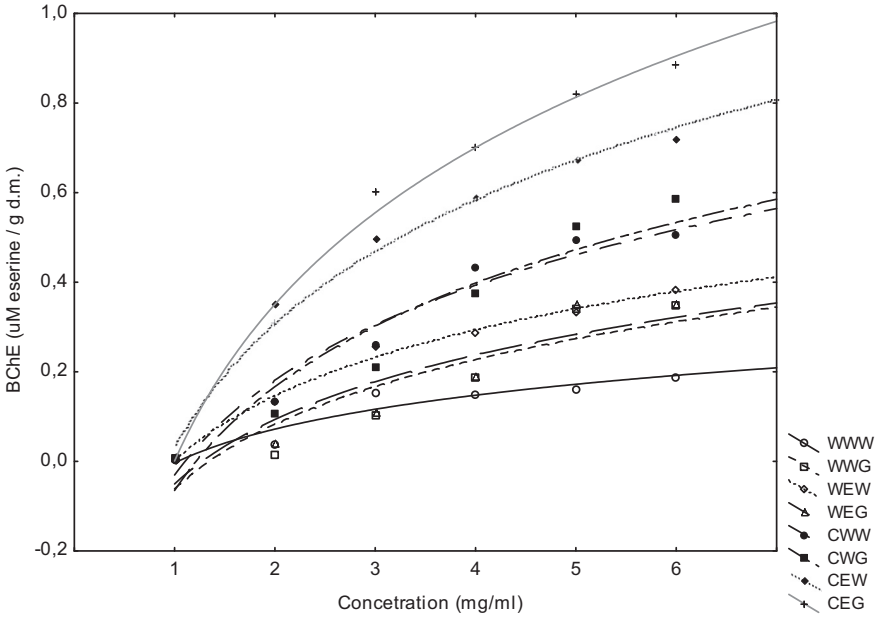

Fig. 4. Activity of *chia seed* extract as an acetylcholinesterase inhibitor. Abbreviation as in Table 1.

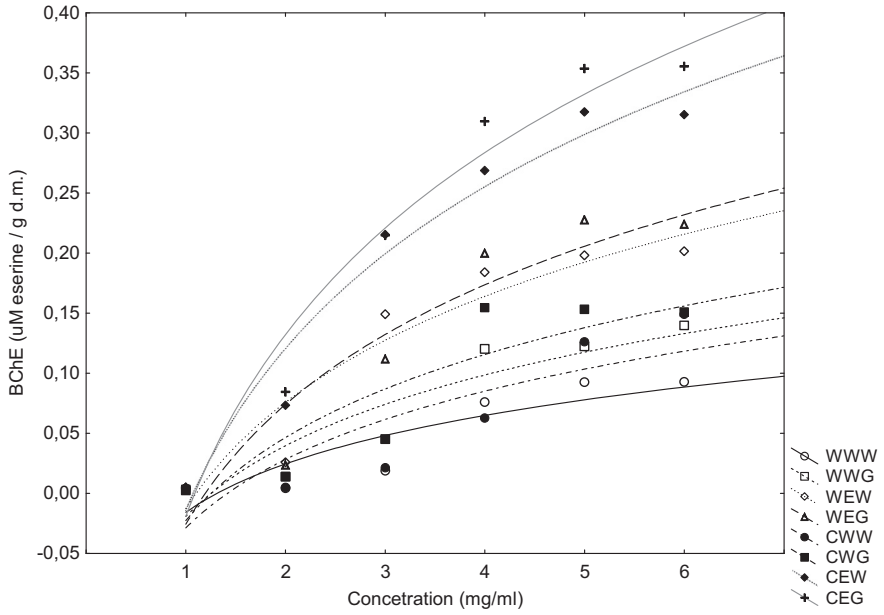

Fig. 5. Activity of chia seed extract as a butyrylcholinesterase inhibitor. Abbreviation as in Table 1.

[35]. On the one hand, the consumption of chia seeds may support the natural system providing protection to the organism because of their content of antioxidants. The presence of chia seeds in readily available food products may increase their microbial stability and prevent contamination without using additional preservatives. Pathogenic strains of the bacteria produce toxin and hydrolytic enzymes. These toxins destroy the cytoskeleton and cause the apoptosis of epithelial cells, induction of pro-inflammatory cytokines, and recruitment of inflammatory cells. They destroy intracellular connections and, together with hydrolytic enzymes, cause colitis, formation of pseudo membranes, and diarrhea [36]. Other human pathogens with experimentally proven sensitivity to the inhibitory effect of phenolic acids, flavonoids, tannins, and anthocyanins are gram-positive bacteria (*S. aureus, L. monocytogenes, M. luteus, E. faecalis, C. botulinum, and B. subtilis*), gram-negative bacteria (*E. coli, S. typhimurium, S. enterica, P. mirabilis, Y. enterocolityca, S. dysenteriae, S. flexneri, P. fluorescens, P. aeruginosa, and V. cholerae*), and the fungal pathogen *C. albicans* [37,38,39]. Kaempferol and quercetin have proven antibacterial properties, although kaempferol is a slightly weaker inhibitor of microbial growth [18]. Thus, we can expect that kaempferol is linked to an enzyme in bacterial cells and blocks the process that is significant for bacterial function. Chia seeds also contain caffeic acid, which exhibits greater antimicrobial activity than p-coumaric acid because of an additional —OH group linked to the phenolic ring in the molecule. Similar observations were made by Figueiredo et al. [40], who demonstrated that the addition of two or more —OH groups increased the antimicrobial activity of benzaldehyde derivatives because they were able to act more effectively on the cell surface by being linked to sulfhydryl groups of proteins [38]. According to Gyawali and Ibrahim [39], free —OH groups can be easily bound with

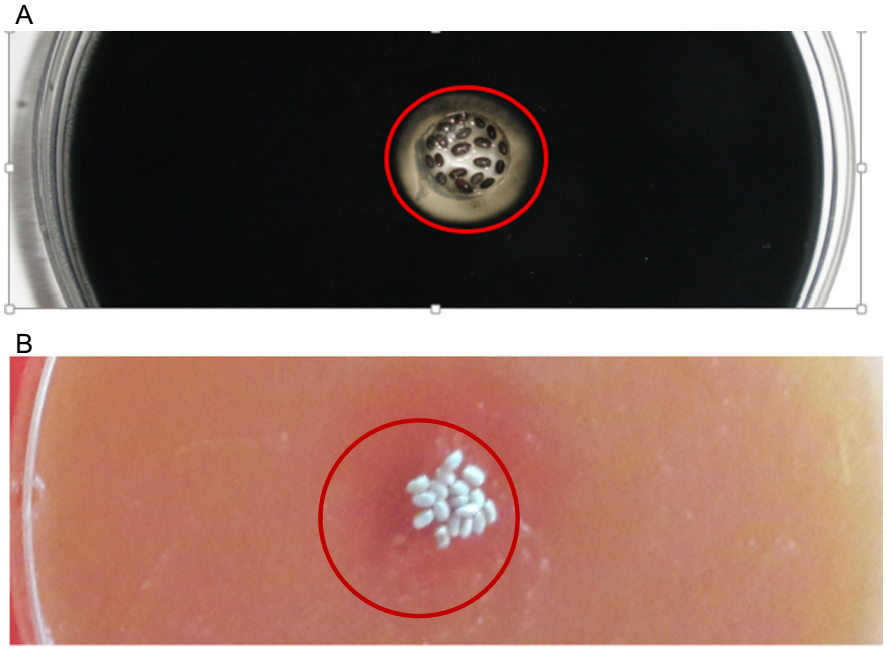

Fig. 6. Inhibitory effect of chia seeds on the bacteria *Clostridium butyricum* (A) and *Lactococcus lactis* (B).

active sites in enzymes, and thus, they can alter the metabolism of microorganisms. The potential of chia seeds against lactic acid fermentation bacteria may be used for the production of fermented milk beverages, where the presence of living bacteria is of key importance for health promotion. According to food technologists and clinicians, it is a favorable phenomenon because bacteria of the genus *Lactobacillus* are a desirable element of the gastrointestinal microbiota. According to scientific reports, like chia seeds, green tea is a source of polyphenols. It is particularly abundant in catechins, gallic acid, caffeic acid, quercetin and its glycosides, and kaempferol. Studies by Duda-Chodak et al. [41] proved that kaempferol and quercetin exhibited a very strong inhibitory effect against microorganisms. As the influence of pure phenolic acids or other flavan-3-ols on these microorganisms was not investigated, it cannot be excluded. What is known from publications is the fact that green tea components inhibit bacteria, chiefly pathogenic species of the genera *Staphylococcus, Streptococcus, Clostridium,* and *Bacillus* as well as *E. coli, H. pylori, M. tuberculosis,* and *P. aeruginosa* [42]. Other researchers proved that the components did not influence *Escherichia* or Eubacterium, whereas at low concentrations, they might stimulate the growth of the lactic acid bacteria *Lactobacillus* and *Bifidobacterium*. The inhibitory effect is chiefly attributed to EGCG, but polymer tannins are also considered. Interestingly, *Lactobacillus* may use these compounds because it is the only microorganism under investigation to produce the tannase enzyme [43]. Gallic acid, which is formed as a result of decomposition of tannins, is easily decomposed into aliphatic acids, which bacteria use for their metabolism in the citric acid cycle (Fig. 6).

4. Conclusions

Several studies conducted by other authors proved that various plant materials and substances contained in extracts inhibited the activity of AChE and/or BChE. This effect might be used for the treatment of Alzheimer's disease. However, to do so, it is necessary to have more data from clinical trials and toxicity tests. In the light of our research, we can say that chia seed extracts are abundant in phenols and they inhibit ChE activity. In view of these facts, their use can be considered in further research on the treatment and prevention of neurodegenerative diseases. In addition, the antibacterial potential of chia seeds has been demonstrated, especially for potentially pathogenic microorganisms, which is an important pro-health attribute of seeds as a potential food additive.

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