Introduction

Alzheimer’s disease (AD) affects the central nervous system (CNS) of persons aged 65 years or older. The typical features of AD include loss of cholinergic neurons and an increase in the activity of butyrylcholinesterase (BChE) and acetylcholinesterase (AChE) (Whitehouse et al., 1981), the enzymes responsible for the rapid hydrolysis of acetylcholine in cholinergic synapses (Rao et al., 2007). The order of effectiveness towards butyrylcholinesterase was: carnosic acid > nordihydroguaiaretic acid > ethyl ferulate > ethyl vanillate > nor-dihydroguaiaretic acid > ethyl 4-hydroxybenzoate = methyl ferulate. The inhibitory activity was dependent on the number/position of OH or/and OCH₃ groups attached to a phenol ring. It can be speculated that OCH₃ substitution in the phenol ring can promote a higher antibutyrylcholinesterase activity (although not statistically confirmed at \( p < 0.05 \)). The presence of a CH=CH-COOH group had a highly favourable effect on the antiacetylcholinesterase activity compared with a CH₂-CH₂-COOH or a COOH group. Methyl and ethyl esters were more potent inhibitors than the corresponding free acids. The molecular weight of the compounds (in the range of \( M = 154.12 \sim 474 \) g/mol) played a minor role in this context.

Key words: Phenolic Acid, Acetylcholinesterase, Butyrylcholinesterase, Alzheimer’s Disease

Anticholinesterase Activity of Phenolic Acids and their Derivatives

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Z. Naturforsch. 68c, 125–132 (2013); received April 2, 2012/February 22, 2013

The ability of 36 phenolic acids and their derivatives to inhibit acetyl- and butyrylcholinesterase was studied. The most efficient acetylcholine inhibitors were: carnosic acid = gentisic acid > 3-hydroxy-4-methoxycinnamic acid = ethyl ferulate = ethyl vanillate = nordihydroguaiaretic acid > ethyl 4-hydroxybenzoate = methyl ferulate. The order of effectiveness towards butyrylcholinesterase was: carnosic acid > nordihydroguaiaretic acid = ethyl ferulate > salicylic acid > gentisic acid > rosmarinic acid = caftaric acid > homogentisic acid. The inhibitory activity was dependent on the number/position of OH or/and OCH₃ groups attached to a phenol ring. It can be speculated that OCH₃ substitution in the phenol ring can promote a higher antibutyrylcholinesterase activity (although not statistically confirmed at \( p < 0.05 \)). The presence of a CH=CH-COOH group had a highly favourable effect on the antiacetylcholinesterase activity compared with a CH₂-CH₂-COOH or a COOH group. Methyl and ethyl esters were more potent inhibitors than the corresponding free acids. The molecular weight of the compounds (in the range of \( M = 154.12 \sim 474 \) g/mol) played a minor role in this context.

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Introduction

Alzheimer’s disease (AD) affects the central nervous system (CNS) of persons aged 65 years or older. The typical features of AD include loss of cholinergic neurons and an increase in the activity of butyrylcholinesterase (BChE) and acetylcholinesterase (AChE) (Whitehouse et al., 1981), the enzymes responsible for the rapid hydrolysis of acetylcholine in cholinergic synapses (Rao et al., 2007). AChE enhances the excessive aggregation of \( \beta \)-amyloid into growing fibrils (\( \beta \)-sheet deposits) (Alvarez et al., 1997; Campos et al., 1998). The positive correlation between \( \beta \)-amyloid fibrils and AD has been repeatedly pointed out by many authors (see review by Seloke and Schenk (2003). The use of cholinesterase inhibitors in AD (Fig. 1) for the reduction of AChE and BChE activities has been investigated since the 1980 s. However, numerous authors, as previously reviewed (Giacobini, 2004; Karczmar, 1998; Sabbagh et al., 2006), have reported a number of side effects after long-term administration of cholinesterase inhibitors to patients. Therefore, the search for new inhibitors as well as new inhibitor-rich food raw materials is a priority.

Phenolic acids are secondary plant metabolites, which occur predominantly as glycosides or esters of organic acids or are bound to cell wall polymers, with only a minority of these compounds present in the free form (Hermann, 1992). Less known phenolic acids are presented in Fig. 2. The \( \text{in vivo} \) neuroprotective role of phenolic acids in the CNS can be distinguished from the \( \text{in vitro} \) antioxidant activity exerted by these compounds. Ferulic, \( p \)-coumaric, nordihydroguaiaretic or rosmarinic acids, respectively, have been previously pointed out as effective neuroprotectants (Yabe et al., 2010; Cheng et al., 2008; Cho et al., 2005; Mamiya et al., 2008). Sinapic acid and ferulic acid attenuated the negative changes in the ACh-signalling system in experimental animals (Yan et al., 2001; Hsieh et al., 2002; Kim et al., 2004, 2007; Karakida et al., 2007). The deposition of \( \beta \)-amyloid plaque was significantly decreased in brains of mice fed with nordihydroguaiaretic and rosmarinic acids. Also, the treatment with rosmarinic acid resulted in an increased content of the non-toxic, soluble \( \beta \)-amyloid (Hamaguchi et al., 2009).

The aim of the present work was to evaluate the \( \text{in vitro} \) anticholinesterase activities of a number of phenolic acids originating from plants. The anticholinesterase activities of the test compounds are discussed in relation to their structures.
Material and Methods

Reagents

Methyl p-coumarate, methyl ferulate, methyl syringate, and methyl vanillate were purchased from Apin Chemicals Ltd. (Abingdon, UK). Other phenolic acids and their derivatives tested in this study as well as acetylthiocholine iodide (ATChI), S-butyrylthiocholine chloride (BTCh), 5,5’-dithiobis-2-nitrobenzoic acid (DTNB), eserine, acetylcholinesterase (AChE), and butyrylcholinesterase (BChE) were purchased from Sigma-Aldrich (Poznań, Poland). Other reagents (HPLC grade) were purchased from P.O.Ch. (Gliwice, Poland).

Test solutions of phenolic acids

Solutions of phenolic acids were freshly prepared in a minimal volume of ethanol (98.0%, v/v) followed by dilution to 0.2 mM in double deionized water. The ethanol content in the test solutions was below 4% and had no influence on the enzyme activity.

Determination of AChE and BChE inhibitory activities

Inhibition of cholinesterases was evaluated using a 96-well microplate reader (Tecan Sunrise, Grödig, Austria) based on the method of Ellman et al. (1961) with modifications. All solutions of reagents were prepared in Tris-HCl buffer (50 mM, pH 8.0). For measurement, 35 μl of ATChI or BTCh (1.5 mM) were mixed with 35 μl of the sample, 80 μl of Tris-HCl buffer (50 mM, pH 8.0), 20 μl of AChE or BChE solution (0.28 units/ml) and 175 μl of 0.3 mM DTNB (containing 10 mM NaCl and 2 mM MgCl₂). Blanks containing eserine (7.27 μM) or Tris-HCl buffer instead of the test sample were also run. Spontaneous hydrolysis of the substrate was determined using blanks containing DTNB and ATChI or BTCh supplemented with Tris-HCl buffer to the final reaction volume. The absorbance at 405 nm (22 °C) was read after 30 min (AChE) or 10 min (BChE). The false-positive effect was determined according to the method of Rhee et al. (2003) with modifications. After the addition of ATChI or BTCh, buffer, and AChE or BChE, the sample was left for 30 min (AChE) or 10 min (BChE) at 22 °C. The solution of the studied compound was then added, followed by direct addition of DTNB and measurement of the absorbance.

The inhibitory activity of the test compounds was expressed in eserine equivalents as previously proposed (Szwajgier and Borowiec, 2012a, b). Eserine, also known as physostigmine, is a very effective, reversible cholinesterase inhibitor. The inhibition of acetyl- and butyrylcholinesterases by eserine (at 6.0 nM–10.0 μM) was measured as described above. The limits of detection of the inhibitory activity of eserine were 6.7 nM (AChE) and 13.0 nM (BChE). The calibration curves (the decrease of absorbance...
versus eserine concentration) were plotted. All results were calculated using these calibration curves in the linear range. Each sample was analysed in eight repeats. Statistical differences were calculated using Tukey's test (STATISTICA 8.0; StatSoft, Kraków, Poland) at $p < 0.05$.

**Results and Discussion**

In Ellman’s spectrophotometric method, a false-positive effect can be exhibited by cholinesterase inhibitors due to the interruption of the reaction between thiocholine and DTNB, rather
than by the actual inhibition of the enzyme activity. In the present study, 15 compounds exhibited a false-positive effect, which was subtracted during the calculation of results (Table I).

As can be seen in Fig. 3, most of the tested compounds inhibited AChE and BChE. The highest anti-AChE activity was exhibited by: carnosic acid \((M = 332.42 \text{ g/mol})\) = gentisic acid \((M = 154.12 \text{ g/mol})\) > 3-hydroxy-4-methoxyxynamic acid \((M = 194.18 \text{ g/mol})\) = ethyl ferulate \((M = 222.24 \text{ g/mol})\) = ethyl vanillate \((M = 196.21 \text{ g/mol})\) = nördihyoguaiatic acid \((M = 302.36 \text{ g/mol})\) > ethyl 4-hydroxybenzoate \((M = 166.17 \text{ g/mol})\) = methyl ferulate \((M = 208.21 \text{ g/mol})\). The order of effectiveness towards BChE was: carnosic acid > nördihyoguaiatic acid = ethyl ferulate > salicylic acid \((M = 138.12 \text{ g/mol})\) > gentisic acid > rosmarinic acid \((M = 360.31 \text{ g/mol})\) = caftaric acid \((M = 312.22 \text{ g/mol})\) > homogentisic acid \((M = 168.15 \text{ g/mol})\).

The first factor that was considered during the evaluation of the inhibitory activity of the test compounds was their molecular weight. It can be noticed that the molecular weights of the most efficient phenolic compounds were lower or similar to the weights of well-known inhibitors \((200–400 \text{ g/mol}, \text{Fig. 1})\). The most efficient inhibitor was carnosic acid \((M = 332.42 \text{ g/mol})\), possessing both hydrophilic and hydrophobic regions in the molecule. Orhan et al. (2007), who studied anticholinesterase activities of 14 phenolic compounds (phenolic acids and flavonoids), detected anti-AChE and anti-BChE activities of gallic acid (at a concentration of 5.88 mM) and anti-BChE activity of chlorogenic acid (at a concentration of 2.82 mM), while no inhibitory activity of caffeic or quinic acids (at concentrations of 5.55 and 5.20 mM, respectively) was detected. Direct comparison between individual phenolic acids was not possible due to the different molar concentrations of the compounds. In another work (Shahwar et al., 2010), ferulic acid exhibited anti-AChE activity at 0.257–1.287 mM (12.38–42.86% of AChE inhibition). The false-positive effect was not estimated in either of those works, however.

Savelev et al. (2003) isolated low-molecular \((M = 136.23–220.35 \text{ g/mol})\) inhibitors from Salvia lavandulaefolia: 1,8-cineole, camphor, \(\alpha\)-pinene, borneol, carophyllene oxide, linalool, and bornyl acetate. The highest anticholinesterase activity was exhibited by 1,8-cineole \([IC_{50} = (0.06 \pm 0.01) \text{ mg/ml}, \ M = 154.25 \text{ g/mol}]\). Synergism was observed between 1,8-cineole and \(\alpha\)-pinene as well as between 1,8-cineole and carophyllene oxide. Moreover, antagonism was detected between camphor and 1,8-cineole (Savelev et al., 2003). Anticholinesterase activities of compounds from Salvia lavandulaefolia as well as synergism or antagonism among them have also been reported by other authors (Perry et al., 2000, 2001). Anticholinesterase activity was exhibited by linalool and \(\alpha\)-terpineol (both \(M = 154.25 \text{ g/mol}\)) from different essential oils (Howes et al., 2003). Miyazawa et al. (1997) studied 17 monoterpenoids (hydrocarbons, alcohols, and ketones) with a \(p\)-menthane skeleton (competitive inhibitors, \(M = 134.22–156.27 \text{ g/mol}\) showing anti-AChE activity. The highest anti-AChE activity was exhibited by (+)-pulegone and \(\alpha\)-terpineol. AChE was more effectively inhibited by terpene ketones, like \((+)-p\)-menth-1-ene, and \(\alpha\)-terpineol, than by terpene alcohols and hydrocarbons, respectively. These findings may indicate that the double bond in the side chain of the monoterpen ketones caused an increase in the anti-AChE activity in comparison to the corresponding compounds with a single bond (including monoterpenoid alcohols). Also, the isopropenyl group increased the inhibitory activity of the compounds, whereas the isopropenyl group exhibited a reverse activity. Unfortunately, the false-positive effect of the test compounds was not taken into consideration in the above-mentioned works. Wang et al. (2007) isolated hydroquinone \((M = 110.11 \text{ g/mol})\) from

<table>
<thead>
<tr>
<th>Compound</th>
<th>False-positive effect [nm of eserine]</th>
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<tbody>
<tr>
<td>Salicylic acid</td>
<td>2.3 ± 0.0</td>
</tr>
<tr>
<td>3-Hydroxybenzoic acid</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>Ethyl 4-hydroxybenzoate</td>
<td>2.7 ± 0.0</td>
</tr>
<tr>
<td>Rosmarinic acid</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>Caftaric acid</td>
<td>6.2 ± 0.1</td>
</tr>
<tr>
<td>Ethyl vanillate</td>
<td>5.8 ± 0.1</td>
</tr>
<tr>
<td>Homovanillic acid</td>
<td>2.6 ± 0.0</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>2.2 ± 0.0</td>
</tr>
<tr>
<td>Gentisic acid</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>Homogentisic acid</td>
<td>7.8 ± 0.1</td>
</tr>
<tr>
<td>4-Hydroxyphenylpyruvic acid</td>
<td>14.9 ± 0.1</td>
</tr>
<tr>
<td>Carnosic acid</td>
<td>10.9 ± 0.2</td>
</tr>
<tr>
<td>Nördihyoguaiatic acid</td>
<td>13.0 ± 0.1</td>
</tr>
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Fig. 3. Anticholinesterase activities of phenolic acids and their derivatives (means ± SEM, n = 8). In the case of each enzyme, different letters denote significant differences between samples (p < 0.05).
the rhizome of *Rhodiola rosea*, which effectively inhibited AChE (*IC*₅₀ = 9.81 · 10⁻³ mg/ml). Hydroquinone exhibited no false-positive effect.

In the present study, an attempt to establish a coherent relationship between the molecular structures of phenolic compounds and their anticholinesterase activities in model systems containing one compound at a time was made. The introduction of an OH or/and an OCH₃ group(s) into the phenol ring increased the anticholinesterase activity (cinnamic acid → all its derivatives). The position of the OH group in coumaric acids had no influence on their anti-AChE and anti-BChE activities. Among hydroxybenzoic acids, on the other hand, the highest anti-BChE activity was observed in the case of 2-hydroxybenzoic acid (salicylic acid), whereas 3- and 4-hydroxybenzoic acids exhibited lower activities. The substitution of the second OH group (coumaric acids → caffeic acid) increased the anti-AChE activity and decreased the anti-BChE activity, however, at *p* > 0.05. The presence of two OH groups in positions 2 and 5 of benzoic acid (gentisic acid) conferred higher efficacy in comparison to protocatechuic acid with OH groups in positions 3 and 4. The introduction of three OH groups attached to the phenol ring (gallic acid) significantly decreased its anti-BChE activity in comparison to other benzoic acids. The presence of a propenoic (CH=CH-COOH) group of caffeic acid (caffeic acid) had a highly favourable effect on the anti-AChE activity compared with CH₂-CH₂-COOH (hydrocaffeic acid) or COOH groups (protocatechuic acid). Methylation of both OH groups in caffeic acid (yielding caffeic acid dimethyl ether) decreased its inhibitory activity (however, at *p* > 0.05). Similarly, at *p* > 0.05, esterification of the carboxy group of caffeic acid with quinic acid, yielding chlorogenic acid, ensured a higher anti-BChE activity than was the case with free caffeic acid. Similarly, rosmarinic acid (the ester of caffeic acid and dihydrocaffeic acid) was a very efficient BChE inhibitor but a weak AChE inhibitor. Caftaric acid exhibited anti-AChE inhibitory activity equal to that of caffeic acid and a high anti-BChE activity, close to that of rosmarinic acid. Last but not least, chicoric acid, a derivative of two caffeic acid and one tartaric acid residue, respectively, was hardly effective, probably due to its high molecular weight and the presence of six OH and four C=O groups located regularly around the molecule (high hydrophilicity). A comparison between caffeic, cichoric, and caftaric acids, respectively, showed that combination of 2 or 3 molecules decreased the inhibitory efficiency. The presence of a CH₂-COOH group (4-hydroxyphenylacetic acid) instead of a CH=CH-COOH group (p-coumaric acid) or a COOH group (4-hydroxybenzoic acid) caused a decrease in anti-BChE activity. However, the presence of a CH₂-CO-COOH group (4-hydroxyphenylpyruvic acid) in the same position instead of a propenoic or a carboxylic acid group ensured greater anti-AChE activity (*p* < 0.05). In this case, the lower anti-BChE activity was not confirmed by statistical calculations (*p* > 0.05). The same relationship was confirmed in the case of vanillic and homovanillic acids (significant differences in the anti-AChE activity) but not in the case of gentisic and homogentisic acids. The results comparing the effect of the presence of a CH₃ group in the *meta* position instead of the hydroxy group (caffeic → ferulic acids, protocatechuic → vanillic acids, gallic → syringic acids) were inconsistent and did not provide clear conclusions. However, it can be speculated that the substitution of the methoxy group in the phenol ring promoted a slightly higher anti-BChE activity (although not confirmed statistically). The high number of OH and C=O groups was probably the reason for the lack of the anticholinesterase activity of ellagic acid because well-known, very active inhibitors contain hydrophobic moiety(ies) in the molecule (Fig. 1). In the course of the study, it was not possible to unequivocally establish whether cinnamic acid derivatives were more efficient than their benzoic acid counterparts. All methyl or ethyl esters of phenolic acids exhibited at least equal (in many cases significantly higher) anticholinesterase activity towards both enzymes in comparison to the corresponding free phenolic acids. However, it must be emphasized that esters of phenolic acids herein studied are hydrolyzed in the intestines upon the action of bacterial esterases (Couteau et al., 2001). Therefore, the anticholinesterase activity as well as other biological activities of free forms of these acids should be studied first.

It has previously been shown that the daily consumption of phenolic acids with food reaches 200 mg (Herrmann, 1989; Scalbert and Williamson, 2000). It has also been demonstrated that the pharmacokinetic properties of phenolic acids are excellent. The highest plasma concentration of ferulic acid after consumption of toma-
toes (360–640 g tomatoes were the only source of this acid in the diet) was obtained after 7–9 h and the recovery was 11–25% of total ferulic acid consumed. Free and total (free + conjugated) levels of ferulic acid excreted by individuals were 0.9–3.0 mg and 2.7–5.5 mg, respectively (Bourne and Rice-Evans, 1998). It can, therefore, be concluded that phenolic acids are effectively absorbed and can reach even peripheral body compartments including brain tissues. There is indirect evidence that sodium ferulate was present in mouse fetal brains after administration in feed (Yu et al., 2006). Although the levels of phenolic acids and their derivatives in the brains of experimental animals have not been studied, reports indirectly suggest that ferulic acid (e.g. Yabe et al., 2010; Yan et al., 2001; Cheng et al., 2008; Cho et al., 2005; Kim et al., 2007) or ethyl ferulate (Joshi et al., 2006) was present in animal cerebral tissues, exerting a positive action.

It can be noticed that phenolic acids are weak inhibitors in comparison to well-known compounds exerting this activity (Karczmar, 1998; Giacobini, 2004; Sabbagh et al., 2006). Eserine is efficient at nanomolar concentrations, whereas phenolic acids are inhibitory only at millimolar concentrations. However, phenolic acids have several advantages over the well-known inhibitors used in the present therapy: they are very broadly distributed in different foods, and their everyday consumption with foods causes no adverse effects. In the present work, phenolic acids were tested singly. However, synergy between individual phenolic acids can occur. Therefore, phenolic acids can be taken into consideration for the nutritional prevention of Alzheimer’s disease rather than for its medical treatment.

In conclusion, anticholinesterase activity of phenolic acids strongly depends on the structure of a given compound, especially the number and/or position of OH and C=O groups, while their molecular weight seems to have a minor significance in this context.

Acknowledgement
This scientific work was supported by the Ministry of Science and Higher Education of the Republic of Poland (Scientific Grant no. 2339/B/PO1/2010/38) and by resources of the Scientific Project POIG 01.01.02-00-061/09.
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