Anticholinesterase Activities of Selected Polyphenols – a Short Report

Dominik Szwajgier*

Department of Biotechnology, Human Nutrition and Food Commodity Sciences, University of Natural Sciences, Skromna 8, 20–704 Lublin, Poland

Key words: Alzheimer’s disease, acetylcholinesterase, butyrylcholinesterase, polyphenols

In this work, anticholinesterase activities of 24 polyphenolic compounds were tested using the modified Ellman’s spectrophotometric method. The most efficient acetyl- and butyrylcholinesterase inhibitors were anthocyanins (pelargonidin, delphinidin and cyanidin), flavones (apigenin and luteolin), flavonols (quercetin, kaempferol and myricetin), as well as dihydrochalcone phloridzin and prenylated chalcone xanthohumol. It was established that all the tested compounds were within a narrow molecular weight range of 254.24–354.40 g/mol, which probably was not discriminative for their inhibitory activity. Among all the classes of polyphenolic compounds, the lowest activities were exerted by flavan-3-ols. The inhibitory activity of the tested polyphenols was decreased by the presence of a 3-hydroxyl group. A simultaneous substitution of a carbonyl group at position 4 and a hydroxyl group at position 3 or a lack of both of these substitutions had no effect on the activity of the investigated compounds. The number and position of other hydroxyl groups in the tested molecules played a minor role in this context. Aglycons were more effective cholinesterase inhibitors than their corresponding glycosylated forms. Overall, the results show that phenolic acids can play a role in neuroprotection. However, further in vitro and in vivo studies involving a larger number of polyphenolic compounds simultaneously with well-known cholinesterase inhibitors should be performed in the nearest future to confirm these findings.

INTRODUCTION

A considerable number of positive activities of polyphenols have been pointed out in the literature. Quercetin and cyanidin 3-glucoside have been shown to exert hypolipidemic and antioxidant effects on erythrocytes in vitro [Duchnowicz et al., 2012]. (+)-Catechin and (-)-epicatechin scavenged hypochlorous acid and peroxyl and hydroxyl radicals as well as inhibited the reduction of cytochrome c, peroxidation of phospholipid liposomes and deoxyribose damage [Scott et al., 1993]. The prenylated chalcones, xanthohumol (XN) and iso-xanthohumol (IXN), exerted antioxidant, antiradical [Miranda et al., 2000b] and anticancer [Gerhäuser, 2005] activities as well as an antiproliferative and cytotoxic effect on human breast, colonal and ovarian cancer cells [Miranda et al., 1999]. XN inhibited cyclooxygenase-1 and cyclooxygenase-2 activity and DNA synthesis, induced apoptosis and cell differentiation arrest and prevented preneoplastic lesions in a mouse mammary gland organ culture [Gerhäuser et al., 2002a]. Also, XN inhibited the mutagenic activation of the potential human carcinogen 2-amino-3-methylimidazo-(4,5-f)-quinioline [Miranda et al., 2000c], decreased the number of 2-amino-3-methylimidazo[4,5-f]quinoline-induced preneoplastic foci in livers and colonas of rats and reduced the rate of DNA damage in colonocytes and hepatocytes [Ferk et al., 2010]. XN lowered the levels of plasma glucose and hepatic triglycerides, reduced the weight of white adipose tissue and increased the levels of plasma adiponecin in diabetic KK-A mice. It also acted on the farnesoid X receptor in vivo through a selective bile acid receptor modulator and was involved in fatty acid synthesis by lowering the expression of gluconeogenic genes [Nozawa, 2005]. Prenylated flavonoids, including XN, inhibited microsomal lipid peroxidation to a higher extent than non-prenylated flavonoids [Rodriguez et al., 2001]. Additionally, XN scavenged reactive oxygen species, including peroxyl and hydroxyl radicals, and inhibited the production of the superoxide anion radical and nitric oxide. IXN moderately inhibited inducible NO synthase and the cytochrome P450 enzyme system responsible for the metabolic activation of chemical carcinogens [Gerhäuser et al., 2002b] as well as inducing quinone reductase in mouse Hepa 1c1c7 cells [Miranda et al., 2000a].

Previously, it was shown that polyphenolic compounds can be effective antioxidants towards neuronal cells in different brain parts [e.g. Ishge et al., 2001]. However, other neuroprotective aspects of polyphenols in the central nervous system (CNS) should be herein reported. Myricetin, morin, quercetin (at concentrations 0.1–1 µmol/L), kaempferol, (+)-catechin and (-)-epicatechin dose-dependently inhibited the formation and extension of β-amyloid (1–40) or (1–42) fibrils in the following order: myricetin = morin = quercetin > kaempferol > (+)-catechin = (-)-epicatechin. Moreover, these compounds destabilized preformed β-amyloid fibrils in a dose-dependent...
manner. β-Amyloid fibrils treated by myricetin were less toxic than intact ones [Ono et al., 2003]. Polyphenols effectively inhibited β-amyloid fibril formation at micromolar concentrations, as previously reviewed by Ono et al. [2006]. Forat et al. [2006] showed that polyphenols (mainly flavonoids) effectively inhibited the formation of fibrillar β-amyloid deposits, with IC₅₀ ranging from 0.1 µmol/L to ~ 30 µmol/L. A number of studies showed the influence of food-originating polyphenols on the different neural cell lines in vitro and in vivo. Genistein and daidzein (at 0.08–1.5 µmol/L) enhanced the AChE activity of the rat neuronal cell line PC12. Both polyphenols exhibited estrogenic activity in tests using breast cancer cell line MCF7 [Isoda et al., 2002]. It was shown that (-)-epigallocatechin gallate and hydroxytyrosol (at 10 µmol/L) exerted a cytoprotective effect on neuroblastoma IMR-32 and lymphoma U937 cell lines by the enhanced resistance of cellular DNA to oxidative damage. Additionally, hydroxytyrosol exhibited a similar effect towards lymphoblastoid cells. Resveratrol and tyrosol were also preliminary tested but these compounds were less efficient antioxidants than (-)-epigallocatechin gallate and hydroxytyrosol [Young et al., 2008]. In another study, epigallocatechin gallate (at 10 µmol/L) effectively elevated the survival of the hippocampal neuronal cells (prepared from 18 days old embryo of Sprague-Dawley rats) previously subjected to β-amyloid (for 48 h). Moreover, this polyphenolic compound decreased the levels of malondialdehyde and decreased the caspase activity abnormally elevated by the pretreatment with β-amyloid. This result suggests the neuroprotective effect of epigallocatechin gallate on neuronal cells ex vivo. The authors pointed out the ability of this polyphenolic compound as free radical scavenger which can be beneficial for the treatment of Alzheimer’s Disease [Choi et al., 2001]. It was shown that cyanidin, kaempferol and epicatechin, (followed by rutin, naringenin, kaempferol, taxifolin and luteolin at 3–300 µmol/L) were the most efficient neuroprotectants towards primary cultures of mouse striatal neurons in the presence of oxidized low-density lipoprotein [Schroeter et al., 2000].

The aim of the present study was to evaluate the anti-acetylcholinesterase (anti-AChE) and anti-butrylcholinesterase (anti-BChE) activities of a number of polyphenols. Two concentrations of polyphenolic compounds were studied. The concentration of 0.2 mmol/L was similar to the content tested by the authors cited above. The concentration of 1 mmol/L was used to unequivocally confirm the ability or disability of a compound to exhibit the inhibitory activity. An attempt to establish the relationship between the structure of the tested compound and their anticholinesterase activities was made.

**MATERIALS AND METHODS**

**Reagents**

Kaempferol (60010), (-)-gallocatechin (G6657), pelargonidin Cl (P1659), quercetin (Q 125), quercetin 3-glucuronide (90733), rutin hydrate (RS143), hesperetin (H 4125), phloretin (P7912), (-)-epicatechin (45300), (-)-epigallocatechin (E3768), phloridzin (P-3449), delphinidin Cl (43725), apigenin (42251), luteolin (L9283), daidzein (D7802), cyanidin (36428) and xanthohumol (X0379) were from Sigma-Aldrich, USA. Procyanidin B2 (42157), myricetin (70050) and (+)-catechin (C1251) were from Fluka. Cyanidin-3-O-glucoside (61361) was from Roth, Germany. Cyanidin-3-rutinoside (0914 S) and pelargonidin-3-glucoside chloride (0907 S) were from Extrasynthese, France. Isoxanthohumol (ALX-350–279-M001) was from Alexis Biochemicals, USA.

Acetycholinesterase (AChE, C3389), butrylcholinesterase (BChE, C7512), acetylthiocholine iodide (ATChI, 01480), S-butyrylthiocholine chloride (BTCh, B3128), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, D8130) and eserine (E8375) were from Sigma-Aldrich, USA. Ethanol (98% v/v) and other reagents (HPLC grade) were purchased from PO.Ch. (Gliwice, Poland).

**Solutions of phenolic compounds**

Standard solutions of polyphenolic compounds (0.2 mmol/L and 1.0 mmol/L) were freshly prepared in a minimal volume of ethanol (or deionized water) and diluted to a final concentration by deionized water. The ethanol content in the final solutions was 40–100 mg/cm³, with no effect on enzyme activity (preliminary results not shown).

**Inhibition of AChE and BChE**

Enzyme activities were measured using a 96-well microplate reader (Tecan Sunrise, Austria) based on Ellman’s method [Ellman et al., 1961] with some modifications described in detail previously [Szwarz & Borowiec, 2012]. The false-positive effect was eliminated by the simultaneous analysis of the false-positive samples according to Rhee et al. [2003] with minor modifications, as described previously [Szwarz & Borowiec, 2012]. The false-positive effect was subtracted during the calculation of the results. Each compound was analysed in eight repeats. Data obtained in the experiment were expressed as mean standard error (± SEM). Statistical differences were calculated using Tukey’s HSD test (STATISTICA 8.0, StatSoft, Poland) with significant differences considered at p<0.05.

**RESULTS AND DISCUSSION**

Twenty four polyphenolic compounds were tested in this study for their ability to inhibit AChE and BChE (Figure 1 and 2). The highest anti-AChE activities were exhibited by quercetin, kaempferol, myricetin, phloretin, delphinidin, pelargonidin and cyanidin at 0.2 mmol/L as well as luteolin, quercetin, kaempferol, myricetin, xanthohumol, delphinidin, pelargonidin and cyanidin at 1.0 mmol/L. In the case of anti-BChE activity, the most effective inhibitors were apigenin, luteolin, quercetin, phloretin and cyanidin (at 0.2 mmol/L) as well as apigenin, luteolin, xanthohumol, phloretin, delphinidin, pelargonidin and cyanidin (at 1.0 mmol/L). Among aglycons, the lowest inhibitory activities were exerted by flavan-3-ols. Various structural differences among the tested phenolics were considered as potential factors leading to the differences in their anticholinesterase activity. No distinct differences were found in the molecular weights between flavan-3-ols (290.27–306.27 g/mol) and other classes of flavonoids tested in this study (254.24–354.40 g/mol). Also, all these compounds, except phloridzin and xanthohumol, consisted of two phenyl rings (A- and B-rings) con-
In this work, structure–activity comparisons were also made within the classes of flavonoids. Introduction of a second hydroxyl group at the 3′-position in flavones (apigenin → luteolin) increased their anti-AChE activity at 0.2 mmol/L but the result was not statistically confirmed (p > 0.05) in other cases. The flavonols quercetin, kaempferol and myricetin exhibited distinct anti-AChE activities, but the differences among them were not statistically significant. The only significantly higher anti-BChE activity was observed for quercetin and myricetin at 1.0 mmol/L and for quercetin at 0.2 mmol/L.

These results are ambiguous and suggest that the number of hydroxyl groups does not influence anti-AChE activity because kaempferol, quercetin and myricetin are substituted with 1, 2 and 3 hydroxyl groups, respectively. The isoflavone daidzein exhibited anti-AChE and anti-BChE activities similar to those of flavones, flavonols or anthocyanins (at both concentrations). The higher anticholinesterase activity of xanthohumol in comparison to isoaxanthohumol was probably due to the differences in the structures of the two prenylated chalcones (lack of the central heterocyclic ring in xanthohumol). Very efficient inhibitors, such as donepezil or phenserine [Araujo et al., 2011], contain a short aliphatic fragment between the phenol rings. The increased bending of the molecule could positively influence the anticholinesterase activity of such compounds. Likewise, the difference in the position of the methyl group in xanthohumol could determine its high anti-AChE activity, but this hypothesis was not further investigated. Similarly, phloretin was an efficient cholinesterase inhibitor probably due to the lack of the central heterocyclic ring. Both xanthohumol and phloretin can be attractive cholinesterase inhibitors. Therefore, a direct comparison between these two polyphenolic compounds and donepezil and phenserine could be useful. In this study, a strong inhibitor eserine (physostigmine) was used as the reference compound. It can be seen that eserine was much more efficient inhibitor than polyphenols (at µmol/L it was as effective as polyphenolic compounds at 0.2 mmol/L or 1mmol/L). However, the polyphenols tested are of nutritional origin and can be consumed nearly without limitation (in a common diet) whereas eserine (as well other pharmaceutical inhibitors like donepezil) should be strictly controlled.

Hesperetin (a flavanone) exhibited, in many cases, lower anti-AChE and anti-BChE activities than flavones, daidzein, phlorizin, flavonols and anthocyanins (especially at 1.0 mmol/L). Four flavan-3-ols were tested in this study. At both test concentrations, (+)-catechin inhibited both enzymes more effectively than (-)-epicatechin (although at p > 0.05). These minor differences in the activities of these two compounds could be a result of their different three-dimensional conformation (at the 3-position). The substitution of the additional hydroxyl group at the 4′-position ((+)-catechin → (-)-gallocatechin and (-)-epicatechin → epigallocatechin) did not cause any significant changes in the anticholinesterase activities of those

FIGURE 1. Anticholinesterase activities of polyphenols at 0.2 mmol/L.

FIGURE 2. Anticholinesterase activities of polyphenols at 1.0 mmol/L.
compounds, with minor exceptions (see Figure 1 and 2). Pro-
cyanidin B2 (4,8’-Bi-[(+)–epicatechin] exerted significantly
lower activities than the monomer. Delphinidin, pelargonidin
and cyanidin exhibited high anticholinesterase activities with
insignificant differences among the individual compounds, ex-
cept the significantly higher anti-BChE activity of cyanidin at
0.2 mmol/L. It is possible that the hydroxylation at positions
3, 5 and 7 as well as at the 4’-position increased the inhibitory
activity of anthocyanins in comparison to other classes of fla-
vonoids. On the other hand, the additional hydroxyl groups in
the B-ring (at positions 3’ and 5’) were probably of minor
significance in view of the fact that pelargonidin, cyanidin
and delphinidin are substituted by 1, 2 and 3 hydroxyl groups
in the B-ring, respectively. In summary, it can be noticed that
the results involving direct comparisons of individual com-
pounds are ambiguous and it is not possible to make any gen-
al conclusions based on these results. However, the minor
role of the number and the position of hydroxyl groups at-
tached to A and B rings can be pragmatized.

It was observed that aglycons were more effective cholines-
terase inhibitors than their corresponding glycosylated forms.
Glycosides have little significance as regards their in vivo activi-
ty in the human organism. They are easily hydrolyzed in the in-
testines due to the activity of intestinal epithelial β-glucosidases
[Nemeth et al., 2003] as well as bacterial enzymes in the intesti-
tines [Simmering et al., 2002]. Also, quercetin 3-glucuronide, a
product of in vivo transformation of quercetin, exerts a signifi-
cantly lower inhibitory activity than free aglycone.

The role of polyphenolic compounds in neuroprotection
has been thoroughly studied previously. It was shown that cy-
anidin, (−)-epicatechin and kaempferol very effectively (over
80% effectiveness at 30 µmol/L) prevented oxidized-LDL in-
duced death of mouse striatal neurons. The overall most ef-
fective protection, in a dose-dependent manner, against a fixed
concentration of oxidized-LDL (12.5 µg LDL protein/mL), was
provided by (−)-epicatechin. In comparison to a control,
(−)-epicatechin very effectively slowed down the DNA frag-
mentation caused by oxLDL, decreased the concentration of lactate dehydrogenase produced by striatal cells, prevented
from the loss of dendrites, nuclear condensation, shrunken
cell bodies and attenuated the death of mouse striatal neu-
rons. Another positive effect of the use of (−)-epicatechin was
attenuation of severe cell damage (loss of dendrites, nuclear
condensation and shrinking of cell bodies). Quercetin partially
reversed the morphological alterations of primary neuronal
cells caused by amyloid-β (1–42) (shrinkage and membrane
blebbing leading to apoptosis and loss of neuronal networks).
It also slightly decreased protein oxidation (protein carbonyl
formation), lipid peroxidation and the formation of 3-nitroty-
rosine [Sultana et al., 2005]. Myricetin and curcumin reduced
β-amyloid deposition similarly to the effect exerted by ros-
marinic acid [Hamaguchi et al., 2009]. Resveratrol increased
heme oxygenase-1 (HO-1) mRNA expression in cultured as-
trocytes without inducing HO-1 protein expression and activi-
ty [Scapagnini et al., 2004]. In contrast to the above-mentioned
results, some food phenolics showed neurotoxic effects in
some studies. Quercetin, epicatechin gallate, (−)-epigallo-
catechin and epigallocatechin gallate (at 30 µmol/L) medi-
ated the loss of more than 25% of mouse striatal neurons
in vitro after 18 h of incubation in the absence of oxidized
LDL. Quercetin was the most toxic compound among all the
structures but its rutinoside (rutin) did not exhibit neu-
rotoxicity probably due to its inability to cross membranes
(glycosylated form). In vitro pre-treatment of striatal neurons
with taxifolin, apigenin or naringenin caused an enhancement
of the toxic effect of oxLDL, but no toxicity towards neu-
ronal cells was exerted by these polyphenols in the absence
of oxLDL. The rank order of cytotoxicity was established as
luteolin > quercetin > naringenin = kaempferol > (−)-epicate-
chin = rutin. Flavonoids were more effective than hydroxy-
cinnamates or vitamin C in the inhibition of the neurotoxicity
caused by the oxidized LDL [Schroeter et al., 2000].

The question can be raised if the cholinergic therapy
can modify the Alzheimer’s disease except the improvement
of the symptoms. Previously, it has been shown that AChE can
bind to β-amyloid and accelerate fibril formation in compari-
ton to the peptide alone. Moreover, the enzyme physically
affected fibril assembly and changes the non-amyloidogenic
form of amyloid to give the toxic β-amyloid [Alvarez et al.,
1995]. Alvarez et al. [1997] showed that AChE promoted the
aggregation of β-amyloid(12–28) and β-amyloid(25–35) but
not β-amyloid(1–16). Cebrian et al. [1997] and Campos
et al. [1998] confirmed that AChE was associated with all
types of fibrillary amyloid deposits in brains. However, some
polyphenolic compounds of natural origin were pointed out as
the inhibitors of fibril formation. Curcumin very effectively
attenuated fibril formation in vitro [Ono et al., 2004]. Riviere
et al. [2007] confirmed this result, moreover, pointed out that
resveratrol and its monoglucoside – piceid exerted a higher in-
hibitory activity towards fibrils formation than curcumin (at
10 µmol/L). Also, catechin, piceatannol, astrinign, resveratrol
diglucoside and resveratrol dimer (viniferin) exhibited signifi-
cant inhibitory activity [Riviere et al., 2007]. Results in presen-
ted as well as in cited works show that selected polyphenolic
compounds can act simultaneously as antioxidants, cholines-
terase inhibitors and anti-fibril agents. It can be supposed that
AChE-polyphenol complexes form a less stable 3-dimension-
al structure in the brain (with AChE attached to β-amyloid
at peripheral sites). These complexes may form less stable
β-amyloid plaques. β-Amyloid-AChE-polyphenol deposits can
probably be considered as weaker AD–promoting factors
because could cause less deformation of neurons in compar-
ison to β-amyloid alone. Also, these polyphenols herein stud-
ied increase the redox potential (act as antioxidants) in place.
The complex study in this area involving neuronal cell lines
and a broad range of polyphenolic compounds is a priority.

As it was mentioned above, the number of known flavo-
noids exceeds 4000. Therefore, the number of studies con-
cerning the in vivo activity of these compounds can be con-
sidered as limited and insufficient. However, the works that
can be found in databases are very promising. Resveratrol ef-
ectively acted as acetylcholinesterase inhibitor in in vivo stud-
ies involving streptozotocin-induced diabetic rats as it was
observed by Schmatz et al. [2009a]. The increase of AChE
activity above normal levels was significantly prevented
in brains of rats (cerebral cortex, hippocampus, and striatum)
supplemented with resveratrol (10 and 20 mg/kg) in compar-
ison to control animals. Similarly to brain regions, the treat-
ment with resveratrol prevented the abnormal rise of AChE activity in whole blood in comparison to diabetic rats. In another study, Schmatz et al. [2009b] showed that resveratrol (10 and 20 mg/kg) prevented from the pathological increase of AChE activity in cerebral cortex synaptosomes in streptozotocin-induced diabetic rats as well as in the control group of animals supplemented with this polyphenol (20 mg/kg). Treatment with quercetin (5–20 mg/kg, twice a day) of streptozotocin-induced diabetic rats slightly attenuated the diabetes markers: blood glucose levels, body weight loss. The performance in Morris water and elevated plus maze was also significantly improved suggesting the beneficial effect of quercetin supplementation on the performance of the central nervous system. At higher quercetin supplementation (40 mg/kg, twice a day), the distinct decrease of escape latency and increased time spent in target quadrant during Morris water maze test was observed. The authors pointed out that this result was comparable to the effect obtained with vitamin C (100 mg/kg, twice a day) and donepezil (3 mg/kg/day 31 – day 35). Nasal administration of quercetin (in the form of liposomes, 0.5 mg of quercetin, once daily, 3 weeks) in a AF64A (ethylcholine mustard aziridinium) rat model of Alzheimer’s disease improved memory deficits (spatial learning and memory) studied in Morris water maze test. Moreover, quercetin liposomes administration reversed the abnormal AChE levels caused by AF64A. Treatment with quercetin completely inhibited the elevation of malondialdehyde, lipid peroxidation, and the decrease of superoxide dismutase and glutathione peroxidase activities in hippocampal homogenates [Tong-Un et al., 2010]. Supplementation with curcumin reversed (nearly to levels observed in controls) negative alterations in cerebral cortex of diabetic rats. The positive effects on neuronal cells included: increased gene expression of muscarinic M1, insulin receptor, superoxide dismutase, choline acetyl transferase and decreased gene expression of muscarinic M3, α7-nicotinic acetylcholine receptor, acetylcholinesterase and glucose transporter 3. Moreover, insulin treatment significantly increased the number of visits and time spent in the novel arm in the Y-maze test [Peeyush Kumar et al., 2011].

CONCLUSIONS

The present study was an attempt to compare the anti-AChE and anti-BChE activities of a number of polyphenols in relation to their structures. In view of the original findings of this report and those cited from the literature, polyphenols can be considered as possible active neuroprotectants with potential application in anti-Alzheimer’s therapy. It has previously been shown that polyphenols can reach the brain. For example, the level of quercetin in brains of mice after quercetin consumption in the diet was 0.28 nmol/g [Huebbe et al., 2010]. Further studies concerning the structure–activity relationships in polyphenols should be carried out in the nearest future using a greater number of polyphenolic compounds.

ACKNOWLEDGEMENTS

This scientific work was supported by the Ministry of Science and Higher Education of the Republic of Poland (Scientific Grant no. 2339/B/P01/2010/38) and co-funded from resources of the scientific project POIG 01.01.02–00–061/09.

REFERENCES

14. Huebbe P., Wagner A.E., Boesch-Saadatmandi C., Sellmer F., Wolfrass S., Rimbach G., Effect of dietary quercetin on brain...
Humulus lupulus

Anticholinesterase Activities of Selected Polyphenols

formation by polyphenols: structural similarity and aromatic

Porat Y., Abramowitz A., Gazit E., Inhibition of amyloid fibril

Ono K., Hasegawa K., Naiki H., Hamada M., Curcumin has

egg-cells and amyloidogenic effects of antioxidants: Implications for the prevention


