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Anticholinesterase Activities of Selected Polyphenols – a Short Report

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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 isoxanthohumol (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)-quinoline [Miranda *et al*., 2000c], decreased the number of 2-amino-3-methylimidazo[4,5-f]quinoline-induced preneoplastic foci in livers and colons 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 adiponectin in diabetic KK-A^y 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 gluconeogenetic 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

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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]. Porat *et al*. [2006] showed that polyphenols (mainly flavonoids) effectively inhibited the formation of fibrillar β-amyloid deposits, with IC_{50} ranging from 0.1 μmol/L to ~ 30 μmol/L. A number of studies showed the infuence 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-butyrylcholinesterase (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 test compounds 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 (R5143), 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.

Acetylcholinesterase (AChE, C3389), butyrylcholinesterase (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 P.O.Ch. (Gliwice, Poland).

Solutions of phenolic compounds

Standard solutions of phenolic 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/cm^3, 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 [Szwajgier & 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 [Szwajgier & 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-

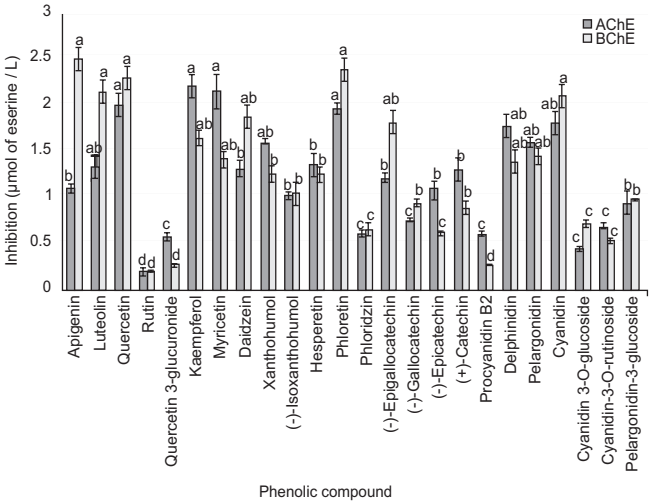

FIGURE 1. Anticholinesterase activities of polyphenols at 0.2 mmol/L.

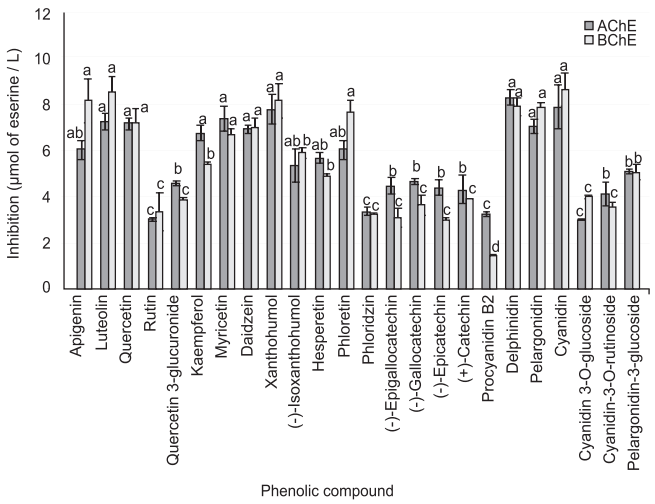

FIGURE 2. Anticholinesterase activities of polyphenols at 1.0 mmol/L.

nected by the heterocyclic C-ring. Therefore, the differences in the activity were hypothesized to correspond to differences in the substitutions of the main skeleton. The fact that flavan-3-ols are substituted by a hydroxyl group at the 3-position was excluded as a cause because the same substitution is observed in flavonols. However, unlike flavonols and flavanones, which are effective cholinesterase inhibitors, flavan-3-ols lack the 4-carbonyl group in the C-ring. In isoflavones and flavones, the B-ring is attached at positions C-3 and C-2, respectively, but this modification does not influence their inhibitory activity. Anticholinesterase activity is not dependent, either, on the simultaneous substitution of the carbonyl group at position 4 and the hydroxyl group at position 3 or on a lack of both of these substitutions. Anthocyanins (based on the flavylium salt structure) without both substitutions were very efficient cholinesterase inhibitors. However, the presence of a 3-hydroxyl group on its own decreased the inhibitory activity of the studied compounds. The present results also show that the number and position of other hydroxyl groups in the tested molecules plays a minor role in this context. Other differences in the structures of the individual classes of flavonoids can also be considered. Flavanones, for instance, lack the double bond between positions 2- and 3- but have a chiral center at the 2-position. These structural features, however, did not influence the activity of hesperetin (a flavanone), which was an efficient inhibitor of cholinesterases. Nevertheless, it must be remembered that because the total number of identified flavonoids exceeds 4000, as thoroughly reviewed by Iwashina [2000], general conclusions must be drawn with caution.

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 isoxanthohumol 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 flavanon) exhibited, in many cases, lower anti-AChE and anti-BChE activities than flavones, daidzein, phloretin, 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

compounds, with minor exceptions (see Figure 1 and 2). Procyanidin 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, except 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 flavonoids. 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 compounds are ambiguous and it is not possible to make any general conclusions based on these results. However, the minor role of the number and the position of hydroxyl groups attached to A and B rings can be pragmatized.

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

The role of polyphenolic compounds in neuroprotection has been thoroughly studied previously. It was shown that cyanidin, (-)-epicatechin and kaempferol very effectively (over 80% effectiveness at 30 μmol/L) prevented oxidized-LDL induced death of mouse striatal neurons. The overall most effective 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 fragmentation 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 neurons. 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-nitrotyrosine [Sultana *et al*., 2005]. Myricetin and curcumin reduced β-amyloid deposition similarly to the effect exerted by rosmarinic acid [Hamaguchi *et al*., 2009]. Resveratrol increased heme oxygenase-1 (HO-1) mRNA expression in cultured astrocytes without inducing HO-1 protein expression and activity [Scapagnini *et al*., 2004]. In contrast to the above-mentioned results, some food phenolics showed neurotoxic effects in some studies. Quercetin, epicatechin gallate, (-)-epigallocatechin and epigallocatechin gallate (at 30 μmol/L) mediated 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 neurotoxicity 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 neuronal cells was exerted by these polyphenols in the absence of oxLDL. The rank order of cytotoxicity was established as luteolin > quercetin > naringenin = kaempferol > (-)-epicatechin = rutin. Flavonoids were more effective than hydroxycinnamates 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 comparison 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 inhibitory activity towards fibrils formation than curcumin (at 10 μmol/L). Also, catechin, piceatannol, astringin, resveratrol diglucoside and resveratrol dimer (viniferin) exhibited significant inhibitory activity [Riviere *et al*., 2007]. Results in presented as well as in cited works show that selected polyphenolic compounds can act simultaneously as antioxidants, cholinesterase inhibitors and anti-fibril agents. It can be supposed that AChE-polyphenol complexes form a less stable 3-dimensional 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 comparison to β-amyloid alone. Also, these polyphenols herein studied 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 flavonoids exceeds 4000. Therefore, the number of studies concerning the *in vivo* activity of these compounds can be considered as limited and insufficient. However, the works that can be found in databases are very promising. Resveratrol effectively acted as acetylcholinsterase inhibitor in *in vivo* studies 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 comparison 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 gluthatione peroxidase activites 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 citied 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.

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