



## Research report

## Effect of a polyphenol-rich wild blueberry extract on cognitive performance of mice, brain antioxidant markers and acetylcholinesterase activity

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## ABSTRACT

The aim of this study was to examine the effect of a polyphenol-rich extract (PrB) of *Vaccinium angustifolium* (wild blueberries) introduced intraperitoneally (i.p.) at 30 (PrB30) and 60 (PrB60) mg/kg body weight for 7 days, on cognitive performance, brain oxidative status and acetylcholinesterase activity in adult, male, 3–4-month-old *Balb-c* mice. Evaluation of rodent learning and memory was assessed by a step-through test on day 6 after a double training and an initial acquisition trial on day 5. Antioxidant status was determined by ferric reducing antioxidant power (FRAP), ascorbic acid concentration (FRASC), malondialdehyde and reduced glutathione levels in whole brain homogenates. Acetylcholinesterase (AChE) activity was determined by Ellman's colorimetric method. Results showed that the PrB60-treated mice exhibited a significant improvement in learning and memory (step-through latency time of  $228 \pm 38$  s compared to  $101 \pm 32$  s of the control group). PrB extract administration also resulted in reduced lipid peroxidation products (38 and 79%) and higher brain ascorbic acid levels (21 and 64%) in both PrB30 and PrB60-treated groups, respectively, and higher glutathione levels (28%) in the PrB60-treated group. Furthermore, salt- and detergent soluble AChE activity significantly decreased in both PrB-treated groups. Thus, the significant cognitive enhancement observed in adult mice after short-term i.p. supplementation with the blueberry extract concentrated in polyphenols, is closely related to higher brain antioxidant properties and inhibition of AChE activity. These findings stress the critical impact of wild blueberry bioactive components on brain function.

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**Abbreviations:** AChE, acetylcholinesterase; ao, ascorbic oxidase; ATCI, acetylthiocholine iodide; BDNF, brain-derived neurotrophic factor; BHT, butylated hydroxytoluene; BW, body weight; BSA, bovine serum albumin; BV2, immortalized mouse microglia; CA1, *Cornu Ammonis* area 1; COX, cyclooxygenase; CREB, cAMP-response element-binding protein; CNS, central nervous system; CSF, cerebrospinal fluid; DS, detergent soluble; DMSO, dimethylsulfoxide; DTNB, 5,5'-dithiobis-2-nitrobenzoate ion; EDTA, ethylenediaminetetraacetic acid; ERK, extracellular signal-related kinase; FRAP, ferric reducing antioxidant power; FRASC, ferric reducing/antioxidant and ascorbic acid; G, globular; GABA,  $\gamma$ -aminobutyric acid; GAE, gallic acid equivalents; GPx, glutathione peroxidase; GSH, glutathione; GST, glutathione S-transferase; IC<sub>50</sub>, median inhibition concentration; IL, initial latency; IGF, insulin-like growth factor; IGF-R, insulin-like growth factor receptor; iNOS, inducible nitric oxide synthase; i.p., intraperitoneal; ISO, isoproterenol; MDA, malondialdehyde; NF- $\kappa$ B, nuclear factor  $\kappa$ B; PBS, phosphate-buffered saline; PrB, polyphenol-rich blueberry extract; RT, room temperature; SS, salt soluble fraction; STL, step-through latency; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances; TCA, trichloroacetic acid; TNF $\alpha$ , tumor necrosis factor- $\alpha$ ; TPZ, 2,4,6-Tris(2-pyridyl)-s-triazine; Tris-HCl, trisaminomethane hydrochloride.

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## 1. Introduction

It has been suggested that fruits and vegetables may play an important role in delaying the onset of Alzheimer's disease, particularly among those who are at high risk for the disease [15]. Furthermore, dietary intake of flavonoids has been inversely related to the risk of dementia [13,34]. High vegetable consumption may also be associated with slower rate of cognitive decline in older age [38]. The main mechanism proposed for the beneficial effect of fruits and vegetables is the potentiation of antioxidant defenses (including enzymatic and non-enzymatic antioxidants) by plant polyphenolic and other antioxidant nutrients. Oxidative stress is linked to neuronal protein misfolding, membrane dysfunction, cell death and glial cell activation that are associated with normal ageing or certain neurodegenerative diseases [1]. Although it is not yet clear whether oxidative stress is the primary cause, an epiphenomenon or a consequence, the brain is particularly vulnerable due to its high metabolic rate, specific features, like the abundant presence of polyunsaturated fatty acids, high levels of iron, and the reduced capacity for cellular regeneration [14].

Blueberries (fruits of various *Vaccinium* species) are among the fruits with high antioxidant power and rich in anthocyanins. In pigs fed wild blueberries for 4 weeks, anthocyanins were detected in the liver, eye, cortex, and cerebellum [28]. Furthermore, in aged rats fed blueberries for 8 weeks, anthocyanins were found in the cerebellum, cortex, hippocampus or striatum in their unmetabolized forms [2]. Interestingly, Williams et al. [51] reported that flavanol levels were higher than anthocyanin levels in both plasma and brain tissue of aged rats supplemented with blueberries for 12 weeks.

Studies have shown that short-term dietary supplementation (8 weeks) of aged rats (19 months old) was effective in reversing age-related deficits in cognitive and motor function [26]. Reactive oxygen species (assayed as 2',7'-dichlorofluorescein diacetate) in the striata from all treated animals were lower than in the control group [26]. It is clear, however, that the significant effects of blueberries on both motor and cognitive behavior (motor behavioral performance on the rod walking and accelorod tasks, learning and memory in the Morris water maze, object recognition memory on the visual paired comparison task) involve a multiplicity of actions, including neuronal signalling and anti-inflammatory effects [42,48]. In particular, short-term dietary supplementation with blueberries of aged rats improved striatal dopamine release and GTPase activity, synaptosomal  $\text{Ca}^{2+}$  recovery after  $\text{H}_2\text{O}_2$  challenge [26,52], cerebellar ISO potentiation of GABAergic inhibition [11], hippocampal plasticity parameters, i.e., higher hippocampal neurogenesis, extracellular receptor kinase activation, and resulted in higher IGF-1 and IGF-1R levels [12]. Additionally, short-term dietary supplementation activated the ERK-CREB-BDNF pathway [51], increased hippocampal heat shock protein 70-mediated neuroprotective response to inflammatory challenge [20], and resulted in lower brain NF- $\kappa$ B levels [23]. Furthermore, blueberry supplementation inhibited the production of the inflammatory mediator, nitric oxide (NO), as well as interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) in cell conditioned media from lipopolysaccharide-activated BV2 microglia and reduced mRNA and protein levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2) [33].

Only a few studies have been performed in young/adult rodents. A 2-month dietary supplementation of rats with blueberries prevented deficits in learning performance induced by bilateral hippocampal injections of kainic acid and prevented the loss of CA1 pyramidal neurons [17] and deficits in cognitive performance, and dopamine release, induced by  $^{56}\text{Fe}$  particle irradiation [47]. Furthermore, adult rats (aged 12 months), that consumed lyophilized berries for 30 days (approximately 3.2 mg/kg day of anthocyanins), had significantly enhanced short-term but not long-term memory in the inhibitory avoidance task and improved working memory in the radial maze [43]. A similar dietary intervention in adult mice (aged 3 months) improved performance in memory tasks and had a protective effect on DNA damage in the hippocampus and cerebral cortex [7].

The central cholinergic system is essential for the regulation of cognitive functions, as evidenced by the extensive loss of cholinergic neurons observed in the forebrain of Alzheimer's disease patients and the learning and memory deficits of anti-cholinergic drugs, such as scopolamine, in a variety of cognitive animal models [45,49,53]. Agonists of cholinergic receptors and inhibitors of acetylcholinesterase (AChE) have been extensively used in order to increase endogenous acetylcholine levels and thus overcome cognitive deficits. Acetylcholinesterase metabolizes acetylcholine to choline and acetyl-CoA. AChE exists into different molecular forms, which can be distinguished on the basis of their shapes, e.g., collagen-tailed asymmetric forms and globular (G) forms [32]. The latter is present in the mammalian brain in different multiples of the monomer subunit, e.g., monomer, dimer and tetramer. It has also been found that these isomeric forms are differen-

tially localized in the neuron. The G1 form is cytosolic and the G4 form is membrane bound by hydrophobic amino acid sequences or glycopospholipids. The detergent soluble (DS) and salt soluble (SS) fraction of AChE contain predominantly the G4 and G1 forms, respectively. Recent studies have shown that dietary supplementation of mice with green tea polyphenols for 7 weeks improved cognitive performance and inhibited AChE activity in scopolamine-induced amnesic mice [31]; however, the effect of blueberries on the above has not been studied.

The aim of this study was to investigate the effect of a 7-day intraperitoneal (i.p.) administration of a polyphenol-rich wild blueberry (*Vaccinium angustifolium*) extract (PrB) on the cognitive performance of adult mice as assessed by a passive avoidance test, brain oxidative status (total antioxidant capacity, ascorbic acid, malondialdehyde (MDA) levels and reduced glutathione (GSH) content) and AChE activity. Results showed that short-term intraperitoneal administration of blueberry polyphenols to healthy, adult mice significantly enhanced cognitive performance in the passive avoidance test and attenuated brain oxidative stress markers. We have documented for the first time that the nootropic action of blueberry polyphenols is related to decreases in brain AChE activity and lipid peroxidation.

## 2. Materials and methods

### 2.1. Plant material and extraction

Wild blueberries were purchased as a composite from Wyman's (Cherryfield, ME), freeze-dried with standard procedures by American Lyophilizer Inc. (Bridgeport, PA, USA) and powdered. Polyphenols were extracted from the above as previously described [21]. In brief, 2 g of blueberry powder was extracted in the dark, under magnetic stirring with 15 mL/g of methanol, acetic acid, and distilled water at a ratio of 25:1:24, respectively, for 2 h. The extract was centrifuged at  $1200 \times g$  for 5 min at room temperature (RT), filtered through a 0.2  $\mu\text{m}$  filter and evaporated to dryness in a Speed Vac system (Freeze dryer 4.5; Labconco Corp., Kansas City, MO, USA). The dry residue was stored at  $-20^\circ\text{C}$  until further use.

The dry residue was re-dissolved with 1 mL of 3% formic acid in water (w/v), centrifuged and the supernatant was absorbed on a C18 Sep-Pak cartridge. The cartridge was washed with methanol, equilibrated with 5 mL of 3% formic acid in water (w/v) and eluted with 5 mL 3% formic acid in 50% methanol (w/v). The polyphenols eluted from the cartridge were evaporated under vacuum until dryness and kept at  $-20^\circ\text{C}$  until use.

### 2.2. Determination of total phenolics and anthocyanins

Total phenolics were measured with the Folin-Ciocalteu reagent method [50]. The total polyphenolic content was expressed as gallic acid equivalents (GAE), using a standard curve with 50–600 mg/L gallic acid. Absorbance was measured at 765 nm with a UV-spectrophotometer (Pharmacia LKB-Biochrom4060). Additionally, total anthocyanin content was estimated according to Giusti and Wrolstad [22] by UV-vis spectroscopy at 538 nm after dissolution in a mixture of methanol and 0.1 M HCl at a ratio of 85:15. Total anthocyanin content was expressed as cyanidin 3-rutinoside equivalents.

### 2.3. In vitro acetylcholinesterase inhibition assay

The assay for AChE activity was performed with the colorimetric method of Ellman et al. [19], utilizing acetylthiocholine iodide (ATCI) as a substrate. The rate of production of thiocholine is determined by the continuous reaction of the thiol with 5,5'-dithiobis-2-nitrobenzoate (DTNB) ion to produce the yellow anion of 5-thio-2-nitro-benzoic acid. Briefly, in the 96 well plates, 25  $\mu\text{L}$  of 15 mM ATCI, 75  $\mu\text{L}$  of 3 mM DTNB and 50  $\mu\text{L}$  of 50 mM Tris-HCl, pH 8.0, containing 0.1% bovine serum albumin (BSA), and 25  $\mu\text{L}$  of the tested phytochemicals were added and the absorbance was measured at 405 nm after 5 min of incubation at RT. After 25  $\mu\text{L}$  of 0.22 U/mL of AChE from electric eel (Sigma-Aldrich Corporation, St. Louis, MO, USA) was added, the absorbance was measured again after 5 min of incubation at RT. Percentage of inhibition was calculated by comparing the rate of enzymic hydrolysis of ATCI for the samples to that of the blank (50% aqueous methanol in buffer). Galanthamine (1–32  $\mu\text{M}$ ) was used as a reference standard and was supplied by Sigma-Aldrich.  $\text{IC}_{50}$  values were determined by GraphPad Prism4.0 (GraphPad Software Inc., USA). All determinations were carried out at least five times, and in triplicate, at each concentration of the standard and samples.

### 2.4. Animals

Male, *Balb-c* mice (34–38 g, BW), 3–4 month-old, were kept in polyacrylic cages (38 cm  $\times$  23 cm  $\times$  10 cm) with nine animals per cage and housed in a room under

controlled temperature (24–26 °C), relative humidity (50–60%); with 12 h light–dark cycles. Mice had *ad libitum* access to food in form of dry pellets and water. The inbred first generation male mice ( $n = 27$ , 4 months old;  $35.6 \pm 1.5$  g BW) were randomly divided into three groups: a vehicle group ( $n = 9$ ) (Control), a PrB-treated group ( $n = 9$ ) with 60 mg PrB/kg BW (PrB60) and a PrB-treated group ( $n = 9$ ) with 30 mg PrB/kg BW (PrB30). All procedures were in accordance with Greek National Laws (Animal Act, PD 160/91) and NIH Animal Care Guidelines.

The PrB extract was administered *i.p.* daily to PrB30 and PrB60 groups ( $n = 9$ /group) at a final volume 20  $\mu$ L, while mice in the C group received 20  $\mu$ L of saline. An aqueous solution of PrB extract was prepared fresh daily by dissolving the dry PrB extracts in double distilled water and by filtering through membrane filters of 0.2  $\mu$ m internal diameter. The PrB extract was administered *i.p.* immediately after preparation.

## 2.5. Behavioral testing: step-through passive avoidance task

Mice were subjected to a step-through test [39,41] on day 6, after a double training and an initial acquisition trial on day 5. The test is based on negative reinforcement to examine long-term memory [29,30]. It was performed according to previously described procedures, using a two-compartment passive avoidance apparatus (white/dark, separated by a black wall with a guillotine door in the middle part) [3], with minor modifications of the time intervals.

In detail, on day 5, the animals were allowed to habituate in the experimental room for 1 h prior to experiments. One hour later, each mouse was placed in the illuminated chamber for the acquisition trial. Each mouse was gently placed in the illuminated compartment, and was left to habituate to the apparatus. One hundred seconds later, the guillotine door was opened and the animal was allowed to enter the dark compartment. The latency with which the animal crossed into the dark compartment was recorded. Animals that waited more than 100 s to enter the dark compartment were eliminated from the experiments. Once the animal crossed with all four paws to the next compartment, the guillotine door was closed and the mouse was taken into its home cage. The trial was repeated after 30 min as in the acquisition trial, where after 5 s the guillotine door was opened and as soon as the animal crossed to the dark compartment the door was closed and a foot shock (25 V, 3 mA, 5 s) was immediately delivered to the grid floor of the dark room. Thereafter, the mouse was immediately removed from the apparatus and returned to its home cage. In this trial, the initial latency (IL) of entrance into the dark chamber was recorded (maximum time allowed was 120 s). Twenty-four hours after training, a retrieval test was performed to determine long-term memory. Each animal was placed in the light compartment for 20 s, the door was opened, and the step-through latency (STL) was measured for entering into the dark compartment. The test session ended when the animal entered the dark compartment or remained in the light compartment for 300 s (criterion for retrieval). During these sessions, no electric shock was applied. All training and testing sessions were carried out during the light phase between 08:00 and 14:00 h.

## 2.6. Tissue preparation

Mice were killed by transcardial perfusion with ice-cold 0.95% NaCl (10 mL/10 g BW) and the whole intact brain was carefully removed and placed on an ice-chilled petri dish for cleaning. The cerebellum was rapidly removed, and the remaining brain was weighed, washed with isotonic saline and homogenized (10% w/v) in 30 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.6 with a glass homogenizer (Thomas, Philadelphia, USA, No B 13957) at a speed of 9500 rpm, thrice. The homogenates were sonicated briefly (15 bursts with a MSE Soniprep 150, Wolf Laboratories Ltd., York, UK) on ice and then centrifuged at  $20,000 \times g$  at 4 °C in a Sorvall superspeed RC2-Bautomat refrigerated centrifuge (Ivan Sorvall Inc., New Jersey, USA) for 2 h to recover a salt soluble fraction (SS). The pellets were re-extracted with an equal volume of 30 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.6, containing 1% Triton X-100 and the suspensions were centrifuged at  $20,000 \times g$  at 4 °C for 2 h to recover a detergent soluble fraction (DS) [16]. The supernatant was collected and stored at –20 °C. Protein concentrations were determined by the Bradford assay with BSA as standard (0.05–1.00 mg/mL).

## 2.7. Ex vivo AChE determination

AChE activity was determined using the colorimetric assay of Ellman, as previously described [19]. Briefly, in the 96 well plates, 25  $\mu$ L of 15 mM ATCI, 75  $\mu$ L of 3 mM DTNB and 75  $\mu$ L of 50 mM Tris–HCl, pH 8.0, containing 0.1% BSA, were added and the absorbance was read at 405 nm after 5 min incubation at RT. Any increase in absorbance due to the spontaneous hydrolysis of the substrate was corrected by subtracting the rate of the reaction before adding the enzyme. Then, 25  $\mu$ L of sample (SS and DS fraction of brain homogenates) was added and the absorbance was read again after 5 min of incubation at RT. The AChE activity is expressed as mol/min/g of tissue protein. All determinations were carried out twice and in triplicate.

## 2.8. FRAP assay

Ferric reducing antioxidant power (FRAP) assay measures the ability of antioxidants to reduce the  $[\text{Fe}(\text{TPTZ})_2]^{3+}$  complex to the blue-colored  $[\text{Fe}(\text{TPTZ})_2]^{2+}$  [8]. Addition of samples (SS brain fraction) to FRAP reagent (10 mM TPTZ and 20 mM

$\text{FeCl}_3$  in 300 mM acetate buffer, pH 3.6) led to increases in absorbance at 490 nm. Ferrrous sulfate ( $\text{FeSO}_4$ ) was used as a standard and the assay showed good linearity (10–1000  $\mu$ M,  $y = 0.0008x - 0.0013$ ,  $R^2 = 0.9997$ ) and sensitivity.

The antioxidant capacity of the brain samples was expressed as  $\mu$ mol  $\text{FeSO}_4$  per g of wet tissue and was calculated by interpolation of the absorbance values to the calibration curve. All determinations were carried out at least three times, and in triplicate.

## 2.9. Determination of ascorbic acid (FRASC assay)

In the above mentioned set-up for the FRAP assay, incubation with ascorbate oxidase from *Curcubita* sp. (Sigma–Aldrich) (4 U/mL), prior to the addition of the FRAP reagent, enables the determination of ascorbic acid [9]. The SS fraction of brain homogenates (60  $\mu$ L) was mixed with 20  $\mu$ L 300 mM acetate buffer, pH 3.6 and placed in two different wells of the 96 well plate. To one of the wells, 32  $\mu$ L of ascorbic oxidase (4 U/mL) solution was added (+ao wells), while 32  $\mu$ L of double distilled water was added to the other well (–ao wells). All samples, including ascorbic acid (between 1 and 250  $\mu$ M, reference standard), were treated in pairs. The paired +ao and –ao wells were incubated at 37 °C for 1 h and after addition of the FRAP reagent, the absorbance was measured at 490 nm. The difference in absorbance values  $\Delta A_{(-ao)} - \Delta A_{(+ao)}$  is attributed to ascorbic acid (ascorbic acid-related  $\Delta A$ ). Ascorbic acid concentration ( $\mu$ M) was calculated by interpolation of the ascorbic acid-related  $\Delta A$  values to the calibration curve. FRASC showed good linearity (5–250  $\mu$ M,  $y = 0.0019x - 0.0053$ ,  $R^2 = 0.9994$ ) and sensitivity with a detection limit of 5  $\mu$ M ascorbic acid. All determinations were carried out twice, and in triplicate.

## 2.10. Determination of malondialdehyde

Malondialdehyde was determined fluorometrically. In brief, 25  $\mu$ L of 90 mM butylated hydroxytoluene (BHT) was added to 250  $\mu$ L of samples (SS fraction of brain homogenates) or MDA standards (0.05–10  $\mu$ M), to prevent further lipid peroxidation [25]. Samples were hydrolysed in mild alkaline conditions at 60 °C for 30 min in order to release bound MDA [24]. Proteins were precipitated with the addition of 3 volumes of 10% trichloroacetic acid (TCA). After being kept on ice for 10 min, mixtures were centrifuged and the supernatants obtained were incubated with 0.8% thiobarbituric acid (TBA) at 90 °C for 90 min. The supernatants were then extracted with *n*-butanol and fluorescence was measured at 553 nm (emission wavelength) after excitation at 515 nm on a Perkin–Elmer LS55 fluorescence spectrometer. All determinations were carried out at least three times and in triplicate.

## 2.11. Determination of reduced glutathione levels

The level of reduced glutathione in the mouse brain was estimated by a specific fluorometric assay of Mokrasch and Teschke [37]. The method is based on the formation of a fluorescent complex after the reaction of *o*-phthalaldehyde with glutathione and histidyl compounds. Briefly, 0.1 mL of brain homogenate (25 mg/mL) were mixed with 0.1 mL buffered formaldehyde (1:4 (v/v) 37% formalin/0.1 M  $\text{Na}_2\text{HPO}_4$ ) and the mixture was left at RT for 5 min to eliminate the interference of endogenous histidine-containing compounds. Then, 1.0 mL of 0.1 M sodium phosphate/5 mM EDTA (pH 8.0) was added to the reaction mixture, followed by 0.1 mL of 1 mg/mL *o*-phthalaldehyde (in methanol). Finally, the reaction mixture was incubated at RT for 45 min in the dark, and the fluorescence was measured in a spectrofluorometer at 345 nm excitation and 425 nm emission wavelengths (Perkin–Elmer LS55 fluorescence spectrometer). Standard solutions of GSH (Sigma–Aldrich) were prepared in 30 mM  $\text{Na}_2\text{HPO}_4$  (pH 7.6). A good linearity was obtained for GSH in the range of 0.5–100  $\mu$ M ( $y = 0.1768x - 0.2811$ ,  $R^2 = 0.9962$ ). The level of GSH was expressed as  $\mu$ mol/g of protein.

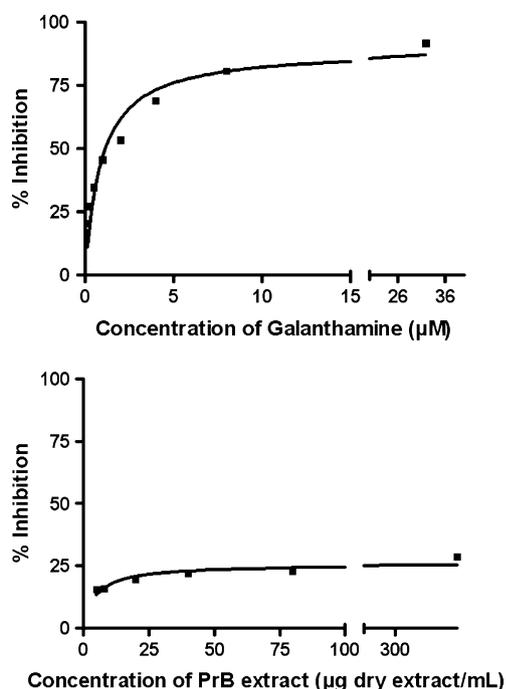
## 2.12. Statistical analysis

Data are presented as mean  $\pm$  S.E. Statistical analysis was performed with GraphPad Instat 3 software (GraphPad Instat Software Inc., USA) using the nonparametric Mann–Whitney test for evaluating statistically significant differences ( $p < 0.05$ ) of each PrB treated group from control.

## 3. Results

### 3.1. Total polyphenol and anthocyanin content of the PrB extract

Total polyphenols are expressed as gallic acid equivalents and total anthocyanins are expressed as cyanidin 3-rutinoside equivalents per gram dry weight. The PrB extract, obtained from solid-phase extraction on Sep–Pak C18, had total polyphenolic content of  $13.01 \pm 0.06$  mg GAE/g dry weight and total anthocyanin content of  $3.78 \pm 0.05$  mg/g dry weight. Analysis of freeze-dried blueberry powder by others [36] has shown total polyphenolic



**Fig. 1.** *In vitro* AChE inhibitory activity of galanthamine and PrB extract at concentrations 1–32  $\mu\text{M}$  and 5–500  $\mu\text{g/mL}$ , respectively ( $n = 5$ ).

content of 2.79 mg GAE/g dry weight and anthocyanin content of 1.16 mg/g dry weight. Thus, the solid-phase extraction concentrated the extract in polyphenols about four times.

### 3.2. The *in vitro* effect of the PrB extract on AChE activity

In order to test if blueberry polyphenols directly inhibit AChE, the effect of PrB extract and galanthamine on electric eel AChE activity was tested *in vitro* and the results are presented in Fig. 1. The PrB extract exhibited moderate AChE inhibitory activity (up to 30%) that was not dose-dependent. Dose-dependent inhibition of AChE was observed for galanthamine, an alkaloid from *Galanthus nivalis*, with an  $\text{IC}_{50}$  of 0.88  $\mu\text{M}$ .

### 3.3. Animal body and brain weight

There were no differences in body weight of treated and control animals before and after the administration of the PrB extract for 7 days. In addition, there were no significant differences in the wet weight of the whole brain between control and PrB groups (Table 1).

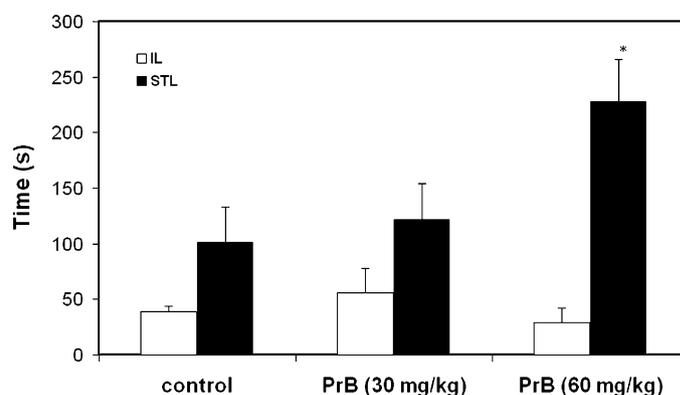
### 3.4. Effect of the PrB extract on the step-through test learning ability

The effect of the PrB extract injected *i.p.* to mice for 6 days on the step-through learning ability is shown in Fig. 2. The scores are expressed as the mean(s) of IL and STL for each group. The STL involves contextual reinforced stimuli and is a direct mea-

**Table 1**  
Mice body weight and wet brain weight before and after *i.p.* administration of 30- (PrB30) and 60 mg (PrB60) PrB extract/kg BW for 7 days<sup>a</sup>.

	Control	PrB30	PrB60
BW before treatment (g)	38.5 $\pm$ 0.9	33.1 $\pm$ 1.5	35.3 $\pm$ 2.0
BW after treatment (g)	39.7 $\pm$ 1.7	33.7 $\pm$ 1.6	34.9 $\pm$ 2.3
Wet brain weight (mg)	361.4 $\pm$ 5.6	361.1 $\pm$ 4.7	358.2 $\pm$ 4.5

<sup>a</sup> Mean  $\pm$  S.E. values ( $n = 9/\text{group}$ ). Control group received *i.p.* injections of saline.



**Fig. 2.** The effect of *i.p.* administration of 30 and 60 mg PrB extract/kg BW on initial latency (IL) and step-through latency (STL) in the double trial step-through test. Asterisk (\*) indicates significant difference from the control littermates ( $n = 9/\text{group}$ ).

sure of passive avoidance behavior. In the control, the PrB30 and PrB60 groups, the IL was not significantly different ( $39 \pm 5$ ,  $56 \pm 22$ , and  $29 \pm 13$  s, respectively), indicating that all groups behaved the same in the training trial. However, the PrB60 group exhibited a significant increase ( $p = 0.03$ ) in STL to  $228 \pm 38$  s, 1 day after the acquisition trial, as compared to the control group ( $101 \pm 32$  s). No significant difference was observed in the PrB30 group in comparison to the control group. Apparently, the PrB extract of 60 mg/kg BW facilitated learning in comparison to the control group, as is evident by the delay of transfer in the dark chamber.

### 3.5. The *ex vivo* effect of the PrB extract on brain AChE activity

In order to determine AChE levels in the brain of mice, the SS and DS fractions of brain homogenates were assayed using the colorimetric method of Ellman (Table 2). Short-term PrB administration (both in PrB30 and PrB60) resulted in a significant decrease in AChE-specific activity in both SS and DS fractions as compared to control. Remarkably, the percentage of inhibition was higher in the brain tissue of PrB30 (66% in SS and 64% in DS AChE) than in the tissues of the PrB60 (31% in SS and 42% in DS AChE) treated mice.

### 3.6. Effect of the PrB extract on brain oxidative status

PrB extract administration significantly altered the concentration of ascorbic acid and MDA in a dose-dependent way, whereas it did not affect the total antioxidant status (FRAP values) (Table 3). In particular, PrB extract administration at 60 mg/kg BW almost doubled the concentration of ascorbic acid and decreased the concentration of MDA in brain tissue by 78%. Additionally, the levels of GSH were significantly ( $p < 0.05$ ) increased in the brain tissue of PrB60-treated mice ( $\sim 28\%$ ), while no difference in the GSH content was observed in the cerebral tissue of PrB30-treated mice.

**Table 2**  
The effect of *i.p.* PrB administration for 7 days on *ex vivo* AChE activity (mol/min/g of tissue protein) in mice brain<sup>a</sup>.

Treatment groups ( $n = 9/\text{group}$ )	Salt soluble (SS)-AChE	Detergent soluble (DS)-AChE
Control	0.210 $\pm$ 0.007	0.814 $\pm$ 0.038
PrB30	0.071 $\pm$ 0.006**	0.291 $\pm$ 0.021**
PrB60	0.145 $\pm$ 0.008*	0.475 $\pm$ 0.031**

<sup>a</sup> The AChE activity for each group denotes mean  $\pm$  S.E. values.

\*  $p < 0.05$  significant difference from control.

\*\*  $p < 0.01$  significant difference from control.

**Table 3**  
Effect of i.p. PrB administration for 7 days in biochemical parameters of mice brain antioxidant status<sup>a</sup>.

	FRAP ( $\mu\text{mol FeSO}_4/\text{g wet tissue}$ )	Ascorbic acid ( $\mu\text{g/g wet tissue}$ )	MDA ( $\mu\text{mol/g protein}$ )	GSH ( $\mu\text{mol/g protein}$ )
Control	8.637 $\pm$ 0.204	134.39 $\pm$ 5.027	2.233 $\pm$ 0.255	37.490 $\pm$ 3.500
PrB30	7.759 $\pm$ 0.459	162.83 $\pm$ 8.393*	1.375 $\pm$ 0.407**	30.784 $\pm$ 8.418
PrB60	7.875 $\pm$ 0.233	221.15 $\pm$ 6.058**	0.472 $\pm$ 0.092**	47.958 $\pm$ 4.859*

<sup>a</sup> The mice brain biochemical parameters are expressed as mean  $\pm$  S.E. values.

\*  $p < 0.05$  significant difference from control littermates ( $n = 9/\text{group}$ ).

\*\*  $p < 0.01$  significant difference from control littermates ( $n = 9/\text{group}$ ).

#### 4. Discussion

The present study has been carried out using mice, since mice trained only once establish a long-lasting memory that lasts at least 24 h in step-through type passive avoidance learning task [30]. We used the passive avoidance task in which animals learn to associate a location with an aversive stimulus. On day 5, the IL to enter the dark chamber is measured as a control for visual ability and motor activity. In our experiment, the mean IL values did not differ among the different groups. The STL is a measure of the memory of the aversive experience. The mean STL of mice treated with the high concentration of the PrB extract (PrB60) were significantly higher than those of the other groups on day 6. These results are in agreement with recent reports on the long-term memory enhancing effect in the inhibition avoidance test of dietary supplementation with *Vaccinium ashei* (Ericaceae) (rabbiteye blueberries) of 3-month-old mice for 30 days [7]. Thus, despite the differences in the route, dose, time-period of administration and the botanical species involved (*ashei* vs *angustifolium*), blueberry administration to adult mice had a profound enhancing effect in the learning and memory, as assayed by avoidance tests. Barros et al. [7] further documented that wild blueberry dietary supplementation induced an increase in the number of crossings during open-field habituation and had an anxiolytic effect in the elevated plus-maze task; both phenomena are related with animal performance in the avoidance tests. The cognitive enhancing effects in adult mice in our experiment are in agreement with previous findings in aged rats (19–21 month-old) as assayed by the performance in Morris water maze [26], in APP+PS1 transgenic mice [27], and in a rodent model of accelerated aging [46].

Previous studies have shown that dietary blueberry flavonoids are bioavailable in the rodent brain and that a relationship exists between the total number of anthocyanins in the cortex, hippocampus and performance in the Morris water maze test [2]. Previous studies on tissue distribution of *Ribes nigrum* (blackcurrant) anthocyanins in rats showed that i.p. administration results in higher absorption and ocular tissue distribution but different kinetics in comparison to oral administration, i.e., the AUC<sub>0–inf</sub> of anthocyanins in the plasma and whole eye were 2.56  $\mu\text{g h/mL}$  and 0.23  $\mu\text{g h/g}$  after oral administration and 12.3  $\mu\text{g h/mL}$  and 25.0  $\mu\text{g h/g}$ , respectively, after i.p. administration, whereas the respective  $t_{1/2}$  were 1.4 and 1.1 h after oral and 2.8 and 2.6 h, after i.p. administration [35]. The absorption and tissue distribution of the PrB extract have not yet been studied, and it is expected that although these may be different from those observed after oral administration, bioactive blueberry polyphenols will reach the rodent brain. Thus, it is suggested that the improvement of rodent performance in the step-through passive avoidance test is related not only to indirect but also direct effects of blueberry polyphenols in specific brain areas.

In the PrB-treated groups, brain AChE activity significantly decreased. Our results of the *in vitro* study of the PrB extract on AChE activity (low inhibitory activity) preclude any direct inhibitory effect of blueberry polyphenols on the enzyme and suggest that other mechanisms might be involved, i.e., decreased gene transcription and translation. The involvement of the cholinergic system in learning/memory has long been established [29,30]. It is expected

that decreased AChE activity may enhance cholinergic activity by raising ACh level (inhibition of metabolism), thereby maintaining/improving cognitive functions. AChE activity decreased in both SS and DS fractions, but in the PrB60-treated mice the percent inhibition was lower than the inhibition detected in the PrB30-treated mice brain, even though cognitive enhancement was only statistically significant in the PrB60 group. This observation needs further investigation, as memory is a complex process requiring the coordination of many different regions of brain and many neurotransmitter systems.

ACh normally has a strong temporal association with the detection of novel or behaviorally significant stimuli and thus excessive ACh release might impair tasks that require learning novel stimuli [5,45]. In accordance, higher AChE inhibition does not necessarily mean better cognitive performance and the findings denote that there is an optimal balance between cholinergic neurotransmission and cognitive performance. Besides, AChE activity is also found in brain regions with low or no cholinergic inputs, such as the substantia nigra, cerebellum, globus pallidus, and hypothalamus, where it exerts non-enzymatic neuromodulatory functions affecting neurite outgrowth and synaptogenesis, modulating the activity of other proteins regional cerebral blood flow, and other functions [49,53]. It has been suggested that hippocampal DS AChE has the most determining effect on cognitive performance [16]. Thus, in order to reach accurate conclusions concerning the relationship of brain AChE inhibition and cognitive performance in mice treated with a PrB extract, knowledge about its brain region-specific effects on DS and SS AChE is necessary.

Evaluation of the effect of blueberry polyphenol administration on rodent brain oxidative stress parameters and antioxidant mechanisms was an important aim of our study, since oxidative stress is involved in almost all pathophysiological cerebral changes. PrB administration decreased brain MDA levels and increased ascorbate in a dose-dependent way. The decreased MDA levels are in agreement with the previously reported decreased striatal reactive oxygen species after dietary blueberry supplementation [26].

In the present study, the levels of brain GSH were significantly increased in the PrB60-treated mice. The protective capacity of GSH is due to its reactive sulfhydryl cysteine moiety, which can scavenge free radicals or bind to electrophilic sites on endogenous toxins, like hydrogen peroxide, lipid hydroperoxides and detoxify them, acting as a substrate for glutathione peroxidase (GPx) and glutathione S-transferase (GST). Diminished GSH status has been linked with normal aging, as well as with neurodegenerative diseases [10]. Previous studies in aged rats after oral administration of grape seed extract (100 mg/kg BW) for 30 days revealed a significant increase in GSH levels [6]. The increase of GSH levels in both experimental designs might be attributed to increases in the expression of  $\gamma$ -glutamyl cysteine synthetase, the rate-limiting enzyme for GSH synthesis, or may be due to the bioavailable polyphenols reaching the brain [2,40,51].

Ascorbate mainly serves as an antioxidant and oxygen-radical scavenger and has been detected in high amounts in brain tissue. Moreover, it has been shown that ascorbate acts as a neuromodulator of both dopamine- and glutamate-mediated neurotransmission and is an essential co-factor for synthesis of noradrenaline and

many neuropeptides [44]. It is also required for the release of norepinephrine and ACh from synaptic vesicles [44]. Thus, the increased brain ascorbate levels in PrB-treated animals may contribute to the enhanced performance in the passive avoidance test. Additionally, it has been shown that short- and long-term supplementation with ascorbic acid has facilitatory effects on acquisition and retrieval processes of passive avoidance learning and memory in rats [46]. A study in young and aged mice supplemented orally with vitamin C alone for 60 days showed no significant differences in performance in passive avoidance test [4]. However, the combination of vitamin C with vitamin E induced significant effects only in aged mice [4]. Ascorbate, belonging to the water-soluble class of vitamins, enters the CNS primarily by active transport from blood plasma at the choroid plexus. It then diffuses from CSF to brain extracellular fluid. Thus, the increased brain ascorbate levels after PrB administration probably represent high plasma ascorbate levels. In healthy adults, cranberry juice consumption (rich in anthocyanins) for 2 weeks significantly increased plasma vitamin C levels, although other indices like FRAP values were not affected [18]. In this study we also observed that cerebral FRAP values were not affected.

In conclusion, a 7-day i.p. administration of a polyphenol-rich wild blueberry extract to healthy adult mice attenuated brain oxidative stress, increased brain ascorbate and GSH levels, and decreased AChE activity. These effects were associated with the enhancement in performance in the passive avoidance behavioral test. Thus, our i.p. study, along with the other dietary studies, stresses the nootropic and neuroprotective potential of blueberry polyphenols. Despite the fact that the mechanisms underlying these effects are still unknown and require more pharmacological, neurochemical and pharmacokinetic research to establish any therapeutic advantage, wild blueberries seem to have the potential to benefit healthy people and contribute to prevention of cognitive decline during aging and neurodegenerative disease.

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