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Aronia melanocarpa (Michx.) Elliot fruit juice reveals neuroprotective effect and improves cognitive and locomotor functions of aged rats

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ABSTRACT

The aim of the study was to investigate the effect of polyphenol-rich *Aronia melanocarpa* (Michx.) Elliot juice (AMJ) on learning ability and memory, and brain morphology of aged rats. A model of healthy male Wistar rats (24 months of age) divided in 2 groups was used: AMJ group supplemented orally with AMJ (10 mL/kg for 105 days) and old control (CO) group without supplementation. Activity cage test showed that AMJ supplemented rats increased the number of vertical movements compared with old controls ($p < 0.05$). In active avoidance test, supplemented rats increased the number of avoidances on 3rd, 4th and 5th days of learning session, compared with the respective day of old controls ($p < 0.05$). AMJ supplementation did not affect the mean neuronal number in the dentate gyrus but significantly increased the density of nerve fibers in the perforant path of the hippocampus ($p < 0.05$). AMJ supplementation increased acetylcholinesterase activity in hippocampus, which is a marker of improved functional activity of the cholinergic neurons. These results indicate that AMJ induced ameliorating changes in the ability of old rats to learn tasks and improved their locomotor functions. AMJ showed a neuroprotective effect by increasing the density of nerve fibers in the hippocampal perforant pathway.

1. Introduction

Aging is the one of the key risk factors for developing neurodegenerative diseases. It is associated with a decrease in cognitive function that significantly affects life quality. The cognitive impairment is one of the signs of aging, which is based on functional, biochemical and morphological changes in brain structures. The oxidative stress is an important event that has been related to the pathogenesis of diseases affecting the central nervous system. This is understandable since nervous tissue is highly sensitive to oxidative stress, due to its high oxygen consumption, high iron and lipid content (especially polyunsaturated fatty acids), and the low activity of antioxidant defences (Koufen and Stark, 2000). Besides, oxidative stress-related low-grade chronic inflammation positively correlates with age, and is among the key factors in the aging process, even in the absence of overt disease (Pawelec et al., 2014). Using natural antioxidants to counteract age-related changes is a strategy of great potential and the idea of aging in a healthy way has led to the extensive study of the anti-aging properties of plant polyphenols. Polyphenols and other natural compounds have been proven as antioxidant sources and acetylcholinesterase (AChE) modulators (Figueira et al., 2017).

The fruit of black chokeberry (*Aronia melanocarpa* (Michx.) Elliot) are among the richest sources of polyphenols, particularly anthocyanins, rendering very high antioxidant activity (Denev et al., 2013).

Abbreviations: AChE, acetylcholinesterase; AMJ, Aronia melanocarpa juice; CA1, cornu ammonis area 1; CA2, cornu ammonis area 2; CA3, cornu ammonis area 3; CO, old controls; DG, dentate gyrus; PP AMJ, perforant path Aronia-supplemented group; PP CO, perforant path old controls group

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Nowadays, because of its health benefits, *A. melanocarpa* is cultivated all over the world. A number of *in vitro* and *in vivo* studies have demonstrated the wide range of applications of the juice, extracts or functional drinks based on *A. melanocarpa* berries because of their anti-inflammatory, anti-mutagenic, anti-carcinogenic, lipid-lowering, anti-diabetic, antihypertensive, hepatoprotective, immunomodulatory effects (Valcheva-Kuzmanova et al., 2004, 2007a, b, c; Denev et al., 2012; Borowska and Brzóska, 2016). Specialized publications provide only limited data on the neuroprotective effect of *A. melanocarpa* and its effect on aging, and age-related brain changes in particular. A recent study has found that black chokeberry juice applied subchronically improves learning ability and memory of young healthy rats (Valcheva-Kuzmanova et al., 2013, 2014). Another study has demonstrated that *A. melanocarpa* extract decreases glutamate-induced death of HT22 cells, reactive oxygen species and intracellular Ca^{2+} levels, and increases the antioxidant status of the mitochondrial membrane potential of HT22 cells (Lee et al., 2017). However, to the best of our knowledge, no studies have been carried out on the neuroprotective effect of *A. melanocarpa* juice or its polyphenols and their impact on the cognitive and locomotor functions in aged mammals. In rats, as in humans, learning abilities and memory performance decline with age, so this rodent species is a suitable model to evaluate strategies of potential clinical value for restoring age-related cognitive deficits. The progressive decline in memory performance of aged rats is associated with and probably preceded by underlying structural, cellular and molecular changes in the hippocampus (Morel et al., 2015). 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 patients with Alzheimer's disease. Acetylcholine exerts acute effects on synaptic plasticity by modulating the spiking activity of neurons and neurotransmitter release (Dannenberg et al., 2015). However, previous studies using a quantitative histochemistry technique for measuring AChE have found a substantial decrease in the AChE content in the cholinergic cell body regions (the ventral pallidum and the medial septal nuclei) and in the projection areas (the cortex and hippocampus) in aged rats, as compared with young controls. In the same group of aged rats a severe deficit in the acquisition of a water maze task has been found (Biegon et al., 1986). Acetylcholine has been shown to have extensive effects on neuronal circuits by affecting neurogenesis, spine and synapse formation (Paez-Gonzalez et al., 2014). The hippocampus has long been implicated in memory functions of humans and other mammals, and is considerably susceptible to oxidative damage due to its high oxygen consumption and higher levels of polyunsaturated fatty acids, which may lead to various neurodegenerative diseases (Selvakumar et al., 2012). Therefore, the aim of the study was to investigate the effect of *A. melanocarpa* juice on the behavioural and locomotor functions in aged rats and their relation to morphological changes in hippocampus and brain AChE activity.

2. Materials and methods

2.1. Chemicals

Folin-Ciocalteu's phenol reagent was provided by Merck (Darmstadt, Germany). Cyanidin-3-O-galactoside chloride, cyanidin-3-O-arabinoside chloride and cyanidin-3-O-arabinoside chloride were purchased from Extrasynthese S.A. (Genay Cedex, France). Gallic acid, chlorogenic acid, 3,4-dihydroxy benzoic acid, p-coumaric acid, caffeic acid, ellagic acid, ferulic acid, catechin, rutin, naringin, naringenin, epicatechin, myrecetin, quercetin-3-glucoside, quercetin, kaempferol, glucose, fructose, sucrose, sorbitol, quinic acid, tartaric acid, malic acid, ascorbic acid, α-keto-glutaric acid, citric acid, shikimic acid, oxalic acid were purchased from Sigma Aldrich (Steinheim, Germany). All other solvents used were of analytical grade and purchased from local distributors.

2.2. Aronia melanocarpa juice

Aronia melanocarpa berries were supplied by the licensed farmer Todor Petkov (Kazanlak, Stara Zagora district, Bulgaria) in the stage of full maturity in August 2017. The fresh berries were placed in polyethylene bags, frozen immediately and stored at −18 °C until juice extraction. Five kilograms of frozen fruit were defrosted at room temperature and homogenized in a laboratory blender. The homogenate was transferred in a brown-glass bottle and incubated in a thermostatic shaking water bath at 60 °C for 1 h. After that the pulp was filtered through a cheesecloth and the liquid fraction obtained was centrifuged and used for the study.

2.3. Experimental animals

The study included 12 male Wistar rats, 24 months of age at the beginning of the experiment, with a body weight of 490–540 g. The animals were provided by the Vivarium of Medical University - Plovdiv where they were maintained under standard laboratory conditions. The rats were divided into 2 groups: a control group (CO, n = 6) and an experimental one (AMJ, n = 6). The former received standard food and drinking water *ad libitum*, whereas the latter was given *A. melanocarpa* juice diluted in a ratio 1:1 with drinking water, at a dose of 10 mL/kg. Previously, this dose showed to be effective in memory improving of healthy rats (Valcheva-Kuzmanova et al., 2014). The experiment lasted for 105 days. Locomotor activity and memory performance were recorded, and at the end of the experimental period the animals were anesthetized with i.m. Ketamin/Xilazine (90 mg/kg/10 mg/kg) and euthanized by cervical decapitation. Immediately after that, the brain of each animal was carefully removed and washed in ice-cold saline, after which it was divided into two symmetrical halves. The right part was fixed in 10% neutral formalin for further histological examination. The hippocampus and frontal cortex were separated from the left part of the brain, frozen in liquid nitrogen and used for biochemical analysis.

The experimental protocol was approved by the Committee on Ethical Treatment of Animals of the Bulgarian Agency for Food Safety (№102/10.07.2014). All animals were treated in compliance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the National Institute of Health (NIH publication No. 86–23, revised 1996).

2.4. High performance liquid chromatography (HPLC) analysis of sugars

HPLC determination of sugars was performed using Agilent 1220 HPLC system (Agilent Technology, USA), with a binary pump and Refractive Index Detector (Agilent Technology, USA). The column was Zorbax Carbohydrate (5 μm, 4.6 × 150 mm, Agilent), connected to a guard column Zorbax Reliance Cartridge (Agilent), 80% acetonitrile was used as eluent, at a flow rate of 1.0 ml/min and temperature 25 °C. The results obtained were expressed as g/L juice.

2.5. HPLC determination of organic acids

HPLC determination of organic acids was performed by Agilent 1220 HPLC system (Agilent Technology, USA), with a binary pump and UV–Vis detector (Agilent Technology, USA). Organic acid separation was performed using an Agilent TC-C18 column (5 μm, 4.6 mm × 250 mm) at 25 °C at 210 nm wavelength. The mobile phase was 25 mM phosphate (K_2HPO_4/H_3PO_4) buffer (pH 2.4), with a flow rate of 1.0 ml/min. The results obtained were expressed as g/L juice.

2.6. HPLC analysis of phenolic compounds

HPLC analysis of phenolic components was performed as described by Denev et al. (2018) using an Agilent 1220 HPLC system (Agilent

Technology, USA), with a binary pump and UV–Vis detector (Agilent Technology, USA). Separation was performed on Agilent TC-C18 column (5 μm, 4.6 mm × 250 mm) at 25 °C using wavelength of 280 nm. The mobile phases used were 0.5% acetic acid (A) and 100% acetonitrile (B), at a flow rate of 0.8 ml/min. The gradient elution started at 14% B, linearly increased to 25% B between the 6-th and 30-th min, and then to 50% B at the 40-th min. The results obtained were expressed as mg/L juice.

2.7. HPLC determination of anthocyanins

Anthocyanins were determined by Agilent 1220 HPLC system (Agilent Technology, Palo Alto, Ca), with a binary pump and UV–Vis detector (Agilent Technology, USA). Wavelength of 520 nm was used. Anthocyanins were separated using an Agilent TC-C18 column (5 μm, 4.6 mm × 250 mm) at 25 °C. The mobile phases used were 5% formic acid (A) and 100% methanol (B) at a flow rate of 1.0 ml/min. The gradient elution started at 15% B and linearly increased to 30% B at the 20-th min. The results were expressed as mg/L juice.

2.8. Total polyphenols analysis

Total polyphenols were determined by the method of Singleton and Rossi (1965), using the Folin-Ciocalteu's reagent. Gallic acid was used for calibration curve and the results were expressed as gallic acid equivalents (GAE) per liter juice.

2.9. Shuttle box test – active avoidance test with negative reinforcement

A shuttle-box automatic reflex conditioner for active avoidance (Ugo Basile, Comerio-Varese, Italy) was used. The duration of the learning session was 5 consecutive days. Each day consisted of 30 trials involving the following parameters: 6 s light and buzzer (670 Hz and 70 dB), 3 sec 0.4 mA foot shock, 12 s pause. A memory retention session with the same parameters excluding foot shock was performed 7 days later (12th day). The number of avoidances during the learning and memory retention sessions was recorded automatically. Before each test the apparatus was wiped clean and dried. The experiments were performed between 9:00 a.m. and 1:00 p.m.

2.10. Activity cage behavioural test for locomotor activity

The horizontal and vertical activity in individual rats was registered by Ugo Basile activity cage apparatus, consisting of an animal cage (with transparent cover) and an electronic unit. Activity was detected by horizontal sensors, designed for the assessment of the ambulatory activity. The movements of the animals were counted and recorded by an electronic unit. The data on horizontal and vertical activity were printed in digital form at pre-set intervals. The activity was recorded for 5 min, starting right after placing the animal into the test cage. The locomotor measurements were performed in a quiet room under normal laboratory lighting. Prior to each test, the cage was wiped clean and dried. The experiments were performed between 9:00 a.m. and 1:00 p.m.

2.11. Histological examination

The Bielschowsky block impregnation method was performed as follows: the dissected brain was cut coronally into 3 mm blocks that were fixed in a 12% solution of neutral formalin for 4–6 weeks. Then the blocks were drained and placed in a 2% silver nitrate solution for 4 days, followed by immersion in ammoniacal silver solution for 3 h. After thorough rinsing with distilled water, they were immersed in a 20% solution of neutral formalin for 18 h. After that they were washed and dehydrated in 40% alcohol for 1 h, 50% alcohol for 1 h, 70% alcohol for 2 h, 85% alcohol for 2 h, 95% alcohol for 24 h, 100% alcohol for 2 h, clarified in cedarwood oil, paraffin-embedded, sliced at a thickness of 12 μm and finally mounted on slides. The slides were deparaffinized in xylene and covered.

2.12. Morphometric analysis

The morphometric analysis involved tissue slices of 12 μm thickness obtained from the cellular layer of the dentate gyrus and the perforant path of the hippocampus. Granular neurons were identified by their position in the granular layer of the dentate gyrus. Coronal brain sections of the Bielschowsky silver stained slices were used to determine the mean number of neuron bodies per unit area (50 μm^2). Nerve fibre density was estimated by counting the number of nerve fibres per unit area (50 μm^2). In all studies five slices per animal were examined and a minimum of five areas of each slice were measured. The measurements were performed using the DP – Soft ver. 3.2 software, Olympus, Japan. Microphotographs were taken using a Nikon Microphot SA microscope (Japan), combined with Camedia-5050Z digital camera (Olympus, Japan).

2.13. Acetylcholinesterase activity

The AChE activity was measured by providing an artificial substrate acetylthiocholine iodide (ATCI), using a modified protocol of Ellman et al. (1961). The method involves the reaction between thiocholine, the cleavage product of ATCI, and 5,5 -dithiobis-(2-nitrobenzoic acid), which results in the formation of a yellow-coloured anion with an absorption maximum at 412 nm. Change of absorbance was measured within 3 min and was used for calculating enzyme activity. The enzyme activity was expressed as micromoles of hydrolysed substrate/min/mg protein.

2.14. Statistical analysis

SPSS 21 was used for the statistical analysis. The results are presented as mean ± standard error of mean (SEM). Paired sample *t*-test, independent sample *t*-test and one-way ANOVA followed by Tukey's test were used for the parametric analysis at a normal distribution level. Wilcoxon signed rank test and Mann-Whitney U tests were used for the non-parametric analysis. $P < 0.05$ was considered as statistically significant.

3. Results and discussion

3.1. Chemical composition of A. melanocarpa juice

The content and composition of sugars and organic acids of the aronia juice used are given in Table 1. The predominant sugar in the juice is sorbitol, which is in agreement with the available literature information (Denev et al., 2018). As far as the organic acids in the juice are concerned, the quinic and malic acid are in highest amounts. The amount of ascorbic acid is significant as well (Denev et al., 2018).

The amount of the individual phenolic components that are likely to contribute to the antioxidant activity of aronia juice are shown in Table 2. As it is evident from the results, it is a source particularly rich in total polyphenols – 11237.4 mg/L. The amount of anthocyanins is significant as well – 2125.1 mg/L, represented mostly by galactoside and arabinoside glycosides of cyanidin. The amount of hydroxycinnamic acids (chlorogenic and neochlorogenic) is even higher, with a cumulative content of 2918.7 mg/L.

3.2. Effect of A. melanocarpa supplementation on cognitive and locomotor functions of aged rats

The regular consumption of foods rich in flavonoids representing the most common group of polyphenolic compounds in human diet, has

Table 1
Sugars and organic acids content of *A. melanocarpa* fruit juice.

Sugars, g/L					
Fructose	Glucose	Sorbitol	Sucrose		
35.8	28.0	105.8	1.1		
Organic acids, g/L					
Quinic acid	Malic acid	Ascorbic acid	Citric acid	Oxalic acid	Tartaric acid
3.25	2.39	0.78	0.37	0.019	0.024

been associated with enhanced cognitive abilities and reduced risk of cognitive decline in aged individuals (Brickman et al., 2014). Memory improvement effects of *A. melanocarpa* juice have been studied experimentally in a passive avoidance test in young rats (Valcheva-Kuzmanova et al., 2014), and revealed that anthocyanins from black chokeberry maintain spatial memory.

In our study, the performance of the control group and the AMJ-supplemented animals subjected to active avoidance test is shown in Fig. 1. The number of avoidances in the CO group determined by the memory retention test was decreased on the second day, as compared with the first day ($p < 0.05$). The group of rats supplemented with 10 mL/kg AMJ increased significantly ($p < 0.05$) the number of avoidances on the 3rd, 4th and 5th day of the learning session, as compared with the behaviour of the controls on the same days. The number of avoidances determined by the memory retention test (day 12) in the AMJ group tended to be increased, as compared with the CO group.

The activity cage test showed that the AMJ group tended to increase the number of relative units of horizontal locomotor activity, as compared with the CO group. The number of relative units of vertical locomotor activity was significantly increased in the AMJ group, as compared with the CO group ($p < 0.05$) (Fig. 2). Spontaneous locomotion and rearing are known to decrease with age as they depend on muscle strength, balance and motor coordination (Sumien et al., 2006). Our results indicated that aronia supplementation improved the psychomotor functions of aged rats.

In order to find out the morphological foundations of cognitive improvement in the experimental animals, we examined the structure of the hippocampus, the memory-associated part of the brain. Bielschowsky silver staining visualizes axons and dendrites and shows details of intracellular neurofibrillary networks in mature neurons. The neuronal cell bodies are visualized in oval shape and light brown colour due to the impregnation of intracellular microfibrils by the silver molecules. The axons and dendrites of neurons are visualized as black intertwined fibres in contrast to the golden brown background of neuropil (Fig. 3).

In the middle part of Fig. 3, the hippocampus in the brain of rats is presented at magnification 40. The pyramidal layer of CA1, CA2 and CA3 is distinct and allows the traceability of these areas of the hippocampus. The neurons from the granular layer of the dentate gyrus delineate it clearly. Fig. 3 presents the CA1, CA2 and CA3 areas, the dentate gyrus (DG) and the perforant path (PP) of rat hippocampus at

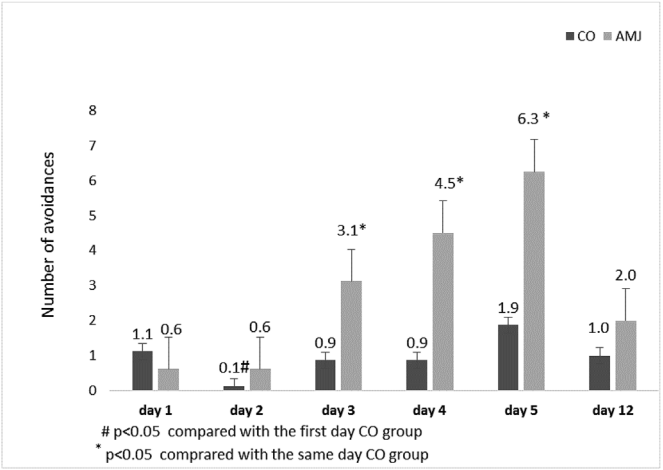

Fig. 1. Effects of *A. melanocarpa* fruit juice supplementation on active avoidance test on rats.

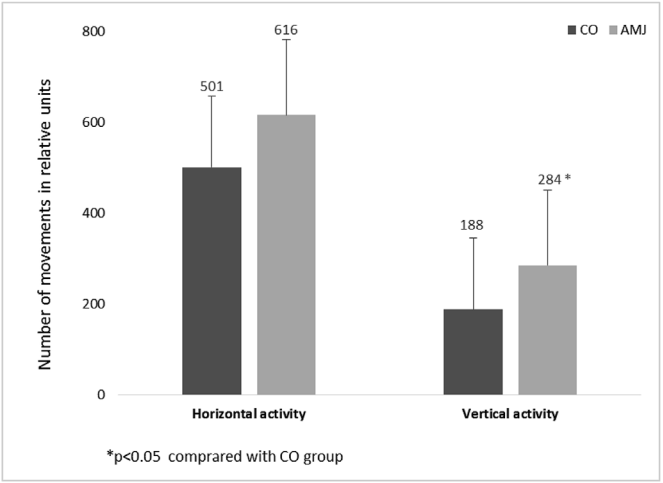

Fig. 2. Effects of *A. melanocarpa* fruit juice supplementation on activity cage test in rats.

Table 2
Content of anthocyanins and other phenolic compounds in *A. melanocarpa* fruit juice.

Anthocyanins, mg/L						
Cyanidin-3-galactoside	Cyanidin-3-glucoside	Cyanidin-3-arabinoside	Cyanidin-3-xyloside			
1498.4	120.1	502.0	4.6			
Phenolic compounds, mg/L						
Chlorogenic acid	Neochlorogenic acid	Epicatechin	Rutin	Quercetin-3-β-glucoside	Quercetin	Total polyphenols
1375.6	1543.1	408.2	446.5	228.9	49.6	11237.4

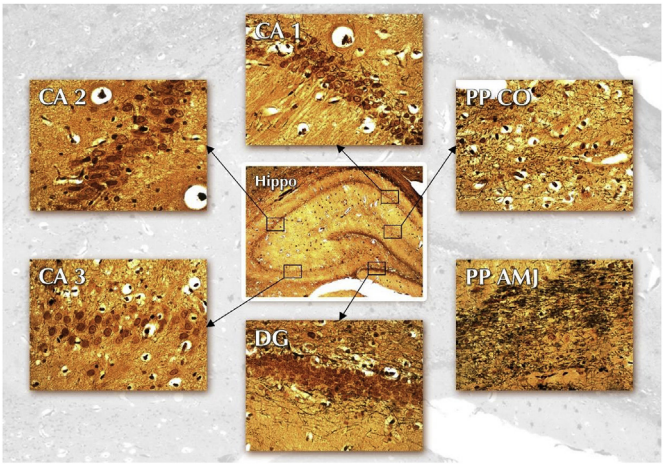

Fig. 3. Rat hippocampus. Bielschowsky silver impregnation. Hippo – hippocampus, magn. x 40; CA1-cornu ammonis area 1; CA2 - cornu ammonis area 2; CA3 - cornu ammonis area 3, DG - dentate gyrus, PP CO - perforant path old controls group, PP AMJ - perforant path aronia-supplemented group, magn x400.

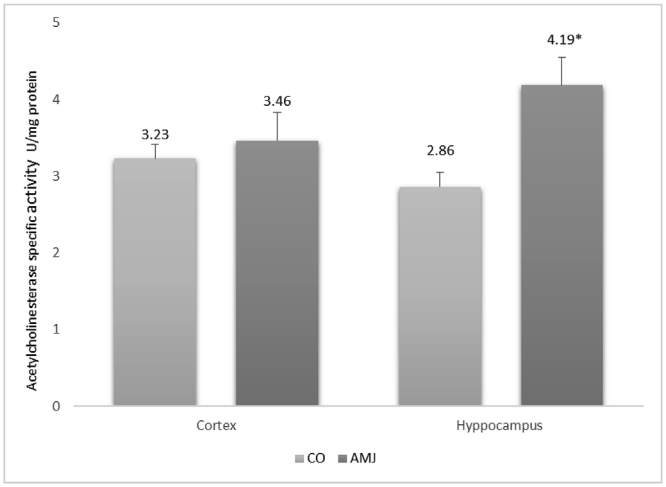

Fig. 5. Acetylcholinesterase specific activity in rat brain in old controls and aronia-supplemented old animals. The asterisk indicates significantly higher ($p < 0.05$) AChE specific activity in aronia-supplemented group in comparison with the old controls.

magnification 400. The PP is the major input to the hippocampus. The PP axons arise at the entorhinal cortex and project to the granule cells of the dentate gyrus and pyramidal cells of the CA3 region, the CA1 region and the subiculum.

The mean neuronal number and nerve fibre density in the hippocampus are shown in Fig. 4. No differences were found in the mean neuronal number of the experimental groups ($p > 0.05$). The nerve fibre density in the perforant pathway in AMJ group was significantly higher ($p < 0.05$) in comparison with the CO group.

The effect of the AMJ supplementation on the activity of AChE in the hippocampus and the prefrontal cortex was determined (Fig. 5). Our results showed that AChE-specific activity in the aronia-supplemented group was significantly higher ($p < 0.05$) in comparison with the aged controls.

The increased activity of AChE observed by us is consistent with the morphological results, since it is an evidence of improved functional activity of the cholinergic neuronal system in the hippocampus. These results can be explained by the increased number of nerve fibres due to the AMJ supplementation. The observed increase in AChE activity is probably not necessarily related to the decrease in acethycholine levels,

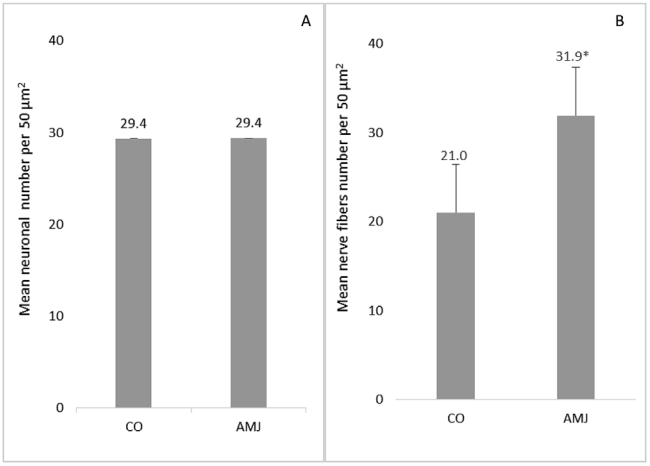

Fig. 4. Mean neuronal amount in dentate gyrus granular layer per unit area (n/50 µm^2) (Panel A) and mean number of nerve fibres per unit area in hippocampal perforant path of rat brain (n/50 µm^2) (Panel B). The asterisk indicates significantly higher ($p < 0.05$) nerve fibre density in the perforant pathway in aronia-supplemented group in comparison with the aged controls.

because the greater number of viable cholinergic nerve fibres are likely to produce higher amounts of the transmitter. Choline acetyltransferase enzyme and AChE are known to be proteins that function as specific markers of the physiological activity of cholinergic neurons. Besides, both play an important role in the homeostasis of neuronal acetylcholine (Fonnum, 1975). Neurotransmission in the cholinergic system is involved in processes, such as learning, memory, sleep, etc. Moreover, cholinergic innervation is directly related to memory consolidation processes (Bowen et al., 1982; Squire et al., 1992) and problems with neurotransmission elicit changes in these functions, which may be one of the causes of senile dementia or Alzheimer disease (Davies, 1983; Orta-Salazar et al., 2014). The selective lesion of cholinergic neurons in the basal forebrain significantly impairs hippocampus-dependent memory function, suggesting a role of septohippocampal cholinergic projections in memory formation (Berger-Sweeney et al., 2001).

A variety of antioxidant compounds derived from natural products (nutraceuticals) have demonstrated a neuroprotective activity in either *in vitro* or *in vivo* models of neuronal cell death or neurodegeneration, respectively. Many of these compounds are generally considered to be antioxidants. They may be classified as such because they either directly scavenge free radicals or indirectly increase endogenous cellular antioxidant defences, for example, via activation of the nuclear factor erythroid-derived 2-related factor 2 transcription factor pathway. Alternative mechanisms of action have also been suggested for the neuroprotective effects of these compounds, such as modulation of signal transduction cascades or effects on gene expression (Kelsey et al., 2010). The potential of polyphenols to improve neurological health appears to be related to a number of mechanisms, including their ability to interact with intracellular neuronal and glial signaling, influence the peripheral and cerebrovascular blood flow, and reduce neuronal damage and loss induced by neurotoxins and neuroinflammation (Spencer, 2010a). Polyphenolic substances from *A. melanocarpa* can accumulate in the brain after long-term consumption and because of their antioxidant action can improve the neuronal cellular antioxidant status. The supplementation of black chokeberry polyphenols is likely to raise the levels of monoamines (norepinephrine, dopamine and 5-hydroxytriptamine) and inhibit the excessive accumulation of inflammatory cytokines, such as cyclooxygenase-2, interleukin-1 and transforming growth factor beta 1, in the brain of aging mice. Apart from that, supplementation can regulate - blood and brain tissue redox balance. Anthocyanins inhibit DNA damage in brain cells and regulate the protein expression in the DNA damage signaling pathway in the brain of aging mice (Wei et al., 2017). Other aspects through which

flavonoids can be neuroprotective include their positive effects on peripheral and cerebrovascular blood flow, ultimately affecting synaptic plasticity processes and cognitive function (Rendeiro et al., 2015). Flavonoids and their physiological metabolites are capable of activating kinases in the signaling pathways by regulating proteins, e.g. cAMP response element-binding protein, and by increasing the production of brain-derived neurotrophic factor required in the processes of learning and retention (Spencer, 2010b). *In vivo* studies have shown that quercetin improves memory and hippocampal synaptic plasticity in models of impairment induced by chronic lead exposure (Hu et al., 2008) and have revealed a neuroprotective effect in cognitive impairment resulting from colchicine administration (Kumar et al., 2008).

4. Conclusion

Our results of the two-way active avoidance test showed that *A. melanocarpa* juice improves significantly the ability of aged rats to learn tasks. Moreover, it improves their locomotor functions. The behavioural and memory changes observed are consistent with the increased number of nerve fibres in the perforant pathway in the hippocampus, which may serve as a marker of increased functional activity of the cholinergic neurons. This is a manifestation of a neuroprotective effect and a probable neuroplasticity effect of *A. melanocarpa* juice.

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References

Berger-Sweeney, J., Stearns, N.A., Murg, S.L., Floerke-Nashner, L.R., Lappi, D.A., Baxter, M.G., 2001. Selective immunolesions of cholinergic neurons in mice: effects on neuroanatomy, neurochemistry, and behavior. J. Neurosci. 21, 8164–8173. https://doi.org/10.1523/JNEUROSCI.21-20-08164.2001.

Biegon, A., Greenberger, V., Segal, M., 1986. Quantitative histochemistry of brain acetylcholinesterase and learning rate in the aged rat. Neurobiol. Aging 7 (3), 215–217. https://doi.org/10.1016/0197-4580(86)90046-1.

Borowska, S., Brzóska, M., 2016. Chokeberries (*Aronia melanocarpa*) and their products as a possible means for the prevention and treatment of noncommunicable diseases and unfavorable health effects due to exposure to xenobiotics. Compr. Rev. Food Sci. F. 15 (6), 982–1017. https://doi.org/10.1111/1541-4337.12221.

Bowen, D., Benton, J., Spillane, J., Smith, C., Allen, S., 1982. Choline acetyltransferase activity and histopathology of frontal neocortex from biopsies of demented patients. J. Neurol. Sci. 57, 191–192. https://doi.org/10.1016/0022-510X(82)90026-0.

Brickman, A., Khan, U., Provenzano, F., Yeung, L., Suzuki, W., Schroeter, H., Wall, M., Sloan, R.P., Small, S.A., 2014. Enhancing dentate gyrus function with dietary flavanols improves cognition in older adults. Nat. Neurosci. 17 (12), 1798–1803. https://doi.org/10.1038/nn.3850.

Dannenberg, H., Pabst, M., Braganza, O., Schoch, S., Niediek, J., Bayraktar, M., Mormann, F., Beck, H., 2015. Synergy of direct and indirect cholinergic septo-hippocampal pathways coordinates firing in hippocampal networks. J. Neurosci. 35, 8394–8410. https://doi.org/10.1523/JNEUROSCI.4460-14.2015.

Davies, P., 1983. Neurotransmitters and neuropeptides in Alzheimer's disease. In: Katzman, R. (Ed.), Biological Aspects of Alzheimer's Disease. Cold Spring Harbor, New York, pp. 255–265.

Denev, P., Kratchanov, Ch, Ciz, M., Lojek, A., Kratchanova, M., 2012. Bioavailability and antioxidant activity of black chokeberry (*Aronia melanocarpa*) polyphenols: in vitro and in vivo evidences and possible mechanisms of action: a review. Compr. Rev. Food Sci. F. 11 (5), 471–489. https://doi.org/10.1111/j.1541-4337.2012.00198.x.

Denev, P., Lojek, A., Ciz, M., Kratchanova, M., 2013. Antioxidant activity and polyphenol content of Bulgarian fruits. Bulg. J. Agric. Sci. 19 (1), 22–27.

Denev, P., Kratchanova, M., Petrova, I., Klisurova, D., Georgiev, Y., Ognyanov, M., Yanakieva, I., 2018. Black chokeberry (*Aronia melanocarpa* (Michx.) Elliot) fruits and functional drinks differ significantly in their chemical composition and antioxidant activity. J. Chem. 13. 2018, Article ID 9574587. https://doi.org/10.1155/2018/9574587.

Ellman, G., Courtney, D., Andres, V., Featherstone, R., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95. https://doi.org/10.1016/0006-2952(61)90145-9.

Figueira, I., Menezes, R., Macedo, D., Costa, I., Dos Santos, C.N., 2017. Polyphenols beyond barriers: a glimpse into the brain. Curr. Neuropharmacol. 15, 562–594. https://doi.org/10.2174/1570159X14666161026151545.

Fonnum, F., 1975. Review of Recent Progress in the Synthesis, Storage, and Release of Acetylcholine in Cholinergic Mechanisms. Raven Press, New York, pp. 145–160.

Hu, P., Wang, M., Chen, W.H., Liu, J., Chen, L., Yin, S.T., Yong, W., Chen, J.T., Wang, H.L., Ruan, D.Y., 2008. Quercetin relieves chronic lead exposure-induced impairment of synaptic plasticity in rat dentate gyrus *in vivo*. Naunyn. Schmiedebergs Arch. Pharmacol. 378, 43–51. https://doi.org/10.1007/s00210-008-0301-z.

Kelsey, N., Wilkins, H., Linseman, D., 2010. Nutraceutical antioxidants as novel neuroprotective agents. Molecules 15, 7792–7814. https://doi.org/10.3390/molecules15117792.

Koufen, P., Stark, G., 2000. Free radical induced inactivation of creatine kinase: sites of interaction, protection, and recovery. Biochim. Biophys. Acta 1501 (1), 44–50. https://doi.org/10.1016/S0925-4439(00)00005-3.

Kumar, A., Sehgal, N., Kumar, P., Padi, S.S., Naidu, P.S., 2008. Protective effect of quercetin against ICV colchicine-induced cognitive dysfunctions and oxidative damage in rats. Phytother Res. 22, 1563–1569. https://doi.org/10.1002/ptr.2454.

Lee, H.Y., Weon, J.B., Ryu, G., Yang, W.S., Kim, N.Y., Kim, M.K., Ma, C.J., 2017. Neuroprotective effect of *Aronia melanocarpa* extract against glutamateinduced oxidative stress in HT22 cells. BMC Complement Altern. Med. 17 (207), 1–7. https://doi.org/10.1186/s12906-017-1716-1.

Morel, G., Andersen, T., Pardo, J., Zuccolilli, G., Cambiaggi, V.L., Hereñú, C.B., Goya, R.G., 2015. Cognitive impairment and morphological changes in the dorsal hippocampus of very old female rats. Neuroscience 10 (303), 189–199. https://doi.org/10.1016/j.neuroscience.2015.06.050.

Orta-Salazar, E., Cuellar-Lemus, C., Díaz-Cintra, S., Feria-Velasco, A.I., 2014. Cholinergic markers in the cortex and hippocampus of some animal species and their correlation to Alzheimer's disease. Neurologia 29 (8), 497–503. https://doi.org/10.1016/j.nrl.2012.10.007.

Paez-Gonzalez, P., Asrican, B., Rodriguez, E., Kuo, C.T., 2014. Identification of distinct ChAT(+) neurons and activity-dependent control of postnatal SVZ neurogenesis. Nat. Neurosci. 17, 934–942. https://doi.org/10.1038/nn.3734.

Pawelec, G., Goldeck, D., Derhovanessian, E., 2014. Inflammation, ageing and chronic disease. Curr. Opin. Immunol. 29, 23–28. https://doi.org/10.1016/j.coi.2014.03.007.

Rendeiro, C., Rhodes, J., Spencer, J., 2015. The mechanisms of action of flavonoids in the brain: direct versus indirect effects. Neurochem. Int. 89, 126–139. https://doi.org/10.1016/j.neuint.2015.08.002.

Selvakumar, K., Bavithra, S., Krishnamoorthy, G., Venkataraman, P., Arunakaran, J., 2012. Polychlorinated biphenyls-induced oxidative stress on rat hippocampus: a neuroprotective role of quercetin. ScientificWorldJournal 980314. 2012. https://doi.org/10.1100/2012/980314.

Singleton, V., Rossi, J., 1965. Colorimetry of total phenolic with phosphomolibdiphosphotungstic acid reagents. Am. J. Enol. Vitic. 16, 144–158.

Spencer, J.P., 2010a. Beyond antioxidants: the cellular and molecular interactions of flavonoids and how these underpin their actions on the brain. Proc. Nutr. Soc. 69, 244–260. https://doi.org/10.1017/S0029665110000054.

Spencer, J.P., 2010b. The impact of fruit flavonoids on memory and cognition. Br. J. Nutr. 104 (3), S40–S70. https://doi.org/10.1017/S0007114510003934.

Squire, L., Ojemann, J., Miezin, F., Petersen, S., Videen, T., Paichle, M., 1992. Activation of the hippocampus in normal humans: a functional anatomical study of memory. Proc. Natl. Acad. Sci. U.S.A. 89, 1837–1841. https://doi.org/10.1073/pnas.89.5.1837.

Sumien, N., Sims, M.N., Taylor, H.J., Forste, M.J., 2006. Profiling psychomotor and cognitive aging in four-way cross mice. Age 28 (3), 265–282. https://doi.org/10.1007/s11357-006-9015-7.

Valcheva-Kuzmanova, S., Borisova, P., Galunska, B., Krasnaliev, I., Belcheva, A., 2004. Hepatoprotective effect of the natural fruit juice from *Aronia melanocarpa* on carbon tetrachloride-induced acute liver damage in rats. Exp. Toxicol. Pathol. 56, 195–201. https://doi.org/10.1016/j.etp.2004.04.012.

Valcheva-Kuzmanova, S., Kuzmanov, K., Tancheva, S., Belcheva, A., 2007a. Hypoglycemic and hypolipidemic effects of *Aronia melanocarpa* fruit juice in streptozotocin-induced diabetic rats. Method. Find. Exp. Clin. 29, 1–5. https://doi.org/10.1358/mf.2007.29.2.1075349.

Valcheva-Kuzmanova, S., Kuzmanov, K., Mihova, V., Krasnaliev, I., Borisova, P., Belcheva, A., 2007b. Antihyperlipidemic effect of *Aronia melanocarpa* fruit juice in rats fed a high-cholesterol diet. Plant Foods Hum. Nutr. (Dordr.) 62, 19–24. https://doi.org/10.1007/s11130-006-0036-2.

Valcheva-Kuzmanova, S., Kuzmanov, K., Tsanova-Savova, S., Mihova, V., Krasnaliev, I., Borisova, P., Belcheva, A., 2007c. Lipid-lowering effects of A*ronia melanocarpa* fruit juice in rats fed cholesterol-containing diets. J. Food Biochem. 31, 589–602. https://doi.org/10.1111/j.1745-4514.2007.00132.x.

Valcheva-Kuzmanova, S., Eftimov, M., Belcheva, I., Tashev, R., Belcheva, S., 2013. Effect of *Aronia melanocarpa* fruit juice on learning and memory in the two-way active avoidance task in rats. J. Biomed. Clin. Res. 6 (1), 18–23. https://doi.org/10.1515/jbcr-2015-0098.

Valcheva-Kuzmanova, S., Eftimov, M., Tashev, R., Belcheva, I., Belcheva, S., 2014. Memory effects of *Aronia melanocarpa* fruit juice in a passive avoidance test in rats. Folia Med. 56 (3), 199–203. https://doi.org/10.2478/folmed-2014-00.

Wei, J., Zhang, G., Zhang, X., Xu, D., Gao, J., Fan, J., Zhou, Z., 2017. Anthocyanins from black chokeberry (*Aronia melanocarpa* Elliot) delayed aging-related degenerative changes of brain. J. Agric. Food Chem. 65, 5973–5984. https://doi.org/10.1021/acs.jafc.7b02136.