NFS Journal 32 (2023) 100146

Contents lists available at ScienceDirect

NFS Journal

journal homepage: www.elsevier.com/locate/nfs

Aronia juice improves working memory and suppresses δ-secretase activity in 5XFAD mice

Takuya Yamane [a,b,f,*], Momoko Imai [a,f], Satoshi Handa [b], Hideo Ihara [c], Tatsuji Sakamoto [b], Tetsuo Ishida [d], Takenori Nakagaki [e], Susumu Uchiyama [a,f]

[a] Department of Biotechnology, Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871, Japan
[b] Department of Biofunctional Chemistry, Graduate School of Agriculture, Osaka Metropolitan University, Sakai, Osaka 599-8531, Japan
[c] Department of Biological Science, Graduate School of Science, Osaka Metropolitan University, Sakai, Osaka 599-8531, Japan
[d] Department of Nutrition, Koshien University, Takarazuka, Hyogo 665-0006, Japan
[e] Nakagaki Consulting Engineer and Co., Ltd, Nishi-Ku, Sakai 593-8328, Japan
[f] Institute of Metabolomics, BYU-Analytica Inc., Suita, Osaka 565-0871, Japan

ARTICLE INFO

Keywords:
Alzheimer's disease
Asparagine endopeptidase
Legumain
Polyphenols
Quercetin

ABSTRACT

Asparagine endopeptidase (AEP) cleaves amyloid precursor protein to facilitate amyloid β (Aβ) production in the brain. Aronia berry (*Aronia melanocarpa*) has various beneficial effects on health through inhibition of enzymes such as dipeptidyl peptidase IV. In this study, to examine neuroprotective effects of aronia juice (AJ), AJ was given to male 5XFAD Alzheimer's disease model mice via drinking bottles over a period from 2 weeks to 12 weeks of age. AJ intake improved the performance of Y-maze test in 5XFAD mice, and suppressed the δ-secretase activity in the mouse brains. AJ intake reduced significantly Aβ deposition in the brain. AJ reduced δ-secretase activity in human neuroblastoma SH-SY5Y cells. Out of five fractions (F1–F5) obtained from AJ, only F5 reduced δ-secretase activity in SH-SY5Y cells in a dose-dependent manner. LC-MS/MS analysis suggested that F5 contains three quercetin glycosides. AJ has neuroprotective function through inhibition of δ-secretase activity of AEP.

1. Introduction

Alzheimer's disease (AD) is the most common and progressive neurodegenerative disorder with devastation of quality-of-life and tremendously high economic costs. It is estimated that fifty-five million people are living with AD and other dementias in worldwide, and AD accounts for 60–80% of all dementia cases [1]. Therefore, it is very important to find means to prevent the onset of AD and to stop the progression of the disease to advanced stages. Neuroprotective roles of fruits and vegetables in AD have been intensively investigated, as recently reviewed [2,3]. For instance, daily drinking pomegranate juice improves behavior in a transgenic AD model mouse [4], and dietary supplementation with blueberry extracts prevents behavioral deficits in a transgenic AD model mouse [5].

Aronia berry (*Aronia melanocarpa*) has many functional components such as anthocyanins and flavonoids [6]. For example, cyanidin 3,5-diglucoside and caffeoylquinic inhibit dipeptidyl peptidase IV (DPP IV) and α-glucosidase [7–11]. Delphinidin-arabinoside-glucoside inhibits HMG-CoA reductase [12], and vicenin-2 suppresses the expression of *arrdc3* gene [13]. In addition, recently, cyanidin 3-*O*-galactoside extracted from aronia berry has been shown to alleviate cognitive impairment in the spontaneous senescence accelerated mouse [14,15]. These studies used SAMP8 mice as age-related cognitive impairment and spontaneous AD model [16,17]. The SAMP mouse develops impairments in learning and memory at about 8 months of age with over production of amyloid precursor protein (APP). On the other hand, 5XFAD transgenic mouse overexpresses five familial Alzheimer's disease mutations [18], and accumulation of intraneuronal β-amyloid 42 ($Aβ_{42}$) in the brain starts at 1.5 months of age [18,19]. At present, effects of aronia berry on this early-onset AD mouse have not been investigated.

In this study, we hypothesized that aronia juice (AJ) contains compounds beneficial effects on the early stage of AD by means of mechanisms hitherto unknown. Therefore, we used 5XFAD mouse as AD mouse model, and AJ was given to the model mice via free drinking AJ in

Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; AEP, asparagine endopeptidase; AJ, aronia juice; APP, amyloid precursor protein; DPP IV, dipeptidyl peptidase IV.

* Corresponding author at: Department of Biotechnology, Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871, Japan.
E-mail address: takuya.yamane@bio.eng.osaka-u.ac.jp (T. Yamane).

https://doi.org/10.1016/j.nfs.2023.100146
Received 19 June 2023; Received in revised form 24 August 2023; Accepted 25 August 2023
Available online 26 August 2023
2352-3646/© 2023 Published by Elsevier GmbH on behalf of Society of Nutrition and Food Science e.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

drinking bottles beginning at 2 weeks of age for 12 weeks. To find mechanisms, we measured asparagine endopeptidase (AEP) activity in the brain because AEP is strongly activated in 5XFAD mouse brain [20]. In the mouse brain, AEP cleaves APP, and this cleavage of APP, called as δ-secretase activity, facilitates the production of Aβ peptides. Effects of AJ and the five fractions obtained from AJ on SH-SY5Y cells (human neuroblastoma) were also examined.

2. Materials and methods

2.1. Materials

AJ was obtained from Nakagaki Consulting Engineer Co Ltd. (Sakai, Japan). BF-188 and anti-Aβ antibody (6E10 antibody) were purchased from Wako Pure Chemicals (Osaka, Japan). Cell counting kit-8 were purchased from Dojindo (Kumamoto, Japan). *Z*-Ala-Ala-Asn-MCA and $A\beta_{25-35}$ peptides were purchased from Peptide Institute (Osaka, Japan). All other chemicals were of analytical grade and were purchased from Wako Pure Chemicals (Osaka, Japan).

2.2. Animal experiments

5XFAD transgenic mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA) [18]. 5XFAD mice were maintained by crossing hemizygous transgenic mice with non-transgenic B6/SJL mice. Before the experiments, genomic DNA was extracted individually from the tail tip of all mice and genotyping was performed by polymerase chain reaction analysis, and non-transgenic littermates were used as controls. One group (5XFAD) consisted of 5 male 5XFAD mice and the mice drank ad libitum water via drinking bottles. Another group (5XFAD-AJ) consisted of 5 male 5XFAD mice and the mice drank ad libitum AJ via drinking bottles from 2 weeks through 12 weeks of age. Control group (WT) consisted of 5 non-transgenic male littermates and the mice drank water. A normal diet (CE-2 diet) was given to all the mice ad libitum. Mice were maintained at 23 ± 2 °C and 55 ± 10% relative humidity under a 12:12 h light:dark cycle. The weight of mice showed no difference throughout the experiments among WT, 5XFAD, and 5XFAD-AJ mice (Fig. S1).

2.3. Y-maze test

Y-maze had three arms made of grey acrylic plates separated 120° apart, and each of them was in the size of 40 cm × 10 cm × 15 cm. After 10 min habitation, each mouse was placed at the center of the Y-maze and allowed to freely explore the different arms in 8 min. The number of arm entries and the sequence of entries were recorded using a video camera. The percentage of alteration was calculated according to the following equation: (number of alteration) × 100/ (number of total arm entries −2). Each mouse of the three groups was subjected to the Y-maze test during the light phase at 14 weeks of age.

2.4. Immunohistochemistry

At 100 days of age, each mouse was anesthetized using isoflurane, and the cerebrum was dissected and fixed in 10% neutrally buffered formalin. The fixed tissues were dehydrated, embedded in paraffin, and sectioned at 5 μm. The sections were deparaffinized, rehydrated, and incubated with 3% hydrogen peroxide solution (in methanol) for 25 min. Each section was incubated with an anti-Aβ antibody (1:500) in PBS overnight at 4 °C. After washing with PBS, the sections were incubated in the secondary antibody for 30 min at room temperature. After reacting with diaminobenzidine, the sections were observed with a microscope (MZ-9300, Keyence, Osaka, Japan).

2.5. BF-188 staining

BF-188, a benzimidazole derivative, is a fluorescent probe that binds strongly to both Aβ and tau protein deposits [21]. Deparaffinized sections were stained with BF-188 according to the manufacturer's protocol. Briefly, the sections were washed with Milli-Q water for 10 min, bleached with 0.25% potassium permanganate solution for 20 min, and washed with PBS twice. The washed sections were immersed in 100 μmol/L BF-188 (in 50% ethanol) for 10 min, washed 5 times by dipping in water for a few seconds, and then observed using a fluorescence microscope (MZ-9300, Keyence, Osaka, Japan).

2.6. Cell cultures and treatments

Human neuroblastoma SH-SY5Y cells were cultured in D-MEM/F-12 medium (Nacarai Tesq, Kyoto, Japan) containing 10% FBS, 1% penicillin-streptomycin under 5% CO_2 at 37 °C. SH-SY5Y cells were seeded in 96-well plates, and the cells were grown up to 80% confluence for 18–24 h before treatments with $A\beta_{25-35}$ peptide, AJ, or the fractions obtained from AJ. Stock solution of $A\beta_{25-35}$ peptide was prepared at 1 mM in bi-distilled water, and the solution was incubated at 37 °C for 3 days to aggregate the peptides. The resultant aggregated-forms of the peptide (toxic $A\beta_{25-35}$ peptide) was added to the culture medium [22,23]. Cell viability of the treated cells was examined using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. The absorbance at 450 nm was measured using a plate reader (Bio-Rad, USA).

2.7. δ-Secretase activity assay

In the present study, δ-secretase activity was assayed as AEP activity using *Z*-Ala-Ala-Asn-MCA as substrate. Assay mixture (1.0 mL) contained 50 mM sodium citrate buffer (pH 5.0), 100 μM *Z*-Ala-Ala-Asn-MCA, 5 mM 2-mercaptoethanol, and enzyme solution (the supernatant of brain tissue homogenates or cell lysates). The reaction was started by the addition of the substrate stock solution (10 mM Z-Ala-Ala-Asn-MCA in dimethyl sulfoxide), and the reaction mixture was incubated at 37 °C for 30 min. Then, reaction was stopped by the addition of 2 mL of 0.2 M acetic acid, and the enzymatically released amino methyl coumarin (AMC) was fluorometrically measured using a fluorescence spectrometer (excitation and emission wavelength of 380 and 440 nm, respectively). A calibration curve was obtained by measuring the fluorescence of assay mixtures (without enzyme solution) containing varying concentrations of AMC. The protein concentration of enzyme solutions was determined using the Bradford assay and bovine serum albumin as a standard protein (Bio-Rad Protein Assays).

2.8. Chromatographic separation of aronia juice

Five fractions (F1–F5) were prepared form AJ using a Wakogel 50C18 column (200 mL gel) as described previously.[8] Briefly, AJ (300 mL) was directly applied to the column preequilibrated with 0.1% aqueous formic acid (solvent A). The column was washed extensively with solvent A, and then the adsorbed compounds were eluted from the column by a stepwise increase in the methanol concentration (methanol: solvent A = 10, 20, 30, 40, and 50% (*v*/v)) to give F1–F5 in this order. Each fraction was evaporated to dryness, weighed, and resolved in 12.5% or 25% aqueous methanol containing 0.1% formic acid to a final concentration of 1–10 mg/mL.

2.9. Liquid chromatography-mass spectrometry (LC-MS)

To examine compounds in the respective fractions, an aliquot of each fraction (1 μL) was injected to an InertSustain C18 column (2.1 mm × 150 mm, GL Science, Tokyo, Japan) preequilibrated with solvent A, and the column was developed at a flow rate of 0.25 mL/min with the

following gradient: 0–5 min with 0% B, 5–35 min with 0–70% B, and 35–35.1 min with 70–100% B, where solvent B was acetonitrile containing 0.1% formic acid. The column temperature was controlled at 40 °C. The eluate was subjected to electrospray ionization (the spray voltage 4 kV, the capillary temperature 320 °C). A mass spectrum of the eluate was recorded between m/z 150 and 1000 in the positive ion mode. The ions with intensity of top five were data-dependently subjected to MS/MS measurement. A dual UHPLC pump (Vanquish, Thermo Fisher Scientific, CA, USA) and an orbitrap mass spectrometer (Q-Exactive, Thermo Fisher Scientific, CA, USA) were used. Data analysis was performed using Compound Discoverer 3.1 software (Thermo Fisher Scientific, CA, USA).

2.10. Statistical analysis

Data are expressed as mean ± S.E. Statistical analyses were performed using Statcel4 software (OMS, Tokyo, Japan). The difference between two groups was evaluated using analysis of variance (one-way ANOVA) followed by unpaired Student's t-test. For comparison of multiple samples, the Tukey-Kramer test was used.

2.11. Ethics

All animal experiments were carried out following the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all of the protocols were approved by the Committee for Animal Research at Osaka Prefecture University (permit number 19–112). During the experimental period, the animals had no significant weight loss, eating disorders, self-injurious behavior, abnormal posture, breathing problems, or anguish symptoms such as crying, diarrhea, bleeding, or vulvar soiling.

3. Results

3.1. Aronia juice improved Y-maze performance of 5XFAD mice

To investigate the effects of AJ intake on working memory of 5XFAD mice, 84 days after the beginning of AJ intake at 2 weeks of age, the performance of Y-maze test was examined for non-transgenic mice (WT group), 5XFAD mice drinking water (5XFAD group), and 5XFAD mice drinking AJ (5XFAD-AJ group) (Fig. 1). The percent alteration of 5XFAD mice was significantly reduced compared to non-transgenic mice, whereas 5XFAD mice taking AJ daily showed the same levels of the alteration as non-transgenic mice (Fig. 1A). As for total arm entries, 5XFAD mice showed a tendency to reduction compared to WT and 5XFAD-AJ mice (Fig. 1B).

3.2. Aronia juice ameliorated Aβ accumulation in the cerebrum

To examine the effects of AJ intake on Aβ accumulation in the cerebrum, Aβ deposition was assayed by immunohistochemistry with a specific antibody against Aβ (6E10). As shown in Fig. 2, the number of Aβ plaques decreased in 5XFAD-AJ mice brain compared to 5XFAD mice. To further assay the levels of Aβ and tau deposit, the brain sections were stained with BF-188 (Fig. S2). The number of BF-188 stained spots were reduced in 5XFAD-AJ mice brain compared to 5XFAD.

3.3. Aronia juice ameliorated increase in δ-secretase activities in the 5XFAD mouse brain

As recently reviewed [24], in mammalian brain, AEP plays a critical role in APP fragmentation and Aβ production. It is known that AEP is activated in normal mice in an age-dependent manner, and is strongly activated in 5XFAD mouse brain [20]. Therefore, we examined the effects of AJ on the AEP activities in the mouse brain (Fig. 3). The AEP activities in 5XFAD mice brains were significantly higher compared to non-transgenic mice. Importantly, 5XFAD-AJ mice brains showed the

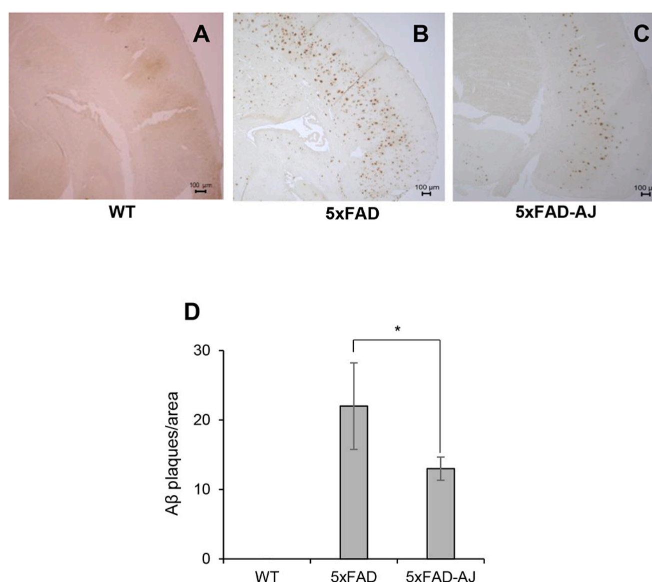

Fig. 2. Amyloid deposition in the cerebrum of WT (A), 5XFAD (B) and 5XFAD-AJ mice (C) at 14 weeks of age. Parasagittal sections of brains from representative 5XFAD and 5XFAD-AJ mice were stained with aniti-Aβ antibody (6E10). The number of plaques for each genotype were quantified (D). *$p < 0.05$, $n = 3$.

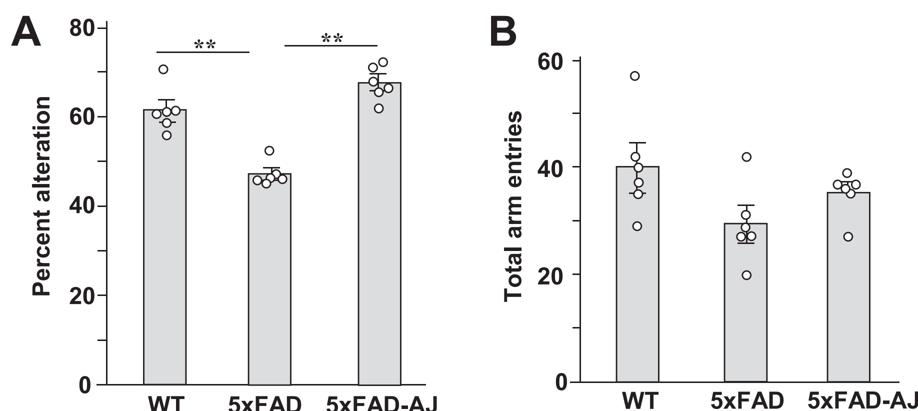

Fig. 1. Effects of aronia juice on Y-maze behavior of 5xFAD mice. Non-transgenic (WT) and 5XFAD mice drank water throughout the experiment, and 5XFAD-AJ mice drank aronia juice instead of water from 2 weeks to 14 weeks of age. (A) Percent alteration. (B) Total arm entries. $n = 6$. **$p < 0.01$.

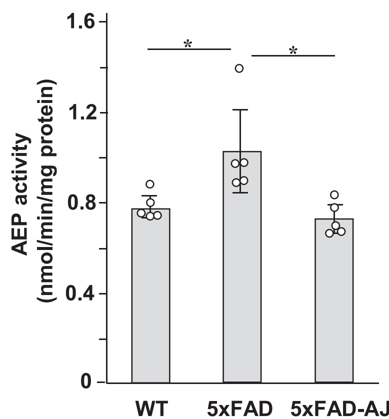

Fig. 3. Effects of aronia juice on AEP activities in the brain of non-transgenic (WT), 5XFAD, and 5XFAD-AJ mice at 14 weeks of age. The AEP activity was assayed using *Z*-Ala-Ala-Asn-MCA as substrate. $n = 5$, $*p < 0.05$.

same levels of the enzyme activities as found for WT mice brains.

3.4. Aronia juice and F5 reduced δ-secretase activities in SH-SY5Y cells

To find mechanisms for the amelioration of the increase in AEP activities in 5XFAD mouse brain by AJ intake, the effects of AJ and the five fractions from AJ on the AEP activities in SH-SY5Y cells, human neuroblastoma cell line, were examined (Fig. 4). The concentrations of AJ and each fraction used for the cell treatments were not toxic to the cells (Fig. S3). AJ reduced AEP activities in a dose-dependent manner (Fig. 4A). Out of the five fractions, only F5 reduced significantly AEP activities of SH-SY5Y cells (Fig. 4B). F5 reduced strongly AEP activities in the cells dose-dependently (Fig. 4C).

3.5. F5 protected SH-SY5Y cells treated with $A\beta_{25-35}$ peptide from cell death

Aggregated $A\beta_{25-35}$ peptide is toxic to neuronal cells. SH-SY5Y cells treated with $A\beta_{25-35}$ showed reduced cell viability (Fig. 5). Only F5 protected significantly SH-SY5Y cells from toxic effects of $A\beta_{25-35}$ peptide.

3.6. LC-MS/MS analysis of F5

Three ions were detected as major ions (Fig. 6). The ion with *m/z* value of 303.05 was produced from all the three ions by CID (Fig. 6C, F, and G). Because the molecular mass of protonated quercetin is 303.05 Da, these three ions seem to be quercetin derivatives. The molecular mass of quercetin 3-O-rutinoside (rutin), quercetin 3-O-galactoside (hyperoside) and quercetin 3-O-glucoside (isoquercetin) is 611.16, 465.465.10, and 465.10, respectively. The ion eluted at 18.2 min seems to be rutin. The ion eluted at 18.62 min (P_1) and that eluted at 18.78 min (P_2) seem to be hyperoside and isoquercetin.

4. Discussion

There are no effective methods to reverse AD or at least stop the progression of AD after the disease progresses over a certain critical level. Therefore, it is important to find means to retain the disease at its early stage. 5XFAD model mouse was made to obtain a very rapid AD amyloid model [18]. In 5XFAD mice, amyloid deposition begins at 2 months of age, and spatial working memory as judged by the alteration performance in Y-maze is significantly impaired at 4 months of age. In the present study, we examined the effects of daily AJ intake on the very early stage of AD in 5XFAD mice over a period from 2 weeks to 12 weeks of age. The Y-maze alteration performance in 5XFAD mice was

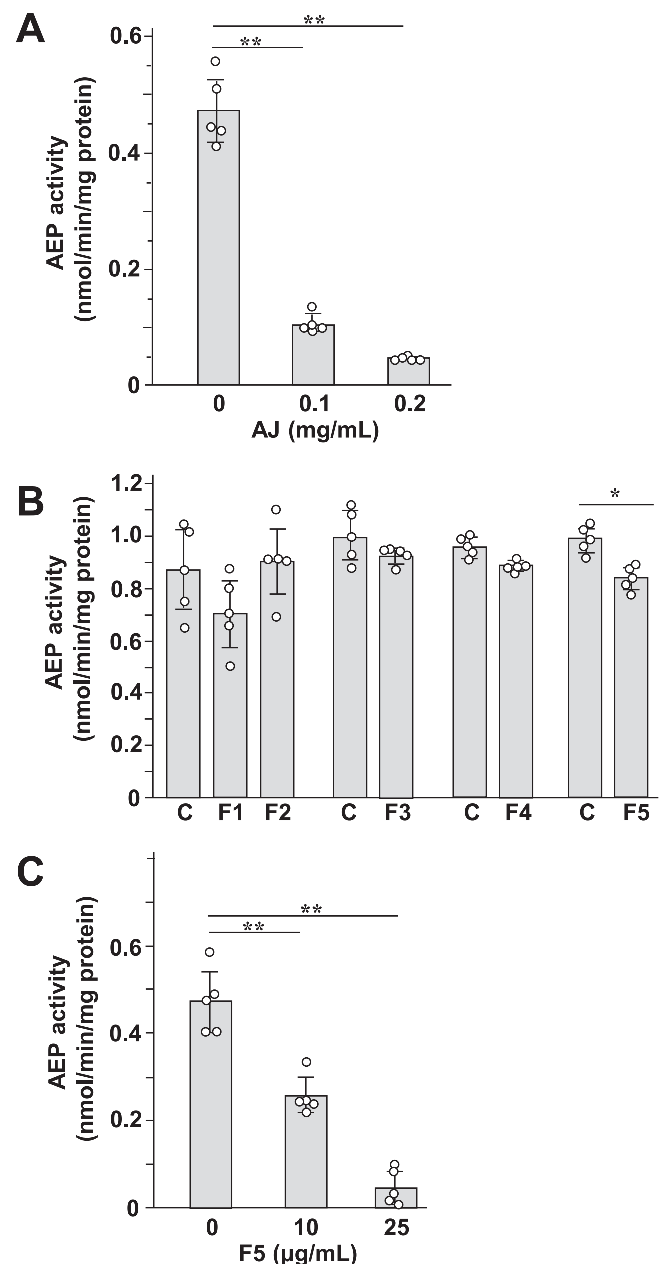

Fig. 4. Effects of aronia juice (A), fractions obtained from the juice (B), and fraction F5 (C) on AEP activities of SH-SY5Y cells. SH-SY5Y cells were cultured for 2 days in the absence and the presence of aronia juice and each fraction, and then the AEP activity of the cells lysates was measured. $n = 5$, $*p < 0.05$, $**p < 0.01$.

significantly impaired at 3 months of age compared to wild type mice, and AJ intake ameliorated the deficits. The deposition of Aβ plaques in 5XFAD mouse brain was apparent at 3 months of age, and AJ intake alleviated the deposition. Because it has been revealed that AEP acts as δ-secretase in the brain to intensify Aβ production [20], we examined the AEP activities in the mouse brain homogenates. Importantly, AJ intake reduced the AEP activities in 5XFAD mice brains. Effects of various natural compounds on the disease progression in 5XFAD mice have been investigated. For example, intraperitoneal injection of carboxy-dehydroevodiamine 5 times a week over a period from 4 months to 6 months of age reduces AD-related pathologies and improves memory deficits in 5XFAD mice [25]. Free drinking of water containing phloroglucinol over a period from 4 months to 6 months of age reduces the Aβ peptides burden and pro-inflammatory cytokines in the

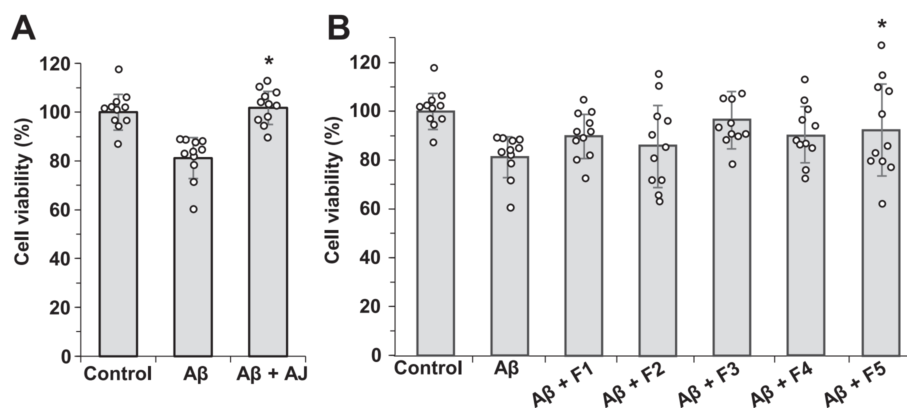

Fig. 5. Effects of aronia juice (A) and its fractions (B) on SH-SY5Y cell death induced by $A\beta_{25-35}$ peptide. $n = 11$. $^{*}p < 0.05$. (A) The cells were incubated with $A\beta_{25-35}$ peptides (25 μM) in the absence (Aβ) and the presence (Aβ + AJ) of 2.5 mg/mL AJ. (B) The cells were incubated with $A\beta_{25-35}$ peptides (25 μM) in the presence of each fraction (2.5 μg/mL).

hippocampus of 5XFAD mice [26]. Administration of diet containing *Centella asiatica* extracts for 4.5 weeks reverses cognitive deficits in 5XFAD mice [27]. Unfortunately, AEP activity was not examined in these studies.

In the present study, we focused on inhibitory effects of AJ on AEP activity (δ-secretase). Fig. 7 shows the partial amino acid sequence of human APP with three familial AD mutations overexpressed in 5XFAD mice and the main cleavage sites of α-, β-, γ- and δ-secretases. As inhibition of β- and γ-secretases or activation of α-secretase can lead to reduce the formation of the $A\beta_{42}$, various natural compounds have been tested to inhibit or activate these secretases [28–36]. Interestingly, Descamps et al. [31] found that rutin (quercetin 3-O-rutinoside) inhibits β-secretase activity, and that treatment of SH-SY5Y cells with rutin (1 μM) decreases the release of the cleaved peptide from APP. In addition, quercetin inhibits β-secretase activity (IC_{50} 25 μM) in vitro [32]. These results suggest possibility that AJ and fraction F5 not only inhibit δ-secretase but also inhibit β-secretase.

γ-Secretase cleaves the transmembrane domain of APP (Fig. 7). Hitherto, no polyphenol is known to directly inhibit γ-secretase activity. Recently, it is proposed that the presence of cholesterol in the membrane is important for substrate recognition by γ-secretase [33]. As polyphenols reduce cholesterol levels in cells [34], it may be possible that AJ inhibits γ-secretase activity indirectly through reducing the cholesterol content in the cell membrane. As for activation of α-secretase (ADAM10), Zhang et al. found that baicalein (5,6,7-trihydroxyflavone) enhances the α-processing of APP by α-secretase through activation of $GABA_A$ receptor signaling [35]. As recently reviewed [36], it is suggested that epigallocatechin-3-gallate, a green tea polyphenol, favors α-secretase processing of APP. Further study is needed to examine effects of AJ on α-, β-, and γ- secretases.

Recently, it is found that C/EBPβ, an inflammation-regulated transcription factor, regulates δ-secretase mRNA and protein levels [37]. Depletion of C/EBPβ from 5XFAD mice diminishes δ-secretase and reduces AD pathologies. In addition, it is found that δ-secretase cleaves BACE1, a rate-limiting aspartyl protease in Aβ generation, to enhance its enzymatic activity, facilitating senile plaques deposit in AD [38]. Importantly, it is demonstrated that gut inflammation increases AEP activity in the colon of a transgenic AD mouse (3XTg AD) in a C/EBPβ dependent manner and triggers gut-to-brain propagation of Aβ and tau fibrils in AD [39]. There is a vicious cycle in which Aβ deposits induce inflammation, inflammation increases active C/EBPβ, C/EBPβ increases active δ-secretase, δ-secretase increases Aβ production, a δ-secretase-truncated APP fragment (C585–695) activates C/EBPβ [40]. In this vicious cycle, δ-secretase plays a key role. In fact, administration of δ-secretase inhibitor, inhibitor 11 [41], via oral gavage for 3 months decreases the brain AEP activity, reduces the generation of Aβ, and ameliorates memory loss in the senescence-accelerated mouse (SAMP8) [42]. Because AJ contains various kinds of polyphenols with strong antioxidant activities, the reduction of the brain AEP activity by drinking AJ may partly due to suppression of inflammation caused by oxidative stress, reducing active C/EBPβ. However, out of the five AJ fractions, only F5 suppressed the AEP activity of SH-SY5Y cells (Fig. 4), suggesting the possibility of direct inhibition of AEP by AJ.

LC-MS/MS analysis of F5 suggested that F5 contains rutin (quercetin-3-*O*-rutinoside), hyperoside (quercetin-3-*O*-galactoside) and isoquercetin (quercetin-3-*O*-glucoside) (Fig. 6). Interestingly, beneficial effects of quercetin and these three quercetin glycosides on AD model animas have been reported. Intraperitoneal administration of quercetin for 3 months on aged transgenic AD model mice (3XTg-AD) ameliorates β-amyloidosis and improves spatial learning and memory [43]. Rutin attenuates Aβ42-induced cytotoxicity in SH-SY5Ycells [44]. Anti-amyloidgenic and fibril-disaggregating effects of quercetin and rutin are also demonstrated in APP695-transfected SH-SY5Y cells [45]. Intake of hyperoside mixed diet for 9 months improves spatial learning and memory in APP/PS1 transgenic mice [46]. Intracerebroventricularly administration of isoquercetin prevents the learning and memory impairment in colchicine-induced AD model rats [47].

Generally, it is proposed that dietary flavonol glycosides are first enzymatically hydrolyzed to liberate the aglycone, and then the aglycone enters into intestinal epithelial cells [48]. In the case of quercetin 3-O-glucoside, quercetin is adsorbed. Importantly, in plasma, quercetin 3-O-glucuronide is detected, but quercetin cannot be detected, indicating that quercetin is conjugated with glucuronic acid in the liver before entering into systemic circulation [49]. Quercetin 3-O-glucuronide is reported to go through the blood-brain barrier (BBB) [50], whereas the polar quercetin 3-O-glucuronide is expected to be less permeable to BBB [51]. It is needed to examine effects of quercetin 3-O-glucuronide on SH-SY5Y cells.

These previous studies suggest that the beneficial effects of AJ on 5XFAD mice are at least partly due to quercetin glycosides in AJ. It is important to examine whether quercetin glycosides inhibit δ-secretase activity in vivo and in vitro AD models.

5. Conclusions

In the present study, the preventive effect of AJ was observed in an animal model of Alzheimer's disease. However, since AJ contains many polyphenols, the beneficial effects of AJ are at least partially due to the quercetin glycosides in AJ. Future studies should investigate whether quercetin glycosides inhibit the activity of δ-secretase in vivo and in vitro AD models. AJ is expected to be widely recognized as a functional food and a nutraceutical through the accumulation of clinical trial results using humans and research data using model animals as in this study.

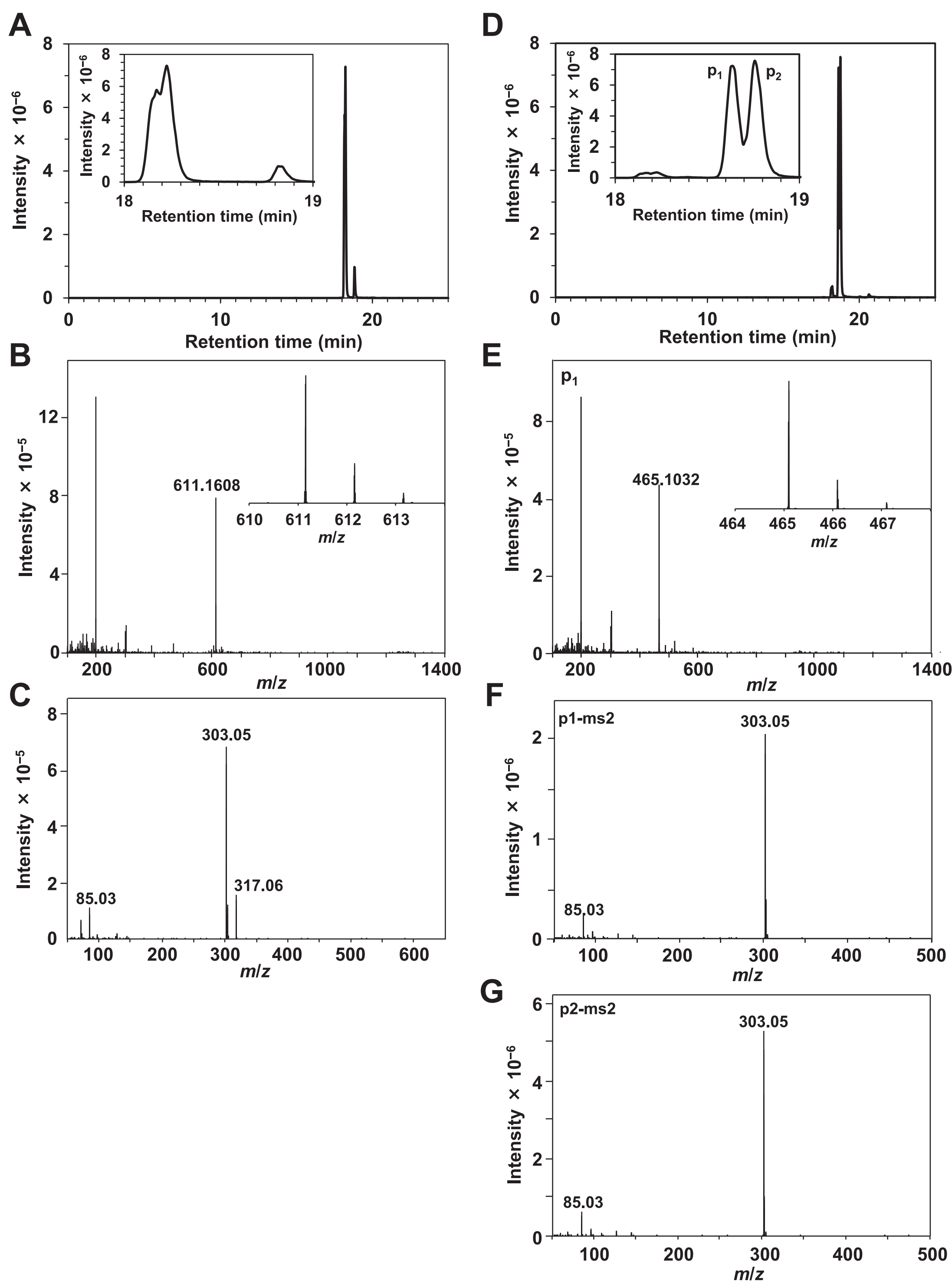

Fig. 6. LC-MS/MS analysis of F5. (A) Extracted chromatogram of the ion with molecular mass of 611.16 Da. (B) Mass spectrum of the ion detected in (A). (C) Mass spectrum of the ions produced from the ion shown in (B) by CID. (D) Extracted chromatogram of the two ions with the same molecular mass of 465.10 Da. (E) Mass spectrum of P_1 ion. (F) Mass spectrum of the ions produced from P_1 by CID. (G) Mass spectrum of the ions produced from P_2 by CID.

Ethical statement

All animal experiments were carried out following the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all of the protocols were approved by the Committee for Animal Research at Osaka Prefecture University (permit number 19-112).

CRediT authorship contribution statement

Takuya Yamane: Conceptualization, Methodology, Investigation, Writing – original draft. **Momoko Imai:** Investigation. **Satoshi Handa:** Investigation. **Hideo Ihara:** Supervision. **Tatsuji Sakamoto:** Supervision. **Tetsuo Ishida:** Writing – review & editing. **Takenori Nakagaki:** Resources. **Susumu Uchiyama:** Supervision.

```
 KNLPKADKKAVIQHFQEKVESLEQEAANERQQLVETHMARVEAMLNDRRRLALENYITALQAVPPRPRHV
421
 FNMLKKYVRAEQKDRQHTLKHFEHVRMVDPKKAAQIRSQVMTHLRVIYERMNQSLSLLYNVPAVAEEIQD
 EVDELLQKEQNYSDDVLANMISEPRISYGNDALMPSLTETKTTVELLPVNGEFSLDDLQPWHSFGADSVP
 ANTENEVEPVDARPAADRGLTTRPGSGLTNIKTEEISEVNLDAEFRHDSGYEVHHQKLVFFAEDVGSNKG
 AIIGLMVGGVVIATVVIITLVMLKKKQYTSIHHGVVEVDAAVTPEERHLSKMQQNGYENPTYKFFEQMQN
                                                                      770
```

Fig. 7. Partial amino acid sequence of the human APP with mutations of K670N, M671L, I716V, and V717I (shown in red letters) overexpressed in 5XFAD mice. The APP transmembrane sequence is highlighted by box. The black and blue bars indicate $A\beta_{42}$, and $A\beta_{25-35}$, respectively. Black arrows indicate the cleavage sites of δ-secretase. Red, blue, and yellow arrows indicate the cleavage site(s) of β-secretase, α-secretase, and γ-secretase, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Not applicable.

Appendix A. Supplementary data

Age-dependent changes in body weight (Fig. S1), BF-188 staining of the mouse brains (Fig. S2), Viability of SH-SY5Y cells in the presence of AJ and fractions of F1-F5 (Fig. S3).

References

[1] Alzheimer''s_Association, Alzheimer's Association 2020 Alzheimer's and Dementia, Available online at, https://www.alz.org/alzheimer_s_dementia, 2020.

[2] M. Singh, M. Arseneault, T. Sanderson, V. Murthy, C. Ramassamy, Challenges for research on polyphenols from foods in Alzheimer's disease: bioavailability, metabolism, and cellular and molecular mechanisms, J. Agric. Food Chem. 56 (2008) 4855–4873.

[3] S.H. Omar, C.J. Scott, A.S. Hamlin, H.K. Obied, The protective role of plant biophenols in mechanisms of Alzheimer's disease, J. Nutr. Biochem. 47 (2017) 1–20.

[4] R.E. Hartman, A. Shah, A.M. Fagan, K.E. Schwetye, M. Parsadanian, R. N. Schulman, M.B. Finn, D.M. Holtzman, Pomegranate juice decreases amyloid load and improves behavior in a mouse model of Alzheimer's disease, Neurobiol. Dis. 24 (2006) 506–515.

[5] J.A. Joseph, N.A. Denisova, G. Arendash, M. Gordon, D. Diamond, B. Shukitt-Hale, D. Morgan, Blueberry supplementation enhances signaling and prevents behavioral deficits in an Alzheimer disease model, Nutr. Neurosci. 6 (2003) 153–162.

[6] A. Sidor, A. Gramza-Michałowska, Black chokeberry *Aronia melanocarpa* L.—A qualitative composition, phenolic profile and antioxidant potential, Molecules 24 (2019) 3710.

[7] M. Kozuka, T. Yamane, Y. Nakano, T. Nakagaki, I. Ohkubo, H. Ariga, Identification and characterization of a dipeptidyl peptidase IV inhibitor from aronia juice, Biochem. Biophys. Res. Commun. 465 (2015) 433–436.

[8] M. Imai, T. Yamane, M. Kozuka, S. Takenaka, T. Sakamoto, T. Ishida, T. Nakagaki, Y. Nakano, H. Inui, Caffeoylquinic acids from aronia juice inhibit both dipeptidyl peptidase IV and α-glucosidase activities, LWT Food Sci. Technol. 129 (2020), 109544.

[9] T. Yamane, M. Kozuka, D. Konda, Y. Nakano, T. Nakagaki, I. Ohkubo, H. Ariga, Improvement of blood glucose levels and obesity in mice given aronia juice by inhibition of dipeptidyl peptidase IV and α-glucosidase, J. Nutr. Biochem. 31 (2016) 106–112.

[10] T. Yamane, M. Kozuka, M. Wada-Yoneta, T. Sakamoto, T. Nakagaki, Y. Nakano, I. Ohkubo, Aronia juice suppresses the elevation of postprandial blood glucose levels in adult healthy Japanese, Clin. Nutr. Exp. 12 (2017) 20–26.

[11] T. Yamane, M. Imai, S. Handa, K. Yamada, T. Sakamoto, T. Ishida, H. Inui, Y. Yamamoto, T. Nakagaki, Y. Nakano, Reduction of blood glucose and HbA1c levels by cyanidin 3,5-diglucoside in KKAy mice, J. Funct. Foods 58 (2019) 21–26.

[12] M. Kozuka, T. Yamane, M. Imai, S. Handa, S. Takenaka, T. Sakamoto, T. Ishida, H. Inui, Y. Yamamoto, T. Nakagaki, Y. Nakano, Isolation of HMG-CoA reductase inhibitors from aronia juice, Food Biosci. 34 (2020), 100535.

[13] T. Yamane, M. Imai, S. Handa, N. Harada, R. Yamaji, T. Sakamoto, T. Ishida, H. Inui, T. Nakagaki, Y. Nakano, Aronia juice supplementation inhibits lipid accumulation in both normal and obesity model mice, PharmaNutrition 14 (2020), 100223.

[14] H. Wen, H. Tian, C. Liu, X. Zhang, Y. Peng, X. Yang, F. Chen, J. Li, Metformin and cyanidin 3-O-galactoside from *Aronia melanocarpa* synergistically alleviate cognitive impairment in SAMP8 mice, Food Funct. 12 (2021) 10994–11008.

[15] Z. Fan, H. Wen, X. Zhang, J. Li, J. Zang, Cyanidin 3-O-β-Galactoside alleviated cognitive impairment in mice by regulating brain energy metabolism during aging, J. Agric. Food Chem. 70 (2022) 1111–1121.

[16] J.E. Morley, H.J. Armbrecht, S.A. Farr, V.B. Kumar, The senescence accelerated mouse (SAMP8) as a model for oxidative stress and Alzheimer's disease, Biochim. Biophys. Acta 2012 (1822) 650–656.

[17] E.M. Rhea, W. Banks, The SAMP8 mouse for investigating memory and the role of insulin in the brain, Exp. Gerontol. 94 (2017) 64–68.

[18] H. Oakley, S.L. Cole, S. Logan, E. Maus, P. Shao, J. Craft, A. Guillozet-Bongaarts, M. Ohno, J. Disterhoft, L. Van Eldik, R. Berry, R. Vassar, Intraneuronal β-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation, J. Neurosci. 26 (2006) 10129–10140.

[19] A.L. Oblak, P.B. Lin, K.P. Kotredes, R.S. Pandey, D. Garceau, H.M. Williams, A. Uyar, R. O'Rourke, S. O'Rourke, C. Ingraham, D. Bednarczyk, M. Belanger, Z. A. Cope, G.J. Little, S.-P.G. Williams, C. Ash, A. Bleckert, T. Ragan, B.A. Logsdon, L. M. Mangravite, S.J.S. Rizzo, P.R. Territo, G.W. Carter, G.R. Howell, M. Sasner, B. T. Lamb, Comprehensive evaluation of the 5XFAD mouse model for preclinical testing applications: a MODEL-AD study, Front. Aging Neurosci. 13 (2021), 713726.

[20] Z. Zhang, M. Song, X. Liu, S.S. Kang, D.M. Duong, N.T. Seyfried, X. Cao, L. Cheng, Y.E. Sun, P.S. Yu, J. Jia, A.I. Levey, K. Ye, Delta-secretase cleaves amyloid precursor protein and regulates the pathogenesis in Alzheimer's disease, Nat. Commun. 6 (2015) 8762.

[21] R. Harada, N. Okamura, S. Furumoto, T. Yoshikawa, H. Arai, K. Yanai, Y. Kudo, Use of a benzimidazole derivative BF-188 in fluorescence multispectral imaging for selective visualization of tau protein fibrils in the Alzheimer's disease brain, Mol. Imaging Biol. 16 (2014) 19–27.

[22] L. Millucci, R. Raggiaschi, D. Franceschini, G. Tresstappen, A. Santucci, Rapid aggregation and assembly in aqueous solution of Aβ(25-35) peptide, J. Biosci. 34 (2009) 293–303.

[23] V. D'Ezio, M. Colasanti, T. Persichini, Amyloid-β25-35 induces neurotoxicity through the up-regulation of astrocytic system xc−, Antioxidants 10 (2021) 1685.

[24] Z. Zhang, Y. Tian, K. Ye, δ-Secretase in neurodegenerative diseases: mechanisms, regulators and therapeutic opportunities, Transl. Neurodegener. 9 (2020) 1.

[25] S. Kan, S. Ha, H. Park, E. Nam, W.H. Suh, Y.H. Suh, K.A. Chang, Effects of a dehydroevodiamine-derivative on synaptic destabilization and memory impairment in the 5xFAD, Alzheimer's disease mouse model, Front. Behav. Neurosci. 12 (2018) 273.

[26] E.J. Yang, U. Mahmood, H. Kim, M. Choi, Y. Choi, J.P. Lee, J.Y. Cho, J.W. Hyun, Y. S. Kim, M.J. Chang, H.S. Kim, Phloroglucinol ameliorates cognitive impairments by reducing the amyloid β peptide burden and pro-inflammatory cytokines in the hippocampus of 5XFAD mice, Free Radic. Biol. Med. 126 (2018) 221–234.

[27] D.G. Mattews, M. Caruso, A.A. Magana, K.M. Wright, C.S. Maier, J.F. Stevens, N. E. Gray, J.F. Quinn, A. Soumyanath, Caffeoylquinic acids in *Centella asiatica* reverse cognitive deficits in male 5XFAD Alzheimer's disease model mice, Nutrients 12 (2020) 3488.

[28] F.I. Baptista, A.G. Henriques, A.M.S. Silva, J. Wiltfang, O.A.B. da Cruz e Silva, Flavonoids as therapeutic compounds targeting key proteins involved in Alzheimer's disease, ACS Chem. Neurosci. 5 (2014) 83–92.

[29] M. Naushad, S.S.K. Durairajan, A.K. Bera, S. Senapati, M. Li, Natural compounds with anti-BACE1 activity as promising therapeutic drugs for treating Alzheimer's disease, Planta Med. 85 (2019) 1316–1325.

[30] K. Youn, Y. Yu, J. Lee, W.S. Jeong, C.T. Ho, M. Jun, Polymethoxyflavones: novel β-secretase (BACE1) inhibitors from citrus peels, Nutrients 9 (2017) 1–12.

[31] O. Descamps, P. Spilman, Q. Zhang, C.P. Libeu, K. Poksay, O. Gorostiza, J. Campagna, B. Jagodzinska, D.E. Bredesen, V. John, AβPP-selective BACE inhibitors (ASBI): novel class of therapeutic agents for Alzheimer's disease, J. Alzheimers Dis. 37 (2013) 343–355.

[32] K. Zhumanova, G. Lee, A. Baiseitova, A.B. Shah, J.H. Kim, J.Y. Kim, K.W. Lee, K. H. Park, Inhibitory mechanism of O-methylated quercetins, highly potent β-secretase inhibitors isolated from Caragana balchaschensis (Kom.), Pojark. J Ethnopharmacol. 272 (2021), 113935.

[33] Ł. Nierzwicki, M. Olewniczak, P. Chodnicki, J. Czub, Role of cholesterol in substrate recognition by γ-secretase, Sci. Rep. 11 (2021) 15213.

[34] S. Dhakal, N. Kushairi, C.W. Phan, B. Adhikari, V. Sabaratnam, I. Macreadie, Dietary polyphenols: a multifactorial strategy to target Alzheimer's disease, Int. J. Mol. Sci. 20 (2019) 5090.

[35] S.-Q. Zhang, D. Obregon, J. Ehrhart, J. Deng, J. Tian, H. Hou, B. Giunta, D. Sawmiller, J. Tan, Baicalein reduces β-amyloid and promotes nonamyloidogenic

amyloid protein processing in an Alzheimer's disease transgenic mouse model, J. Neurosci. Res. 91 (2013) 1239–1246.
[36] P.R. Manzine, M. Ettcheto, A. Cano, O. Busquets, E. Marcello, S. Pelucchi, M. Di Luca, K. Endres, J. Olloquequi, A. Camins, M.R. Cominetti, ADAM10 in Alzheimer's disease: pharmacological modulation by natural compounds and its role as a peripheral marker, Biomed. Pharmacother. 113 (2019), 108661.
[37] Z.H. Wang, K. Gong, X. Liu, Z. Zhang, X. Sun, Z.Z. Wei, S.P. Yu, F.P. Manfredsson, I. M. Sandoval, P.F. Johnson, J. Jia, J.X. Wang, K. Ye, C/EBPβ regulates delta-secretase expression and mediates pathogenesis in mouse models of Alzheimer's disease, Nat. Commun. 9 (2018) 1784.
[38] Y. Xia, Z.H. Wang, Z. Zhang, Z.X. Liu, S.P. Yu, J.Z. Wang, X.C. Wang, K. Ye, Delta- and beta- secretases crosstalk amplifies the amyloidgenic pathway in Alzheimer's disease, Prog. Neurobiol. 204 (2021), 102113.
[39] C. Chen, Y. Zhou, H. Wang, A. Alam, S.S. Kang, E.H. Ahn, X. Liu, J. Jia, K. Ye, Gut inflammation triggers C/EBPβ/δ-secretase-dependent gut-to-brain propagation of Aβ and tau fibrils in Alzheimer's disease, EMBO J. 40 (2021), e106320.
[40] Y. Yao, S.S. Kang, Y. Xia, Z.H. Wang, X. Liu, T. Muller, Y.E. Sun, K.A. Ye, delta-secretase-truncated APP fragment activates CEBPB, mediating Alzheimer's disease pathologies, Brain 144 (2021) 1833–1852.
[41] Z. Zhang, O. Obianyo, E. Dall, Y. Du, H. Fu, X. Liu, S.S. Kang, M. Song, S.P. Yu, C. Cabrele, M. Schubert, X. Li, J.Z. Wang, H. Brandstetter, K. Ye, Inhibition of delta-secretase improves cognitive functions in mouse models of Alzheimer's disease, Nat. Commun. 8 (2017) 14740.
[42] J. Wang, H.J. Hu, Z.K. Liu, S.S. Wang, Q. Cheng, H.Z. Chen, M. Song, Pharmacological inhibition of asparaginyl endopeptidase by δ-secretase inhibitor 11 mitigates Alzheimer's disease-related pathologies in a senescence-accelerated mouse model, Transl. Neurodegener. 10 (2021) 12.
[43] A.M. Sabogal-Guáqueta, J.I. Muñoz-Manco, J.R. Ramírez-Pineda, M. Lamprea-Rodriguez, E. Osorio, G.P. Cardona-Gómez, The flavonoid quercetin ameliorates Alzheimer's disease pathology and protects cognitive and emotional function in aged triple transgenic Alzheimer's disease model mice, Neuropharmacol. 93 (2015) 134–145.
[44] S.W. Wang, Y.J. Wang, Y.J. Su, W.W. Zhou, S.G. Yang, R. Zhang, M. Zhao, Y.N. Li, Z.P. Zhang, D.W. Zhan, R.T. Liu, Rutin inhibits β-amyloid aggregation and cytotoxicity, attenuates oxidative stress, and decreases the production of nitric oxide and proinflammatory cytokines, Neurotoxicol. 23 (2012) 482–490.
[45] K. Jiménez-Aliaga, P. Bermejo-Bescós, J. Benedí, S. Martín-Aragón, Quercetin and rutin exhibit antiamyloidogenic and fibril-disaggregating effects in vitro and potent antioxidant activity in APPswe cells, Life Sci. 89 (2011) 939–945.
[46] L. Chen, Y.P. Zhou, H.Y. Liu, J.H. Gu, X.F. Zhou, Z. Yue-Qin, Long-term oral administration of hyperoside ameliorates AD-related neuropathology and improves cognitive impairment in APP/PS1 transgenic mice, Neurochem. Int. 151 (2021), 105196.
[47] Q. Yang, Z. Kang, J. Zhang, F. Qu, B. Song, Neuroprotective effects of isoquercetin: an in vitro and in vivo study, Cell J. 23 (2021) 355–365.
[48] C. Manach, J.L. Donovan, Pharmacokinetics and metabolism of dietary flavonoids in humans, Free Radic. Res. 38 (2004) 771–785.
[49] A.J. Day, F. Mellon, D. Barron, G. Sarrazin, M.R. Morgan, G. Williamson, Human metabolism of dietary flavonoids: identification of plasma metabolites of quercetin, Free Radic. Res. 35 (2001) 941–952.
[50] A. Ishisaka, R. Mukai, J. Terao, N. Shibata, Y. Kawai, Specific localization of quercetin-3-O-glucuronide in human brain, Arch. Biochem. Biophys. 557 (2014) 11–17.
[51] K.A. Youdim, M.S. Dobbie, G. Kuhnle, A.R. Proteggente, N.J. Abbott, C. Rice-Evans, Interaction between flavonoids and the blood-brain barrier: in vitro studies, J. Neurochem. 85 (2003) 180–192.