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Deer antler extracts reduce amyloid-beta toxicity in a *Caenorhabditis elegans* model of Alzheimer's disease

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ABSTRACT

Ethnopharmacological relevance: Velvet antler extracts (VAE) are composed of a variety of active substances and growth factors, and have been reported to improve sleep quality and memory.
Aim of the study: We aimed to explore the protective effects and mechanism of action for VAE on Alzheimer's disease (AD) using a transgenic *Caenorhabditis elegans* model.
Materials and methods: C. elegans were cultivated at 40% relative humidity on solid nematode growth medium (NGM) containing live *E. coli* (OP50) as the food source, with Strain N2 (normal) held at 20 °C and the CL4176s (transgenic) held at 16 °C. AD-like aggregation of Aβ peptide in the CL4176s strain is induced by lifting the temperature to 25 °C. Nematodes were treated with three types of VAEs and Resveratrol (positive control). Analyses included qRT-PCR for quantification of gene transcripts of interest; ELISA for measuring levels of amyloid-β protein; Thioflavin T fluorescent staining for localizing Aβ depositions; assays for reactive oxygen species (ROS) and superoxide dismutase activity (SOD).
Results: VAEs reduced β-amyloid peptide (Aβ) toxicity in the transgenic *C. elegans* model. An enzymatically-digested VAE (EDVAE) was superior to both a cold-water VAE (CWVAE) and a hot-water VAE (HWVAE) from the same velvet antler. EDVAE treatment reduced the severity of the Aβ-induced paralysis phenotype and decreased the amount of Aβ deposits in the AD model nematodes, and these effects were found to be significantly better than that of the positive control Resveratrol. In addition, EDVAE treatment reduced production of ROS (induced by Aβ), enhanced SOD activity, and elevated expression levels of antioxidant-related transcription factors, although it is not known whether these effects were achieved directly or indirectly.
Conclusion: EDVAE had a protective role in Aβ-induced toxicity in the transgenic AD nematodes, possibly through reducing accumulation of toxic Aβ and enhancing the ability of nematodes to resist oxidative stress. Thus, EDVAE has potential to be an effective treatment to relieve the symptoms of AD.

1. Introduction

Alzheimer's disease (AD) is the most prevalent of the dementias for which there are currently no effective therapies. The condition is progressive and significantly impacts on cognition, function, lifespan of sufferers, and society healthcare costs (Waite, 2015). With the aging population growing rapidly worldwide, the incidence of AD is increasing, and AD has become one of the leading causes of disability and death in the elderly (Gaugler et al., 2016). Although major efforts have been invested to develop effective drugs for treating AD, the developed drugs thus far can only relieve symptoms at best, but as yet, cannot provide a cure (Awasthi et al., 2016; Rezaee et al., 2021).

AD is characterized by progressive impairment of memory and cognition, and this symptom is associated with neuronal death, deposition of amyloid-β (Aβ) and intracellular neurofibrillary tangles (Abbas and Wink, 2010; Yang et al., 2016). Abnormal aggregation of Aβ in the brain is considered to be the leading cause of AD development (Armbrust et al., 2021). Much effort has focused on control of abnormal Aβ

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aggregation for the prevention and treatment of AD (Wolozin et al., 2011).

Although how the accumulation of Aβ product exactly affects the pathological progression of AD is unknown, it is now widely accepted that Aβ-aggregation increases oxidative stress and then causes neuron apoptosis/death (Yu et al., 2014; Zhang et al., 2017). Redox impairment occurs when there is an imbalance between the production and quenching of reactive oxygen/reactive nitrogen species (ROS/RNS) (Koopman et al., 2010). ROS enhances the formation and aggregation of Aβ and Tau hyperphosphorylation, and in turn, the accumulated Aβ and hyperphosphorylated Tau cause an increase in oxidative stress. Preventing the deposition of Aβ oligomers by reduction of oxidative stress or activation of disease modifying pathways could reduce the incidence of AD (Butterfield et al., 2007, Lemere and Masliah, 2010).

The AD transgenic *Caenorhabditis elegans* (*C. elegans*; CL4176 strain) has become a very popular model to investigate environmentally-induced neurotoxicity and neurodegeneration (Jia et al., 2021) and has contributed to understanding the pathogenesis of mammalian AD (Griffin et al., 2017, Zhang et al., 2017). In the CL4176s model, expression of $A\beta_{1\text{-}42}$ peptide can be induced in the muscle cells by elevating the temperature to 25 °C, which causes deposition of Aβ and eventually leads to an observable paralyzed phenotype/behavior of the CL4176s (Vassar et al., 1999; Wang et al., 2017).

A variety of substances have an inhibitory effect on Aβ aggregation such as the antibody aducanumab, which can reduce Aβ plaques in the brain (Sevigny et al., 2016), and non-peptidic drugs (synthetic derivatives of licochalcone A and coumarin), which can inhibit Aβ aggregation (Lee et al., 2018). Certain natural products also show anti-Aβ effects, e.g. *Panax Notoginseng* Saponins (Zhou et al., 2019a,b), *Oroxylum indicum* extract (Mairuae et al., 2019), Adzuki bean (*Vigna angularis*) extract (Miyazaki et al., 2019), and Pasteurized Orange Juice (Caland et al., 2019).

Velvet antler, an animal-derived traditional medicine, has been widely used in China for more than 2,000 years (Zhigang et al., 2014). Velvet antlers are removed from the male sika deer or wapiti during their growth phase in the late spring and summer period (Li et al., 2009), and are often used for the enhancement of immune function and slowing down the aging process (Hong et al., 2004).

Studies have shown that velvet antler extracts (VAE) have significant effects on antioxidant (Hardy and Selkoe, 2002; Iwatsubo et al., 1994), anti-aging, anti-inflammation (Hardy and Selkoe. 2002; Kim et al., 2004), and anti-amnesic effects (Kim et al., 2014). It is reported that VAE can effectively induce differentiation of a human nerve progenitor cell line, SK-N-SH *in vitro*, and are more potent than retinoic acid and nerve growth factors (Li, 2012). Given that the rate of antler growth can exceed 2 cm/day including nerve fibers (Li, 2012), such a neuro-protective effect may not be surprising. Overall, these studies indicate that VAE has effects on promoting nerve growth and is neuroprotective, and thus may have potential to be developed as a compound for the treatment or prevention of neurodegenerative diseases such as AD.

In the present study, we used the *C. elegans* CL4176s model to study the protective effects of VAEs on the AD-like phenotype in this model. We found that VAEs effectively alleviated some of the symptoms of AD in the nematodes, possibly via reducing aggregation of Aβ and enhancing the ability to resist oxidative stress.

2. Materials and methods

This study includes a number of experiments, for easier reference we briefly listed each of them in Supplementary Table S1.

2.1. C. elegans and strain maintenance

C. elegans strains N2 (wild type) and CL4176s dvIs27 [pAF29(myo-3/A-Beta 1-42/let UTR) + pRF4(rol-6(su1006))] were obtained from the *C. elegans* Genetic Center, CGC (University of Minnesota, Minneapolis, MN, USA). All strains were maintained at 40% relative humidity on solid nematode growth medium containing live *E. coli* (OP50) as the food source, with Strain N2 held at 20 °C and the CL4176s held at 16 °C (Song et al., 2018).

2.2. Preparation of VAE

Three whole traditionally-dried two-branched velvet antlers (weighing 181, 201 and 208 g) from sika deer (*Cervus nippon*) were purchased from the local commercial market, sliced, and then ground to powder (Inversion discharge type vibration mill, JNNAN BILLION, China). Three methods were used to prepare velvet antler extracts, namely enzymatically-digested extract (EDVAE), cold water extract (CWVAE), and hot water extract (HWVAE). For detailed extraction procedures, refer to the Supplementary Methods.

Three different concentrations were selected for each type of VAE: 0.1, 1.0 and 10.0 mg/mL (Min et al., 2016); double-distilled pure water (ddH2O) was used as the negative control (N control) for each extraction; and resveratrol (Res) used as the positive control with the concentration of 22.8 μg/mL (best effective dosage) based on a previous report (Regitz et al., 2016).

2.3. High-performance liquid chromatography (HPLC) analysis

Cytochrome C (MW 12500), antibacterial peptide (MW 6512), bacitracin (MW 1423), Gly-Gly-Arg-Tyr (MW 451) and Gly-Gly-Gly ($C_6H_{11}N_3O_4$, MW 189) (Shanghai Ziqibio Co., Ltd, Shanghai, China) were used as the standards for the relative molecular mass calibration. Each protein standard above was made up to 1 mg/mL in the mobile phase solution and filtered through 0.45 μm membrane filter (Millipore, Milford, MA).

HPLC (TSKgel G2000 SWXL 300 mm × 7.8 mm (GEL LOT 502R)) was used for the separation of the compounds of these VAEs. For detailed fractionation procedures refer to the Supplementary Methods.

2.4. Synchronization of nematodes

Mature CL4176s (20 per treatment) were allocated to each of five treatments: N control, EDVAE, CWVAE, HWVAE and Res in the sample-pretreated 60-mm-dishes. The Mature CL4176s were removed 2 h after dish cultivation. Eggs laid during this 2 h period were cultivated at 16 °C for 48 h to the L3 stage. N2 nematodes were synchronized following the same way for CL4176s, but their eggs were cultivated at 20 °C.

2.5. Paralysis assay

The experimental procedure for CL4176s paralysis was slightly modified from Song's method (Song et al., 2018). As described above, the eggs laid by the CL4176s in each dish were cultivated at 16 °C for 48 h to the L3 stage, and then the temperature was increased to 25 °C to induce $A\beta_{1\text{-}42}$ expression in their body wall muscle cells. CL4176s were observed at 4 h intervals until all CL4176s were found to become paralyzed. The criteria used to judge paralysis were: the failure to roll-over in response to a touching stimulus with a platinum loop, or they retain the ability to move their heads only (determined by the phenomenon that the cultivation surface formed a bacteria-free fan-shaped blank area; (Young-Il et al., 2014). The assay was repeated three times and there were at least 80 nematodes used per treatment.

Based on results from the paralysis assay, 10 mg/ml EDVAE had the best therapeutic effect and it was used for the following experiments.

2.6. Lifespan assay

The lifespan assay was performed using N2 (wildtype) as previously described (Dulovic et al., 2016) with slight modifications for nematodes

Table 1
Timing (h) recorded when 50% and 90% of nematodes exhibited paralysis.

Extract type	Concentration	50% paralysis (mean ± s.d.)	90% paralysis (mean ± s.d.)	P value	the number of nematodes
Ctrl		51.43±0.73	55.75±0.47		≥80
Resveratrol	22.8 μg/mL	53.50±0.87	60.73±1.96	<0.001	≥80
EDVAE	0.1 mg/mL	52.90±0.57	58.00±1.13	<0.001	≥80
	1 mg/mL	53.60 ±0.85	60.85±0.21	<0.001	≥80
	10 mg/mL	56.25±0.35	65.00±0.28	<0.001	≥80
	Dose effect	P<0.05	P<0.001		
CWVAE	0.1 mg/mL	51.60±0.57	57.80±0.28	>0.05	≥80
	1 mg/mL	55.15±0.92	59.60±0.42	<0.05	≥80
	10 mg/mL	54.30±0.71	59.95±1.34	>0.05	≥80
	Dose effect	NS	NS		
HWVAE	0.1 mg/mL	52.75±1.06	58.75±0.64	>0.05	≥80
	1 mg/mL	55.25±0.78	61.25±0.64	<0.001	≥80
	10 mg/mL	54.80±0.28	61.00±0.57	<0.05	≥80
	Dose effect	NS	NS		

being maintained at 20 °C. Nematodes from the N control, 10 mg/ml EDVAE or Res pre-treated dishes were synchronized. When hermaphrodites reached the L4 stage, they were transferred to fresh sample-pretreated dishes with dishes changed daily until the time the nematodes stopped producing eggs. Nematodes were defined as dead when they did not move in response to a touching stimulus. A nematode was excluded from the assay if it was missing, had burrowed, crawled off the dish, displayed internal hatching, or vulval protrusion. Each lifespan assay was proceeded independently at 20 °C and repeated at least three times. At least 80 nematodes were used per treatment.

2.7. Brood size assay

The brood size assay was performed as previously described with slight modifications (Dulovic et al., 2016). Briefly, N2 nematodes from the three allocated groups (N control, 10 mg/ml EDVAE and Res) were synchronized as described above. Each group had 10 nematodes and each hermaphrodite was transferred to the fresh N control, EDVAE or Res pre-treated dishes at the L4 stage. This transfer was repeated at 24-h intervals until the time when nematodes no longer producing eggs. The brood size of each nematode was defined as the total number of hatched off-spring during the assay. Unhatched eggs were not counted, and the criteria for exclusion were as per the lifespan assay above. Each brood size assay was performed three times at 20 °C.

2.8. Movement assay

We divided nematode phenotypes/behaviors into four categories under stimulation as below. Type A: could move in a standard sinusoidal manner; Type B: could move in an irregular manner; Type C: could not move but their heads could still swing; and Type D: dead. Observations were carried out on days 6, 10, 14 and 18 following the synchronization. The criteria for exclusion were as per the lifespan assay above.

2.9. Thioflavin T staining and ELISA of Aβ

Thioflavin T staining was performed to detect Aβ aggregates in the body wall muscle of the CL4176s nematodes as described previously (Song et al., 2018). At 48 h after the temperature shift from 16 °C to 25 °C, all nematodes on the N control, 10 mg/ml EDVAE or Res pre-treated dishes were collected for Thioflavin T staining assay. Detailed protocol for this staining is provided in the Supplementary Methods.

For the Aβ ELISA assay, nematodes were cultivated using the method similar to Thioflavin T staining assay. To measure the concentration of Aβ, total protein in CL4176s nematodes was extracted using sonication in RIPA Lysis Buffer (GenStar). Detailed protocol for this assay is provided in the Supplementary Methods.

2.10. Measurement of ROS levels

Measurement of ROS in the CL4176s was performed using the 2′,7-dichlorofluorescein diacetate (H2DCF-DA) method (Zhou et al., 2019a, b) with minor modifications. Briefly, synchronized nematodes on the N control, EDVAE or Res pre-treated dishes were cultivated at 16 °C to L3 stage, and then the temperature increased to 25 °C to induce $A\beta_{1\text{-}42}$ expression in their body wall muscle cells. The nematodes were collected at 48h after cultivation for the measurement of ROS. Detailed protocol for this measurement is provided in the Supplementary Methods.

2.11. Measurement of superoxide dismutase (SOD) activity

For this assay, the CL4176s were cultivated using a similar method to the ELISA assay:100 nematodes were collected from each treatment group and total protein of these nematodes was extracted using sonication in RIPA Lysis Buffer (GenStar). SOD activity was measured using a Total Superoxide Dismutase Assay Kit with WST-8 (Beyotime, Shanghai, China).

2.12. Real-time quantitative PCR (qRT-PCR)

For CL4176s cultivated under conditions leading to expression of Aβ protein species, the expression levels of Aβ transgene (*amy-1)*, stress induced transcription factor *(skn-1)*, heat shock factor protein 1 (*hsf-1*), and forkhead transcription factor (*daf-16*) were measured. Detailed procedure for RNA isolation and reverse transcription procedures are provided in the Supplementary Methods. The qRT-PCR primers are listed in Supplementary Table S1.

2.13. Statistical analysis

Statistical analysis was performed using Prism GraphPad (GraphPad Software Inc., San Diego, CA, USA). Data obtained from paralysis and lifespan for nematodes cultured in the absence or presence of EDVAE and Res were compared between groups using the two-tailed, unpaired Student's t-test. Data other than paralysis and lifespan were analyzed using one-way analysis of variance (ANOVA). Results were expressed as the mean ± standard deviation of three independent experiments. $p<0.05$ was taken as statistically significant.

3. Results

3.1. EDVAE significantly alleviated symptoms of paralysis induced by $A\beta_{1\text{-}42}$ in CL4176s

To evaluate the effects of different types of VAE on Aβ-induced toxicity, CL4176s were treated with different concentrations (0.1, 1.0

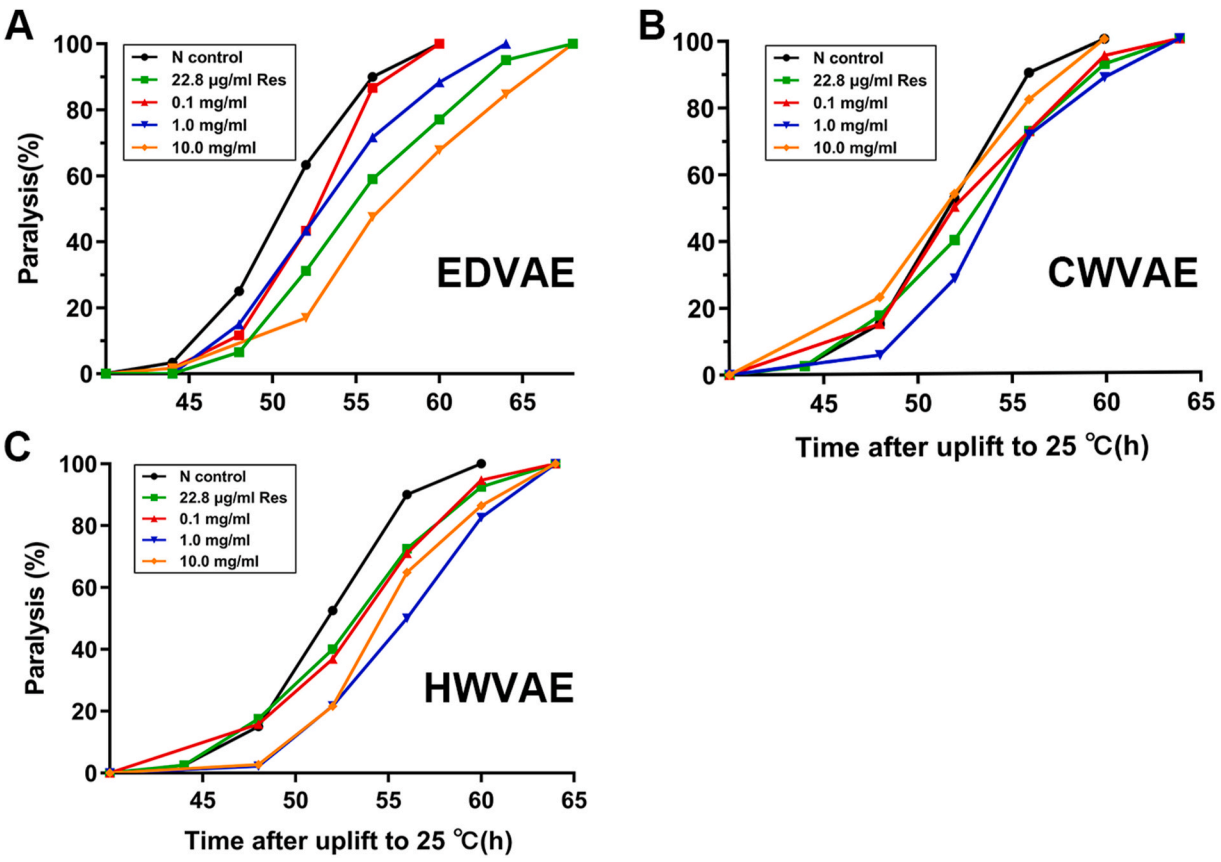

Fig. 1. Effects of the velvet antler extracts (VAEs; 0.1; 1.0; 10.0 mg/mL) on the rate of paralysis in CL4176s. Time represents hours after the temperature uplift to 25 °C. (A) EDVAE alleviated symptoms of paralysis induced by $A\beta_{1\text{-}42}$ in CL4176s (control vs. 0.1, 1.0, 10.0 mg/mL EDVAE and 22.8 μg/mL Res, $p<0.001$). (B) 1.0 mg/mL CWVAE alleviated symptoms of paralysis induced by Aβ1-42 in CL4176s (control vs. 0.1 and 10.0 mg/mL CWVAE, $p>0.05$; control vs. 1.0 mg/mL CWVAE and 22.8 μg/mL Res, $p<0.05$). (C) 1.0 mg/mL and 10.0 mg/mL HWVAE alleviated symptoms of paralysis induced by Aβ1-42 in CL4176s (control vs. 0.1 mg/mL HWVAE, $p>0.05$; control vs. 1.0 mg/mL HWVAE, $p<0.001$; control vs. 10.0 mg/mL HWVAE and 22.8 μg/mL Res, $p<0.05$).

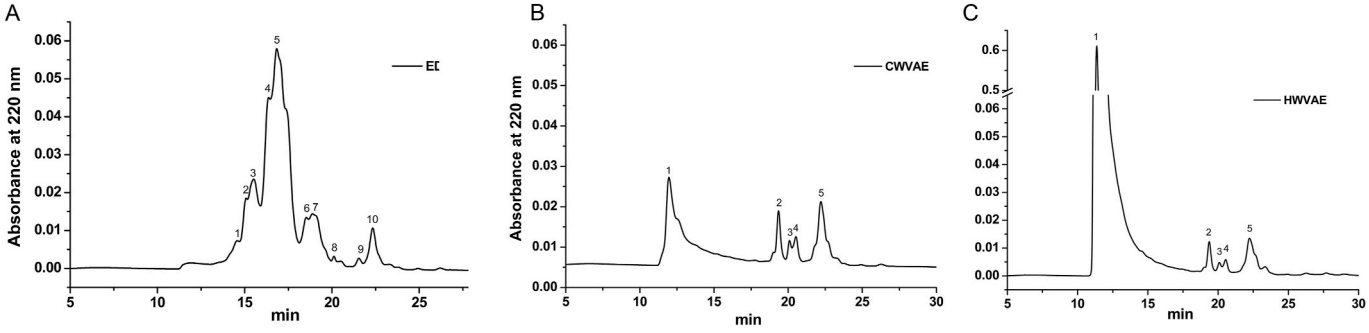

Fig. 2. HPLC analysis of EDVAE, CWVAE and HWVAE at 220 nm absorbance. (A) The chromatogram at 2 mg/mL EDVAE. (B) The chromatogram at 2 mg/mL CWVAE. (C) The chromatogram at 2 mg/mL HWVAE.

and 10.0 mg/mL) of the three types of extracts, respectively. We found that EDVAE significantly delayed the timing of initiation of paralysis, and the protective effects were in a dose-dependent manner (Table 1 and Fig. 1A). Furthermore, EDVAE at 10 mg/mL was found to be more effective than the positive control (Res at 22.8 μg/mL). The effects of CWVAE or HWVAE at certain concentrations were also found to have a significant effect on the Aβ-induced paralysis in CL4176s (Table 1 and Fig. 1B and C), but to a less extent than that of EDVAE (Fig. 1A).

Since EDVAE at 10.0 mg/mL had the greatest effect among the VAE groups, we further fractionated the components of EDVAE and compared this with the other VAEs using HPLC. A standard curve was established based on the retention time and molecular weight of the five

Table 2
Percentage of different molecular size in the VAEs through HPLC (2 mg/mL).

Molecular size distribution	EDVAE	CWVAE	HWVAE
	Solid content of the original extract (mg/mL)		
	97.0	20.0	15.6
>18,000 Da	0	59.2%	96.1%
2,500–4,000 Da	8.5%	0	0
1,000–2,500 Da	26.2%	0	0
350–1,000 Da	48.8%	0	0
131–350 Da	12.0%	10.7%	0.9%
60–130 Da	0.2%	8.8%	0.7%
<60 Da	4.3%	21.3%	2.3%

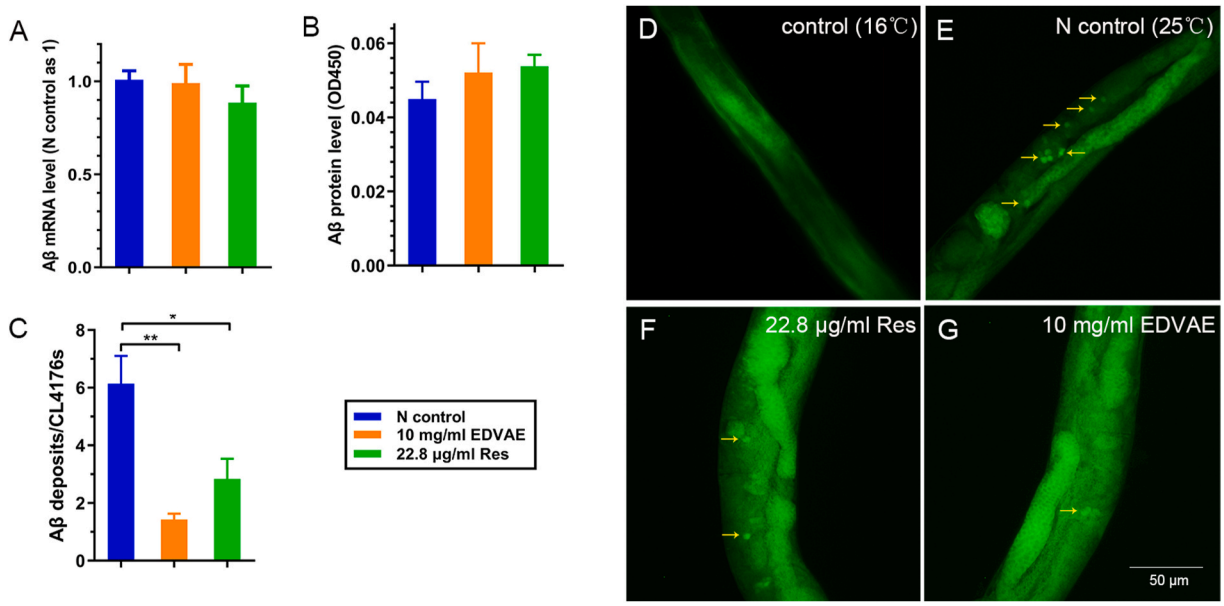

Fig. 3. Effects of EDVAE (10 mg/mL) treatment on Aβ expression and Aβ accumulation. Quantitative real-time PCR and ELISA were preformed using the CL4176s model 48 h after temperature uplift to 25 °C. The results showed that EDVAE had no significant effects on the expression of Aβ mRNA (A) and Aβ protein (B). The wall muscle in the mid of the bodies were focused in all photos of the nematodes, and the small arrows indicate the Aβ deposits. (C). The photomicrographs of thioflavin T staining (green) on nematodes from the different groups. Nematodes that were maintained at a permissive temperature (16 °C) were used as negative controls (C–G). Statistics of the number of Aβ deposits in nematodes from the different groups (10 nematodes/group) (*p<0.05, **p<0.01). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

used protein standards (Supplementary Figs. S1A and S1B). Components of the EDVAE, CWVAE and HWVAE, fractionated using HPLC, are shown in Fig. 2A, B and 2C respectively (with molecular weights calculated using the standard curve). We found that the EDVAE comprised mainly low molecular weight substances, whereas the CWVAE and the HWVAE contained much higher weight components (summarized in Table 2, with data in Tables S1–S3). These low molecular substances are likely to be amino acids and peptides, which had greater effects on the alleviation of the paralytic symptoms in the CL4176s.

3.2. EDVAE decreased Aβ deposition/aggregation in the CL4176s

As the 10 mg/mL EDVAE was the most effective dose, this dosage was selected for the further investigation of the possible mechanism underlying alleviation of the paralysis symptoms. We first determined whether EDVAE would affect expression levels of the mRNA and/or the protein of Aβ using both qPCR and ELISA. The results showed that, neither the mRNA nor the protein expression level was significantly affected compared to the N control (Fig. 3A and B).

Next, we determined whether EDVAE could impair aggregation of Aβ through examination of the Aβ deposits in the CL4176s using thioflavin T staining. The results showed that the amount of Aβ deposits in CL4176s was significantly increased when the temperature was lifted from 16 °C (Figure 3D) to 25 °C (Fig. 3E); no significant difference in Aβ deposits was detected between the EDVAE treatment group and the Res positive control group (Fig. 3F; Fig. 3G), but both were significantly lower than that in the N control group at 25 °C (Fig. 3C).

At 48 h after Aβ induction, EDVAE treatment reduced amyloid deposits by 76% when compared with the N control group (N control vs EDVAE: 6.14 vs 1.43, p<0.01; N control vs Res: 6.14 vs 2.83, p<0.05) (Fig. 3C). Overall, these data indicate that effects of EDVAE treatment on alleviation of the paralytic symptoms of the CL4176s may be due to inhibition of abnormal aggregation of Aβ deposits, rather than impaired expression of Aβ.

3.3. EDVAE reduced ROS and increased SOD activity in the CL4176s

We next investigated the effects of EDVAE on ROS production using a fluorescence assay. As shown in Fig. 4A, significant responses in both the EDVAE and the Res (positive control) treated groups were detected at 48 h after temperature up-lift (ROS reduced by approximately 40% and 55% respectively, both p<0.05 compared with the N control group). Given that SOD-3 is a well-known scavenger enzyme of ROS in oxidative stress, and catalyzes the conversion of active superoxide anions to molecular oxygen (Dong et al., 2013), we measured the SOD activity. Compared with the N control group, treatment with 10 mg/mL EDVAE significantly increased SOD activity (p<0.05) in CL4176s; the similar trend achieved with Res treatment, but did not reach significance (Fig. 4B).

3.4. EDVAE increased expression of stress response genes in CL4176s

Previous research reported that *skn-1, hsf-1* and *daf-16* played important roles in regulating Aβ aggregation (Guo et al., 2016). To study the possible molecular pathway underlying the effects of EDVAE, we measured the expression levels of *skn-1*, *hsf-1* and *daf-16*. The results showed that the expression levels of *skn-1*, *hsf-1* and *daf-16* were increased by 20%, 65%, and 70% respectively in the EDVAE treated CL4176s, compared to the N control, while the effect of Res was not significant except for *daf-16* (P<0.05) (Fig. 4C). Since expression levels of these transcriptional factors were significantly increased, we further tested the expression of their downstream genes (Guo et al., 2016; Zhou et al., 2018). As shown in Fig. 4D, compared to the N control, EDVAE-treated CL4176s showed *sod-3,* the downstream gene of *daf-16,* was significantly upregulated.

3.5. EDVAE did not show visible toxicity and significantly extended lifespan of N2 C. elegans

Overall, treatment with 10 mg/mL EDVAE did not show any toxicity to N2 nematodes (Fig. 5). Lifespan was extended by 4% (Fig. 5A), there

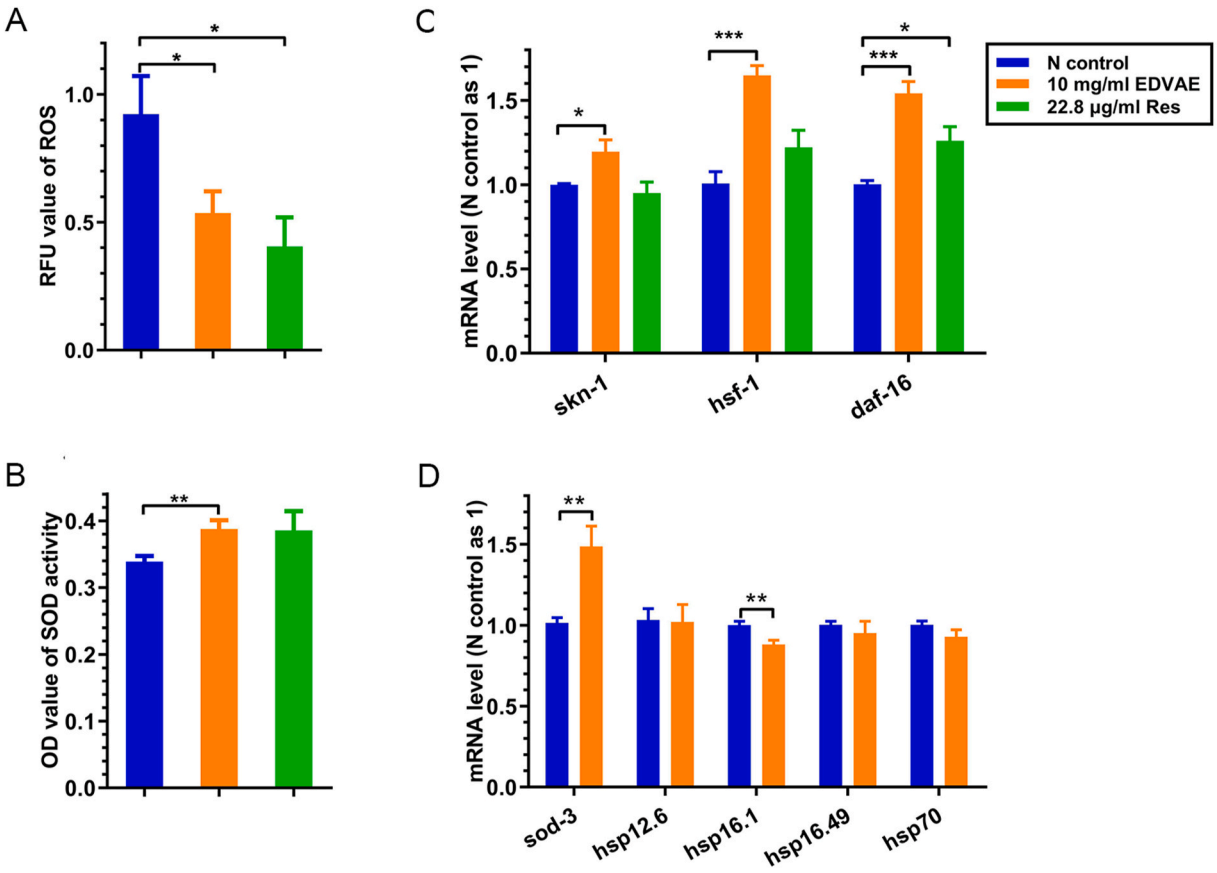

Fig. 4. Enhancement of the ability to resist oxidative stress in the nematodes by EDVAE. (A) ROS was measured in nematodes from the different groups at 48 h after temperature uplift to 25 °C using 2′,7′-dichlorofluorescein diacetate. Results were expressed as DCF (2′,7′-dichlorofluorescein) fluorescence relative to the untreated control (*p<0.05, **p<0.01). (B) The SOD activity in nematodes from the different groups. The SOD activity was significantly increased after the treatment of EDVAE (*p<0.05). (C) The relative expression of *skn-1*, *hsf-1* and *daf-16*. (D) The relative expression of *hsf-1* and *daf-16* downstream genes. The relative gene expression level was calculated using the method of $2^{-\triangle\triangle CT}$ and the gene *β-actin* was used as the internal reference. Data represent an average from three independent experiments (***p<0.001, *p<0.05).

was no effect on reproduction (Fig. 5B). Besides, the movement ability was enhanced by EDVAE, which indicated by more type A nematodes and less type D (died) nemotodes in EDVAE-treated group than Ctrl group at 10 days, 14 days and 18days after treatment (Fig. 5C). Moreover, the effect of EDVAE on extending the lifespan of the N2 nematodes was found to be greater than that of the Res treatment.

3.6. Composition of the extracts

The fractionation data are summarized in Table 2. The results showed that the EDVAE contained a wide spectrum of relatively low molecular weight substances: those less than 75 Da (4%) were small chemical compounds, between 75 and 130 Da (0.2%) are likely single amino acids, between 190 and 300 Da (12%) are likely di- and tri-peptides, and between 900 and 1,300 Da (63%) are likely polypeptides (likely to be bioactive ingredients). By contrast, the compounds of the water extracts were dominated by large molecular substances (59% in the CWVAE and 96% in the HWVAE >18,000 Da, with only 20% and 2% of the fractions in the amino acid and dipeptide range (75–300 Da). Based on the size distribution, the concentration of putative peptides and polypeptides (75–4,000 Da) is estimated at 93, 4 and 0.2 mg/mL in the original extracts of EDVAE, CWVAE and HWVAE respectively.

4. Discussion

With the growth of the aging population in our society, the number of patients diagnosed with AD increases year after year (Gaugler et al., 2016). Aβ is the major component of senile plaques and one of the most significant pathological features of AD. The imbalance between the production and clearance of Aβ in the central nervous system results in abnormal accumulation of Aβ, which has been shown to stimulate a variety of cell signaling pathways, eventually leading to synaptic degeneration, loss of neurons, and decreased cognitive function (Dillin et al., 2011; Li et al., 2018). Due to the limited effectiveness of drugs so far approved (e.g. by the US FDA) for the treatment of AD, there is a need to actively search for new compounds that have the potential for treating/alleviating AD.

C. elegans is an ideal animal model for screening and testing such compounds because their complete genetic information is available, and 60% of their genes are homologues with humans (Finkel and HolbrookOxidants, 2000). Using transgenic nematodes as an alternative AD model, such as CL4176s, it is possible to study the toxicity mechanisms of AD and the potential therapeutic effects of candidate drugs and nutritional supplements (Link, 1995, Link, 2006). CL4176s has been used thus far to explore the efficacy and mechanism of anti-AD using coffee extract (Dostal et al., 2010), fucoidan (Wang et al., 2017), a polysaccharide from *Coptis chinensis* Franch (Li et al., 2018) and cranberry extract (Guo et al., 2015).

In this study, we used the CL4176s model to investigate the effects of VAEs on alleviating symptoms of nematode paralysis and found that all three types of VAEs were effective in reducing Aβ-induced toxicity with the most significant effects achieved by EDVAE at 10 mg/ml (Figs. 1A and 3C). To produce EDVAE, we used an acid-pepsin digestion followed by alkaline trypsin to treat the VA powder in order to mimic the

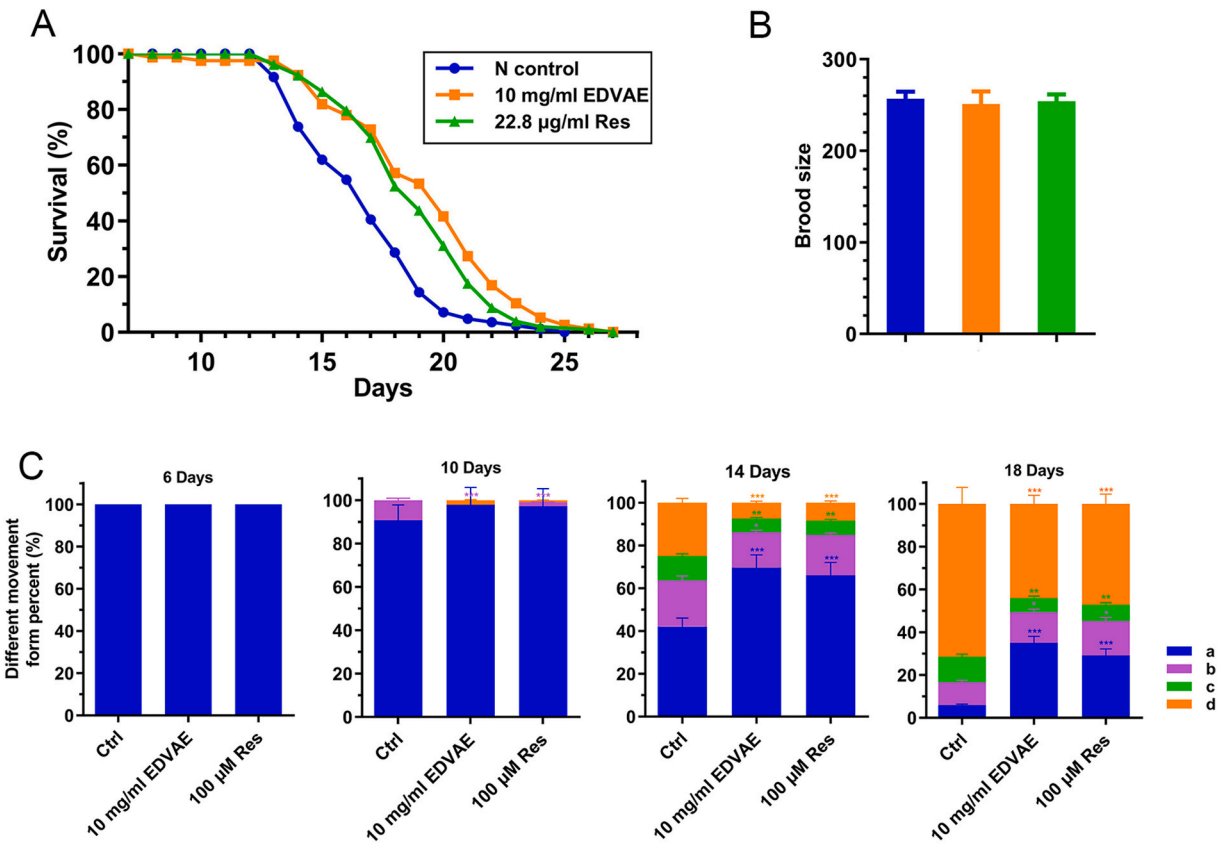

Fig. 5. No toxic effects of EDVAE were detected in *C. elegans*. (A) EDVAE significantly extended the lifespan of nematodes (control vs. 10 mg/mL EDVAE and 22.8 μg/mL Res, $p<0.001$). (B) No toxic effects of EDVAE were found on brood size of N2 nematodes. (C) EDVAE significantly enhanced the motor ability of N2 nematodes.

digestive processes in the stomach and intestine in the human body. The results demonstrated that through the digestion of human digestive enzymes, VAEs not only maintained bioactivity, but also their effects on reducing Aβ-induced toxicity were significantly enhanced, suggesting that VAEs have the potential to be developed as an oral drug/dietary supplement for alleviating AD symptoms.

The data from the EDVAE fractionation showed that this extract contained a wide spectrum of relatively low molecular weight substances (Fig. 2A, Table 2). Based on the molecular size distribution, the concentration of putative effective peptides and polypeptides (75–4,000 Da) is estimated at 93, 4 and 0.2 mg/mL of the original extracts of EDVAE, CWVAE and HWVAE respectively. These fractions of peptides

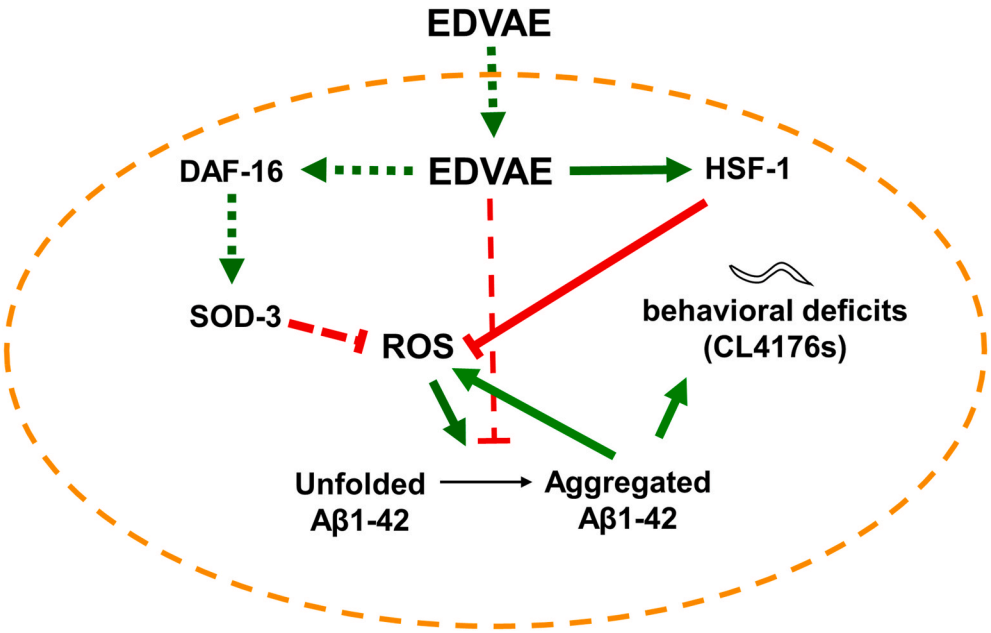

Fig. 6. Hypothetical model of the mode of action of EDVAE on CL4176s. The yellow circle represents cytomembrane. After entering the cytomembrane, EDVAE likely down-regulates ROS level via up-regulating DAF-16, which in turn likely activates SOD-3 expression; or via up-regulating HSF-1. Reduction in ROS level inhibits aggregation of Aβ, consequently improving the behavioral abnormality of CL4176s. Green lines: signals for up-regulating or activating gene expression; red lines: signals for inhibition of gene expression; dashed lines: hypothetical pathways to be elucidated. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and polypeptides are likely to contain key factors for the observed effects. As shown previously, peptide size and sequence are two critical factors that determine their potency, bioavailability, and cellular mechanisms (Fan et al., 2019). Compared to the water extractions in the present study, EDVAE mainly consisted of low molecular weight proteins, including a variety of peptides, and had more significant effects at relieving the AD-like phenotype in CL4176s. This may be due to the hydrolysis of large proteins into small peptides by enzymatic digestion, which increased their bioavailability. However, given that some significant effects were also shown with the CWVAE and HWVAE (Fig. 1), then the higher molecular weight compounds cannot be completely excluded.

As Aβ is an important biomarker in the development of AD, we investigated whether EDVAE treatment could affect the expression levels of mRNA and protein of Aβ. The results showed that Aβ did not show any significant differences at both mRNA and protein expression levels between the different treatment groups and the N control group (Fig. 3). However, the Aβ deposits/aggregates in the transgenic *C. elegans* was significantly reduced after the EDVAE treatment, and this reduction was even greater than the positive control (Res). Therefore, we propose that a reduction in Aβ aggregation may be the main reason for the improvement of the phenotype/behavior of CL4176s following the EDVAE treatment, although the underlying molecular mechanism requires further investigation.

Oxidative stress plays a key role in the pathological development of AD. Experimental evidence suggests that cellular damage caused by free radicals of oxygen might be a major contributor to Aβ toxicity (Markesbery, 1997; Shukla et al., 2011). Here, we observed that EDVAE treatment significantly reduced Aβ-induced ROS production and significantly increased the activity of SOD in the Aβ transgenic nematodes (Fig. 4A and B). The symptom improvement after EDVAE treatment may be through activation of antioxidant signaling pathways in the nematodes, however, the underlying mechanism needs to be further elucidated. To test this hypothesis further in this study, we determined whether some of the relevant regulators (*skn-1*, *hsf-1, and daf-16*), which have been reported to play key roles in the regulation of Aβ toxicity through activation of antioxidant pathways (Dillin and Cohen. 2011; Dostal et al., 2010; Guo et al., 2015), participated in the EDVAE-mediated mitigation of Aβ toxicity. Up-regulation of *skn-1*, *hsf-1* and *daf-16* expression in the EDVAE group was observed (Fig. 4C). We further determined the expression levels of the several major downstream target genes for *hsf-1* and *daf-16* and showed that *sod-3* was significantly upregulated in the CL4176s treated with EDVAE, compared to the control (Fig. 4D). These results indicate that EDVAE might play a role in regulating antioxidants via activating *daf-16* and its downstream *sod-3*. The hypothetical signaling pathway of the EDVAE function in the CL4176s is outlined in Fig. 6.

For any substance to be developed as a potential drug to treat/alleviate AD, toxicity must be firstly evaluated. Our results showed no evidence of toxicity for EDVAE based on reproductive performance at the dose of 10 mg/mL, and the treatment also increased the lifespan and the exercise ability of nematodes (Fig. 5). Previous studies showed that the longevity-promoting activity of natural products was closely linked to their antioxidant activities (Chen et al., 2013). In addition, it is reported that increased ROS accumulation could cause damage to proteins, DNA, and lipids, resulting in acceleration of aging and age-related diseases (Finkel and HolbrookOxidants, 2000), such as AD. Our results have shown that treatment with10 mg/mL EDVAE prolonged the lifespan of nematodes, which was consistent with the result of delaying the occurrence of paralysis symptoms.

5. Conclusion

In summary, treatment with EDVAE (enzymatically digested velvet antler extract) significantly reduced the Aβ-induced toxicity in the transgenic *C. elegans* model of AD, and this effect may be via reducing Aβ aggregation and mitigating oxidative stress, although these effects need to be validated to determine whether being realized through direct or indirect way. Further work along this line would lead to the identification of active compounds from EDVAE for neuroprotection, and development of a pharmaceutical or functional food for treating neurodegenerative disease such as AD in clinics.

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CRediT authorship contribution statement

Fangzhou Du: Conceptualization, Methodology, did experiments, Formal analysis, prepared the manuscript. **Haiping Zhao:** Formal analysis. **Mengjie Yao:** did experiments. **Yanyan Yang:** did experiments. **Jingxue Jiao:** did experiments. **Chunyi Li:** Conceptualization, Methodology, prepared the manuscript, All authors read and approved the final version of the manuscript.

Declaration of competing interest

The work described has not been published previously, and it is not under consideration for publication elsewhere. Its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out. The present study was performed according to international, national and institutional rules considering animal experiments, clinical studies and biodiversity rights.

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Abbreviations

Alzheimer's disease AD
β-amyloid peptide Aβ
Enzymatically-digested extract from the velvet antler EDVAE
Cold-water extract from the velvet antler CWVAE
Hot water extract from the velvet antler HWVAE
Reactive oxygen species ROS
The AD transgenic *Caenorhabditis elegans* CL4176s
2′,7-dichlorofluorescein diacetate (H2DCF-DA)
Superoxide Dismutase (SOD)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2021.114850.

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