frontiers | Frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
published: 09 June 2022
doi: 10.3389/fnmol.2022.901953

Eucommia ulmoides Olive Male Flower Extracts Ameliorate Alzheimer's Disease-Like Pathology in Zebrafish *via* Regulating Autophagy, Acetylcholinesterase, and the Dopamine Transporter

Chen Sun[1,2†], Shanshan Zhang[1,2†], Shuaikang Ba[1,2†], Jiao Dang[1,2], Qingyu Ren[1,2], Yongqiang Zhu[1,2], Kechun Liu[1,2] and Meng Jin[1,2]*

[1] Biology Institute, Qilu University of Technology (Shandong Academy of Sciences), Jinan, China, [2] Key Laboratory for Drug Screening Technology, Shandong Academy of Sciences, Jinan, China

OPEN ACCESS

Edited by:
Claire Russell,
Royal Veterinary College (RVC),
United Kingdom

Reviewed by:
Dileep Kalarickal Vijayan,
RIKEN, Japan
Enes Akyuz,
University of Health Sciences, Turkey

***Correspondence:**
Meng Jin
mjin1985@hotmail.com

† These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Methods and Model Organisms, a section of the journal Frontiers in Molecular Neuroscience

Received: 22 March 2022
Accepted: 09 May 2022
Published: 09 June 2022

Citation:
Sun C, Zhang S, Ba S, Dang J, Ren Q, Zhu Y, Liu K and Jin M (2022) Eucommia ulmoides Olive Male Flower Extracts Ameliorate Alzheimer's Disease-Like Pathology in Zebrafish via Regulating Autophagy, Acetylcholinesterase, and the Dopamine Transporter. Front. Mol. Neurosci. 15:901953. doi: 10.3389/fnmol.2022.901953

Alzheimer's disease (AD) is the most prevalent neural disorder. However, the therapeutic agents for AD are limited. *Eucommia ulmoides* Olive (EUO) is widely used as a traditional Chinese herb to treat various neurodegenerative disorders. Therefore, we investigated whether the extracts of EUO male flower (EUMF) have therapeutic effects against AD. We focused on the flavonoids of EUMF and identified the composition using a targeted HPLC-MS analysis. As a result, 125 flavonoids and flavanols, 32 flavanones, 22 isoflavonoids, 11 chalcones and dihydrochalcones, and 17 anthocyanins were identified. Then, the anti-AD effects of the EUMF were tested by using zebrafish AD model. The behavioral changes were detected by automated video-tracking system. Aβ deposition was assayed by thioflavin S staining. Ache activity and cell apoptosis in zebrafish were tested by, Acetylcholine Assay Kit and TUNEL assay, respectively. The results showed that EUMF significantly rescued the dyskinesia of zebrafish and inhibited Aβ deposition, Ache activity, and occurrence of cell apoptosis in the head of zebrafish induced by $AlCl_3$. We also investigated the mechanism underlying anti-AD effects of EUMF by RT-qPCR and found that EUMF ameliorated AD-like symptoms possibly through inhibiting excessive autophagy and the abnormal expressions of *ache* and *slc6a3* genes. In summary, our findings suggested EUMF can be a therapeutic candidate for AD treatment.

Keywords: AD, $AlCl_3$, Ache, *slc6a3*, flavonoids

INTRODUCTION

Alzheimer's disease (AD) is a common neurodegenerative disease which is age-related. Patients with AD are characterized by the progressive loss of acquired knowledge and memory decline. The loss of neurons, formation of neurofibrillary tangles, tau protein aggregation, amyloid β-protein (Aβ) deposition, and low levels of acetylcholine (ACh) are the main clinical hallmarks of AD (Kepp, 2016; Sanabria-Castro et al., 2017).

The aging tendency of the population is leading to an increased prevalence of AD. Currently, over 47 million people have been diagnosed with AD, and this has caused heavy burdens for families

and society (Neves et al., 2021). To deal with this situation, many AD drugs have been developed, such as anti-tau, an amyloid β-protein (Aβ) aggregation inhibitor, and cholinergic-enhancing and anti-inflammatory drugs. Unfortunately, these drugs are not able to prevent the progression of AD and only can improve cognitive function and memory to a certain extent (Pearson, 2001; Huang and Mucke, 2012). Therefore, the development of AD drugs is an urgent task.

Because of their novel structures and extensive physiological activities, natural products from plants have been always an important source of drug development. *Eucommia ulmoides* Olive (EUO), also named Du-zhong, is a deciduous tree in the family of *Eucommiaceae* (Yan et al., 2018). It is also the traditional Chinese herb. The leaf and bark of EUO are officially documented in the Chinese Pharmacopeia. The leaf extracts of EUO are reported to be treat AD, aging, diabetes, hypertension, and osteoporosis (He et al., 2014). However, studies investigating the male flowers of EUO began relatively late. Currently, many studies have shown that the male flowers, like the leaf and bark of EUO, also contain many bioactive components including lignans, megastigmane glycosides, iridoids, phenolics, and flavonoids. These bioactive constituents have typically exhibited neuroprotective, anti-oxidant, anti-tumor, anti-inflammatory, anti-hypertensive, anti-aging, immunity promotion, and other activities (Luo et al., 2010; Kobayashi et al., 2012; Zhang et al., 2012; Niu et al., 2015; Hao et al., 2016; Yan et al., 2018). There are similar bioactive constituents between the male flower and leaf of EUO. Hence, we hypothesize that extracts of the EUO male flower (hereafter referred to as EUMF) may have anti-AD activity.

Zebrafish is an ideal model system for human disease and drug development. They possess a high homology to humans and have rapid development and small sizes (Kerstin et al., 2013; Hong et al., 2020). Many studies have reported that a zebrafish AD model can be established by using $AlCl_3$, an *in vivo* animal model that can mirror the primary characteristic pathological changes of patients with AD. Various clinical hallmarks of AD can be detected in this model (Huang et al., 2016; Pan et al., 2019). But unfortunately, an important clinical hallmark-Aβ deposition has not yet been successfully detected in zebrafish AD model.

In summary, in this study we isolated and purified the EUMF and identified the chemical compositions. To verify our hypothesis mentioned in the previous paragraph, the zebrafish AD model was used to investigate the therapeutic effect of EUMF on AD symptoms. In addition, Aβ deposition detection was used innovatively in our zebrafish AD model. Finally, we further tested the mRNA expressions of key factors involved in autophagy and the regulation of neurotransmitters to reveal the underlying mechanism.

MATERIALS AND METHODS

Animals

The adult wild-type zebrafish (AB strain) were maintained in a zebrafish facility at 28.5°C ± 0.5°C with a 14 h light/10 h dark cycle photoperiod at the Key Laboratory for Drug Screening Technology of the Shandong Academy of Sciences. Larvae were obtained from natural mating. Zebrafish larvae at 3 days post-fertilization (dpf) were used this study. All experiments were conducted in compliance with the standard ethical guidelines and under the control of the Biology Institute, the Qilu University of Technology of Animal Ethics Committee.

Preparation of the *Eucommia ulmoides* Olive Male Flower

The hydrothermal extraction method is used to prepare EUMF. Approximately 20 g of dried EUMF powder was placed into a flask, and 2,000 mL of ultrapure water was added. Then the flask was placed into an electric jacket for extraction by heat reflux three times, 2 h each time. The supernatant was obtained by centrifugation at 5,000 rpm for 10 min. The combined extraction solution was concentrated by rotary evaporation and then freeze-dried to obtain the EUMF.

Identification of Flavonoid Compounds Using HPLC-MS

A targeted HPLC-MS analysis of the flavonoid compounds was performed on SCIEX Qtrap 6500 + system (SCIEX, United States). The Xselect HSS T3 C_{18} column (2.1 × 150 mm, 2.5 μm) was used for sample separation. Distilled water containing 0.1% formic acid was used as solvent A, and acetonitrile containing 0.1% formic acid was used as solvent B. The elution condition was maintained at 2% B for 2 min, from 2 to 100% B for 13 min, maintained at 100% B for 2 min, and equilibrated with the initial elution solvent for 3 min. The flow rate was 0.4 mL/min. The injection volume of the sample was 1 μL. The column temperature was set to be 50°C. Mass spectrometry was performed in both the positive and negative ion modes. The optimal positive MS parameters were a curtain gas pressure of 35 psig and an ion spray voltage of 5,500 V at a temperature of 550°C. For the negative MS mode, the ion spray voltage was set as −4,500 V and the other parameter was the same as the positive mode. All of the compounds were identified according to LC and MS information and compared with flavonoid compound databases that were supplied by the Novogene Co., Ltd. (Tianjin, China).

Establishment of Zebrafish Alzheimer's Disease Model

The establishment of the zebrafish AD model referenced to the previous studies (Huang et al., 2016; Pan et al., 2019) with a slight modification. In brief, 3 dpf larval zebrafish were randomly transferred to six-well cell culture plates with a density of approximately 20 larvae per well. Then they were treated with 80 μM $AlCl_3$ from 3 to 6 dpf to generate the zebrafish AD models.

Eucommia ulmoides Olive Male Flower and Donepezil Treatments

The larvae were treated with different concentrations of EUMF (100, 200, 300, 400, 500, 600, 700, 800, and 1,600 μg/mL) from

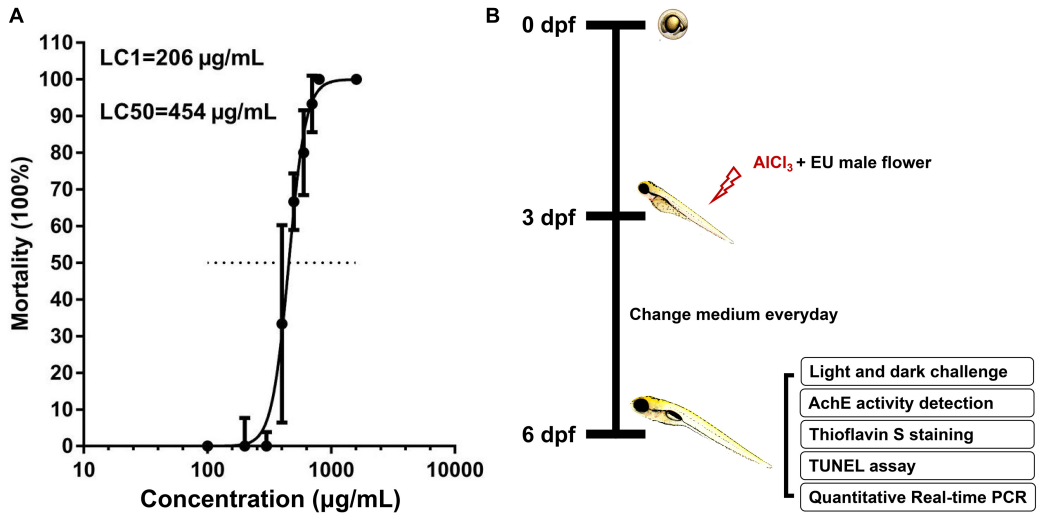

FIGURE 1 | Mortality curve and experimental workflow chart. **(A)** Larval zebrafish were exposed to different concentrations of EUMF (100, 200, 300, 400, 500, 600, 700, 800, and 1,600 μg/mL) from 3 to 6 dpf. The mortality was recorded within each group at 3, 4, 5, and 6 dpf. Dead larvae were judged using missing heartbeats. **(B)** Larvae at 3 dpf were co-exposed to $AlCl_3$ and three different concentrations of EUMF from 3 to 6 dpf. At 6 dpf the zebrafish were subjected to a behavioral test. In addition, we also evaluated the AchE activity, Aβ deposition, and apoptosis in the brain and performed RT-qPCR.

3 to 6 dpf. We found that the LC1 and LC50 of the EUMF were 206 and 454 μg/mL, respectively, based on the EUMF lethality curve of **Figure 1A**. LC_1 is typically regarded as a no-observed-effect concentration value. Therefore, we tested the anti-AD activity of the EUMF at concentrations below LC_1 (206 μg/mL). The zebrafish larvae were co-treated with 80 μM $AlCl_3$ and EUMF at three different concentrations (50, 100, and 200 μg/mL) from 3 to 6 dpf (**Figure 1B**). Donepezil which is the inhibitor of acetylcholinesterase (Ache) was used as the positive drug. In the positive group, the larvae were co-treated with 80 μM $AlCl_3$ and 4.0 μM donepezil from 3 to 6 dpf. After treatment, 10 larvae from each group were randomly selected for the image acquisition.

Behavioral Analysis

The larvae from each group were randomly collected, and cleaned using an embryo medium (1 mM $MgSO_4$, 0.5 mM KCl, 15 mM NaCl, 0.05 mM $(NH4)_3PO_4$, 0.15 mM KH_2PO_4, 0.7 mM $NaHCO_3$, and 1 mM $CaCl_2$). They were then placed in 48-well plates. After a 20-min acclimation period, the locomotor activity for each larva was recorded using an automated computerized video-tracking system (Viewpoint, Lyon, France). The behavioral tests contained three alternating light-dark cycles with 60 min (10 min illumination, 10 min darkness alternately). Zeblab software (Viewpoint, Lyon, France) was used to recorded and analyzed the zebrafish movement distance and speed change to light-dark and dark-light cycles.

Detection of the Amyloid β-Protein Deposition

The zebrafish larvae were fixed using 4% paraformaldehyde. All of the fixed zebrafish were processed by embedding in the optimal cutting temperature compound (OCT Compound, SAKURA, United States) and frozen at −20°C until sectioning. Subsequently, the tissue sections were used for thioflavin S staining (Chao et al., 2018). In brief, the sections were washed with 0.01 M phosphate buffered solution (PBS) for 30 min at room temperature. Next, 0.3% thioflavin S (Sigma-Aldrich, Darmstadt, Germany) was introduced, and the sections were incubated for 8 min at room temperature in the dark. Finally, the sections were washed with 0.01 M PBS for 30 min in dark, and a fluorescence microscope (Zeiss, Jena, Germany) was used to analyze the sections. The fluorescence intensity of the

TABLE 1 | The sequences of primer pairs used in real-time quantitative PCR assay.

No	Gene symbol	Forward primer	Reverse primer
1	*ambra1a*	TAACCAGGAAACTGGCCAAC	AATATGCTGCAGGGGACAAC
2	*atg5*	AGGGGATAACAGCACAAACG	CTTCTTATGCAGCGTGTCCA
3	*ulk1b*	AGGCCGAAAGTCTCACTTCA	AGCCATGTACATCGGAGACC
4	*lc3b*	CCTCCAACTCAACTCCAACC	GCCGTCTTCGTCTCTTTCC
5	*ache*	TCTTGCCCACTGTGCTACTC	TCTTGTACCCTGCACTCTGC
6	*slc6a3*	CTAATCGCCTTCTCCAGCTACA	GGCCACGTTGTGTTTCTGTGACAT
7	*rpl13a*	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG

TABLE 2 | Flavonoids compounds identified in EUMF by LC-MS.

No	RT (min)	Molecular Weight	Formula	Name	Relative content (%)	Class
1	0.680	274.084	$C_{15}H_{14}O_5$	Afzelechin	0.0854	Flavonoids
2	0.700	420.454	$C_{25}H_{24}O_6$	Kuwanon A	0.0064	Flavones and Flavanols
3	0.710	448.400	$C_{22}H_{22}O_{11}$	Methylluteolin C-hexoside	0.0210	Flavones and Flavanols
4	0.720	418.100	$C_{21}H_{22}O_9$	O-methylnaringenin C-pentoside	0.2106	Flavanones
5	0.720	418.394	$C_{21}H_{22}O_9$	Methylnaringenin C-pentoside	0.2024	Flavanones
6	0.730	402.350	$C_{20}H_{18}O_9$	Apigenin C-pentoside	0.0411	Flavones and Flavanols
7	0.730	446.404	$C_{22}H_{22}O_{10}$	Methylapigenin C-hexoside	0.0837	Flavones and Flavanols
8	0.730	550.460	$C_{25}H_{26}O_{14}$	di-C, C-pentosyl-luteolin	0.0554	Flavones and Flavanols
9	0.740	478.400	$C_{22}H_{22}O_{12}$	Selgin C-hexoside	0.0150	Flavones and Flavanols
10	0.780	342.343	$C_{19}H_{18}O_6$	Methylophiopogonanone A	0.0025	Isoflavonoids
11	0.940	478.400	$C_{22}H_{22}O_{12}$	Selgin 5-O-hexoside	0.0348	Flavones and Flavanols
12	0.950	272.069	$C_{15}H_{12}O_5$	Butein	0.0093	Chalcones and dihydrochalcones
13	0.970	476.430	$C_{23}H_{24}O_{11}$	Irisolidone 7-O-beta-d-glucoside	0.0106	Isoflavonoids
14	0.970	476.430	$C_{23}H_{24}O_{11}$	Methylchrysoeriol 5-O-hexoside	0.0136	Flavones and Flavanols
15	0.970	508.430	$C_{23}H_{24}O_{13}$	Limocitrin O-hexoside	0.0236	Flavones and Flavanols
16	0.980	756.660	$C_{33}H_{40}O_{20}$	C-hexosyl-apigenin O-hexosyl-O-hexoside	0.0048	Flavones and Flavanols
17	0.980	479.000	$C_{22}H_{23}O_{12}$	Petunidin 3-O-glucoside	0.0489	Anthocyanins
18	1.000	624.552	$C_{28}H_{32}O_{16}$	C-hexosyl-chrysoeriol O-hexoside	0.0105	Flavones and Flavanols
19	1.010	286.279	$C_{16}H_{14}O_5$	Sakuranetin	0.0118	Flavanones
20	1.010	416.378	$C_{21}H_{20}O_9$	Methylapigenin C-pentoside	0.0043	Flavones and Flavanols
21	1.040	430.405	$C_{22}H_{22}O_9$	Ononin	0.0468	Isoflavonoids
22	1.130	868.702	$C_{43}H_{32}O_{20}$	8-Gingerol	0.0069	Flavonoids
23	1.220	576.500	$C_{30}H_{24}O_{12}$	Procyanidin A1	0.0009	Anthocyanins
24	1.260	576.500	$C_{30}H_{24}O_{12}$	Procyanidin A2	0.0011	Anthocyanins
25	1.284	254.240	$C_{15}H_{10}O_4$	Chrysin	0.0004	Flavones and Flavanols
26	2.600	332.262	$C_{16}H_{12}O_8$	Laricitrin	0.4216	Flavones and Flavanols
27	4.440	302.279	$C_{16}H_{14}O_6$	Homoeriodictyol	0.0003	Flavanones
28	4.560	302.043	$C_{15}H_{10}O_7$	Tricetin	0.0003	Flavones and Flavanols
29	5.098	306.270	$C_{15}H_{14}O_7$	(-)-epigallocatechin	0.0005	Flavonoids
30	5.210	484.840	$C_{21}H_{21}ClO_{11}$	Cyanidin 3-O-glucoside	0.0111	Anthocyanins
31	5.213	484.840	$C_{21}H_{21}ClO_{11}$	Idaein chloride	0.0081	Anthocyanins
32	5.310	466.392	$C_{21}H_{22}O_{12}$	Taxifolin O-glucoside	0.0064	Flavanones
33	5.380	356.332	$C_{19}H_{16}O_7$	Ophiopogonanone C	0.2663	Flavanones
34	5.390	626.520	$C_{27}H_{30}O_{17}$	Quercetin-3,4′-O-di-beta-glucopyranoside	0.3593	Flavones and Flavanols
35	5.420	809.120	$C_{33}H_{41}O_{21}C_{/1}$	Delphinidin 3-sophoroside-5-rhamnoside	0.0329	Anthocyanins
36	5.442	468.840	$C_{21}H_{21}ClO_{10}$	Callistephin chloride	0.0005	Anthocyanins
37	5.515	528.890	$C_{23}H_{25}ClO_{12}$	Malvidin 3-galactoside chloride	0.0005	Anthocyanins
38	5.572	528.890	$C_{23}H_{25}ClO_{12}$	Oenin chloride	0.0022	Anthocyanins
39	5.602	338.700	$C_{15}H_{11}ClO_7$	Delphinidin chloride	0.0009	Anthocyanins
40	5.680	610.518	$C_{27}H_{30}O_{16}$	C-hexosyl-luteolin O-hexoside	0.0012	Flavones and Flavanols
41	5.730	594.518	$C_{27}H_{30}O_{15}$	Apigenin-6,8-di-C-glycoside	0.1253	Flavones and Flavanols
42	5.745	432.380	$C_{21}H_{20}O_{10}$	Puerarin	0.0031	Isoflavonoids
43	5.810	624.544	$C_{28}H_{32}O_{16}$	di-C,C-hexosyl-methylluteolin	0.1441	Flavones and Flavanols
44	5.820	594.518	$C_{27}H_{30}O_{15}$	di-C,C-hexosyl-apigenin	0.2993	Flavones and Flavanols
45	5.890	288.252	$C_{15}H_{12}O_6$	Fustin	0.0043	Flavanones
46	6.030	564.499	$C_{26}H_{28}O_{14}$	Isoschaftoside	0.0142	Flavones and Flavanols
47	6.040	594.526	$C_{27}H_{30}O_{15}$	C-pentosyl-chrysoeriol O-hexoside	0.0236	Flavones and Flavanols
48	6.050	564.490	$C_{26}H_{28}O_{14}$	C-pentosyl-C-hexosyl-apigenin	0.0028	Flavones and Flavanols
49	6.074	564.492	$C_{26}H_{28}O_{14}$	Schaftoside	0.0828	Flavones and Flavanols
50	6.080	430.628	$C_{27}H_{42}O_4$	Hecogenin	0.0367	Flavonoids
51	6.090	466.398	$C_{21}H_{22}O_{12}$	Plantagoside	0.0256	Flavanones
52	6.100	448.400	$C_{21}H_{20}O_{11}$	Luteolin C-hexoside derivative	0.2109	Flavones and Flavanols
53	6.174	448.380	$C_{21}H_{20}O_{11}$	Isoorientin	0.0972	Flavones and Flavanols
54	6.230	416.378	$C_{21}H_{20}O_9$	Toringin	0.0272	Flavones and Flavanols

(Continued)

TABLE 2 | (Continued)

No	RT (min)	Molecular Weight	Formula	Name	Relative content (%)	Class
55	6.230	446.121	$C_{22}H_{22}O_{10}$	Sissotrin	0.0007	Isoflavonoids
56	6.250	448.377	$C_{21}H_{20}O_{11}$	Orientin	0.0972	Flavones and Flavanols
57	6.263	322.700	$C_{15}H_{11}ClO_6$	Cyanidin chloride	0.0007	Anthocyanins
58	6.270	594.518	$C_{27}H_{30}O_{15}$	Saponarin	0.0360	Flavones and Flavanols
59	6.280	610.525	$C_{27}H_{30}O_{16}$	Kaempferol-3-gentiobioside	0.0200	Flavones and Flavanols
60	6.280	594.518	$C_{27}H_{30}O_{15}$	4′-O-Glucosylvitexin	0.0360	Flavones and Flavanols
61	6.300	578.519	$C_{27}H_{30}O_{14}$	6"-O-xylosyl-glycitin	0.0023	Isoflavonoids
62	6.350	611.500	$C_{27}H_{31}O_{16}$	Tulipanin	0.3871	Anthocyanins
63	6.360	610.520	$C_{27}H_{30}O_{16}.xH_2O$	Rutin hydrate	8.0753	Flavones and Flavanols
64	6.380	596.542	$C_{27}H_{32}O_{15}$	Neoeriocitrin	0.4294	Flavanones
65	6.390	434.121	$C_{21}H_{22}O_{10}$	Isohemiphloin	0.0171	Flavanones
66	6.411	578.520	$C_{27}H_{30}O_{14}$	Vitexin-2-O-rhaMnoside	0.0026	Flavones and Flavanols
67	6.430	612.576	$C_{28}H_{36}O_{15}$	Neohesperidin dihydrochalcone	0.1784	Chalcones and dihydrochalcones
68	6.430	596.534	$C_{27}H_{32}O_{15}$	Eriocitrin	0.0007	Flavanones
69	6.451	610.518	$C_{27}H_{30}O_{16}$	Rutin	7.9072	Flavones and Flavanols
70	6.460	432.113	$C_{21}H_{20}O_{10}$	Apigenin C-glucoside	0.0009	Flavones and Flavanols
71	6.470	208.255	$C_{15}H_{12}O$	Chalcone	0.5983	Chalcones and dihydrochalcones
72	6.510	594.526	$C_{27}H_{30}O_{15}$	Kaempferol-3-O-rutinoside	0.0309	Flavones and Flavanols
73	6.530	462.366	$C_{21}H_{18}O_{12}$	Luteolin-7-O-beta-D-glucuronide	0.0017	Flavones and Flavanols
74	6.530	464.382	$C_{21}H_{20}O_{12}$	Quercetin-3′-O-glucoside	9.1850	Flavones and Flavanols
75	6.530	464.469	$C_{23}H_{28}O_{10}$	Isomucronulatol-7-O-glucoside	3.3727	Isoflavonoids
76	6.533	432.378	$C_{21}H_{20}O_{10}$	Isovitexin	0.0007	Flavones and Flavanols
77	6.540	464.376	$C_{21}H_{20}O_{12}$	Quercetin-O-glucoside	0.4630	Flavones and Flavanols
78	6.547	464.380	$C_{21}H_{20}O_{12}$	Myricitrin	4.7113	Flavones and Flavanols
79	6.550	594.159	$C_{27}H_{30}O_{15}$	Kaempferol 3-O-robinobioside	0.4242	Flavones and Flavanols
80	6.554	464.380	$C_{21}H_{20}O_{12}$	Hyperoside	2.9062	Flavones and Flavanols
81	6.559	432.380	$C_{21}H_{20}O_{10}$	Vitexin	0.0006	Flavones and Flavanols
82	6.581	446.404	$C_{22}H_{22}O_{10}$	Calycosin-7-O-beta-D-glucoside	0.0027	Isoflavonoids
83	6.590	464.419	$C_{22}H_{24}O_{11}$	Hesperetin 5-O-glucoside	1.6133	Flavanones
84	6.590	464.096	$C_{21}H_{20}O_{12}$	Spiraeoside	7.5218	Flavones and Flavanols
85	6.590	464.096	$C_{21}H_{20}O_{12}$	Isotrifoliin	7.3683	Flavones and Flavanols
86	6.590	462.360	$C_{21}H_{18}O_{12}$	Scutellarin	0.0010	Flavones and Flavanols
87	6.596	464.380	$C_{21}H_{20}O_{12}$	Isoquercitrin	8.8025	Flavones and Flavanols
88	6.620	448.101	$C_{21}H_{20}O_{11}$	Trifolin	0.1696	Flavones and Flavanols
89	6.620	448.383	$C_{21}H_{20}O_{11}$	Luteoloside	0.1774	Flavones and Flavanols
90	6.650	448.377	$C_{21}H_{20}O_{11}$	Kaempferol7-O-beta-D-glucopyranoside	0.1172	Flavones and Flavanols
91	6.664	448.380	$C_{21}H_{20}O_{11}$	Luteolin 7-O-glucoside	0.1331	Flavones and Flavanols
92	6.680	432.380	$C_{21}H_{20}O_{10}$	Apigenin 5-O-glucoside	0.0666	Flavones and Flavanols
93	6.720	462.404	$C_{22}H_{22}O_{11}$	Chrysoeriol C-hexoside	0.0445	Flavones and Flavanols
94	6.730	594.526	$C_{27}H_{30}O_{15}$	Lonicerin	1.4742	Flavones and Flavanols
95	6.780	625.560	$C_{28}H_{33}O_{16}$	Petunidin 3-O-rutinoside	0.0030	Anthocyanins
96	6.780	432.380	$C_{21}H_{20}O_{10}$	Genistin	0.0018	Isoflavonoids
97	6.790	624.552	$C_{28}H_{32}O_{16}$	Isorhamnetin-3-O-neohespeidoside	6.5757	Flavones and Flavanols
98	6.825	624.544	$C_{28}H_{32}O_{16}$	Narcissoside	1.5707	Flavones and Flavanols
99	6.837	580.535	$C_{27}H_{32}O_{14}$	Narirutin	0.0171	Flavanones
100	6.840	578.520	$C_{27}H_{30}O_{14}$	Isorhoifolin	0.1097	Flavones and Flavanols
101	6.860	462.410	$C_{22}H_{22}O_{11}$	Pratensein-7-O-glucoside	0.1006	Isoflavonoids
102	6.864	462.400	$C_{22}H_{22}O_{11}$	Tectoridin	0.0013	Isoflavonoids
103	6.880	316.262	$C_{16}H_{12}O_7$	Rhamnetin	0.0394	Flavones and Flavanols
104	6.885	304.250	$C_{15}H_{12}O_7$	Taxifolin	0.2520	Flavones and Flavanols
105	6.900	268.269	$C_{16}H_{12}O_4$	Tectochrysin	0.0005	Flavones and Flavanols
106	6.910	306.700	$C_{15}H_{11}ClO_5$	Pelargonidin chloride	0.0008	Anthocyanins
107	6.940	608.545	$C_{28}H_{32}O_{15}$	Chrysoeriol 7-O-rutinoside	0.0021	Flavones and Flavanols
108	6.940	578.520	$C_{27}H_{30}O_{14}$	Rhoifolin	0.2416	Flavones and Flavanols

(Continued)

TABLE 2 | (Continued)

No	RT (min)	Molecular Weight	Formula	Name	Relative content (%)	Class
109	6.970	270.241	$C_{15}H_{10}O_5$	6,7,4′-Trihydroxyisoflavone	0.0012	Isoflavonoids
110	6.970	448.383	$C_{21}H_{20}O_{11}$	Vincetoxicoside B	0.0033	Flavones and Flavanols
111	6.979	580.530	$C_{27}H_{32}O_{14}$	Naringin	0.0110	Flavanones
112	6.980	418.390	$C_{21}H_{22}O_9$	Liquiritin	0.0054	Flavanones
113	7.010	502.200	$C_{26}H_{30}O_{10}$	Phellodensin F	0.0034	Flavanones
114	7.020	434.121	$C_{21}H_{22}O_{10}$	Prunin	0.0868	Flavanones
115	7.038	432.380	$C_{21}H_{20}O_{10}$	Sophoricoside	0.0075	Isoflavonoids
116	7.060	272.069	$C_{15}H_{12}O_5$	Pinobanksin	0.1659	Flavanones
117	7.080	446.367	$C_{21}H_{18}O_{11}$	Apigenin7-O-beta-D-glucuronide	0.0006	Flavones and Flavanols
118	7.080	418.351	$C_{20}H_{18}O_{10}$	Kaempferol 3-A-L-Arabinopyranoside	0.0060	Flavones and Flavanols
119	7.080	432.420	$C_{22}H_{24}O_9$	Heptamethoxyflavone	0.0076	Flavones and Flavanols
120	7.080	434.400	$C_{21}H_{20}O_{10}$	Resokaempferol 7-O-hexoside	0.0077	Flavones and Flavanols
121	7.120	608.545	$C_{28}H_{32}O_{15}$	Neodiosmin	0.0006	Flavones and Flavanols
122	7.120	536.000	$C_{24}H_{24}O_{14}$	Eriodictyol O-malonylhexoside	0.0030	Flavanones
123	7.180	462.404	$C_{22}H_{22}O_{11}$	Chrysoeriol 5-O-hexoside	0.0047	Flavones and Flavanols
124	7.186	462.403	$C_{22}H_{22}O_{11}$	Homoplantaginin	0.0058	Flavones and Flavanols
125	7.190	480.376	$C_{21}H_{20}O_{13}$	Myricetin 3-O-galactoside	0.0185	Flavones and Flavanols
126	7.190	492.430	$C_{23}H_{24}O_{12}$	Tricin 5-O-hexoside	0.0017	Flavones and Flavanols
127	7.200	610.560	$C_{28}H_{34}O_{15}$	Neohesperidin	0.0461	Flavanones
128	7.200	448.400	$C_{22}H_{22}O_{11}$	Chrysoeriol 7-O-hexoside	0.0087	Flavones and Flavanols
129	7.240	448.380	$C_{21}H_{20}O_{11}$	Quercitrin	0.0074	Flavones and Flavanols
130	7.240	436.410	$C_{21}H_{24}O_{10}$	Phlorizin	0.0718	Chalcones and dihydrochalcones
131	7.300	476.430	$C_{23}H_{24}O_{11}$	Methylchrysoeriol C-hexoside	0.0030	Flavones and Flavanols
132	7.320	526.490	$C_{27}H_{26}O_{11}$	Tricin 4′-O-(beta-guaiacylglyceryl) ether	0.0316	Flavones and Flavanols
133	7.340	318.240	$C_{15}H_{10}O_8$	Myricetin	0.0088	Flavones and Flavanols
134	7.350	432.106	$C_{21}H_{20}O_{10}$	Kaempferin	0.0009	Flavones and Flavanols
135	7.360	432.106	$C_{21}H_{20}O_{10}$	Kaempferol 7-O-rhamnoside	0.0007	Flavones and Flavanols
136	7.372	582.550	$C_{27}H_{34}O_{14}$	Naringin dihydrochalcone	0.0001	Flavanones
137	7.400	688.639	$C_{33}H_{36}O_{16}$	Tricin 4′-O-(β-guaiacylglyceryl) ether O-hexoside	0.0165	Flavones and Flavanols
138	7.417	286.240	$C_{15}H_{10}O_6$	Fisetin	0.0190	Flavones and Flavanols
139	7.471	418.394	$C_{21}H_{22}O_9$	Isoliquiritin	0.0068	Chalcones and dihydrochalcones
140	7.500	330.289	$C_{17}H_{14}O_7$	Tricin	0.1054	Flavones and Flavanols
141	7.500	578.470	$C_{26}H_{26}O_{15}$	Tricin O-malonylhexoside	0.0295	Flavones and Flavanols
142	7.510	688.630	$C_{33}H_{36}O_{16}$	Tricin 4′-O-(beta-guaiacylglyceryl) ether 5-O-hexoside	0.0137	Flavones and Flavanols
143	7.523	436.409	$C_{21}H_{24}O_{10}$	Trilobatin	0.0356	Chalcones and dihydrochalcones
144	7.575	446.360	$C_{21}H_{18}O_{11}$	Baicalin	0.0095	Flavones and Flavanols
145	7.635	286.236	$C_{15}H_{10}O_6$	Scutellarein	0.0022	Flavones and Flavanols
146	7.660	314.289	$C_{17}H_{14}O_6$	Kumatakenin	0.0134	Flavones and Flavanols
147	7.660	254.240	$C_{15}H_{10}O_4$	4′,7-Dihydroxyflavone	0.0164	Flavones and Flavanols
148	7.670	534.420	$C_{24}H_{22}O_{14}$	Tricin 5-O-hexoside derivative	0.0073	Flavones and Flavanols
149	7.790	592.553	$C_{28}H_{32}O_{14}$	Linarin	0.0019	Flavones and Flavanols
150	7.860	622.571	$C_{29}H_{34}O_{15}$	Pectolinarin	0.0015	Flavones and Flavanols
151	7.879	594.520	$C_{30}H_{26}O_{13}$	Tiliroside	0.0020	Flavones and Flavanols
152	7.971	416.000	$C_{21}H_{20}O_9$	Apigenin 4-O-rhamnoside	0.0002	Flavones and Flavanols
153	7.999	288.252	$C_{15}H_{12}O_6$	Eriodictyol	0.3773	Flavanones
154	8.020	286.048	$C_{15}H_{10}O_6$	2′-Hydroxygenistein	0.0155	Isoflavonoids
155	8.049	594.561	$C_{28}H_{34}O_{14}$	Poncirin	0.0011	Flavanones
156	8.050	302.043	$C_{15}H_{10}O_7$	Morin	1.3804	Flavones and Flavanols
157	8.060	286.240	$C_{15}H_{10}O_6$	Luteolin	0.0010	Flavones and Flavanols
158	8.100	668.600	$C_{33}H_{32}O_{15}$	Tricin O-sinapoylpentoside	0.0003	Flavones and Flavanols
159	8.180	284.263	$C_{16}H_{12}O_5$	Calycosin	0.0329	Isoflavonoids
160	8.229	314.246	$C_{16}H_{10}O_7$	Wedelolactone	0.0004	Isoflavonoids
161	8.260	460.388	$C_{22}H_{20}O_{11}$	Wogonoside	0.0027	Flavones and Flavanols
162	8.518	272.253	$C_{15}H_{12}O_5$	Naringenin chalcone	5.6733	Chalcones and dihydrochalcones

(Continued)

TABLE 2 | (Continued)

No	RT (min)	Molecular Weight	Formula	Name	Relative content (%)	Class
163	8.519	500.840	$C_{21}H_{21}ClO_{12}$	Myrtillin chloride	0.0002	Anthocyanins
164	8.598	274.270	$C_{15}H_{14}O_5$	Phloretin	0.2532	Chalcones and dihydrochalcones
165	8.610	272.069	$C_{15}H_{12}O_5$	Butin	4.9779	Flavanones
166	8.645	270.280	$C_{16}H_{14}O_4$	Echinatin	0.0001	Chalcones and dihydrochalcones
167	8.683	272.250	$C_{15}H_{12}O_5$	Naringenin	5.7225	Flavanones
168	8.700	270.240	$C_{15}H_{10}O_5$	Apigenin	0.0057	Flavones and Flavanols
169	8.720	270.240	$C_{15}H_{10}O_5$	Genistein	0.0002	Isoflavonoids
170	8.800	302.327	$C_{17}H_{18}O_5$	Isomucronulatol	0.0010	Isoflavonoids
171	8.811	286.240	$C_{15}H_{10}O_6$	Kaempferol	0.0106	Flavones and Flavanols
172	8.849	300.263	$C_{16}H_{12}O_6$	Tectorigenin	0.0009	Isoflavonoids
173	8.860	302.079	$C_{16}H_{14}O_6$	7-O-Methyleriodictyol	0.0017	Flavanones
174	8.911	300.260	$C_{16}H_{12}O_6$	Diosmetin	0.0031	Flavones and Flavanols
175	8.960	302.236	$C_{15}H_{10}O_7$	Quercetin	0.0197	Flavones and Flavanols
176	8.969	316.262	$C_{16}H_{12}O_7$	Isorhamnetin	0.0097	Flavones and Flavanols
177	8.972	302.270	$C_{16}H_{14}O_6$	Hesperetin	0.0073	Flavanones
178	9.020	330.074	$C_{17}H_{14}O_7$	3,7-Di-O-methylquercetin	0.0008	Flavones and Flavanols
179	9.160	360.320	$C_{18}H_{16}O_8$	5,7,3′-trihydroxy-6,4′,5′-trimethoxyflavone	0.0000	Flavones and Flavanols
180	9.190	330.100	$C_{17}H_{14}O_7$	Di-O-methylquercetin	1.5690	Flavones and Flavanols
181	9.370	372.375	$C_{20}H_{20}O_7$	Isosinensetin	0.0001	Flavones and Flavanols
182	9.550	372.370	$C_{20}H_{20}O_7$	Sinensetin	0.0002	Flavones and Flavanols
183	9.590	338.360	$C_{20}H_{18}O_5$	Wighteone	0.0004	Isoflavonoids
184	9.594	300.263	$C_{16}H_{12}O_6$	Hydroxygenkwanin	0.6367	Flavones and Flavanols
185	9.870	284.263	$C_{16}H_{12}O_5$	Maackiain	0.0168	Isoflavonoids
186	9.935	300.310	$C_{17}H_{16}O_5$	Farrerol	0.0002	Flavanones
187	10.004	344.320	$C_{18}H_{16}O_7$	Eupatilin	0.0001	Flavones and Flavanols
188	10.110	284.225	$C_{15}H_8O_6$	Rhein	0.0004	Anthocyanins
189	10.287	284.260	$C_{16}H_{12O5}$	Wogonin	0.0014	Flavones and Flavanols
190	10.315	286.279	$C_{16}H_{14}O_5$	Isosakuranetin	0.0023	Flavanones
191	10.340	374.347	$C_{19}H_{18}O_8$	Chrysosplenetin B	0.0001	Flavones and Flavanols
192	10.372	256.250	$C_{15}H_{12}O_4$	Pinocembrin	0.0015	Flavanones
193	10.373	514.520	$C_{27}H_{30}O_{10}$	Baohuoside I	0.0004	Flavones and Flavanols
194	10.374	374.341	$C_{19}H_{18}O_8$	Casticin	0.0005	Flavones and Flavanols
195	10.400	286.328	$C_{17}H_{18}O_4$	Loureirin A	0.0010	Chalcones and dihydrochalcones
196	10.518	402.390	$C_{21}H_{22}O_8$	Nobiletin	0.0046	Flavones and Flavanols
197	10.520	356.332	$C_{19}H_{16}O_7$	6-Formyl-isoophiopogonanone A	0.0022	Flavanones
198	10.550	284.263	$C_{16}H_{12}O_5$	Oroxylin A	0.0005	Flavones and Flavanols
199	10.780	314.295	$C_{17}H_{14}O_6$	Pectolinarigenin	0.0002	Flavones and Flavanols
200	11.410	372.370	$C_{20}H_{20}O_7$	Tangeretin	0.0030	Flavones and Flavanols
201	11.419	336.720	$C_{16}H_{13}ClO_6$	Peonidin chloride	0.0007	Anthocyanins
202	11.520	388.368	$C_{20}H_{20}O_8$	Demethylnobiletin	0.0001	Flavones and Flavanols
203	11.564	224.250	$C_{15}H_{12}O_2$	Flavanone	0.0007	Flavanones
204	11.740	298.295	$C_{17}H_{14}O_5$	Mosloflavone	0.0009	Flavones and Flavanols
205	11.930	324.370	$C_{20}H_{20}O_4$	Isobavachalcone	0.0005	Chalcones and dihydrochalcones
206	12.143	394.420	$C_{23}H_{22}O_6$	Deguelin	0.0001	Isoflavonoids
207	12.670	368.380	$C_{21}H_{20}O_6$	Anhydroicaritin	0.0000	Flavones and Flavanols

Aβ deposition in the head was measured using Image-Pro Plus version 5.1.

Determination of Ache Activity

After co-treatment with $AlCl_3$ and EUMF, zebrafish larvae at 6 dpf were killed by tricaine (Sigma-Aldrich, Darmstadt, Germany). Cold physiological saline was added to the larvae in a 2 mL tube at a ratio of 1:9 (mass:volume) without any additional water. Next, the samples were homogenized using automated tissue homogenization, followed by centrifuged at 2,500 rpm for 10 min at 0°C. The supernatant was collected for the assay. The enzyme activity of Ache was determined by using the Amplite^{TM} Fluorimetric Acetylcholinesterase Assay Kit (AAT Bioquest, California, United States) according to the manufacturer's instructions with a slight modification as follows. The acetylthiocholine reaction mixture was 50 μM.

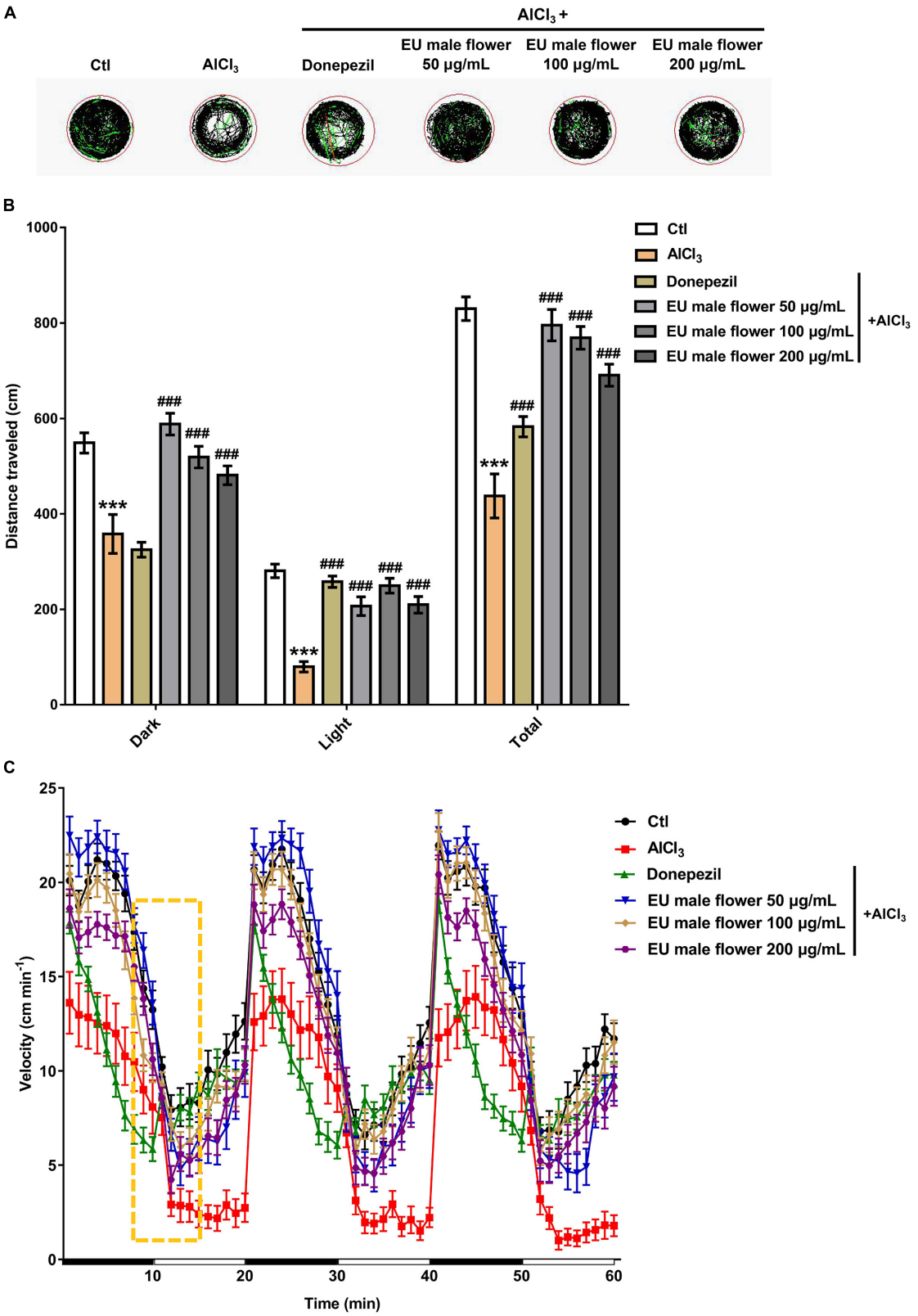

FIGURE 2 | Effect of EUMF on $AlCl_3$-induced locomotion impairments in zebrafish. **(A)** Dgital track map. The red, green, and black lines depict fast, medium, and slow movements, respectively (n = 10). **(B)** Total distance moved in the Ctl, $AlCl_3$, and $AlCl_3$ + EUMF groups ($^{***}P < 0.001$ vs. Ctl; $^{###}P < 0.001$ vs. $AlCl_3$; n = 10). **(C)** Speed change in the Ctl, $AlCl_3$, and $AlCl_3$ + EUMF groups (the speed change after light stimulus is demarcated by the frame; n = 10).

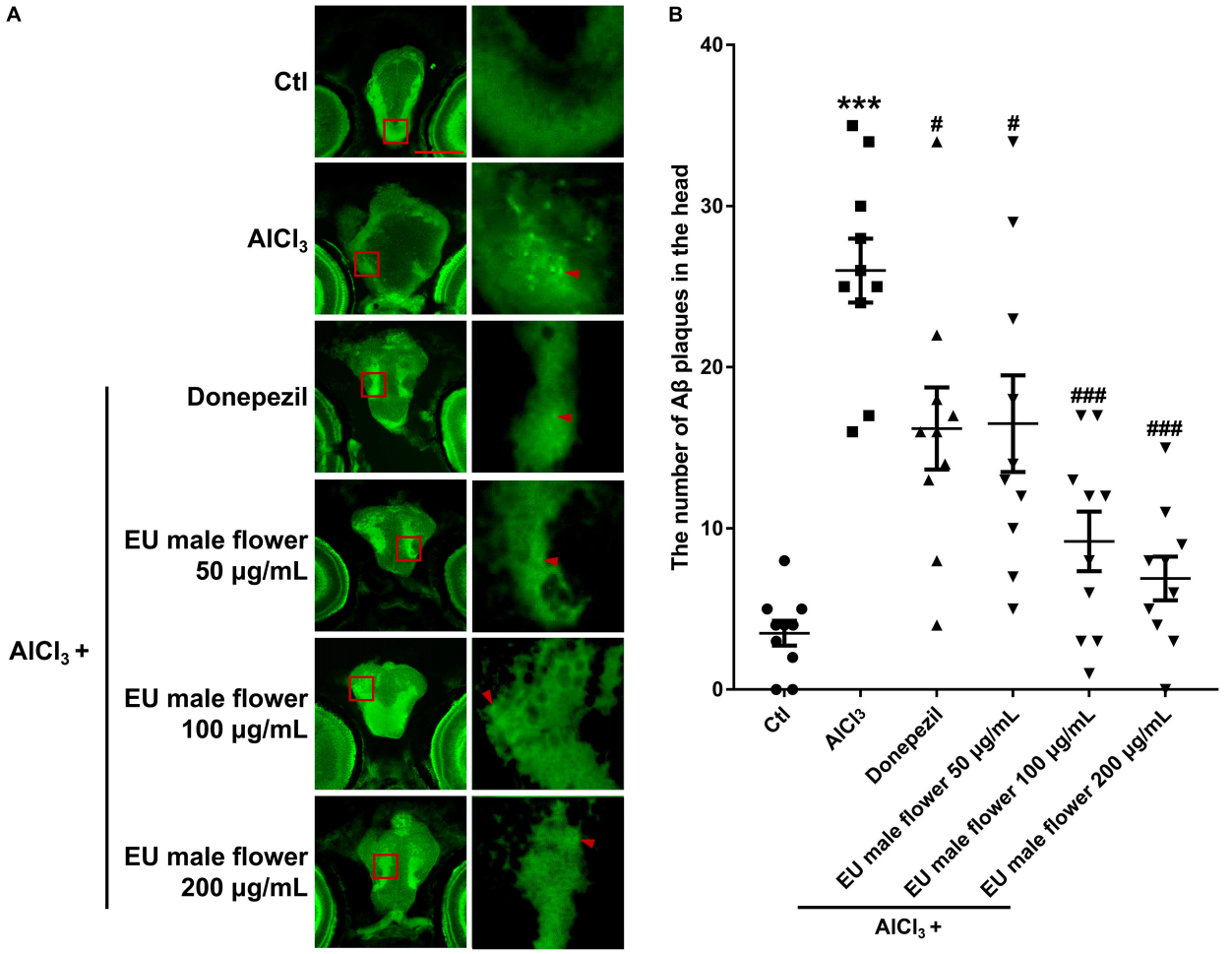

FIGURE 3 | Inhibition of EUMF on Aβ aggregation in zebrafish. **(A)** The Aβ plaques in the brain region were stained using thioflavin S in the Ctl, $AlCl_3$, and $AlCl_3$ + EUMF groups (Aβ is demarcated by arrows; scale bar = 100 μm). **(B)** Statistical analysis of the Aβ plaque count in each group (***$P < 0.001$ vs. Ctl; $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ vs. $AlCl_3$; $n = 10$).

The test samples addition added into the acetylthiocholine reaction mixture was also 50 μM. The fluorescence at Ex/Em = 490/520 was monitored.

Apoptosis Assessment

Apoptotic cells in the head were assessed using the One Step TUNEL Apoptosis Assay Kit (Beyotime, Jiangsu, China). Briefly, the zebrafish larvae at 6 dpf were fixed in 4% paraformaldehyde. Next, they were blocked with 3% hydrogen peroxide in methanol and incubated with the TUNEL reaction mixture. The larvae were photographed by using a fluorescence microscope (Zeiss, Jena, Germany). The fluorescence intensities of apoptotic cells in the head were measured using Image-Pro Plus version 5.1.

Detection of Gene Expression

The expression of six genes: *autophagy and beclin 1 regulator 1a* (*ambra1a*), *autophagy-related gene 5* (*atg5*), *unc-51 like autophagy activating kinase 1* (*ulk1b*), *autophagy-related ubiquitin-like modifier LC3 B* (*lc3b*), *acetylcholinesterase* (*ache*), and *solute carrier family 6 member 3* (*slc6a3*) were detected in the zebrafish larvae using RT-qPCR. The total RNA was extracted from the larval tissue using the EASY spin Plus RNA Mini Kit (Aidlab Biotechnologies, Beijing, China) according to manufacturer instructions. Next, RNA was reverse transcribed into cDNA using the PrimeScript™ RT Master Mix (Takara Biomedical Technology Co., Ltd., Beijing, China), The RT-qPCR was conducted using the SYBR® Premix DimerEraser™ (Takara Biomedical Technology Co., Ltd., Beijing, China). The housekeeping gene, *rpl13a,* was used as a reference gene. The primer sequences of the above genes are shown in **Table 1**.

Statistical Analysis

The data are presented as mean ± SEM. The statistical analyses were conducted using Graph Pad Prism 8.0 (GraphPad Software; San Diego, CA, United States) by a one-way ANOVA followed by the Dunnett's multiple comparison test. If the *P*-value was less than 0.05, the difference was considered as significant.

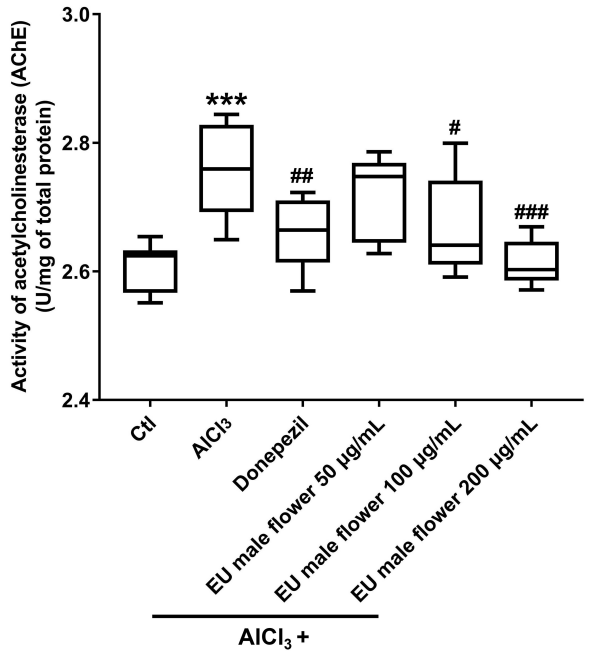

FIGURE 4 | Inhibition of EUMF on the AChE activity in zebrafish (***$P < 0.001$ vs. Ctl; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. $AlCl_3$; $n = 10$).

RESULTS

Flavonoids Compounds Analysis of the *Eucommia ulmoides* Olive Male Flower

A large number of literature studies have shown that flavonoids show neuroprotective effects against AD (Remya et al., 2012; Remya et al., 2014; Bakhtiari et al., 2017; Zhao et al., 2019; Li et al., 2021; Noori et al., 2021; Pragya and Arun, 2021). Thus, the flavonoids were selected as the primary components of EUMF for further study. The total contents of the EUMF flavonoids were determined according to the obtained standard curves of the total flavonoids which was reported in a previous study from our lab (Zhang et al., 2020). According to regression equations ($y = 0.0003x + 0.0107$), the total contents of the EUMF flavonoids were 45.99 ± 0.5853 mg/g. Moreover, the targeted LC-MS analysis of the flavonoids showed that total 206 compounds were detected (**Table 2**). Among them, 125 flavonoids and flavanols, 32 flavanones, 22 isoflavonoids, 11 chalcones and dihydrochalcones, and 17 anthocyanins were identified. In addition, quercetin-3′-O-glucoside (relative content of 9.1850%), isoquercitrin (8.8025%), rutin hydrate (8.0753%), rutin (7.9072%), spiraeoside (7.5218%), isotrifoliin (7.3683%), isorhamnetin-3-O-neohespeidoside (6.5757%), naringenin (5.7225%), naringenin chalcone (5.6733%), butin, myricitrin, isomucronulatol-7-O-glucoside, hyperoside, hesperetin 5-O-glucoside, narcissoside, di-O-methylquercetin, lonicerin and morin were the primary components of EUMF. The relative content of these 18 components accounted for greater than 90% of the total flavonoids.

Dyskinesia Rehabilitation Effects of *Eucommia ulmoides* Olive Male Flower in Zebrafish Larvae

Behavioral tests were performed on the zebrafish larvae at 6 dpf. As shown in **Figure 2A**, The black lines, green lines, and red lines indicate slow, medium, and fast movements, respectively. We found that the distance traveled by zebrafish in the AD model group was significantly shorter than the zebrafish in the untreated group, whether in light or dark environments (**Figure 2B**). The speed change of the zebrafish in the AD model group was also notably weakened after light stimulus alteration compared with the zebrafish in the untreated group (**Figure 2C**). These results indicated that $AlCl_3$ lessened the locomotor capacity of the zebrafish, and this was consistent with the previous study (Pan et al., 2019; Li et al., 2020). Accordingly, the establishment of zebrafish AD model was successful. After treatment with 4.0 μM donepezil, the distance traveled and speed change of the zebrafish both increased compared with the zebrafish in the AD group (**Figures 2B,C**). This implied that donepezil improved the dyskinesia of zebrafish induced by $AlCl_3$. Interestingly, a similar trend of behavioral change in the positive group was also observed in the EUMF treatment groups. When the zebrafish were co-treated with $AlCl_3$ and different concentrations of the EUMF (50, 100, and 200 μg/mL), their dyskinesias were also reduced. In particular, the EUMF treatment correlated with a longer distance in dark environments than that of the donepezil group (**Figures 2B,C**). The above results indicated that the EUMF improved the exercise capacity and may play a protective role against $AlCl_3$-induced AD-like symptoms in zebrafish.

Inhibition the Amyloid β-Protein Aggregation Effects of *Eucommia ulmoides* Olive Male Flower in the Zebrafish Larvae

Amyloid β-protein deposition is an important clinical hallmark in AD patients (Meldolesi, 2017). To further identify the anti-AD activity of the EUMF, the Aβ plaques in the heads of zebrafish were quantitatively determined. As shown in **Figure 3**, only a few of the Aβ plaques were observed in the brain of the untreated group. In contrast, there were many large Aβ plaques in the brain of the AD model group. Compared with the AD group, larval treatment with donepezil or EUMF (50, 100, and 200 μg/mL) significantly reduced the Aβ plaque count. These results implied that EUMF had anti-AD activity.

Inhibitory Activity of *Eucommia ulmoides* Olive Male Flower on the Ache Activity

Ache is an enzyme that can degrade ACh. Many studies have proposed that a reduced level of ACh may be the primary etiology of AD. Hence, Ache has also been proposed to be related to the formation of AD (Remya et al., 2013; Hu et al., 2019). Based on this, we assessed the activity of Ache to explore the protective mechanism of EUMF on AD. As shown in **Figure 4**, the AD model group showed a higher activity of Ache compared with the untreated group. However, the groups co-treated with

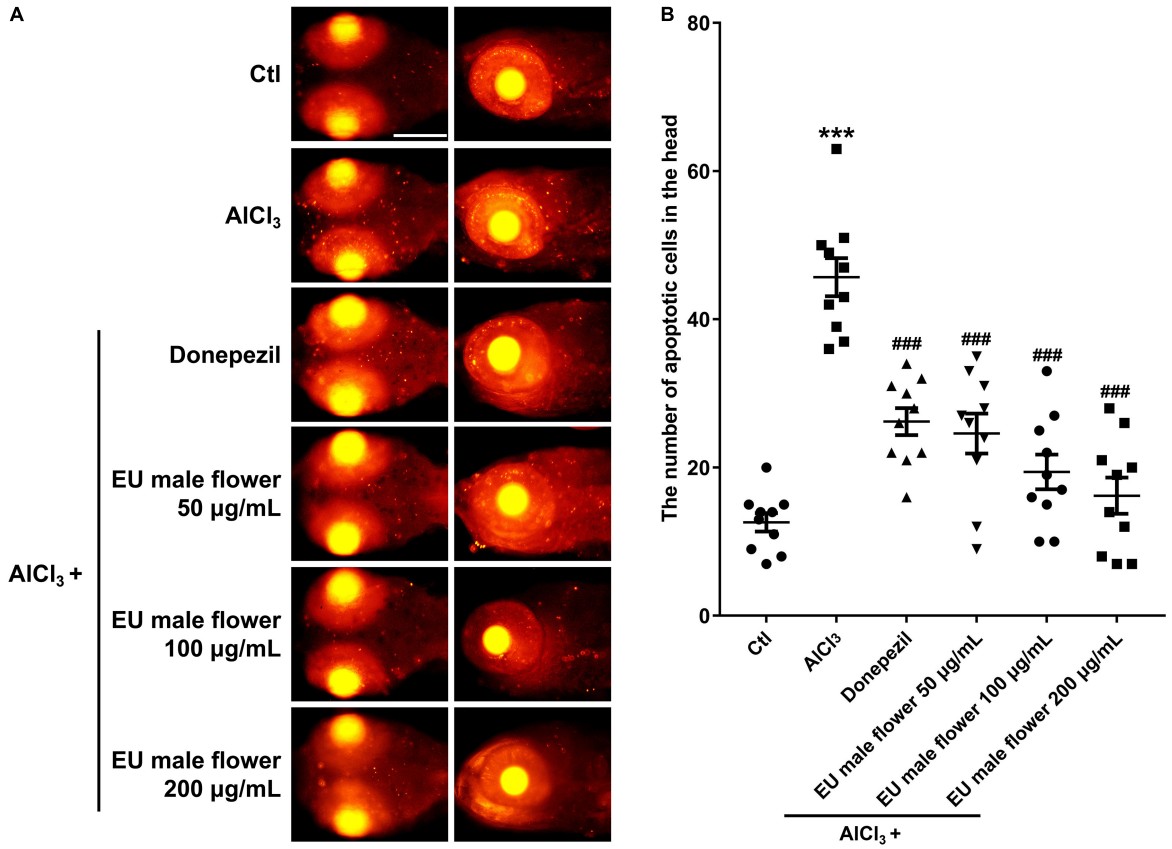

FIGURE 5 | Effect of EUMF on apoptosis in the brains of the $AlCl_3$-modeled zebrafish. **(A)** The apoptotic cells were stained with TUNEL (scale bar = 100 μm). **(B)** Statistical analysis of the apoptotic cells count in the larvae heads ($^{***}P < 0.001$ vs. Ctl; $^{###}P < 0.001$ vs. $AlCl_3$; $n = 10$).

both $AlCl_3$ and donepezil or different concentrations of EUMF showed reduced activity of Ache compared with the AD model group. Our results indicated that the EUMF may be an effective therapeutic agent for AD by suppressing the activity of Ache.

Effect of *Eucommia ulmoides* Olive Male Flower on $AlCl_3$-Induced Apoptosis in the Brain

We found many apoptotic cells that appeared primarily in the brain region in the zebrafish AD model. In contrast, no obvious apoptotic cells were observed in the control group. Donepezil or different concentrations of the EUMF treatment significantly reduced the number of apoptotic cells in the zebrafish brains (**Figure 5**). The above results suggested that EUMF suppressed the apoptosis induced by $AlCl_3$ in zebrafish brain.

Effect of *Eucommia ulmoides* Olive Male Flower on the Expression of Autophagy-Related and Neurotransmitter-Related Genes

Many lines of evidence have suggested that dysregulated autophagy is implicated in a pathogenic role in the neurological diseases (Sheng et al., 2010; Chu, 2018). Therefore, we assayed the expression of autophagy-related genes to investigate whether EUMF protected against AD-like symptoms by regulating autophagy. *Ambra1a*, *atg5*, *ulk1b*, and *lc3b* are core members involved in autophagy (Kang et al., 2011; Jiang et al., 2013). We found that transcript levels of aforementioned genes were significantly upregulated in the AD model group compared with the control, while when the EUMF reached a certain concentration, it reversed the increases (**Figure 6**). In addition, we also found that the EUMF treatment under a certain concentration downregulated the expression level of *ache* and *slc6a3*, and these were drastically increased after treatment with $AlCl_3$ (**Figure 6**).

DISCUSSION

Alzheimer's disease is the most common clinical degenerative disease associated with aging. The complex pathogenetic factors of AD have limited its effective treatment. EUO is a traditional Chinese medicine. It has been reported that the extracts of EUO leaf can be used to treat AD. Therefore, we investigated the therapeutic effects of its male flowers on AD like symptoms using zebrafish. We found that the dyskinesia in the zebrafish AD model was significantly improved by EUMF. The Aβ plaques

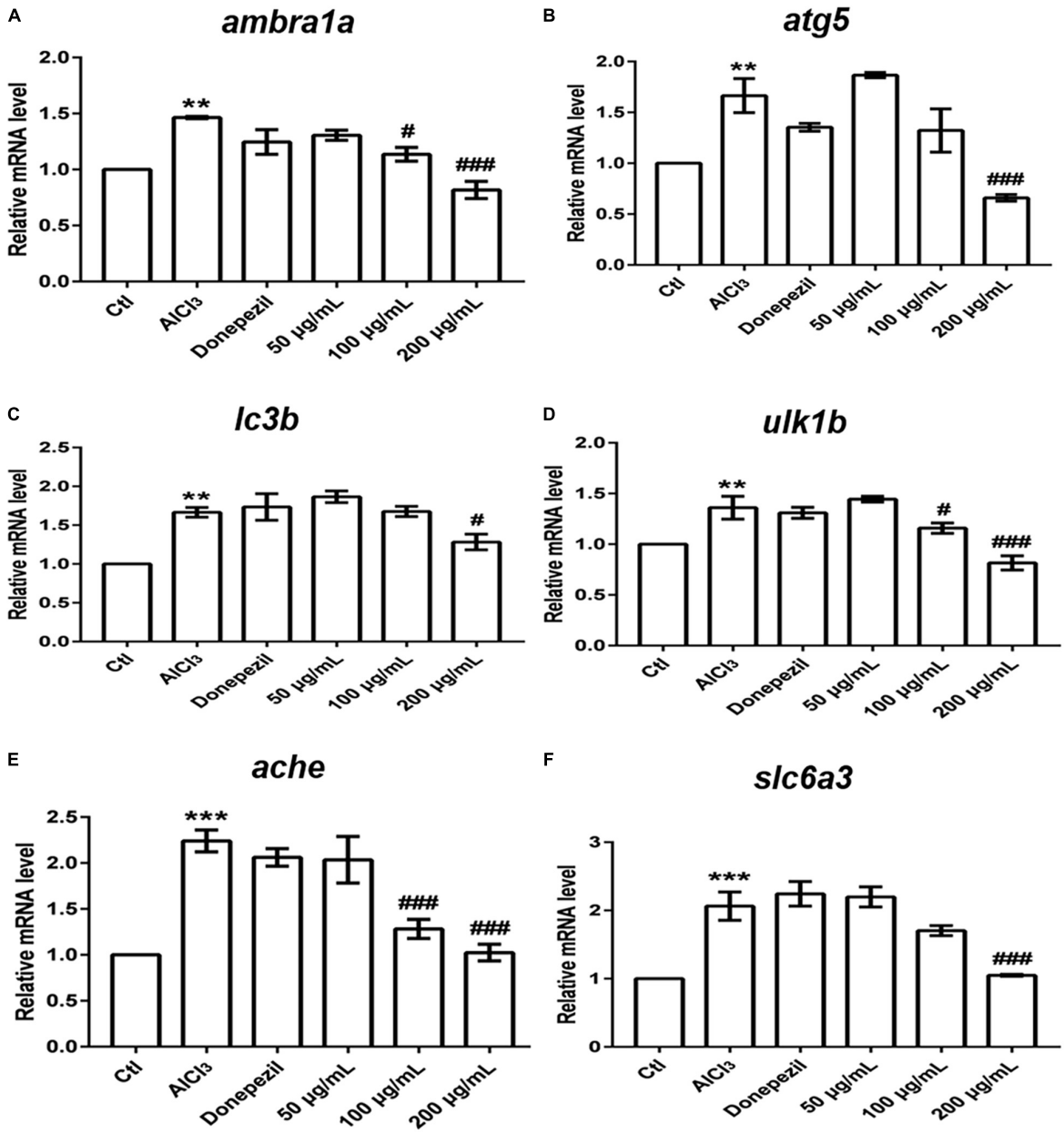

FIGURE 6 | Transcriptional alterations of genes. The amount of gene expression was exhibited as the relative expression (shown as fold) compared with the Ctl. ($^{**}P < 0.01$, $^{***}P < 0.001$ vs. Ctl; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. $AlCl_3$). **(A–D)** Expressions of genes involved in autophagy. **(E)** Transcript levels of *ache*. **(F)** Transcript levels of *slc6a3*.

count, Ache activity, and number of apoptotic cells in the zebrafish AD model were also clearly reduced by EUMF. The above results indicated that the EUMF may be an agent for AD treatment. In addition, mechanism investigation revealed that the anti-AD activity of the EUMF may be related to its inhibition of excessive autophagy and abnormal expressions of *ache* and *slc6a3* genes.

Autophagy is an important biological process by which cellular material is degraded by lysosomes or vacuoles and recycled. Paradoxically, it has the characteristics of a double-edged sword. Autophagy can serve to protect the nervous system by clearing degrading damaged organelles or accumulated misfolded proteins in neurons, but it may also induce neuron death and damage the nervous system (Shintani and Klionsky, 2004). Previous studies have shown that autophagy influences the secretion of Aβ to the extracellular space in neurons through either excretory or exocytic mechanisms, and hence it plays a critical role in Aβ plaque formation. Furthermore, extracellular Aβ plaques accumulation is an important pathogenic factor leading to AD (Nilsson et al., 2015). Based on these facts,

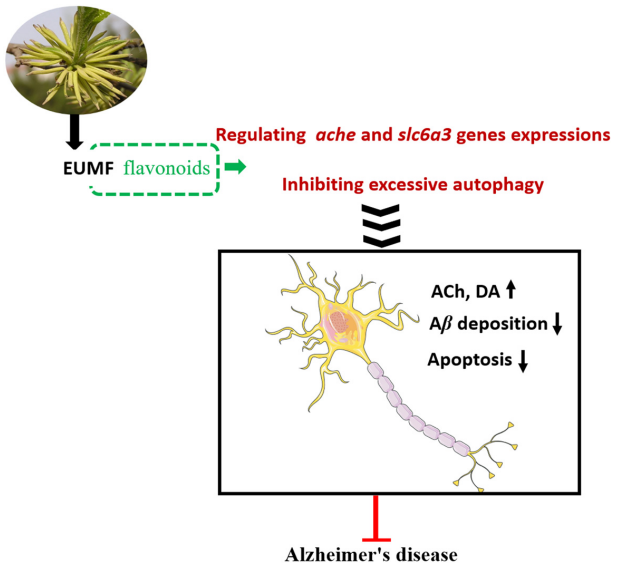

FIGURE 7 | The proposed mechanism underlying the anti-AD effect of EUMF. EUMF inhibits the excessive autophagy and abnormal expressions of the *ache* and *slc6a3* genes to exert the therapeutic effects against AD-like symptoms. Flavonoids in EUMF may contribute to this biological process.

we hypothesized that $AlCl_3$ may activate abnormal excessive autophagy by upregulating the expression of *ambra1a*, *atg5*, *ulk1b*, and *lc3b* in zebrafish. Then further damage, referring to the deposition of extracellular Aβ plaques induced by abnormal excessive autophagy, would occur. Finally, AD-like symptoms in the zebrafish were induced. However, EUMF restored high expressions of *ambra1a*, *atg5*, *ulk1b*, and *lc3b* induced by $AlCl_3$. Thus, autophagy was not excessively activated. Accordingly, this reduced the extracellular Aβ plaque count and reversed AD's disease-like pathology in zebrafish.

Ache is the gene that encode Ache that inactivates the neurotransmitter ACh by catalyzing its hydrolysis to choline and acetic acid (Hu et al., 2019). *Slc6a3* is the gene that encode the dopamine transporter (Dat) that can provide rapid clearance of dopamine (DA) (Dedic et al., 2018). The primary function of ACh is to complete the transmission of neural signals. Once the synthesis and decomposition of ACh is abnormal, neural signaling transition may be blocked. To some extent, AD will be the result (Hu et al., 2019). DA is also a neurotransmitter that is critically implicated in cognitive function. Previous studies have found that the restoration of DA transmission plays a role in learning and memory in the mouse model of AD. DA dysfunction has a pathogenic role in the cognitive decline symptoms of AD (Martorana and Koch, 2014). Because Ache and Dat are inhibitors of ACh and DA, respectively, it is conceivable that they also play a critical role in the occurrence of AD. Interestingly, our results showed that both the expressions of *ache* and *slc6a3* genes were upregulated in the AD zebrafish model. However, treatment with EUMF reduced these increased expressions. Collectively, we suggest that beside of inhibiting the abnormal excessive autophagy, EUMF also reverse AD-like pathology in zebrafish by regulating the expressions of *ache* and *slc6a3* at the transcript levels. Definitely, we will perform gene expression test of other neurotransmitters including glutamate in the future to further investigate the underlying mechanism.

Flavonoids are a group of plant metabolites which can improve the cognitive functions. They can work within the processes associated with AD (Kaur et al., 2022; Maccioni et al., 2022). For example, quercetin belonging to the subcategory of flavonoids can significantly mitigate memory deficits in scopolamine mice model via protection against neuroinflammation and neurodegeneration (Olayinka et al., 2022). Eriodictyol which is a natural flavonoid compound can ameliorate cognitive dysfunction in APP/PS1 mice by inhibiting ferroptosis (Li L. et al., 2022). Anthocyanins can reduce the neuronal damage in *in vivo* and *in vitro* models of AD (Li H. et al., 2022). Here, we identified many flavonoids including quercetin-3′-O-glucoside, isoquercitrin, rutin hydrate, rutin, spiraeoside, isotrifoliin, isorhamnetin-3′-O-neohespeidoside, naringenin, naringenin chalcone, butin, myricitrin, isomucronulatol-7-O-glucoside, hyperoside, hesperetin 5-O-glucoside, narcissoside, di-O-methylquercetin, lonicerin, and morin in EUMF. Therefore, flavonoids in EUMF may contribute to its anti-AD effects. However, one limitation of this study is that the exact compounds of flavonoids in EUMF, which act as a promising agent against AD need further investigation. In the further work, we will analyze the composition and activity of the flavonoid compounds in EUMF to thoroughly understand the anti-AD activity of EUMF.

CONCLUSION

In conclusion, our study provided evidence that EUMF had anti-AD activity. EUMF ameliorated AD-like pathology in zebrafish possibly by inhibiting excessive autophagy and the abnormal expressions of *ache* and *slc6a3*. Flavonoid compounds in the EUMF may contribute to this biological process (**Figure 7**). Our data implied that EUMF is an attractive therapeutic candidate for AD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committe of Biology Institute, Shandong Academy of Sciences.

AUTHOR CONTRIBUTIONS

MJ conceptualized the idea and supervised the entire study. CS, SZ, SB, and JD performed the study and analyzed the results. MJ, QR, and YZ analyzed the results. CS and SZ

wrote the manuscript. MJ and KL revised the manuscript and contributed to the final form. All authors read and approved the final manuscript.

FUNDING

This work was supported by the Belt and Road Innovative Foreign Experts Project (No. DL2021023004L), the National Natural Science Foundation of China (No. 42006090), and the Jinan Talent Project for Universities (Nos. 2021GXRC106, 2021GXRC111, and 2020GXRC031). We are also grateful for grants from Shandong High-end Foreign Experts Recruitment Program (Nos. WST2019006 and WST2020008), Key R&D Project of Shandong Province (No. 2021CXGC010507), and Science, Education, and Industry Integration Innovation Pilot Project of Qilu University of Technology (Shandong Academy of Sciences) (No. 2020KJC-ZD10).

REFERENCES

Bakhtiari, M., Panahi, Y., Ameli, J., and Darvishi, B. (2017). Protective effects of flavonoids against Alzheimer's disease-related neural dysfunctions. *Biomed. Pharmacother.* 93, 218–229. doi: 10.1016/j.biopha.2017.06.010

Chao, F., Jiang, L., Zhang, Y., Zhou, C., Zhang, L., Tang, J., et al. (2018). Stereological investigation of the effects of treadmill running exercise on the hippocampal neurons in middle-aged APP/PS1 transgenic mice. *J. Alzheimers Dis.* 63, 689–703. doi: 10.3233/JAD-171017

Chu, C. T. (2018). Autophagy in neurological diseases: an update. *Neurobiol. Dis.* 122, 1–2. doi: 10.1016/j.nbd.2018.10.010

Dedic, N., Kühne, C., Jakovcevski, M., Hartmann, J., Genewsky, A. J., Gomes, K. S., et al. (2018). Deussing, chronic CRH depletion from GABAergic, long-range projection neurons in the extended amygdala reduces dopamine release and increases anxiety. *Nat. Neurosci.* 21, 803–807. doi: 10.1038/s41593-018-0151-z

Hao, S., Xiao, Y., Lin, Y., Mo, Z. T., Chen, Y. X., Peng, F., et al. (2016). Chlorogenic acid-enriched extract from *Eucommia ulmoides* leaves inhibits hepatic lipid accumulation through regulation of cholesterol metabolism in HepG2 cells. *Pharm. Biol.* 54, 251–259. doi: 10.3109/13880209.2015.1029054

He, X., Wang, J., Li, M., Hao, D., Yang, Y., Zhang, C. R., et al. (2014). Eucommia ulmoides Oliv.: ethnopharmacology, phytochemistry and pharmacology of an important traditional Chinese medicine. *J. Ethnopharmacol.* 151, 78–92. doi: 10.1016/j.jep.2013.11.023

Hong, Y., Yu, L., Tingting, L., Lee, H. J., Fang, L., Wang, Y., et al. (2020). A map of cis-regulatory elements and 3D genome structures in zebrafish. *Nature* 588, 337–343. doi: 10.1038/s41586-020-2962-9

Hu, Y. H., Yang, J., Zhang, Y., Liu, K. C., Liu, T., Sun, J., et al. (2019). Synthesis and biological evaluation of 3–(4-aminophenyl)-coumarin derivatives as potential anti-Alzheimer's disease agents. *J. Enzyme Inhib. Med. Chem.* 34, 1083–1092. doi: 10.1080/14756366.2019.1615484

Huang, W., Li, C., Shen, Z., Zhu, X., Xia, B., and Li, C. (2016). Development of a zebrafish model for rapid drug screening against Alzheimer's disease. *J. Pharm. Pharmacol.* 4, 174–185. doi: 10.17265/2328-2150/2016.04.003

Huang, Y., and Mucke, L. (2012). Alzheimer mechanisms and therapeutic strategies. *Cell* 48, 1204–1222. doi: 10.1016/j.cell.2012.02.040

Jiang, T. F., Zhang, Y. J., Zhou, H. Y., Wang, H. M., Tian, L. P., Liu, J., et al. (2013). Curcumin ameliorates the neurodegenerative pathology in A53T alpha-synuclein cell model of Parkinson's disease through the downregulation of mTOR/p70S6K signaling and the recovery of macroautophagy. *J. Neuroimmune Pharmacol.* 8, 356–369. doi: 10.1007/s11481-012-9431-7

Kang, R., Zeh, H. J., Lotze, M. T., and Tang, D. (2011). The beclin1 network regulates autophagy and apoptosis. *Cell Death Differ.* 18, 571–580. doi: 10.1038/cdd.2010.191

Kaur, R., Sood, A., Lang, D. K., Bhatia, S., Al-Harrasi, A., Aleya, L., et al. (2022). Potential of favonoids as anti-Alzheimer's agents: bench to bedside. *Environ. Sci. Pollut. Res.* 29, 26063–26077. doi: 10.1007/s11356-021-18165-z

Kepp, K. P. (2016). Alzheimer's disease due to loss of function: a new synthesis of the available data. *Prog. Neurobiol.* 143, 36–60. doi: 10.1016/j.pneurobio.2016.06.004

Kerstin, H., Matthew, D. C., Carlos, T., Torrance, J., Berthelot, C., Muffato, M., et al. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 496, 498–503. doi: 10.1038/nature12111

Kobayashi, Y., Hiroi, T., Araki, M., Hirokawa, T., Miyazawa, M., Aoki, N., et al. (2012). Facilitative effects of *Eucommia ulmoides* on fatty acid oxidation in hypertriglyceridaemic rats. *J. Sci. Food Agric.* 92, 358–365. doi: 10.1002/jsfa.4586

Li, H., Zheng, T. T., Lian, F. Z., Xu, T., Yin, W. Y., and Jiang, Y. G. (2022). Anthocyanin-rich blueberry extracts and anthocyanin metabolite protocatechuic acid promote autophagy-lysosomal pathway and alleviate neurons damage in in vivo and in vitro models of Alzheimer's disease. *Nutrition* 93:111473. doi: 10.1016/j.nut.2021.111473

Li, L., Li, W. J., Zheng, X. R., Liu, Q. L., Du, Q., Jie, Y., et al. (2022). Eriodictyol ameliorates cognitive dysfunction in APP/PS1 mice by inhibiting ferroptosis via vitamin D receptor-mediated Nrf2 activation. *Mol. Med.* 28, 11–30. doi: 10.1186/s10020-022-00442-3

Li, Y., Qin, J., Cheng, Y., Ai, Y., Han, Z., Li, M., et al. (2021). Polysaccharide from *Patinopecten yessoensis* skirt boosts immune response via modulation of gut microbiota and short-chain fatty acids metabolism in mice. *Foods* 10:2478. doi: 10.3390/foods10102478

Li, Y. Q., Chen, Y., Fang, J. Y., Jiang, S. Q., Li, P., and Li, F. (2020). Integrated network pharmacology and zebrafish model to investigate dualeffects components of *Cistanche tubulosa* for treating both osteoporosis and Alzheimer's disease. *J. Ethnopharmacol.* 254:112764. doi: 10.1016/j.jep.2020.112764

Luo, L. F., Wu, W. H., Zhou, Y. J., Yan, J., Yang, G. P., and Ouyang, D. S. (2010). Antihypertensive effect of *Eucommia ulmoides* Oliv. extracts in spontaneously hypertensive rats. *J. Ethnopharmacol.* 129, 238–243. doi: 10.1016/j.jep.2010.03.019

Maccioni, R. B., Calfio, C., Gonzalez, A., and Luttges, V. (2022). Novel nutraceutical compounds in Alzheimer prevention. *Biomolecules* 12, 249–264. doi: 10.3390/biom12020249

Martorana, A., and Koch, G. (2014). Is dopamine involved in Alzheimer's disease? *Front. Aging Neurosci.* 6:252. doi: 10.3389/fnagi.2014.00252

Meldolesi, J. (2017). Neurotrophin receptors in the pathogenesis, diagnosis and therapy of neurodegenerative diseases. *Pharmacol. Res.* 121, 129–137. doi: 10.1016/j.phrs.2017.04.024

Neves, S., Macdonald, A., Poole, M., and Dening, K. H. (2021). "Participatory co-design: approaches to enable people living with challenging health conditions to participate in design research," in *Perspectives on Design and Digital Communication II. Springer Series in Design and Innovation*, Vol. 14, eds N. Martins, D. Brandão, and F. Moreira da Silva (Cham: Springer). doi: 10.1177/14713012211065377

Nilsson, P., Loganathan, K., Sekiguchi, M., Matsuba, Y., Hui, K., Tsubuki, S., et al. (2015). Aβ secretion and plaque formation depend on autophagy. *Cell Rep.* 5, 61–69. doi: 10.1016/j.celrep.2013.08.04234

Niu, X. L., Xu, D. R., Luo, J., and Kong, L. Y. (2015). Main iridoid glycosides and HPLC/DAD-Q-TOF-MS/MS profile of glycosides from the antioxidant extract of *Eucommia ulmoides* Oliverse seeds. *Indust. Crops Prod.* 79, 160–169. doi: 10.1016/j.indcrop.2015.11.027

Noori, T., Dehpour, A. R., Sureda, A., Sobarzo-Sanchez, E., and Shirooie, S. (2021). Role of natural products for the treatment of Alzheimer's disease. *Eur. J. Pharmacol.* 898:173974. doi: 10.1016/j.ejphar.2021.173974

Olayinka, J., Eduviere, A., Adeoluwa, O., Fafure, A., Adebanjo, A., and Ozolua, R. (2022). Quercetin mitigates memory deficits in scopolamine mice model via protection against neuroinflammation and neurodegeneration. *Life Sci.* 292:120326. doi: 10.1016/j.lfs.2022.120326

Pan, H., Zhang, J., Wang, Y., Cui, K., Cao, Y., Wang, L., et al. (2019). Linarin improves the dyskinesia recovery in Alzheimer's disease zebrafish by inhibiting the acetylcholinesterase activity. *Life Sci.* 222, 112–116. doi: 10.1016/j.lfs.2019.02.046

Pearson, V. E. (2001). Galantamine: a new Alzheimer drug with a past life. *Ann. Pharmacother.* 35, 1406–1413. doi: 10.1345/aph.1A092

Pragya, P., and Arun, U. (2021). Flavonoid-based nanomedicines in Alzheimer's disease therapeutics: promises made, a long way to go. *ACS Pharmacol. Transl. Sci.* 4, 74–95. doi: 10.1021/acsptsci.0c00224

Remya, C., Dileep, K. V., Tintu, I., Variyar, E. J., and Sadasivan, C. (2012). Design of potent inhibitors of acetylcholinesterase using morin as the starting compound. *Front. Life Sci.* 6:107–117. doi: 10.1080/21553769.2013.815137

Remya, C., Dileep, K. V., Tintu, I., Variyar, E. J., and Sadasivan, C. (2013). In vitro inhibitory profile of NDGA against AChE and its in silico structural modifications based on ADME profile. *J. Mol. Model.* 19, 1179–1194. doi: 10.1007/s00894-012-1656-0

Remya, C., Dileep, K. V., Tintu, I., Variyar, E. J., and Sadasivan, C. (2014). Flavanone glycosides as acetylcholinesterase inhibitors: computational and experimental evidence. *Indian J. Pharm. Sci.* 76, 567–570.

Sanabria-Castro, A., Alvarado-Echeverría, I., and Monge-Bonilla, C. (2017). Molecular pathogenesis of Alzheimer's disease: an update. *Ann. Neurosci.* 4, 46–54. doi: 10.1159/000464422

Sheng, R., Zhang, L. S., Han, R., Liu, X. Q., Gao, B., and Qin, Z. H. (2010). Autophagy activation is associated with neuroprotection in a rat model of focal cerebral ischemic preconditioning. *Autophagy* 6, 482–494. doi: 10.4161/auto.6.4.11737

Shintani, T., and Klionsky, D. J. (2004). Autophagy in health and disease: a double-edged sword. *Science* 306, 990–995. doi: 10.1126/science.1099993

Yan, J. K., Shi, X. L., Donkor, P. O., Ding, L. Q., and Qiu, F. (2018). Megastigmane glycosides from leaves of *Eucommia ulmoides* oliver with ACE inhibitory activity. *Fitoterapia* 116, 121–125. doi: 10.1016/j.fitote.2016.12.001

Zhang, S. S., Yu, Z. P., Xia, J. Y., Zhang, X. M., Liu, K. C., Attila, S., et al. (2020). Anti-parkinson's disease activity of phenolic acids from *Eucommia ulmoides* oliver leaf extracts and their autophagy activation mechanism. *Food Funct.* 11, 1425–1440. doi: 10.1039/c9fo02288k

Zhang, W. P., Fujikawa, T., Mizuno, K., Ishida, T., Ooi, K., Hirata, T., et al. (2012). Eucommia leaf extract (ELE) prevents OVX-induced osteoporosis and obesity in rats. *Am. J. Chin. Med.* 40, 735–752. doi: 10.1142/S0192415X12500553

Zhao, S., Zhang, L., Yang, C., Li, Z., and Rong, S. (2019). Procyanidins and Alzheimer's disease. *Mol. Neurobiol.* 56, 5556–5567. doi: 10.1007/s12035-019-1469-6

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Sun, Zhang, Ba, Dang, Ren, Zhu, Liu and Jin. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.