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Exploring the Therapeutic Potential of *Camellia longii* Orel & Luu Leaf Extracts for Memory Loss in Alzheimer's Disease: Novel Findings and Functional Food Applications

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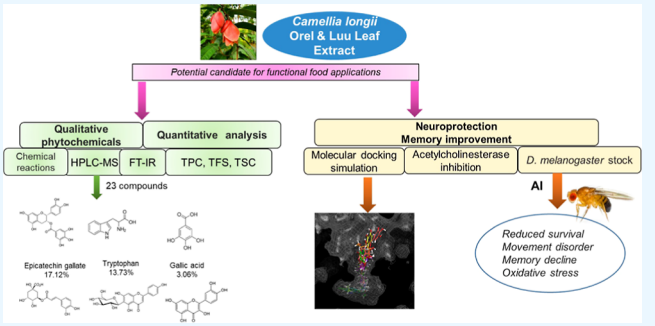
ABSTRACT: Novel research on the chemical compositions and biochemical activities of *Camellia longii* Orel and Luu leaf extracts revealed valuable resources with potential applications in Alzheimer's disease treatment. Qualitative phytochemicals detected various compound groups, including polyphenols, saponins, tannins, flavonoids, alkaloids, amino acids, coumarins, and polysaccharides. HPLC-MS identified 23 compounds in *C. longii* leaves with compounds found at significant levels, including epicatechin gallate (17.12%), tryptophan (13.73%), isovitexin (12.91%), gallic acid (3.06%), and quercetin (3.06%). Interestingly, the ethanol extract (CLL-Ew) exhibited the highest extraction yield (26.6%) and potent antioxidant and acetylcholinesterase (AChE) inhibitory effects in vitro. In the *Drosophila melanogaster* model, CLL-Ew improved longevity, movement, and memory by reducing malondialdehyde and increasing glutathione levels. Docking simulations suggested that the above compounds bind tightly to AChE's active site, potentially contributing to memory enhancement. Interestingly, observations of male and female mice after administration of a dose of 5000 mg/kg *C. longii* leaf extract were recorded normally throughout the 14 day experiment. These findings highlight the potential of *C. longii* leaf extracts in functional foods and therapeutic interventions for memory impairment prevention and treatment.



1. INTRODUCTION

Camellia longii Orel and Luu, commonly named Hong tra, Tra hoa do, belongs to the *Camelia* genus and Theaceae family, one of Asia's most traditional and well-known plant families. There are approximately 500 species in the world, 18 different genuses, of which the *Camellia* genus has about 120−300 species.[1] *C. longii* was first discovered in 2010, morphologically published in 2014,[2] and was ranked a critically endangered species. To date, a comprehensive investigation into the chemical compositions and biological activities of *C. longii* leaves, particularly concerning Alzheimer's disease (AD), has not yet been studied in scientific literature.

Recently, several species of *Camellia* were studied, including *Camellia quephongnensis*;[3] *Camellia chrysantha* indicated the high levels of bioorganic compounds, bioactivities, and potential for treating memory loss in AD;[4,5] *Camellia nitidissima; and Camellia euphorbia*.[6] Many compounds have been isolated and identified and belong to polyphenols, flavonoids, amino acids, tannins, vitamins, and saponins. Among them, polyphenol and flavonoid compounds, typically compounds like catechin, rutin, quercetin, and vitexin, could lower blood pressure, regulate blood glucose, lower cholesterol, reduce blood lipids, antitumor, boost the immune system, and treat symptoms of neurological disorders such as AD or Parkinson's disease.[6−9] Polyphenols have demonstrated the potential to enhance cognitive function and mitigate brain neuropathology in animal models of AD via many mechanisms.[10] Catechin and catechin derivatives are marked compounds in the Theaceae family, including catechin (C), epicatechin (EC), epigallocatechin 3-gallate (EGCG), and epicatechin 3-gallate (ECG), which can prevent several dangerous diseases such as cancer, obesity, myocardial infarction, cardiovascular, and neurodegenerative diseases.[11] Epigallocatechin-3-gallate has been indicated to increase glutathione (GSH) peroxidase activity, inhibit acetylcholinesterase (AChE) activity, and suppress NO metabolite formation and ROS generation in streptozotocin-induced dementia in mice.[8,9] Additionally, it enhanced memory formation and

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inhibited γ-secretase enzyme activity in mutant PS2 Alzheimer mice.[12] Vitexin (apigenin-8-C-glucoside) is also a critical flavonoid, which has been shown to have anti-inflammatory, antiophthalmic, and neuroprotective effects.[13] Quercetin improves circulation, boosts memory, and positively affects the treatment of AD. Furthermore, quercetin is also known for critical biological effects such as anti-inflammatory, cardiovascular disease prevention, support in the treatment of symptoms of neurological disorders such as AD or Parkinson's disease, cancer resistance, gastrointestinal ulcer, duodenitis, antibacterial, antifungal, allergy, and fever treatment.[14,15] The results of species of *Camellia* showed the potential application of *C. longii* to support and treat diseases related to free radicals and memory loss, specifically AD.

One of the most recent studies was related to the flowers of *C. longii* collected from Nam Cat Tien National Park, Lam Dong, Vietnam.[16] This research indicated that the extracts contained high total phenolic content (206.8−378.6 mg of gallic acid equivalents/g of crude extract) and flavonoid content (298.5−390.3 mg of rutin equivalents/g of crude extract) and had moderate potentials in antioxidant activity and potent α-glucosidase inhibition. Once again, the finding provides further evidence of the promising prospects for investigating chemical compositions and biological activities, particularly through comprehensive studies on the leaves of *C. longii* for supporting and treating memory loss associated with AD.

Our research comprehensively studied the chemical composition of crude extract and fractionated extracts prepared from *C. longii* leaves using classical and modern methods such as chemical reactions, Fourier transform infrared (FT-IR), HPLC-EIS-MS, and UV−vis. Besides, biological activities, including antioxidant activity and memory improvement, have been intensely studied in vitro, in silico, and in vivo to prove the applicability of *C. longii* leaves in the treatment of memory loss in AD, the foremost cause of dementia in late adult life, and expected to accelerate from 26.6 million cases in 2006 to 106.8 million by 2050.[10] Therefore, this research is necessary to provide scientific evidence of the chemical compositions, bioactivities of a new species, pharmacological values, and functional food applications of this plant species and contribute to the breeding and preservation of this plant species in Vietnam.

2. MATERIALS AND METHODS

2.1. Materials. Aluminum chloride ($AlCl_3$), potassium ferricyanide ($K_3[Fe(CN)_6]$), and trichloroacetic acid (TCA) were purchased from Merk, Germany. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), Folin−Ciocalteu reagent (FC), donepezil hydrochloride (Alzepil 5 mg, Egis Pharmaceuticals Private Limited Company), dithiobisnitrobenzoic (DTNB), AChE, acetylcholine iodide (ACTI), gallic acid, and vitamin C (ascorbic acid, AA) were obtained from Sigma Chemical Co. (St. Louis, USA). Solvents and other chemicals were in analytical grades.

2.2. Plant Extractions. *C. longii* Orel and Luu leaves were collected from Bu Gia Map National Park, Binh Phuoc province, Vietnam, in September 2022. *C. longii* was identified by the master Khuong Huu Thang. The voucher samples are preserved with analytical number CLL-01 at the Department of Chemical Technology, Faculty of Chemical and Food Technology, HCMC University of Technology and Education, Ho Chi Minh City, Vietnam. He is an expert in plant and wildlife conservation. Ethanol extract (CLL-Ew) was prepared by maceration with an ethanol solvent 70° (*v*/*v*). Next, four fractional extracts (CLL-H, CLL-Ea, CLL-B, and CLL-W) were prepared using liquid−liquid extraction with *n*-hexane, ethyl acetate, *n*-butanol, and water. All extracted solutions were rotary-evaporated at 50 °C, 110 rpm in 20−30 min and then dried at 45−50 °C until unchanged mass, humidity and extraction yields were determined, and the solutions were preserved in the cold condition (2−4 °C).[17]

2.3. Phytochemical Screening. *2.3.1. Qualitative Phytochemical Analysis.* The extracts were preliminary investigated functional groups of polyphenols, flavonoids, saponins, coumarins, polysaccharides, amino acids, tannins, alkaloids, terpenoids, organic acids, and carotenoids based on the characteristic reactions of functional groups with reagents.[16]

2.3.2. Fourier Transform Infrared. The FT-IR spectra were recorded using a Jasco FT/IR-4700 spectrometer equipped with a DLATGS detector and Spectra ManagerTM II software. The spectral range covered 400−4000 cm^{-1}, with a resolution of 0.4 cm^{-1} and an accuracy of ±0.01 cm^{-1}.[17] Samples were mixed and finely ground with KBr (IR grade, Fisher Scientific) with a ratio of 1 mg of sample/900 mg of KBr to make transparent KBr pellets. Prior to each sample FT-IR measurement, a background spectrum was obtained by measuring a pure KBr pellet. The samples were scanned and represented the average spectra of three replicate experiments.

2.3.3. Determination of TPC, TFC, and TSC. The total compound contents were determined based on UV−vis analysis by measuring the absorption of colored complexes at the maximum wavelength and calculating data based on the calibration curve of the standard substances.[18,19] The total polyphenol content (TPC), total flavonoid content (TFC), and total saponin content (TSC) were represented by the mg gallic acid equivalent/g of dry weight (mg GAE/g DW), mg catechin equivalent/g of dry weight (mg CAE/g DW), and mg oleanolic equivalent/g DW (mg OAE/g DW), respectively. The detailed experimental procedure is presented in the Supporting Information.

2.3.4. Liquid Chromatography−Mass Spectrometry. Extract samples were dissolved in methanol solvent and filtered before injection into a mass spectrometry system. In detail, we accurately weighed about 0.1 g of sample into a 10 mL extraction tube, added 10 mL of MeOH solvent, ultrasonicated for 10 min, and centrifuged at 6000 rpm for 5 min. Afterward, the sample is filtered through a 0.22 μm filter membrane. Chemical compositions of the extracts were analyzed by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (HPLC-ESI-MS) using an ACE3-C18 (4.6 × 150 mm; 3.5 μm) column at 40 °C, a flow rate of 1 mL/min, and a volume sample of 20 μL. The gradient mobile phase was composed of solution A (0.1% formic acid in deionized water) and solution B (0.1% formic acid in acetonitrile) as 0 min 95:5; 2 min 95:5; 25 min 0:100; and 30 min 0:100 (*v*/*v*). For ESI-MS, full-scan mass spectra were scanned from 150 *m*/*z* 150 to 2000. High-purity nitrogen was used as a nebulizer gas at 1.2 bar, 200 °C, and 0.8 mL/min flow rate. Precision ion *m*/*z* was adjusted in the ESI-L turning mix by direct syringe pump of 200 μL/h.

2.4. Bioactivities. *2.4.1. In Vitro Antioxidant Assays.* The antioxidant activity of extracts of *C. longii* leaves was evaluated by ferric reducing antioxidant power (FRAP) assay and DPPH free radical scavenging assay.[17] The experiments were repeated three times for each sample and are described in detail in the

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Supporting Information. Ascorbic acid was employed as the positive control in two methods, exhibiting an EC_{50} of 13.15 μg/mL in the FRAP assay and an IC_{50} value of 10.27 μg/mL in the DPPH assay.

2.4.2. In Vitro AChE Inhibitory Assay. The AChE inhibitory assay was carried out following an adaptation of the spectrophotometric method reported by ref 20 and described in detail in the Supporting Information. Donepezil was used as a positive control, with an IC_{50} value of 21.16 ng/mL.

2.4.3. Drosophila melanogaster Stock and Culture In Vivo. The wild-type strain Canton-S acquired from the Bloomington Drosophila Stock Center (BDSC) was used in this study. Flies of both genders were grown on standard food (Sf) comprising 5% dry yeast, 5% sucrose, 3% milk powder, and 0.85% agar at constant temperature and humidity (25 °C; 60% relative humidity) under 12 h dark/light cycle conditions. Undergoing growth and metamorphosis, 1 day-old adult fruit flies were transferred to new culture media to continue the experiment.

2.4.4. Experimental Design. *Drosophila melanogaster* was divided into six groups, including Group 1 (Control), which was placed on an Sf alone and served as the physiological group, Group 2 ($AlCl_3$) was placed on an Sf plus 40 mM $AlCl_3$ and served as the pathological group, while Group 3−6 ($AlCl_3$ + DO, $AlCl_3$ + 0.125, $AlCl_3$ + 0.25, and $AlCl_3$ + 0.5, respectively) were placed on Sf containing $AlCl_3$ plus either donepezil (DO, 0.1 mg/mL) or the extract (0.125, 0.25, and 0,5 mg/mL). The media was changed every 2 days, and throughout testing, they were kept away from light to prevent their antioxidant activity from diminishing. The doses of the extract were selected from a preliminary investigation, and the selected doses did not cause any significant toxicity in the flies. The concentration of $AlCl_3$ was based on previously published data.[21] Donepezil, being an FDA-approved drug for the treatment of early stages of AD by inhibiting cholinesterase, was used as a positive control, and the dose of donepezil was selected based on previous publications. The flies were exposed to these treatments for 5 days.

2.4.5. Survival Test. The flies were observed, and the dead flies were counted every 4 h until they died. The survival rate was expressed as a survival curve (the Kaplan−Meier survival test).

2.4.6. Climbing Assay. The protocol was thoroughly outlined in the Supporting Information and carried out following the procedures previously described by Ly et al.[22]

2.4.7. Olfactory Learning Assay. Protocol was carried out as described previously with some slightly modifications[23] and described in the Supporting Information.

2.4.8. Biochemical Assays.[21,24] Flies were anesthetized, weighed, and frozen until experimentation. Frozen flies were homogenized in 0.1 M phosphate buffer, pH 7.4, using a homogenizer. After centrifugation (10,000×*g*, 10 min, 4 °C), the supernatants were collected and used for biochemical assays with a microplate reader (Biotek).

2.4.8.1. Malondialdehyde Measurement. In brief, 12 μL of fly homogenization was mixed with 120 μL of 0.37% TBA. The mixture was incubated at 95 °C for 1 h. After that, 108 μL of n-butanol was added. The absorbance was recorded at 532 nm, and the malondialdehyde (MDA) equivalent was calculated.

2.4.8.2. Measurement of GSH. In brief, 10 μL of fly homogenate was added to 115 μL of PBS (pH = 7.4) and 25 μL of DTNB. The mixture was incubated in the dark for 5 min. The absorbance was measured at 412 nm, and the GSH equivalent was calculated.

2.5. Acute Toxicity Study in ICR Mice. ICR mice (6−7 weeks old) were purchased from the Biotechnology Center of Ho Chi Minh City, Vietnam. Mice were housed in polypropylene plastic cages in the breeding room at a temperature of 25 ± 1 °C, a humidity of 65 ± 5%, and a 12 h light/dark cycle. Mice were stably maintained in the animal laboratory for 1 week before the experiment.

The acute oral toxicity of the extract was determined based on OECD guideline no. 423, 2002. The extract was mixed in distilled water to reach 5000 mg/kg dose. The mice were divided into two groups: control group—distilled water (oral administration, *p.o.*)—20 mL/kg—6 mice per sex; and treated group—extract (*p.o.*)—5000 mg/kg—6 mice per sex. The mice were monitored continuously for 30 min, then intermittently for 4 h, and after that throughout 24 h to observe for behavioral changes, toxicity, and death symptoms following administration. The mice were provided food and water and observed daily for 14 days for body weight, mortality, and clinical signs of toxicity. If any animal dies, the animal's organs were examined macroscopically and microscopically.

2.6. Molecular Docking Simulation. Potential interactions between the protein AchE and different ligands were evaluated by using molecular docking. The 3D structure of the compounds was retrieved from PubChem. The compounds' potential protonation and tautomeric states were generated using Gypsum-DL tool.[25] This study uses the crystal structure of *Torpedo californica* acetylcholinesterase (TcAChE) bound with compound 5i, a potent inhibitor,[26] which was obtained from the RCSB Protein Data Bank, no. 7AIX. The structure of TcAChE was processed in Chimera to remove water outside of the binding cavity and to minimize the protein structure.[27] The processed protein and compound structures were sent to the DockThor server[28] to perform docking using the Standard default setting, and water molecules inside the binding cavity were treated as a cofactor. The docking pose with the best (lowest) score of each compound was visualized in PyMol 2.5 (Schrödinger, Inc.), and the protein−ligand interaction was analyzed using Free Maestro (Schrödinger, Inc.).

2.6.1. ADMET Predictions and Analysis. The compounds' chemical absorption, distribution, metabolism, elimination (ADME), physiochemical properties, and drug-likeliness were predicted using the SwissADME server.[29] The toxicity of the compound was predicted using the ProTox-II server (https://tox-new.charite.de/protox_II/).[30]

2.7. Data Analysis. The data were collected and calculated by Microsoft Excel (Microsoft, USA). The results were given as means ± standard deviation (SD) if normal distribution and as interquartile range if non-normal distribution. The data were checked for normality by the Anderson−Darling test and Shapiro−Wilk test. The results were statistically analyzed and graphed by GraphPad Prism 8.0.2 software (Inc., La Jolla, CA, USA) using log-rank (Mantel−Cox) test for survival rate, one-way analysis of variance (ANOVA), followed by Tukey's test for homogeneity of variance, Dunnett's test for heterogeneity of variance in the case of normal distribution, while Kruskal−Wallis test followed by Dunn post hoc test in the case of non-normal distribution. Means were considered significantly different at $p < 0.05$.

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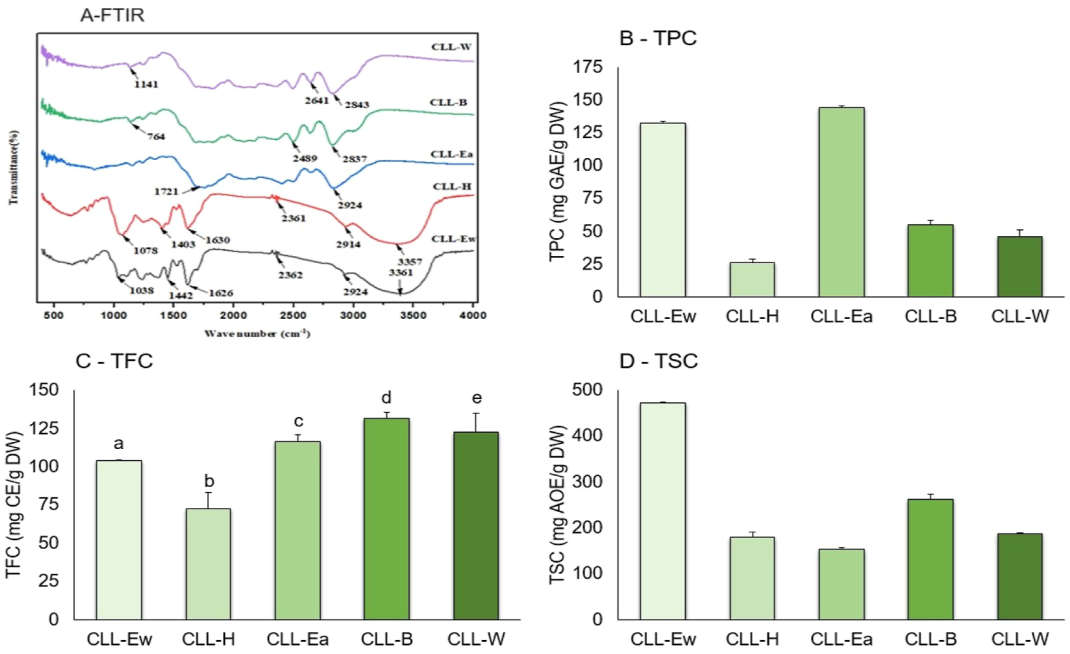

Figure 1. FT-IR spectrum (A) and TPC, TFC, and TSC values (B−D) of extracts prepared from *C. longii* leaves. Means were presented as mean ± standard deviation (n = 3) and followed by different letters that are significantly different at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Extraction Yields. Extraction yield and moisture are two important parameters for evaluating the extraction capacity of organic compounds. CLL-Ew total high-segment magnetic extraction performance was highest at 26.20% from dried leaves, followed by 23.11% CLL-Ea, 22.20% BLL-B, 15.75% CLL-H, and 24.09% from the crude extract. The high humidity of *C. longii* extracts was relatively high, ranging from 5.74 to 18.93%. Of these, CLL-B had the highest humidity level at 18.93%, followed by CLL-H (11.74%), CLL-W (11.13%), CLL-Ea (7.82%), and CLL-Ew (5.74%). The results indicated that the more polarized organic solvents increased the extraction efficiency. For CLL-H extract, *n*-hexane is a nonpolarized solvent, so it can extract fat compounds like steroids and waxes. However, these fats can retain moisture and have high viscosity, so the humidity is high. Ethyl acetate solvent has quite a moderate polarity, so that it can extract polyphenols, flavonoids, and alkaloids. Last, *n*-butanol has high polarity and is suitable for extracting saponin glucoside compounds from the long-circular groups.[31]

3.2. Chemical Compositions of *C. longii* Leaf Extracts. *3.2.1. Qualitative Phytochemical Analysis.* The preliminary qualitative functional group results in Table S1 show the presence of compound groups such as polyphenols, flavonoids, alkaloids, saponins, and tannins. Among them, saponins, flavonoids, and tannins had a high positive response in CLL-Ew, CLL-Ea, CLL-B, and CLL-W. For cardiac glycosides, carotenes, and organic acids, the qualitative results are relatively less clear, so they are doubtful. Coumarins were highly present in CLL-H and CLL-Ea but not absent or doubtful in the remaining extracts. Conversely, alkaloids occur in CLL-Ew, CLL-B, and CLL-W but are not missing in CLL-H and CLL-Ea. The differences in solvents with different polarities leading to the extraction of the various compounds will also be distinct.[31]

3.2.2. Fourier Transform Infrared. Similar to the preliminary determination of functional groups by chemical reactions, the FT-IR spectral results also show that all five extracts are present with polyphenols, saponins, tannins, flavonoids, and alkaloids. In addition, the functional groups of amino acids, coumarins, and polysaccharides were detected based on specific signals (Figure 1A). According to previous publications, the absorption of signals at wavelengths around 700−900 cm^{-1} is a signal of C−H binding of polyphenol rings in the coumarin group.[32] The absorption peak within 1060 cm^{-1} is the oscillating signal of the polysaccharide's C−O deoxyribose binding range. Bindings of the C═O group with 1630−1665 cm^{-1} absorption in extractive height are usually flavonoid group signals. The absorption of oligosaccharide binding to sapogenin, which is C−O−C, reaches approximately 1045 cm^{-1} in ethanol extract.[33] Besides, C−N bond signals are also found through the signal of the absorption peak at 1250 cm^{-1}, which is commonly found in grade 3 amides. The 1400−1450 cm^{-1} absorption peak signals CH_3 binding in lipids and proteins and COO binding in polysaccharides, pectins, and amino acids. The absorption peaks between 1500 and 1600 and 1620 cm^{-1} are, respectively, the signals of C═C binding in aromatic and alkene rings. The absorption peaks around 2100−2300 cm^{-1} are the signal of the C≡N and C≡C links.[34] The absorption peaks between 2928 and 2930 cm^{-1} signal the Csp^3−H bond of alkane or polysaccharide.[33] The broad-nose signals of the absorption peak in the range of 3360−3340 cm^{-1} are of the O−H bonds fluctuating in alcohol or polyphenols, and N−H oscillates in amide.[34]

3.2.3. Quantitative Analysis of TPC, TFC, and TSC. The TPC, TFC, and TSC of five extracts prepared from *C. longii* leaves are shown in Figure 1. For the TPC and TFC values, CLL-Ew and CLL-Ea show a high amount of total contents as CLL-EW (132.61 mg GAE/g DW and 104.22 mg GAE/g DW) and CLL-Ea (144.34 mg GAES/g DW and 116.63 mg CE/g DW). CLL-B has a TPC of 54.89 mg GAE/g DW, but the highest TFC of 131.44 mg CE/g DW. The extract containing the least biocompounds is CLL-H, with a TPC of 26.42 mgGAE/g DW and a TFC of 26.42 mgGAE/g DW.

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Table 1. Chemical Compositions of *C. longii* Leaf Extracts Were Identified by HPLC-EIS-MS

no.	samples	compounds	*m/z*	formula	retention time (s)	% area	scientific name	bioactivities
1	CLL-Ew	epicatechin gallate (ECG) (**1**)	442.0879	$C_{22}H_{18}O_{10}$	11.299	17.12	C. sinensis	anticancer, cardiovascular, and neurodegenerative diseases, AD[11]
2		*β*-sitosterol (**2**)	437.3719	$C_{29}H_{50}O$	10.421	15.79	Camellia dalatensis	regulate blood sugar and diabetes[39]
3		tryptophan (**3**)	205.0589	$C_{11}H_{12}N_2O_2$	9.060	13.73	C. chrysantha	neurotransmitter and neuromodulator[40]
4		isovitexin (**4**)	433.2576	$C_{21}H_{20}O_{10}$	8.524	12.91	C. euphlebia	antioxidant, anticancer, anti-inflammatory, antihyperalgesic, and neuroprotective effects[7]
5		gallic acid (**5**)	171.0493	$C_7H_6O_5$	2.151	3.26	C. quephongnensis	antioxidant activity[3]
6		quercetin (**6**)	302.9635	$C_{15}H_{10}O_7$	2.021	3.06	C. euphlebia	antioxidant activity, diabetes, enhances memory[3]
7		ellagic acid (**7**)	302.9535	$C_{14}H_6O_8$	1.562	2.37	C. sinensis	antioxidant activity, anti-inflammatory[6]
8		amyl nitrite (**8**)	118.0856	$C_5H_{11}N0_2$	1.562	2.37	Camellia genus	cardiovascular support[41]
9		diisopropylamine (**9**)	102.1269	$C_6H_{15}N$	1.559	2.16	Camellia genus	cardiovascular support[41]
10	CLL-H	olibanumol L (**10**)	502.3169	$C_{32}H_{52}O_3$	13.92	3.08	C. nitidissima	anti-inflammatory, anticancer[3]
11		1,2,3,4-tetra-*O*-acetyl-*β*-D-glucopyranose (**11**)	348.2143	$C_{14}H_{20}O_{10}$	14.465	1.42	Camellia genus	anticancer[41]
12		isolariciresinol (**12**)	361.2376	$C_{20}H_{24}O_6$	13.973	1.20	Camellia bugiamapensis	antioxidant activity, cardiovascular support activity[42]
13		3-hydroxy-*β*-ionone (**13**)	209.1563	$C_{13}H_{20}O_2$	7.722	0.58	C. bugiamapensis	antioxidant activity[42]
14		caffeic acid (**14**)	181.1228	$C_9H_8O_4$	8.281	0.36	C. sinensis	antioxidant activity[43]
15		catechol (**15**)	111.0419	$C_6H_6O_2$	1.215	0.02	C. sinensis	antioxidant activity[43]
16	CLL-Ea	epicatechin gallate (**1**)	442.0879	$C_{22}H_{18}O_{10}$	5.247	0.82	C. sinensis	anticancer, cardiovascular, and neurodegenerative diseases, AD[11]
17		olibanumol L (**10**)	502.3169	$C_{32}H_{52}O_3$	13.92	0.81	C. nitidissima	antioxidant activity[35]
18		icariside B1 (**16**)	409.1828	$C_{19}H_{30}O_8$	4.343	0.79	C. bugiamapensis	antioxidant activity activity[42]
19		(6S, 9R)-roseoside (**17**)	409.1828	$C_{19}H_{30}O_8$	6.792	0.79	C. dalatensis	antirectal cancer activity[42]
20		caffeic acid (**14**)	181.1228	$C_9H_8O_4$	8.291	0.07	C. sinensis	antioxidant activity[43]
21		teasperol (**18**)	301.1475	$C_{14}H_{14}O_6$	12.696	0.28	C. sinensis	antioxidant activity[8]
22		3-hydroxy-*β*-ionone (**13**)	209.1563	$C_{13}H_{20}O_2$	7.824	0.12	C. bugiamapensis	antioxidant activity, anti-inflammatory activity[42]
23		phloroglucinol (**19**)	127.0155	$C_6H_6O_3$	22.165	0.69	C. sinensis	antioxidant activity, AchE inhibition, reduce cholesterol[44]
24	CLL-B	chlorogenic acid (**20**)	355.2722	$C_{16}H_{18}O_9$	14.791	0.88	Camellia genus	antioxidant activity[39]
25		3-hydroxy-*β*-ionone (**13**)	209.1563	$C_{13}H_{20}O_2$	11.738	0.39	C. bugiamapensis	antioxidant activity, anti-inflammatory[43]
26		*α*-linolenic acid (**21**)	301.1415	$C_{18}H_{30}O_2$	12.686	0.73	C. sinensis	cardiovascular support[43]
27	CLL-W	olibanumol L (**10**)	502.3166	$C_{13}H_{20}O_3$	13.909	9.04	C. nitidissima	anticancer[35]
28		3-hydroxy-*β*-ionone (**13**)	209.1563	$C_{13}H_{20}O_2$	11.739	1.91	C. bugiamapensis	antioxidant activity, anti-inflammatory[43]
29		catechol (**15**)	111.0419	$C_6H_6O_2$	1.228	1.17	C. sinensis	antioxidant activity[43]
30		stigmasterol (**22**)	413.2671	$C_{29}H_{48}O$	15.521	0.98	C. dalatensis	regulate blood sugar and diabetes[39]
31		phloroglucinol (**19**)	127.0155	$C_6H_6O_3$	22.163	0.68	C. sinensis	antioxidant activity, AchE inhibition[44]
32		ellagic acid (**7**)	325.2715	$C_{14}H_6O_8$	14.980	0.53	C. quephongnensis	antioxidant activity, anti-inflammatory[3]
33		gallic acid (**5**)	171.0504	$C_7H_6O_5$	1.087	0.12	C. quephongnensis	antioxidant activity, anti-inflammatory[41]
34		*α*-linolenic acid (**21**)	301.1415	$C_{18}H_{30}O_2$	12.675	0.12	C. sinensis	cardiovascular support[43]
35		teasperol (**18**)	301.1419	$C_{14}H_{14}O_6$	12.675	0.12	C. quephongnensis	antioxidant activity[41]
36		3*β*-acetoxy-20-lupanol (**23**)	127.0156	$C_{32}H_{54}O_3$	16.176	0.03	C. nitidissima	regulate blood sugar and diabetes[35]
37	polyphenol: **1, 4, 5, 6, 7, 12, 14, 15, 18, 19, 20** acid amine: **3** sesquiterpenoid: **17** steroid: **2, 22** triterpenoid: **10, 23** monoterpene: **11, 13, 16** fatty acid: **21** compound containing nitrogen: **8, 9**							

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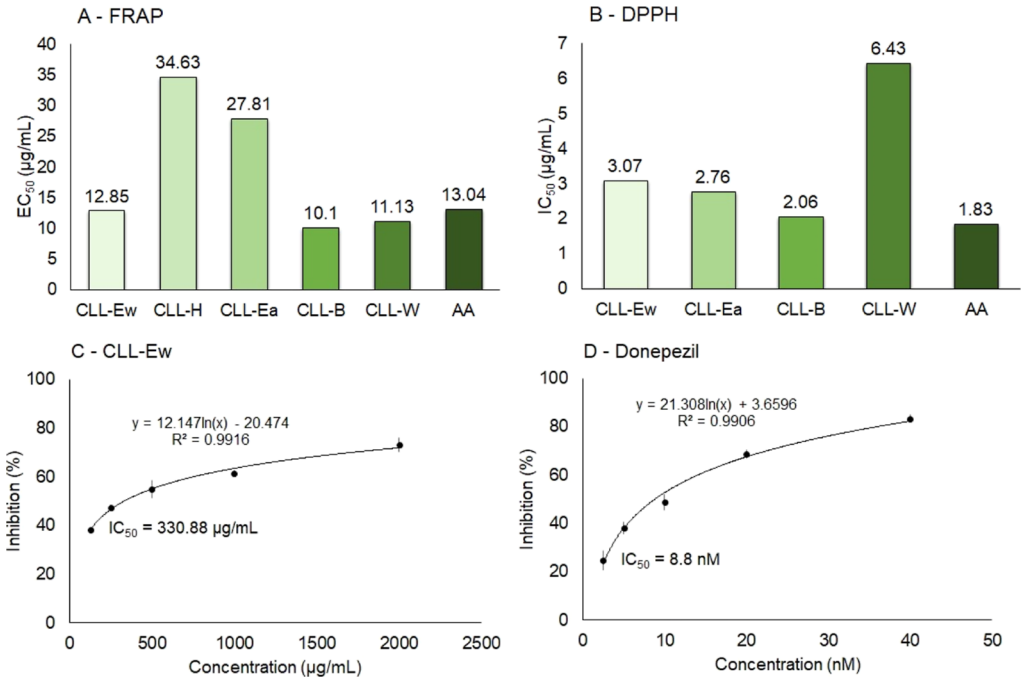

Figure 2. In vitro bioactivities of *C. longii* leaf extract and its fractions. (A, B) EC_{50} and IC_{50} values of *C. longii* leaf extract and its fractions in FRAP and DPPH assays, respectively. (C, D) AChE inhibitory activity of *C. longii* leaf extract and donepezil, respectively.

Interestingly, CLL-W has a high TFC of 122.81 mg CE/g DW, suggesting the potential of CLL-W instead of disposal after extraction. The results of TPC and TFC are constructive with the qualitative analysis of the functional group. Among them, the highest positive response was CLL-W (++++), CLL-Ea (+++), and CLL-B (++), with TFCs ranging from 104.22 to 131.44 mg CE/g DW, respectively. Furthermore, the LC−MS results also revealed flavonoid compounds quercetin, vitexin, and epicatechin that appeared in CLL-Ew, CLL-B, and CLL-W. The TPC of some species of *Camellia nitidissima*, *C. chrysantha*, and *Camellia euphlebia* is 281.04 mg GAE/g DW, 69.12 mg GAE/g DW, and 11.52 mg GA E/g DW.[6,35] From there, the leaf specimen of *C. longii* was identified as a potential source of polyphenol and flavonoid extracts with strong antioxidant capabilities.[36] On the other hand, the results of TSC were relatively high, and there were statistical differences between samples (p < 0,05). Of these, the highest extract (CLL-Ew) was 474.38 (mg OAE/g DW), followed by (CLL-B) 262.82 mg OAAE/g DW, (CLL-W) 186.38 mg OAEs/g DW, and (CLL-H) 179.75 mg OAEs/g DW, and the lowest (CLL-Ea) was 153.67 mg OAP/g DW. These results were similar to qualitative phytochemical analysis using chemical reactions and FT-IR analysis. Besides, this is a first study of quantitative TSC based on vanillin−sulfuric testing. Compared to previous reports, the TSC of the *C. longii* leaves was 21−99 times higher than that of *Chenopodium quinoa* Willd (7.449 mg OAE/g DW).[37] This indicated the high TSC of *C. longii* leaf extract and suggested a potential source of raw materials with high saponin content and support for treating memory loss in AD.[11]

3.2.4. High-Performance Liquid Chromatography Coupled with Electrospray Ionization Tandem Mass Spectrometry. Twenty-three compounds (Table 1) were identified in *C. longi* leaves by HPLC-EIS-MS, *m/z*, and error mass (ppm), referring to the previous studies on the chemical composition of the *Camellia* genus. Of these, 11/23 compounds belong to polyphenol compounds, including epicatechin gallate (CLL-Ew, 17.12%; CLL-Ea, 0.82%), isovitexin (CLL-E, 12.91%), gallic acid (CLL-E, 3.26%), quercetin (CLL-E, 3.06%), ellagic acid (CLL-E, 2.37%; CLL-W, 0.53%), isolariciresinol (CLL-H, 1.22%), catechol (CLL-H, 1.2%; CLL-W, 1.17%), caffeic acid (CLL-H, 0.36%; CLL-Ea, 0.07%), phloroglucinol (CLL-W, 0.98%; CLL-Ea, 0.69%), chlorogenic acid (CLL-B, 0.88%), and teasperol (CLL-Ea, 0.28%). Other compounds comprise one acid amin (tryptophan, CLL-EW, 13.73%), two steroids, six terpenoids, one fatty acid, and two nitrogen compounds. Among the above substances, epicatechin gallate (ECG), quercetin, ellagic acid, and gallic acid are found in green tea *Camellia sinensis* L, *C. nitidissima*, *C. euphlebia*, and *C. chrysantha* leaves.[1,2,38]

These compounds are classified into five groups: polyphenols, flavonoids, monoterpenes, triterpenes, and amino acids. Substances that exhibit valuable biological activities such as antioxidant and anti-inflammatory, improving circulation; enhancing memory; treating neurocellular diseases such as Alzheimer's or Parkinson's, cancer, especially colon and breast cancer, and diabetes mellitus; reducing symptoms of pneumonia; preventing the development of various arthritis, including arthrosis; cardiovascular protection; and lowering cholesterol (Table 1).[11,13,36] This serves as the foundational scientific groundwork for subsequent investigations into bioactivity in general, specifically into AD.

3.3. Bioactivities. *3.3.1. In Vitro Antioxidant Activity.* The results of FRAP and DPPH assays showed that *C. longii* leaf extract and its fractions could reduce Fe^{3+} and inhibit DPPH free radicals in various concentrations (p < 0.05) (Tables S2 and S3 and Figure 2). This means that bioactivity compounds in extracts inhibited antioxidant activities based on two mechanisms, including hydrogen atom transfer (HAT) and electron transfer (ET).[45]

The EC_{50} values of the extracts in the FRAP assay were in the range of 9.98−34.64 μg/mL (Figure 2A), in which the *n*-

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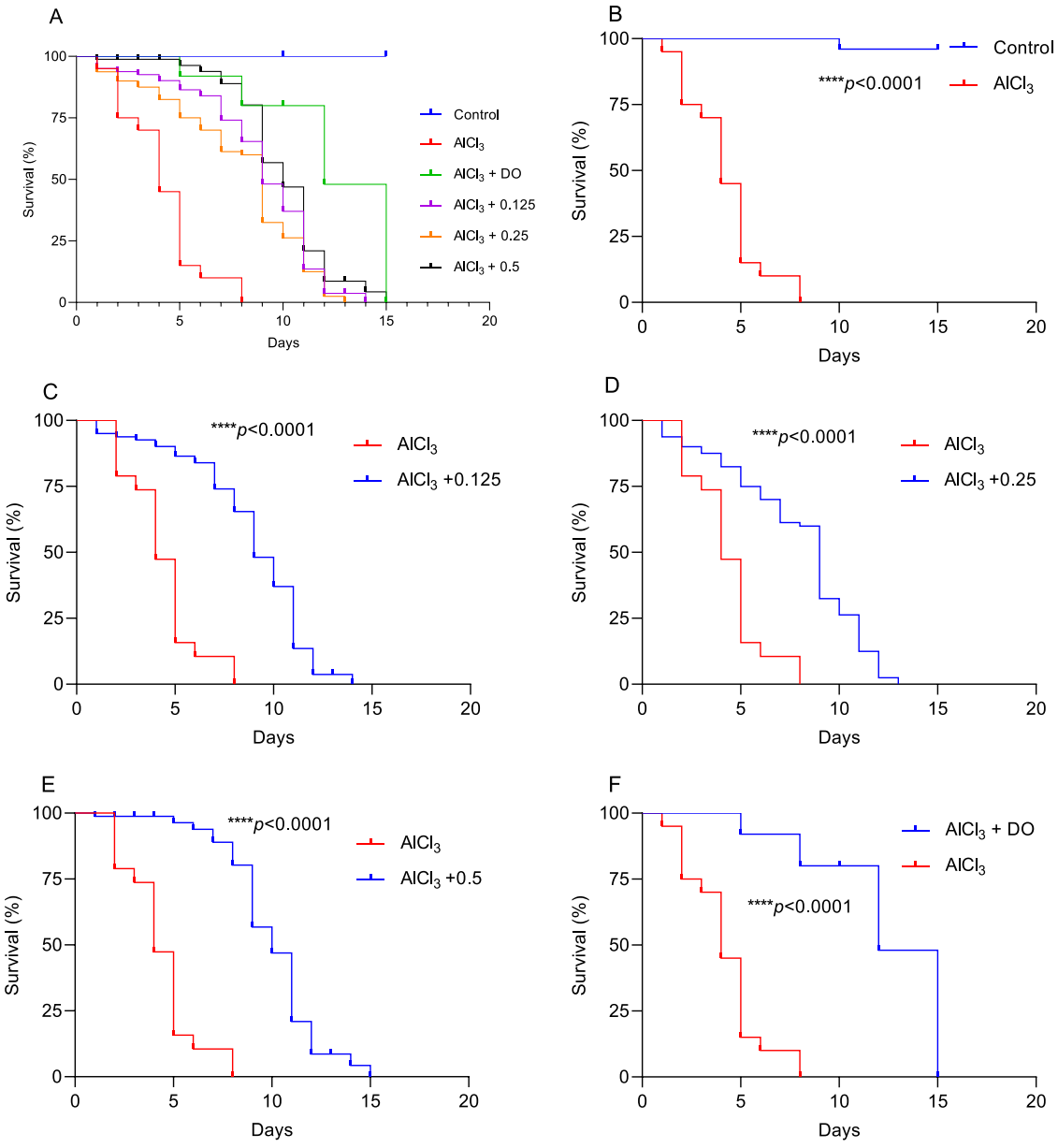

Figure 3. Effect of the extract on the survival rate of male flies. (A) Comparison of all groups and (B−F) comparison of each group with the $AlCl_3$ group. Log-rank (Mantel−Cox) test (n = 80).

butanol fraction (CLL-B) had the most vigorous activity with an EC_{50} of 9.98 μg/mL, compared with AA at 13.15 μg/mL. The reducing capacity of the *n*-hexane fraction (CLL-H) was the lowest among the five samples. The sequence for reducing power was CLL-B > CLL-W > AA > CLL-Ew > CLL-Ea > CLL-H. Interestingly, these results of the DPPH assay were quite similar to the results of the FRAP assay with the order of IC_{50} of the extracts of CLL-B < CLL-Ea < CLL-Ew < CLL-W. The IC_{50} values of the extract and its fractions were 2.09−6.28 μg/mL. The IC_{50} value of scavenging DPPH radicals for the *n*-butanol fraction (CLL-B) was 2.09 μg/mL; it has the best activity among the five samples, while the IC_{50} value of AA was 1.27 μg/mL. In contrast, the DPPH scavenging capacity of the *n*-hexane fraction (CLL-H) was relatively low; it inhibited less than 50% of DPPH free radicals at 100 μg/mL concentration, so the IC_{50} value was not determined. The results suggested that *C. longii* leaves may contain components with good scavenging capabilities and reducing Fe^{3+} using donating electron capabilities.

Besides, TPC had a moderate to strong Pearson correlation with IC_{50} in the DPPH assay (r = 0.61114) and EC_{50} in the FRAP assay (r = 0.70201). TFC showed a weak correlation with DPPH (0.02142) but a strong correlation with FRAP (0.78685). The strong correlation between TFC and FRAP could be explained based on the significant chelated capacities of flavonoids. From that, chelating compounds can also sterically hinder the formation of hydroperoxide metal complexes.[45] TSC showed a weak correlation on both antioxidant test models (r = 0.28162 and r = −0.4868). Polyphenol and flavonoid have proven their antioxidant activities because the hydroxyl groups are responsible for neutralizing free radicals by donating hydrogen (H•).[46] The LC−MS results also indicated that polyphenols and flavonoids were found, such as epicatechin gallate (ECG), gallic acid, isovitexin, quercetin, ellagic acid, and phloroglucinol, in *C.*

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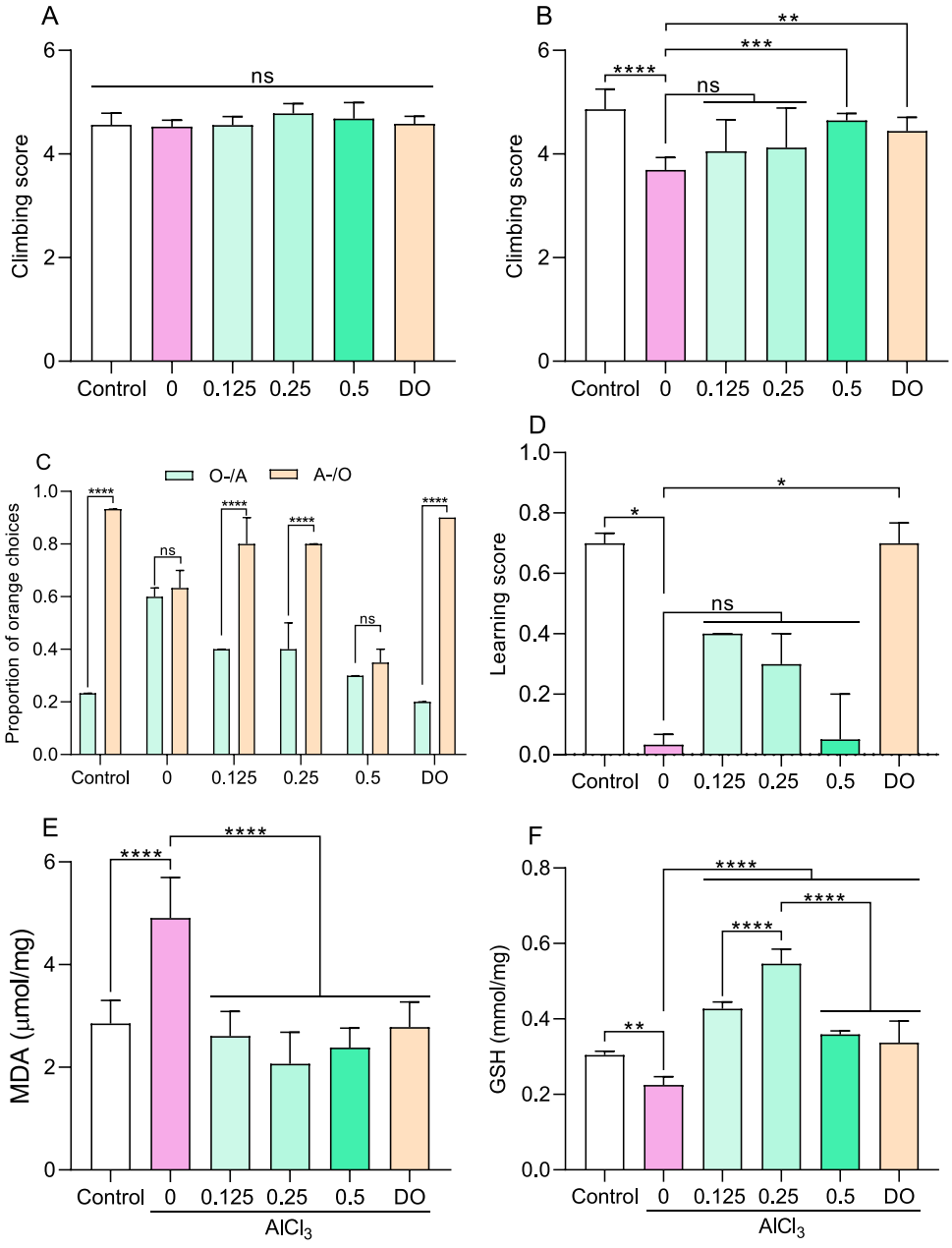

Figure 4. Effects of the extract on flies' locomotor ability, short-term memory, and oxidative stress markers after 5 day exposure. The effect of the extract on locomotor ability of 1 day-old (A) and 5 day-old (B) adult flies via climbing score (mean ± SD). Population size $N = 10$ and biological replication $n = 8$; $^{ns}p > 0.05$: not significant, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$: significant by Dunnett's test. The effect of the extract on short-term memory of 5 day-old adult flies via odor choice (C) and learning score (D) (median with interquartile range). Population size $N = 30$ and biological replication $n = 3$; $^{ns}p > 0.05$: not significant, $^{*}p < 0.05$: significant by Dunn post hoc test, $^{****}p < 0.0001$: significant by Holm-Šídák test. MDA (E) and GSH (F) contents in fly homogenate (mean ± SD, $n = 8$). $^{**}p < 0.01$ and $^{****}p < 0.0001$: significant by Tukey's test.

longii leaf extracts. Chlorogenic acid found in the *n*-butanol fraction was also evaluated to be a very strong free radical scavenger (DPPH) with an IC_{50} value of 12.3 μM.[47] Moreover, the quantitative results also showed that in extracts with polar solvents such as ethanol, ethyl acetate, *n*-butanol, and water, TPC and TFC were more dominant than those of the less polar fraction (CLL-H). The results were similar to the study of *C. sinensis*, which also showed the highest DPPH inhibition in the 50% ethanol extract (IC_{50}, 15.89 μg/mL).[48] Besides, the results of a previous study in some species of the genus *Camellia* collected in Northern Thailand showed that the reducing power tends to be significantly higher in extracts by polar solvents such as acetone and ethanol.[49] This suggested that the antioxidant activity may be closely related to the content of bioactive constituents, of which proanthocyanidins, polyphenols, and flavonoids are the main groups responsible for ferric reduction activity.

Several studies indicate that numerous plants rich in polyphenols, flavonoids, and saponins, which exhibit potent antioxidant properties, also possess the capability to inhibit AD effectively.[26,50] These natural antioxidants, considered secondary metabolites, can support the body's antioxidant defenses, combating the harmful effects of free radicals generated during metabolism and for which radical levels may be exacerbated in AD.[50] Based on the results, it was found that the CLL-Ew extract showed both high biocompound contents (TPC,

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132.61 mg of GEA/g of DW; TFC, 104.22 mg of CAE/g of DW; TSC, 471.38 mg of OAE/g of DW), strong antioxidant activities (DPPH, IC_{50}, 3.07; FRAP, EC_{50}, 12.85 μg/mL), and high extraction yield (26.20%). Therefore, the CLL-Ew sample was chosen to study the AChE inhibitory activity in vitro, in vivo, and in silico.

3.3.2. In Vitro AChE Inhibition Activity. In Figure 2C,D, the in vitro AChE inhibitions (%) of CLL-Ew at 500 and 2000 μg/mL were 54.90 ± 3.47 and 73.17 ± 2.79%, respectively. The IC_{50} values of CLL-Ew and donepezil (positive control) on AChE were 330.88 μg/mL and 8.580 nM (21.16 ng/mL), respectively. The results in this study were consistent with previous reports on AChE inhibition of some plants that belong to *Camellia*. Among them, of genus *Camellia*, green and black tea (*C. sinensis*) were known to inhibit AChE with IC_{50} values of 300 μg/mL and 600 μg/mL, respectively.[13] Compared to the above study, the AChE inhibition ability of *C. longii* leaf extract is nearly 2 times higher than that of black tea, and the inhibition ability is almost equivalent to that of *C. sinensis* L. green tea. *Camellia* species are rich in chemicals, especially polyphenols and flavonoids, which are the main active ingredients with superior inhibitory effects (AChE), in which polyphenol compounds interact with amino acid radicals that determine the active location of AChE through hydrogen bonding, hydrocarbonation, and $\pi-\pi$ interactions.[51]

3.3.3. Ameliorative Role of C. longii Leaf Extract in a Drosophila melanogaster Model of Aluminum Chloride-Induced Neurotoxicity. The *C. longii* leaf extract has good antioxidant and AChE inhibitory effects; this study continued to investigate the protective role of the extract in the *D. melanogaster* model of toxicity caused by aluminum chloride ($AlCl_3$), an environmental and industrial neurotoxicant known to induce oxidative damage and inflammation in tissues.

Results showed that $AlCl_3$ shortened the survival curve of male and female flies at a concentration of 40 mM compared to the control group. In male flies, donepezil hydrochloride (0.1 mg/mL) and the extract lengthened the survival curves of prophylaxis groups at concentrations of 0.125, 0.25, and 0.5 mg/mL (Figure 3). In female flies, the extract (0.125, 0.25, and 0.5 mg/mL) also increased the vitality of the positive control group when compared to 0 mg/mL, while donepezil hydrochloride (0.1 mg/mL) showed no changes in females (Figure S1). In general, the extract showed better effect in improving the vitality of male flies than female flies.

Figure 4A,B illustrates the impact of the *C. longii* leaf extract on the climbing ability of *D. melanogaster*. The findings demonstrate that the extract at 0.5 mg/mL dose and donepezil hydrochloride (0.1 mg/mL) improved $AlCl_3$-induced climbing activity comparable to the pathological group. Conversely, the administration of the extract at concentrations of 0.125 and 0.25 mg/mL did not yield significant differences compared with the $AlCl_3$ group. The results suggested that depending on the dose, *C. longii* leaf extract could ameliorate the locomotor dysfunction in $AlCl_3$-induced fruit flies.

As depicted in Figure 4C, the control group, donepezil group, and prophylaxis groups with the extract (0.125 and 0.25 mg/mL) displayed the ability to learn, memorize, and differentiate between the scents of oranges and apples (LI > 0). However, for the $AlCl_3$ group, there was no significant difference in distinguishing the two types of scents. Therefore, it can be concluded that $AlCl_3$ at a concentration of 40 mM has a neurotoxic effect on memory impairment in fruit flies. Besides, for the learning score recorded in the groups (Figure 4D), the fruit fly group applied prophylaxis 0.125 and 0.25 mg/mL of leaf extract had an impact on improving memory comparable with the $AlCl_3$ group but not significant.

Figure 4E,F indicates the $AlCl_3$-induced increase in MDA content and decreased GSH content compared to the control group. The leaf extract had the potential to reduce the amount of MDA and increase GSH content, with 0.25 mg/mL showing the best effect, even better than donepezil hydrochloride 0.1 mg/mL.

Acetylcholine within brain cells plays a pivotal role in memory processes. AChE facilitates the breakdown of acetylcholine into choline and acetate. Consequently, inhibiting AChE activity is a key strategy for enhancing memory function in AD. Findings from both in vitro and in vivo studies elucidated the AChE inhibitory properties of CLL-Ew, indicating its potential efficacy in mitigating AChE activity.

The findings of this study revealed that aluminum, donepezil, and *C. longii* leaf extract affect the survival, behavior, and biochemical parameters of *D. melanogaster*. The accumulation of aluminum in the tissues of the investigated species was the cause of the alterations. The $AlCl_3$-induced *D. melanogaster* shows a poorer survival rate and shorter life span than the groups treated with donepezil or leaf extract and the control group. These results indicate that aluminum has a cumulative impact and affects the life span. The mixture of antioxidants in *C. longii* leaf extract has the ability to lengthen the life span significantly. Phytochemical screening provides information about the chemical makeup of the compounds in plant extracts that have biological effects. Polyphenols have excellent antioxidant capabilities; eating them may help protect against neurological illnesses.[52] Phloroglucinol found in the *C. longii* (Table 1) and other species in *Camellia* genus was capable of treating AD by reducing ROS levels in cells at a concentration of 10 μg/mL of fluoroglycinol for 1 h, which was supplemented with 8 μM Aβ1−42 in HT-22 neurons.[53] DCF-DA fluorescence intensity in phloroglucinol-pretreated cells was significantly lower (1.59 ± 0.52, $n = 9$, $p < 0.01$) compared to phloroglucinol-untreated Aβ1−42.[53]

Flies given $AlCl_3$ had poor movement (negative geotaxis), which is a sign of neurodegeneration. Because of the relation between lower locomotion and poor cholinergic transmission, the activity of AChE, an enzyme involved in cholinergic neurotransmission.[54] Hence, it may be hypothesized that the AChE activity of flies exposed to $AlCl_3$ would rise dramatically. Considering that an increase or decrease in AChE activity may disrupt locomotor activity and other behaviors, the substantial increase in AChE activity in flies exposed to $AlCl_3$ may be the cause of their locomotor impairment. Intriguingly, *C. longii* leaf extract (PLE) lowered AChE activity when fed to flies with more mobility than did the diseased group.

The T-maze test is a behavioral test to assess the ability to improve memory and cognitive processes formed through the spatial location-dependent working and learning process of stupefied *D. melanogaster* memory loss. *C. longii* leaf extract has the effect of improving memory loss on *D. melanogaster* with dementia. According to studies on people with AD, the deposition of amyloid plaques creates senile plaques and neural plexuses, leading to neuronal death, synaptic loss, and inflammatory reactions. These specific lesions occur in different areas of the brain, especially the cerebral cortex, the basal frontal cortex, and the temporal lobe hippocampus, neurotransmitters, especially acetylcholine, which is considered to play a fundamental role in brain biochemical disorders.

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However, AChE is broken down by enzyme AChE to choline and acetic acid. AChE has been extensively studied in related tissues. These studies revealed changes in AChE activity and alterations in its polymorphism in the brain as well as in cerebrospinal fluid and in the blood. The simultaneous presence of enzymes in plaques also reinforces evidence of these unusual features. Studies have shown that AChE forms a complex when bound to components in plaque at its negatively charged portion. Furthermore, the neurotoxicity of amyloid plaques was enhanced in the presence of AChE. The abnormal glycosylation of some forms of AChE in AD is closely related to the presence of amyloid plaques. Some of the AChE inhibitors currently used in the treatment of AD are donepezil. Therefore, the extract's AChE inhibitory effect also contributes to the memory improvement effect of fruit flies caused by $AlCl_3$.

The neurological toxicity caused by aluminum chloride is related to oxidative stress. An increase in MDA and a decrease in GSH caused by aluminum are another mechanism that contributes to superoxide production. The result of this research is relevant to the theory due to the change of MDA and GSH caused by $AlCl_3$ at a concentration of 40 mM. MDA and GSH were utilized as markers of lipid peroxidation and free radical production in the brain. The results showed that *C. longii* leaf extract has potent antioxidant action against $AlCl_3$-induced oxidative damage by reducing MDA content and increasing GSH content.

3.4. Acute Oral Toxicity of *C. longii* Leaf Extract. The study result showed that no animals died after administration of the extract at a limit dose of 5000 mg/kg (fatal fraction of 0%). Mice were observed with particular attention during the first 30 min and then for 4 h. Observations were recorded, and the results are summarized in Tables 2 and 3.

Table 2. Mouse Body Weight during Testing

gender	day	body weight (g)	
		control	extract
female	**1**	19.57 ± 1.41	20.25 ± 0.63
	7	24.73 ± 1.11	25.08 ± 0.97
	14	28.70 ± 1.14	28.35 ± 1.89
male	**1**	19.98 ± 0.95	19.95 ± 1.37
	7	25.17 ± 0.93	25.27 ± 1.38
	14	30.06 ± 1.72	29.20 ± 2.47

Mean ± SD (n = 6).

The body weight of the experimental mice in both the control and extract-treated groups gradually increased throughout the study period, and there was no significant difference between the two groups ($p > 0.05$) (Table 2). This shows that the extract did not affect the development of the mice.

Observations of male mice after administration of a dose of 5000 mg/kg *C. longii* leaf extract were recorded normally throughout the experiment. Meanwhile, the female mice were a little more active and slept less in the first few hours. In detail, the behavior of female mice after administration of a dose of 5000 mg/kg of *C. longii* leaf extract showed a slight increase in somatomotor activity in the first 30 min. Drowsiness and sleepy effects were decreased in the extract-treated group during the initial 4 h. Other observations were recorded normally throughout the experiment (Table 3). It was suggested that the female mice were more sensitive than male mice and that the extract had a psychoactive effect on the female mice. More in-depth research is needed for the new detection in the future, and it promises significant value.

3.5. Molecular Docking Simulation. In our knowledge, the chemical components are closely related to the biological effects of plant extracts. Therefore, to explore which compounds in the *C. longii* leaf extract could inhibit AChE and contribute to the in vivo memory-improving effect of the extract, the in silico AChE inhibition ability of the compounds found in the extract was investigated. To evaluate the reliability of the docking methods, ligand 5i was redocked to the TcAChE structure. The overlay of the crystal structure and bested redocked pose of 5i in TcAChE is shown in Figure S2. The two poses are largely overlapped with an RMSD of 0.156, with the parts inside the binding pocket, which is also the active site, being virtually identical. The results illustrate the accuracy of the docking methods. A total of 25 compounds, including the control donepezil and 5i, were docked to the TcAChE structure. The seven top compounds with their best docking score are shown Figure 5A. As shown in Figure S2, the best binding poses of all seven compounds are inside the active site of TcAChE, similar to the ligand 5i. The ligand 5i has the best (lowest) of −21.13, which is understandable since this compound is the native ligand of the used TcAChE structure. Isovitexin has the next lowest score, followed by tryptophan, chlorogenic acid, donepezil, epicatechin gallate, and quercetin (Figure 5A). All compounds show a wide range of interactions with the residues in the binding pocket, with the majority being hydrogen bonds and the Pi−Pi stacking interaction. The residues participate in interactions that vary between compounds. Interestingly, for the top 4 compounds, water molecules were shown to play a role as hydrogen bond relay between the compound and residues in the cavity (Figure 5B−H).

3.5.1. ADMET Prediction and Analysis. The physicochemical properties, drug-likeliness, and ADMET of the seven top compounds, including the control donepezil, are shown in Table 4.

The physiochemical properties of tryptophan, donepezil, and quercetin satisfy all of Lipinski's rules, while isovitexin, chlorogenic acid, and epicatechin gallate each have one violation.[55] Three compounds trigger alerts for Pan Assay Interference Compounds (PAINS), compounds that can interfere with assay readouts.[56]

Tryptophan, donepezil, and quercetin have high gastrointestinal absorption. All compounds except donepezil are not substrates of *P*-glycoprotein. Donepezil and quercetin are substrates of at least one cytochrome P450 enzyme. These are preferable properties for druggability. All compounds except donepezil have poor blood−brain barrier penetration, which could be a problem for AChE inhibitor drugs that are active in brain cells. Tryptophan, chlorogenic acid, and epicatechin gallate have low acute toxicity, which is indicated by the high LD_{50} values of more than 1000 mg/kg. Except for tryptophan and epicatechin gallate, other compounds trigger at least one of the four toxicity end points: carcinogenicity, immunotoxicity, mutagenicity, or cytotoxicity.

AChE is a well-studied target for the management of dementia. Docking analysis of the 23 compounds identified from the *C. longii* extract predicted five top compounds that potentially bind to the active pocket of AchE. The active pocket has a peripheral site connected to a catalytic center by a

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Table 3. Behavioral Patterns of Female Mice and Male Mice in the *C. longii* Leaf Extract Administration[a]

	Observations																							
	30 min				4 h				24 h				48 h				7 days				14 days			
	m		f		m		f		m		f		m		f		m		f		m		f	
parameters	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E
fur, skin	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
eyes	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
salivation	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
respiration	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
urination (color)	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
faces consistency	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
somatomotor activity, behavior pattern	N	↑	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
sleep	N	↓	N	N	N	↓	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
mucous membrane	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
convulsions, tremors	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
itching	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
coma	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
mortality	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF

[a]m = male, f = female, C = control, E = extract, N = normal, ↑ = increased, ↓ = decreased, and NF = not found.

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A.

Compound	Docking score
5i	-21.13
Isovitexin	-10.70
Tryptophan	-10.36
Chlorogenic acid	-9.90
Donepezil	-8.98
Epicatechin gallate	-8.78
Quercetin	-8.48

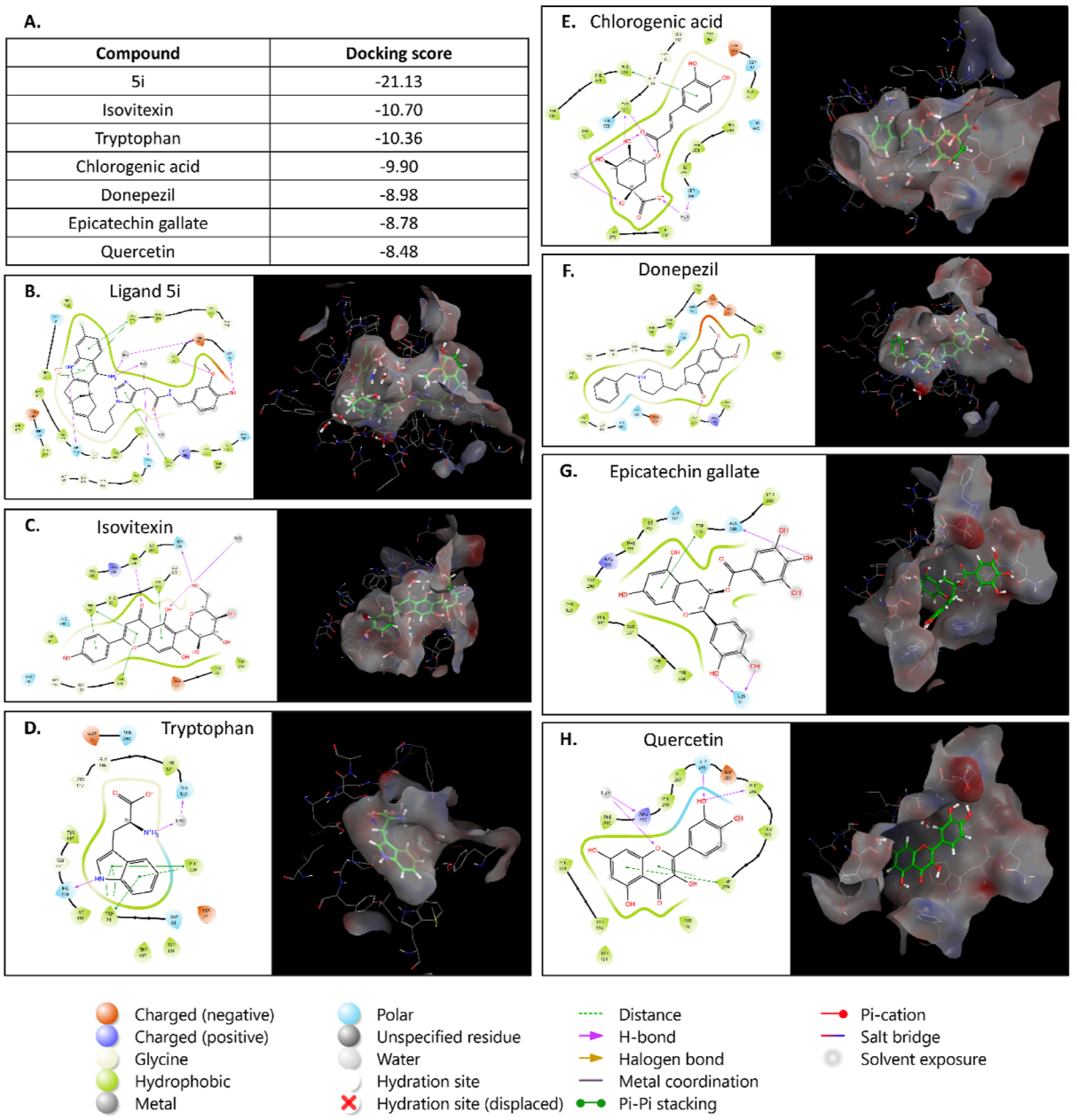

Figure 5. TcACh compound docking. (A) Docking score of seven compounds to the TcAChE structure. (B−H) 2D and 3D representations of the best docking pose of the compounds and their interaction with TcAChE.

narrow aromatic gorge. The catalytic center consisted of two important sites: the anionic sites (PHE330 and TRP84 in TcAChE) and the esteratic site (GLU327, HIS440, and SER200 in TcAChE).[57] Many available dementia drugs are reversible AchE inhibitors that can occupy both sites, such as donepezil[58] and rivastigmine.[59] The native structure of ligand 5i spanned from the peripheral site to the catalytic center; it exhibits strong interaction with TRP84, PHE330, and HIS440 (Figure 5B). Docking of donepezil into the TcAChE structure also showed interactions with HIS440, SER200, and TRRP84 (Figure 5F). Among the top five compounds in the extract, isovitexin, tryptophan, and chlorogenic acid bind to the catalytic center and interact with residues of the anionic and esteratic sites. Epicatechin gallate and quercetin are the two compounds with higher (not as good) docking scores; they bind to the peripheral site and the aromatic gorge but not to the catalytic center, which could affect the inhibition activity. Interestingly, epicatechin gallate, tryptophan, and isovitexin are three major compounds in the extract, which consist of 17.12, 13.7, and 12.9%, respectively, of the extract by weight. These three compounds, especially tryptophan and isovitexin, might be responsible for the majority of the AchE activity of the extract.

Isovitexin and quercetin have the highest predicted acute toxicity, with a LD value of 159 (mg/kg). This property is not inhibitive since quercetin has been commonly used in supplements, while studies also report antioxidant, anticancer, anti-inflammatory, antihyperalgesic, and neuroprotective effects of isovitexin.[7] Isovitexin has low gastrointestinal absorption and blood−brain barrier permeability, which can hinder its potential as a medication. Tryptophan, on the other hand, is a common amino acid with preferable ADMET and

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Table 4. Physiochemical, Drug-Likeliness, and ADMET Properties of Isovitexin, Tryptophan, Chlorogenic Acid, Donepezil, Epicatechin Gallate, and Quercetin

molecule		isovitexin	tryptophan	chlorogenic acid	donepezil	epicatechin gallate	quercetin
physiochemical properties	MW	432.38	204.23	354.31	379.49	442.37	302.24
	rotatable bonds	3	3	5	6	4	1
	H-bond acceptors	10	3	9	4	10	7
	H-bond donors	7	3	6	0	7	5
	consensus Log P	−0.02	0.18	−0.39	4	1.3	1.23
	ESOL Log S	−2.84	−0.68	−1.62	−4.81	−3.7	−3.16
	ESOL class	soluble	very soluble	very soluble	moderately soluble	soluble	soluble
drug-likeliness	Lipinski violations	1	0	1	0	1	0
	bioavailability score	0.55	0.55	0.11	0.55	0.55	0.55
	PAINS alerts	0	0	1	0	1	1
absorption	GI absorption	low	high	low	high	low	high
	Pgp substrate	no	no	no	yes	no	no
distribution	skin permeation (cm/s)	−8.79	−8.3	−8.76	−5.58	−7.91	−7.05
	BBB permeant	no	no	no	yes	no	no
metabolism	CYP1A2 inhibitor	no	no	no	no	no	yes
	CYP2C19 inhibitor	no	no	no	no	no	no
	CYP2C9 inhibitor	no	no	no	no	no	no
	CYP2D6 inhibitor	no	no	no	yes	no	yes
	CYP3A4 inhibitor	no	no	no	yes	no	yes
toxicity	LD_{50} (mg/kg)	159	2000	5000	505	1000	159
	hepatotoxicity	inactive	inactive	inactive	inactive	inactive	inactive
	carcinogenicity	inactive	inactive	inactive	active	inactive	active
	immunotoxicity	inactive	inactive	active	active	inactive	inactive
	mutagenicity	active	inactive	inactive	inactive	inactive	active
	cytotoxicity	inactive	inactive	inactive	active	inactive	inactive

toxicity properties. Tryptophan-based amides have been previously investigated as potential inhibitors for butyrylcholinesterase (BChE), a closely related protein to AChE.[60] Further investigations of the extract and its constituents might reveal their potential as a treatment for dementia.

4. CONCLUSIONS

In summary, five extracts prepared from *C. longii* Orel and Luu leaves in Bu Gia Map National Park, Vietnam, have comprehensively studied chemical constituents, antioxidant activities, and anti-AD related to activities of the crude extract (CLL-Ew). Interestingly, not the fractionated extract but the crude extract has high antioxidant capacities and a high content of polyphenols, saponins, and flavonoids, along with high extraction efficiency. This is advantageous for product production due to high efficiency and simple process, thereby reducing product costs and producing various functional products such as tea bags, drinking water, etc. Furthermore, studies on the ability to support memory loss in three categories confirm this extract's effectiveness and potential application for treating memory loss in AD. The present results highlighted *C. longii* leaf compounds as future anti-Alzheimer chemical leads and the potential for use as functional foods or in therapeutics for the prevention and possible treatment of memory impairment. Therefore, this research is necessary to provide scientific evidence of the chemical compositions, the activities of a new species, and the discovery of pharmacological values and functional food and contribute to the breeding and preservation of this plant species in Vietnam.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c02980.

Methods for determining total polyphenol, flavonoid, and saponin contents; in vitro antioxidant assays; *D. melanogaster* stock and culture in vivo; results of phytochemical screening of ethanol, *n*-hexane, ethyl acetate, *n*-butanol, and aqueous extracts of *C. longii* leaves; absorbance and EC_{50} values of vitamin C and *C. longii* extracts in FRAP assay; inhibition percentage (%) and IC_{50} values of vitamin C and *C. longii* extracts in DPPH assay; and information on the extract's effect on female flies' survival rate and the docked poses of compounds (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.
Animal ethics statement: The university's scientific committee has ethically approved of all experiments in vivo. Animals were given access to food and water ad libitum. Animal experiments were conducted following the National Research Council's Guide for the Care and Use of Laboratory Animals (NIH publication #85−23, revised in 1985). The animals were also maintained, controlled, and studied according to the approved guidelines and regulations of the Ministry of Health, Vietnam (no. 141/Q-K2T).

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