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Molecular docking studies of phlorotannins from *Eisenia bicyclis* with BACE1 inhibitory activity

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ABSTRACT

In our consecutive research on an anti-AD remedy derived from maritime plants, the BACE1 inhibitory activities of *Eisenia bicyclis* and its isolated phlorotannins were evaluated. The *E. bicyclis* extract and its fractions exhibited predominant BACE1 inhibition. With the exception of phloroglucinol (**1**), all test phlorotannins isolated from the most active EtOAc soluble fraction, showed significant and non-competitive inhibition against BACE1:dioxinodehydroeckol (**2**, IC_{50} = 5.35 μM; K_i = 8.0); eckol (**3**, IC_{50} = 12.20 μM; K_i = 13.9); phlorofurofucoeckol-A (**4**, IC_{50} = 2.13 μM; K_i = 1.3); dieckol (**5**, IC_{50} = 2.21 μM; K_i = 1.5); triphloroethol A (**6**, IC_{50} = 11.68 μM; K_i = 12.1); 7-phloroethol (**7**, IC_{50} = 8.59 μM; K_i = 7.2). In addition, plausible protein–ligand interactions of **3**, **4**, and **5** were similar and may occur primarily through the TYR132 and THR133 of BACE1 via molecular docking simulations (AUTODOCK 4.0 and FRED 2.0 programs). As a result, the *E. bicyclis* extract and the phlorotannins contained therein would clearly have beneficial use in the development of therapeutic and preventive agents for AD and suggest potential guidelines for the design of BACE-selective inhibitors.

Alzheimer's disease (AD) is a neurodegenerative disorder, the susceptibility of which is surging in the elderly. Dementia is the most prevalent symptom of AD and has the characterization of progressive cognitive decline, memory loss, extensive neuronal loss, decrease in cholinergic transmission, and the presence of senile plaques. AD pathogenesis is reported to associate with two major molecular mechanism pathways, the cholinergic and the β-amyloid cascade.[1] Amyloid β peptide (Aβ), a major and crucial component of senile plaques, is formed through the cleavage of amyloid precursor protein (APP) by β- and γ-secretases. Aβ has been reported to exert neurotoxicity, increase protein oxidation, with protein carbonyls and 3-nitrotyrosine as markers, and accelerate the cross-linking of advanced glycation endproducts.[2] Since the formation and accumulation of APP-Aβ plays a crucial histophysiologic role in AD pathogenesis, interest in the β-site APP cleaving enzyme 1 (BACE1, β-secretase, aspartyl protease, Asp2, memapsin2) has arisen as a new approach towards AD treatment.[3]

Currently, marine plants are intriguing materials as nutraceuticals and pharmaceuticals given their numerous biological and phytochemical benefits.[4] *Eisenia bicyclis* (Kijellman) Setchell, one of the phlorotannin-rich natural resources, is a common perennial brown alga belonging to the family Laminariaceae that inhabits the middle Pacific coast around Korea and Japan.[5] This alga is frequently consumed as a foodstuff, along with *Ecklonica stolonifera*, *Laminaria japonica* and *Undaria pinnatifida* and is also used as the raw material for sodium alginate.[6] In particular, phlorotannins, isolated mainly from the *Ecklonia* and *Eisenia* species, are responsible for a variety of bioactivities, including anti-diabetic complications,[5,7] antitumor,[8] hepatoprotective,[9] anti-plasmin inhibitory,[10] algicidal,[11] tyrosinase inhibitory,[12] anti-inflammatory,[13] nitrite-scavenging,[14] anti-skin aging,[15] antioxidant,[16] anti-hyperlipidemic,[17] anti-allergic,[18] and angiotensive converting enzyme-inhibitory activities.[19]

Although several reports on the in vitro anti-acetylcholinesterases inhibitory and in vivo memory-enhancing activities of *Ecklonia* species and phlorotannins have been reported,[20] the BACE1 inhibitory activities of *E. bicyclis* and its phlorotannins were first evaluated as an anti-AD remedy in this study. In addition, prediction of the protein–ligand conformation was carried out with two docking programs, AUTODOCK 4.0 and Fast Rigid Exhaustive Docking (FRED) 2.0 for the first time, in order to confirm the BACE1 inhibitory activities and propose phlorotannin guidelines as BACE1 inhibitors in drug discovery.

To evaluate the potential of *E. bicyclis* and phlorotannins as anti-AD agents, the BACE1 inhibitory activities were measured according to the manufacturer protocol.[21] As shown in Table 1, the MeOH extract of *E. bicyclis* exerted significant inhibitory activities with an IC_{50} value of 4.87 μg/mL in the BACE1 assay. Upon bioactivity-guided fractionation of the *E. bicyclis* extract, the EtOAc, *n*-BuOH, and H_2O fractions in the same assay exhibited potent inhibitory activities

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Table 1
BACE1 inhibitory activities of extract and fractions from *E. bicyclis*

Extract and fractions	IC_{50}[a](μg/mL)
MeOH extract	4.87 ± 0.61
CH_2Cl_2 fraction	>125
EtOAc fraction	0.19 ± 0.00
n-BuOH fraction	0.50 ± 0.08
H_2O fraction	1.03 ± 0.02
Sophoraflavanone G[b]	0.60 ± 0.07

[a] Final concentration of test samples were 125 μg/mL, dissolved in 10% DMSO. 50% Inhibition concentrations expressed as the mean ± S.E.M. of triplicates.
[b] Sophoraflavanone G used as a positive control.

Table 2
BACE1 inhibitory activities and inhibition constants of phlorotannins from *E. bicyclis*

Compounds	IC_{50} (μM)[a]	K_i[b]
Phloroglucinol (**1**)	36.47 ± 1.04	
Dioxinodehydroeckol (**2**)	5.35 ± 0.53	8.0
Eckol (**3**)	12.20 ± 0.46	13.9
Phlorofucofuroeckol-A (**4**)	2.13 ± 0.08	1.3
Dieckol (**5**)	2.21 ± 0.16	1.5
Triphloroethol A (**6**)	11.68 ± 0.80	12.1
7-Phloroethol (**7**)	8.59 ± 0.28	7.2
Quercetin[c]	10.82 ± 0.32	14.0

[a] Final concentration of test samples were 100 μM, dissolved in 10% DMSO. 50% Inhibition concentrations expressed as the mean ± S.E.M. of triplicates.
[b] Inhibition constants (K_i) determined by interpretation of the Dixon plot.
[c] Quercetin used as a positive control.

with IC_{50} values of 0.19, 0.50, and 1.03 μg/mL, respectively, as compared with the positive control of sophoraflavanone G (0.60 μg/mL). Since the EtOAc fraction, with the most predominant BACE1 inhibitory activity, is commonly known as the phlorotannin-rich fraction, repeated chromatography of this fraction with silica gel, RP-18, and Sephadex LH20 column afforded: phloroglucinol (**1**); dioxinodehydroeckol (**2**); eckol (**3**); phlorofurofucoeckol-A (**4**); dieckol (**5**); triphloroethol A (**6**); 7-phloroethol (**7**) (Fig. 1).[22] As demonstrated in Table 2, several phlorotannins isolated from *E. bicyclis* exhibited significant BACE1 inhibition in a dose-dependent manner, as compared with: quercetin (10.82 μM); **1** (IC_{50} = 36.47 μM); **2** (IC_{50} = 5.35 μM); **3** (IC_{50} = 12.20 μM); **4** (IC_{50} = 2.13 μM); **5** (IC_{50} = 2.21 μM); **6** (IC_{50} = 11.68 μM); **7** (IC_{50} = 8.59 μM). In accordance with previous findings on in vitro anti-acetylcholinesterase inhibitory and in vivo memory-enhancing activities of phlorotannins,[20] the present results indicate that *E. bicyclis* phlorotannins **1**–**7** have a strong potential of inhibition and prevention of AD through inhibition of BACE1.

In an attempt to explain this manner of BACE1 inhibition, kinetic analysis was investigated at different substrate (150, 250, and 375 nM) and inhibitor concentrations. The inhibition constants (K_i) were determined by interpretation of the Dixon plot, where the value of the *x*-axis implies $-K_i$. The Dixon plot is a graphical

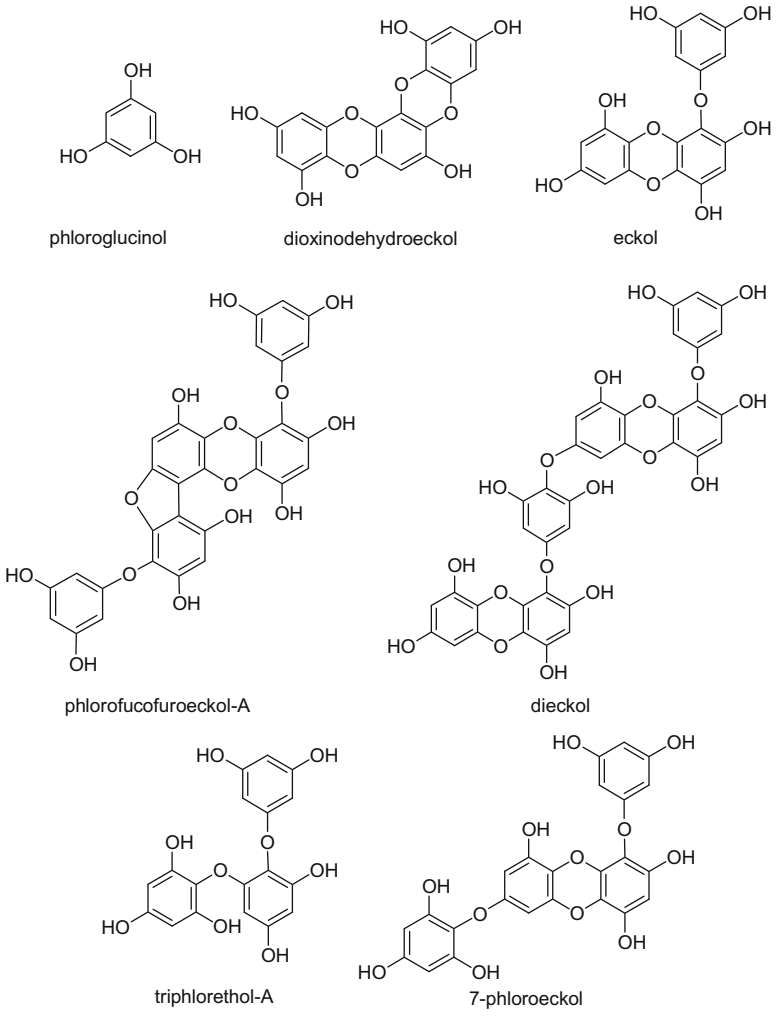

Figure 1. Structures of phlorotannins from *E. bicyclis*.

method [plot of 1/enzyme velocity (*1/V*) against inhibitor concentration (I)] for determination of the type of enzyme inhibition and was used to determine the dissociation or inhibition constant (K_i) for the enzyme–inhibitor complex.[23] Test concentrations of phlorotannins are as follows: **1** (20 and 8 μM); **2** (25, 10, 5 μM); **3** (25, 10, 5 μM); **4** (8 and 2 μM); **5** (10 and 5 μM); **6** (25, 10, 5 μM); **7** (20 and 10 μM). The reaction mixture consisted of the same, aforementioned BACE1 assay method. In the case of competitive inhibition, the *x*-axis implies $-K_i$ when $1/V = 1/V_{max}$. In non-competitive inhibition, the *x*-axis implies $-K_i$ when $1/V = 0$. As shown in Figure 2, **2** (K_i = 8.0), **3** (K_i = 13.9), **4** (K_i = 1.3), **5** (K_i = 1.5), **6** (K_i = 12.1), and **7** (K_i = 7.2) showed non-competitive inhibition with a substrate in the Dixon plots, except for a mixed type of **1**. Usually, the lower the K_i value, the tighter the binding with the enzyme, and the more effective an inhibitor is, indicating that **4** and **5** may be crucial candidates as BACE1 inhibitors.

Among the BACE1 inhibitory phlorotannins **1**–**7**, molecular docking analysis of **3**–**5** was executed to confirm the inhibition mode of the selective BACE1 inhibitors. Generally, structure-based virtual screening (SBVS) can be performed using the protein target coordinates or the ligand coordinates of the bioactive conformation.[24] In order to estimate the conformation of the protein–ligand complex and to increase accuracy, repeatability, and reliability of the docking results, two programs: AUTODOCK 4.0 (AUTODOCK4 and AUTODOCKTOOLS4); FRED 2.0 (OpenEye Scientific Software, Santa Fe, NM, USA) were utilized. Twelve ligand structures were constructed and minimized using Chemschetch 3.5 and Omega 2.0 software (OpenEye Scientific Software, USA), for 2D and 3D conformation, respectively. The docking programs were used to dock the compounds into the binding sites of the crystallographic structures defined as all residues 5–6 Å away from the inhibitor in the original complex. For docking studies, the crystal structure of the protein targets (NCBI GI 167887469) was allocated from the protein sequence alignment [Brookhaven Protein Data Bank (PDB ID 2qk5 chain A)]. The predicted protein ligand complexes were optimized and ranked according to the empirical scoring function ScreenScore, which estimates the binding free energy of the ligand receptor complex. Automated docking is widely used as an effective tool capable of quickly and accurately predicting biomolecular conformations and binding energies of protein–ligands complexes in molecular design. In particular, AUTODOCK 4.0 uses a semiempirical free energy force field to predict binding free energies of protein–ligand complexes of a known structure and binding energy for both the bound and unbound states.[25] On the basis of the rigid rotations and translations of each conformer within the binding site, FRED 2.0 was employed. The approach of the Fred software is to thoroughly dock/score all possible positions of each ligand in the binding site, exhaustively test all poses of the ligand inside the defined binding site, and maintain the protein–ligand complex as rigid during most of the docking process, leading to compensation for target flexibility.[26] The docking of the BACE1-phlorotannin molecule was successful as indicated by the statistically significant scores. As illustrated in Figure 3, the three BACE1-phlorotannin complexes were well performed with three phlorotannins (**3**–**5**) stably posed in the pocket of the BACE1 by AUTODOCK 4.0 (pink) and FRED 2.0 (blue). As for **3**, the binding site predicted by AUTODOCK 4.0 was formed by residues: ASP93; TYR129; PRO131; TYR132; THR133; GLN134; ASP289; THR292, while that by FRED 2.0 by residues: ASP93; TYR129; PRO131; TYR132; THR133; GLN134; ASP289; THR292; ARG296. In the case of BACE–**4** conformation, the binding site predicted by AUTODOCK 4.0 was formed by residues: GLY72; ASP93; SER96; TYR129; PRO131; TYR132; THR133; GLN134; ILE287; ASP289; GLY291; THR293; ASN294, while that by FRED 2.0 by residues: GLU73; GLY74; GLY95; VAL130; TYR132; THR133; GLN134; THR259; ILE287; THR292. As for **5**, the binding site predicted by AUTODOCK 4.0 was formed by residues: SER96; ASN98; TYR129; PRO131; THR133; ILE187; ARG189; THR292, while that by FRED 2.0 by residues: ASP93; TYR132; THR133; GLN134; THR292; ARG296. Moreover, the docking analysis indicated that the lowest energy conformation of the most proposed complex has to be taken into account when using the AUTODOCK 4.0 (−8.37, −11.01, −13.25 kcal/mol) and FRED 2.0 programs (−62.8034, −59.5194, −67.8853 kcal/mol) for **3**–**5**, respectively. These molecular docking studies have pointed out that TYR132 and THR133 are the crucial residues responsible for BACE1 inhibition. Both the in vitro evaluation and molecular docking data indicate that *E. bicyclis* phlorotannins have a strong potential of inhibition and prevention of AD through β-amyloids pathways.

In addition to the significant BACE1 inhibition, phlorotannins as potent candidates for anti-Alzheimer drugs would possess diverse advantages: ease of blood–brain barrier (BBB) penetration; prevention and compensation of BBB dysfunction. Preliminary require-

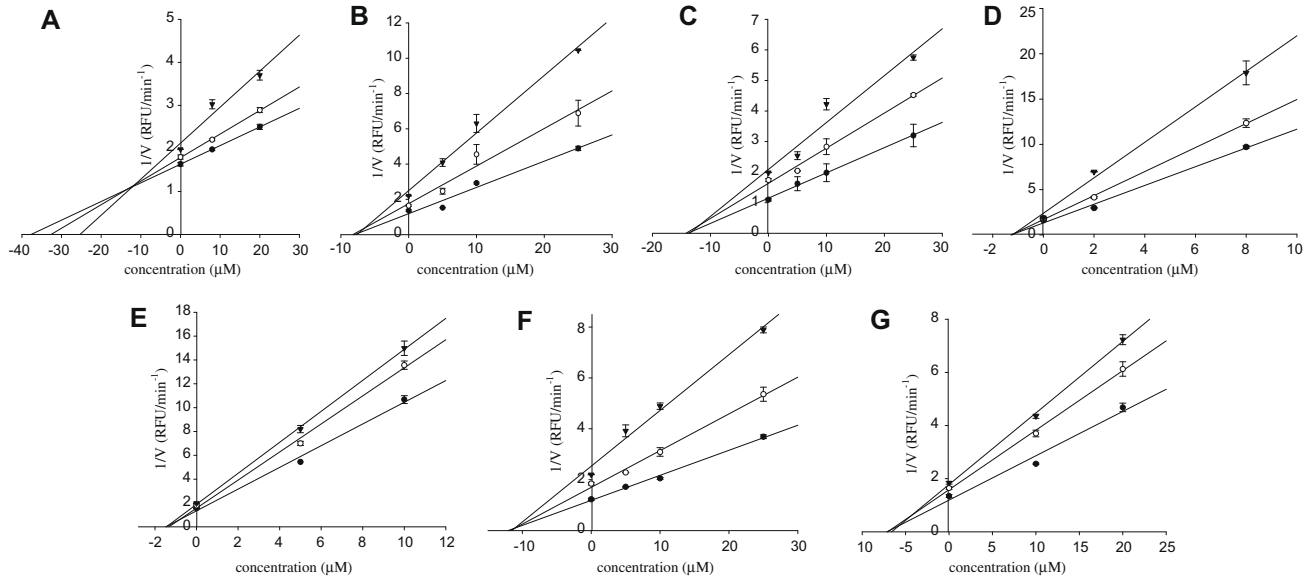

Figure 2. Dixon plots for inhibition of BACE1 by phlorotannins in the presence of different concentrations of substrate: 375 nM (●); 250 nM (○); 150 nM (▼). phloroglucinol (A), dioxinodehydroeckol (B), eckol (C), phlorofucofuroeckol-A (D), dieckol (E), triphloroethol A (F), 7-phloroethol (G).

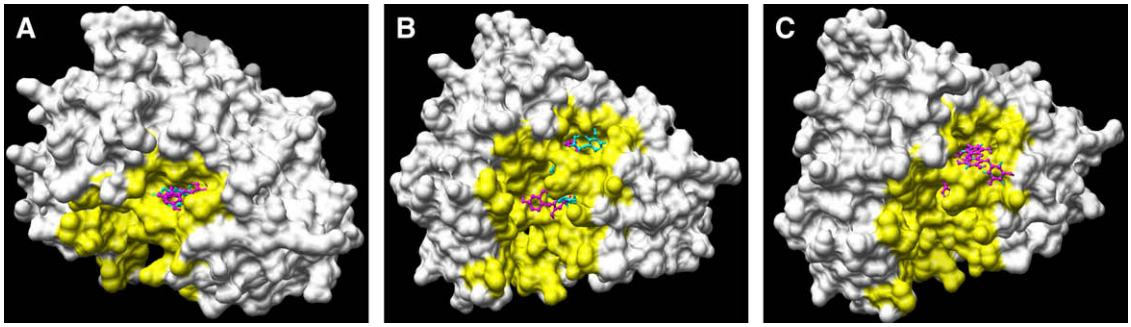

Figure 3. Molecular docking models of BACE1-phlorotannins complex Three selected phlorotannins, including: eckol (A); phlorofurofucoeckol-A (B); dieckol (C), were carried out by two docking programs. The structural ligands were performed by AUTODOCK 4.0 (pink) and FRED 2.0 (blue). Yellow portions indicate positions of the cleft or pocket between the two BACE1 domains.

ments of anti-AD candidates must be the bioavailability and penetration through the BBB and plasma membrane; therefore, proposed BACE1 inhibitors must be of relatively low-molecular-weight and high lipophilicity. The predicted BBB penetration values of phlorotannins depend on molecular weight. Although the high-molecular-weight phlorotannins, such as **4** and **5**, presume low BBB penetration values, the low-molecular-weight phlorotannins, such as **1**–**3**, **6**, and **7** predict relatively high BBB penetration values. Moreover, Prasain et al. suggested that select high-molecular weight catechins can penetrate the BBB and potentially exert neuroprotective effects.[27]

Recently, Aβ plagues were implicated in oxidative stress-induced BBB dysfunction, leading to neurotoxicity. The AD brain is characterized by extensive oxidative stress due to drastically reduced levels of antioxidants and elevated levels of resultant biomarkers, including 8-hydroxy-deoxyguanosine, thiobarbituric acid reactive substances (TBARS), 4-hydroxy-2-trans-nonenal (HNE), protein carbonyls, and 3-nitrotyrosine.[2,28] In particular, Aβ fragments, including Aβ(25–35), Aβ(1–40), and Aβ(1–42) are reported to directly produce free radicals and extensive oxidative stress and induce memory deficits.[29] Such Aβ plagues can also form pores in neuronal membranes, enriched in lipid peroxidation-vulnerable polyunsaturated fatty acid, leading to changes in neuronal and endothelial cell membrane permeability.[2] In addition, Aβ accumulation might play important roles in the neuroinflammatory responses, concomitant with the activation of NF-κB as a pivotal transcription factor and the accelerated release of inflammatory mediators, including COX-2, iNOS, cytokines, as well as the growing formation of reactive oxygen species (ROS) and nitric oxide.[28,30] All these alterations likely contribute to Aβ neurotoxicity. Previous reports have revealed that phlorotannins possess potent inhibitory activities against peroxynitrite and ROS in in vitro antioxidant assays.[16,19] As such, antioxidant phlorotannins possess beneficial and pivotal anti-AD properties by virtue of scavenging free radicals and inhibition of reactive oxygen species in neuronal cells, as well as inhibition of the Aβ-inducing inflammatory response and oxidative stress in AD brain.

In conclusion, the MeOH extract and polar fractions of *E. bicyclis* exhibited significant efficacies in the BACE1 inhibitory assay. Accordingly, seven phlorotannins from the most active EtOAc fraction have been identified. Although a more detailed explanation has yet to be proffered, most of the isolated phlorotannins exerted significant and non-competitive inhibitory activities against BACE1 through TYR132 and THR133 residues. Therefore, the *E. bicyclis* extract and the phlorotannins contained therein would clearly have beneficial uses in the development of therapeutic and preventive agents for AD.

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21. BACE1 fluorescence resonance energy transfer (FRET) assay kit (β-Secretase, human recombinant) was purchased from the PanVera Co. (Madison, WI, USA). The assay was carried out according to the supplied protocol with select modifications. Briefly, a mixture of 10 μL of the assay buffer (50 mM sodium acetate, pH 4.5), 10 μL of BACE1 (1.0 U/mL), 10 μL of the substrate (750 nM Rh-

EVNLDAEFK-Quencher in 50 mM, ammonium bicarbonate), and 10 μL of the tested samples (final concentration (f.c.) 125 μg/mL for extract and fractions; f.c. 100 μM for phlorotannins) dissolved in 10% DMSO was incubated for 60 min at 25 °C in the dark. The proteolysis of two fluorophores (Rh-EVNLDAEFK-Quencher) by BACE1 was monitored by the formation of the fluorescent donor (Rh-EVNL), which increases in fluorescence wavelengths at 530–545 nm (excitation) and 570–590 nm (emission), respectively. Fluorescence was measured with a microplate spectrofluorometer (Molecular devices, Sunnyvale, CA, USA). The mixture was irradiated at 545 nm and the emission intensity was recorded at 585 nm. The percent inhibition (%) was obtained by the following equation: % Inhibition = $[1 - (S_{60} - S_0)/(C_{60} - C_0)] \times 100$, where C_{60} was the fluorescence of the control (enzyme, buffer, substrate) after 60 min of incubation, C_0 the initial fluorescence of the control, S_{60} the fluorescence of the tested samples (enzyme, sample solution, substrate) after 60 min of incubation, and S_0 the initial fluorescence of the tested samples. To allow for the quenching effect of the samples, the sample solution was added to reaction mixture, and any reduction in fluorescence by the sample was then investigated. The BACE1 inhibitory activity of each sample was expressed in terms of the IC_{50} value (μg/mL and μM required to inhibit the proteolysis of the BACE1 substrate by 50%), as calculated from the log–dose inhibition curve. Quercetin and sophoraflavanone G were used as the positive controls.

22. A lyophilized powder of *E. bicyclis* (4 kg) was extracted with MeOH (18 L) three times for 3 h under reflux, and the solvent was evaporated in vacuo to give MeOH extract (1400 g). This extract was followed by partitioning with organic solvents, yielding CH_2Cl_2 (134.4 g), EtOAc (412.6 g) and *n*-BuOH (198.5 g) fractions. A portion of the EtOAc fraction (300 g) was subjected to chromatography on a silica gel column, with EtOAc–MeOH (50:1–5:1) as eluent, yielding 10 subfractions (EF01–EF10). Repeated column chromatography of EF01 (40 g) was conducted with a solvent mixture of *n*-hexane and EtOAc, yielding 11 subfractions (EF0101–EF0111). Compound **1** (150 mg) was purified from EF0104 (3.2 g) on an RP-18 column, eluted with aqueous MeOH (20% MeOH to 100% MeOH, gradient elution). RP-18 column chromatography of EF0105 (20.5 g), using identical solvent conditions, led to the isolation of **2** (95 mg) and **3** (1.0 g). Compounds **4** (1.2 g), **5** (1.7 g), and **7** (50 mg) were purified from EF0106 (12.5 g), on RP-18 (20% MeOH to 100% MeOH, gradient elution) and Sephadex LH-20 columns (100% MeOH). EF02 (10.5 g) was subjected to chromatography on a silica gel column, with CH_2Cl_2–MeOH (18:1) as eluent, yielding eight subfractions (EF0201–EF0208). Successive chromatography of EF0204 (800 mg) on an RP-18 column, with 20% MeOH as eluent, yielded five subfractions (F020401–F020405). Repeated column chromatography of EF020401 (225 mg) on a silica gel column, with CH_2Cl_2–MeOH (18:1) as eluent, resulted in the isolation of **6** (58 mg). All isolated compounds **1**–**7** were identified and characterized as phloroglucinol,[16] dioxinodehydroeckol,[16] eckol,[10] phlorofurofucoeckol-A,[10] dieckol,[16] triphloroethol-A,[10] and 7-phloroethol,[5] by spectroscopic methods, as well as by comparison with published data.

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