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journal homepage: <http://ees.elsevier.com/apjtm>Original research <http://dx.doi.org/10.1016/j.apjtm.2016.01.014>Anti-Alzheimer's disease potential of coumarins from *Angelica decursiva* and *Artemisia capillaris* and structure-activity analysisMd. Yousof Ali¹, Susoma Jannat¹, Hyun Ah Jung^{2*}, Ran Joo Choi³, Anupom Roy¹, Jae Sue Choi^{1*}¹Department of Food and Life Science, Pukyong National University, Busan 608-737, Republic of Korea²Department of Food Science and Human Nutrition, Chonbuk National University, Jeonju 561-756, Republic of Korea³Angiogenesis & Chinese Medicine Laboratory, Department of Pharmacology, University of Cambridge, Cambridge, UK

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ABSTRACT

Objective: To use structure-activity analysis to study the anti-Alzheimer's disease (anti-AD) activity of natural coumarins isolated from *Angelica decursiva* and *Artemisia capillaris*, along with one purchased coumarin (daphnetin).

Methods: Umbelliferone, umbelliferone 6-carboxylic acid, scopoletin, isoscapoletin, 7-methoxy coumarin, scoparone, scopolin, and esculetin have been previously isolated; however 2'-isopropyl psoralene was isolated from *Angelica decursiva* for the first time to evaluate their inhibitory effects against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and β -site amyloid precursor protein cleaving enzyme 1 (BACE1) enzyme activity. We scrutinized the potentials of coumarins as cholinesterase and BACE1 inhibitors via enzyme kinetics and molecular docking simulation.

Results: Among the test compounds, umbelliferone 6-carboxylic acid, esculetin and daphnetin exhibited potent inhibitory activity against AChE, BChE and BACE1. Both esculetin and daphnetin have a catechol group and exhibit significant anti-AD activity against AChE and BChE. In contrast, presence of a sugar moiety and methoxylation markedly reduced the anti-AD activity of the coumarins investigated in this study. With respect to BACE1 inhibition, umbelliferone 6-carboxylic acid, esculetin and daphnetin contained carboxyl or catechol groups, which significantly contributed to their anti-AD activities. To further investigate these results, we generated a 3D structure of BACE1 using Autodock 4.2 and simulated binding of umbelliferone 6-carboxylic acid, esculetin and daphnetin. Docking simulations showed that different residues of BACE1 interacted with hydroxyl and carboxylic groups, and the binding energies of umbelliferone 6-carboxylic acid, esculetin and daphnetin were negative (−4.58, −6.25 and −6.37 kcal/mol respectively).

Conclusions: Taken together, our results suggest that umbelliferone 6-carboxylic acid, esculetin and daphnetin have anti-AD effects by inhibiting AChE, BChE and BACE1, which might be useful against AD.

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1. Introduction

Alzheimer's disease (AD) is the major form of dementia and is a deadly neurodegenerative disease that is progressive in nature and develops through a multifactorial process. AD is the fourth leading cause of death in developed countries, and predominates in Europe and USA following cardiovascular disease, cancer, and stroke [1]. The two most common hypotheses used to describe the pathology of AD are known as the 'cholinergic hypothesis' and 'amyloid hypothesis'. The cholinergic hypothesis suggests that AD is caused by a deficiency in the brain levels of the cerebral neurotransmitter acetylcholine (ACh), which is hydrolyzed by acetylcholinesterase (AChE) [2]. Similarly, butyrylcholinesterase (BChE) activity is increased by 40%–90% during the progression of AD [3], and BChE inhibition is considered a potentially important aspect of treating AD. In addition to the AChE and BChE hypotheses, accumulation of amyloid- β peptide (A β) in the brain is widely considered to be critically involved in the pathogenesis of AD [4]. A β plaques emerge roughly 15 years before the symptoms of AD appear [5], and once AD develops the cognitive decline caused by neuronal damage cannot be reversed, even after A β levels in the brain are lowered by immunotherapy [6]. Thus, prevention of A β accumulation is considered an important part of preventing AD. A β is excised from amyloid- β precursor protein through sequential cleavage by aspartic protease β -secretase 1 (BACE1) [4]. Thus, because BACE1 initiates A β processing, inhibition of BACE1 activity may be an effective way to prevent A β accumulation [7].

Coumarins are an important class of natural compounds and are used as additives in both foods and cosmetics [8]. Coumarin has been reported to have antibacterial [9], anti-oxidant [10], anti-inflammatory and anticoagulant [11], and anti-AD activities [12]. Coumarins are characterized a fused benzene and α -pyrone ring that serves as the structural nucleus. Importantly, a number of studies have demonstrated the ability of coumarins to inhibit AChE [12–14], and the benzopyrone structural nucleus of coumarins is an essential aspect of hybrid molecules capable of simultaneously inhibiting AChE and AChE-induced β -amyloid aggregation [15]. Likewise, the possibility of numerous chemical substitutions afforded by the structural nucleus makes coumarins interesting molecules for drug discovery.

As part of our continuing efforts to identify compounds from natural resources that inhibit AChE, BChE, and BACE1, we recently found that the methanolic extract of *Angelica decursiva* (*A. decursiva*) (Umbelliferae) fulfills these criteria [16]. This plant is used traditionally as an anti-inflammatory, diuretic, expectorant, and diaphoretic, as well as a remedy for colds, influenza, hepatitis, arthritis, indigestion, coughs, chronic bronchitis, pleurisy, typhoid, headaches, flatulence, fever, colic, travel sickness, rheumatism, bacterial and fungal infections, and diseases of the urinary organs [17,18]. Furthermore, extensive investigations of different species of this genus have been carried out in the last decade, and as a result many classes of compounds have been isolated including different types of coumarin derivatives [19]. In addition, *Artemisia capillaris* (*A. capillaris*) is commonly distributed in sandy areas along the Korean coastline. This plant has been frequently used in the treatment of liver disease, including hepatitis, jaundice, fatty liver and bilious disorder. Infusions of the buds, stems and whole plant of *A. capillaris* have been used in traditional Chinese medicine primarily as a choleric, anti-inflammatory,

antipyretic, and diuretic agent for treating epidemic hepatitis [20,21]. This study deals with the anti-AD activities and the structure–activity relationship of coumarins isolated from *A. decursiva* and *A. capillaris*. Specifically, because there is currently no detailed information regarding the molecular interactions between umbelliferone 6-carboxylic acid, esculetin, daphnetin and BACE1, we performed molecular docking analysis and detailed enzyme kinetic analysis in order to investigate the possibility of using compounds umbelliferone 6-carboxylic acid, esculetin and daphnetin as potent anti-AD drug candidates.

2. Materials and methods

2.1. General experimental procedures

The ^1H - and ^{13}C -NMR spectra were determined using a JEOL JNM ECP-400 spectrometer (Tokyo, Japan) at 400 MHz for ^1H and 100 MHz for ^{13}C in deuterated chloroform (CDCl_3). Column chromatography was conducted using silica gel 60 (70–230 mesh, Merck, Darmstadt, Germany). All TLC analyses were conducted using pre-coated Merck Kieselgel 60 F₂₅₄ plates (20 cm \times 20 cm, 0.25 mm) and using 50% H_2SO_4 as the spray reagent.

2.2. Chemicals and reagents

Electric-eel acetylcholinesterase (AChE, EC3.1.1.7), horse-serum butyrylcholinesterase (BChE, EC 3.1.1.8), acetyl thiocholine iodide (ACh), butyrylthiocholine chloride (BCh), 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB), quercetin, daphnetin and berberine were purchased from E. Merck, Fluka, or Sigma–Aldrich unless otherwise stated. The BACE1 FRET assay kit (β -secretase) was purchased from Pan Vera Co. (Madison, WI, USA). All chemicals and solvents used for column chromatography were of reagent grade, purchased from commercial sources, and used as received.

2.3. Isolation of coumarins

Umbelliferone, umbelliferone 6-carboxylic acid, scopoletin, isoscapoletin, 7-methoxy coumarin, scoparone, scopolin and esculetin were isolated from *A. decursiva* and *A. capillaris*, according to the method described by Zhao *et al.* [18] and Islam *et al.* [21], respectively. 2'-Isopropyl psoralene was isolated from subfraction-2 of dichloromethane fraction from *A. decursiva*, and identified by spectroscopic evidence including ^1H and ^{13}C -NMR, as well as by comparison with spectral published data [22].

2.4. In vitro ChE enzyme assay

The inhibitory activities of the isolated coumarins towards ChE were measured using the spectrophotometric method developed by Ellman *et al.* [23]. ACh and BCh were used as substrates to assay the inhibition of AChE and BChE, respectively. Each reaction mixture consisted of 140 μL sodium phosphate buffer (pH 8.0), 20 μL of test sample solution at a final concentration of 100 μM for all compounds, and 20 μL of either AChE or BChE solution, which were then combined and incubated for 15 min at room temperature. All test samples and positive control (berberine) were dissolved in

10% DMSO. Reactions were initiated upon addition of 10 μ L of DTNB and 10 μ L of either ACh or BCh, respectively. The enzymatic hydrolysis mediated by AChE or BChE was monitored according to the formation of the yellow 5-thio-2-nitrobenzoate anion at 412 nm for 15 min, which was due to the reaction of DTNB with thiocholine released from ACh or BCh, respectively. All reactions were performed in 96-well plates in triplicate and recorded using a microplate spectrophotometer (Molecular Devices).

2.5. *In vitro* BACE1 enzyme assay

The *in vitro* BACE1 enzyme assay was carried out according to the manufacturers recommended protocol with minor modifications. Briefly, a mixture of 10 μ L of assay buffer (50 mM sodium acetate, pH 4.5), 10 μ L of BACE1 (1.0 U/mL), 10 μ L of the substrate (750 nM Rh-EVNLDAEFK-Quencher in 50 mM ammonium bicarbonate) and 10 μ L of the test samples were dissolved in 10% DMSO and incubated for 60 min at 25 °C in the dark. The proteolysis of two fluorophores (Rh-EVNLDAEFK-Quencher) by BACE1 was determined by monitoring formation of the fluorescent donor (Rh-EVNL) at wave lengths of 530–545 nm for excitation and 570–590 nm for emission. Fluorescence was measured with a microplate spectrofluorometer (Gemini EM, Molecular Devices, and Sunnyvale, CA, USA). Specifically, each reaction was excited at 545 nm and the emission intensity was recorded at 585 nm.

2.6. Kinetic parameters of different coumarins towards AChE, BChE and BACE1 inhibition

In order to determine the mechanism of inhibition of the different coumarins, AChE and BChE inhibition was evaluated by monitoring the effects of different concentrations of substrate (0.6–0.1 mM for different samples). Specifically, AChE and BChE inhibition assays were performed as described above, but the substrate concentration was varied. Reactions were initiated upon addition of 10 μ L of DTNB and 10 μ L of either Ach or Bch at the indicated concentrations. In addition, to determine the kinetic mechanisms of the different coumarins towards BACE1, we employed two complementary kinetic methods, namely Lineweaver–Burk and Dixon plots [24,25]. Specifically, Dixon plots for inhibition of BACE1 by umbelliferone 6-carboxylic acid, esculetin and daphnetin were obtained in the presence of different concentrations of substrate: 375 nM (\blacktriangledown); 250 nM (\circ) and 150 nM (\bullet). Likewise, the test concentrations of umbelliferone 6-carboxylic acid, esculetin and daphnetin for the BACE1 inhibition kinetic analysis were 0.1 μ M (\blacktriangledown), 1 μ M (\circ), and 10 μ M (\bullet) for umbelliferone 6-carboxylic acid; 2.5 μ M (\blacktriangledown), 12.5 μ M (\circ), and 62.5 μ M (\bullet) for esculetin; and 2.5 μ M (\blacktriangledown), 25 μ M (\circ), and 100 μ M (\bullet) for daphnetin. Inhibition constants (K_i) were determined by interpretation of Dixon plots, where the value of the x-axis intercept was taken as K_i [25,26].

2.7. BACE1 molecular docking simulations

To estimate the structure of the enzyme–inhibitor complex and to ensure accuracy, repeatability, and reliability of docking results, we employed Autodock 4.2 software. In our study, the test concentrations of coumarins for AChE were 100 μ M, 50 μ M, and 10 μ M for umbelliferone 6-carboxylic acid,

100 μ M, 20 μ M, and 4 μ M for esculetin; and 100 μ M, 50 μ M, and 25 μ M for daphnetin. Likewise, the test concentrations for BChE were 100 μ M, 50 μ M and 25 μ M for umbelliferone 6-carboxylic acid; 100 μ M, 20 μ M, and 1 μ M for esculetin; and 50 μ M, 25 μ M, and 10 μ M for daphnetin. Specifically, we used Autodock 4.2 to dock different coumarins into the binding site of the BACE1 crystallographic structure, which was defined as all residues 5–6 Å from the inhibitor in the original complex. Autodock 4.2 uses a semi-empirical free energy force field to predict binding free energies of protein–ligand complexes of known structures and binding energy for both bound and unbound states. Twelve ligand structures were constructed and minimized using Chemdraw and LigandScout software for 2D and 3D conformations, respectively. For docking studies, the crystal structure of the BACE1 protein target was obtained from the protein sequence alignment [Protein Data Bank (PDB ID 2wjo)]. The 3D structures of umbelliferone 6-carboxylic acid, esculetin and daphnetin were generated and minimized using Chemdraw and LigandScout, for 2D and 3D conformation, respectively.

2.8. Statistical analysis

All results are expressed as the mean \pm SEM of triplicate samples. Results were analyzed using one-way ANOVA and Student's *t*-test where appropriate (Systat Inc., Evanston, IL, USA). Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Inhibitory activity of coumarins against AChE, BChE and BACE1

In order to evaluate the anti-AD activity of the isolated coumarins, we first evaluated their abilities to inhibit AChE, BChE, and BACE1. A total of 10 coumarin derivatives were screened for their *in vitro* AChE, BChE and BACE1 inhibitory capacities (Table 1). Our results showed that esculetin and daphnetin exhibited good AChE inhibitory activity with IC_{50} values of 6.13 and 11.57 μ M, while umbelliferone 6-carboxylic acid, umbelliferone, scopoletin, isoscapoletin, 2'-isopropyl psoralene, as well as 7-methoxy coumarin showed moderate inhibitory activities with IC_{50} values of 104.12, 145.19, 150.28, 153.77, 173.89, and 186.47 μ M, respectively. Next we investigated the BChE inhibitory activities of the isolated coumarins (Table 1). The coumarins daphnetin, esculetin, and umbelliferone 6-carboxylic acid exhibited promising BChE inhibitory activity with IC_{50} values of 8.66, 9.29, and 27.19 μ M, which compared favorably to the positive control berberine (IC_{50} of 7.01 μ M). On the other hand, umbelliferone, isoscapoletin, 7-methoxy coumarin, scopoletin, and 2'-isopropyl psoralene possessed only moderate inhibitory activity towards BChE with IC_{50} values of 105.28, 110.30, 119.39, 159.30, and 179.22 μ M, respectively. Finally, with respect to BACE1 inhibitory activity, umbelliferone 6-carboxylic acid, esculetin and daphnetin exhibited strong inhibitory activity with IC_{50} values of 0.34, 7.67, and 11.19 μ M compared to the positive control quercetin 13.98 μ M. On the other hand, isoscapoletin, umbelliferone, and 7-methoxy coumarin exhibited moderate inhibitory activities with IC_{50} values of 146.43, 143.1, and 172.26 μ M respectively.

Table 1ChEs and BACE1 inhibitory activities of coumarins isolated from *A. decursiva* and *A. capillaris* (IC₅₀ (μM)) (mean ± SEM).

Test compounds	AChE ^b	BChE ^c	SI ^d	BACE1 ^e
Umbelliferone	145.19 ± 0.18	105.28 ± 1.02	0.72	143.1 ± 1.89
Umbelliferone 6-carboxylic acid	104.12 ± 0.11	27.19 ± 1.20	0.26	0.34 ± 0.01
Esculetin	6.13 ± 0.83	9.29 ± 0.18	1.51	7.67 ± 0.91
Daphnetin	11.57 ± 0.49	8.66 ± 0.18	0.74	11.19 ± 0.93
Scopoletin	150.28 ± 0.27	159.30 ± 1.02	1.06	>100
Isoscapoletin	153.77 ± 1.93	110.30 ± 0.39	0.71	146.43 ± 1.08
7-Methoxy coumarin	186.47 ± 0.86	119.39 ± 0.99	0.64	172.26 ± 0.96
Scoparone	259.47 ± 0.97	273.39 ± 1.94	1.05	>100
Scopolin	>200	>200	–	>100
2'-Isopropyl psoralene	173.89 ± 1.19	179.22 ± 0.91	1.03	>100
Berberine ^f	0.717 ± 0.01	7.01 ± 0.31	–	–
Quercetin ^g	–	–	–	13.98 ± 0.51

^{b,c,e} Final concentration of test samples were 100 μM, dissolved in 10% DMSO; ^dSI: selectivity index (BChE/AChE). ^fBerberine and ^gquercetin were used as positive controls for the ChE and BACE1 assays, respectively.

All of the other compounds were inactive at the concentrations tested.

3.2. Kinetic parameters of coumarins on AChE and BChE inhibition

In order to determine the manner of AChE and BChE inhibition by the different isolated coumarins, we performed kinetics analyses using different concentrations of substrate (0.2 mM–0.6 mM) and inhibitors, and evaluated the results using Dixon plots, which are a graphical method for determining the type of enzyme inhibition. Dixon plots are generated by plotting the reciprocal enzyme velocity (1/V) against inhibitor concentration

(I), and can be used to determine the dissociation or K_i for the enzyme–inhibitor complex, in which the value of the x-axis intercept is the K_i [26]. As shown in Figure 1A–C and Table 2, the manner of inhibition of umbelliferone 6-carboxylic acid and esculetin was noncompetitive with K_i values of 80.99 μM and 25.09 μM, while compound daphnetin exhibited mixed type inhibition with a K_i value of 19.98 μM for AChE. Similarly, Figure 1D–F and Table 2, indicated that the type of inhibition of umbelliferone 6-carboxylic acid was noncompetitive with a K_i value of 49.78 μM, whereas esculetin and daphnetin exhibited mixed type inhibition with K_i values of 16.11 μM and 7.18 μM, respectively.

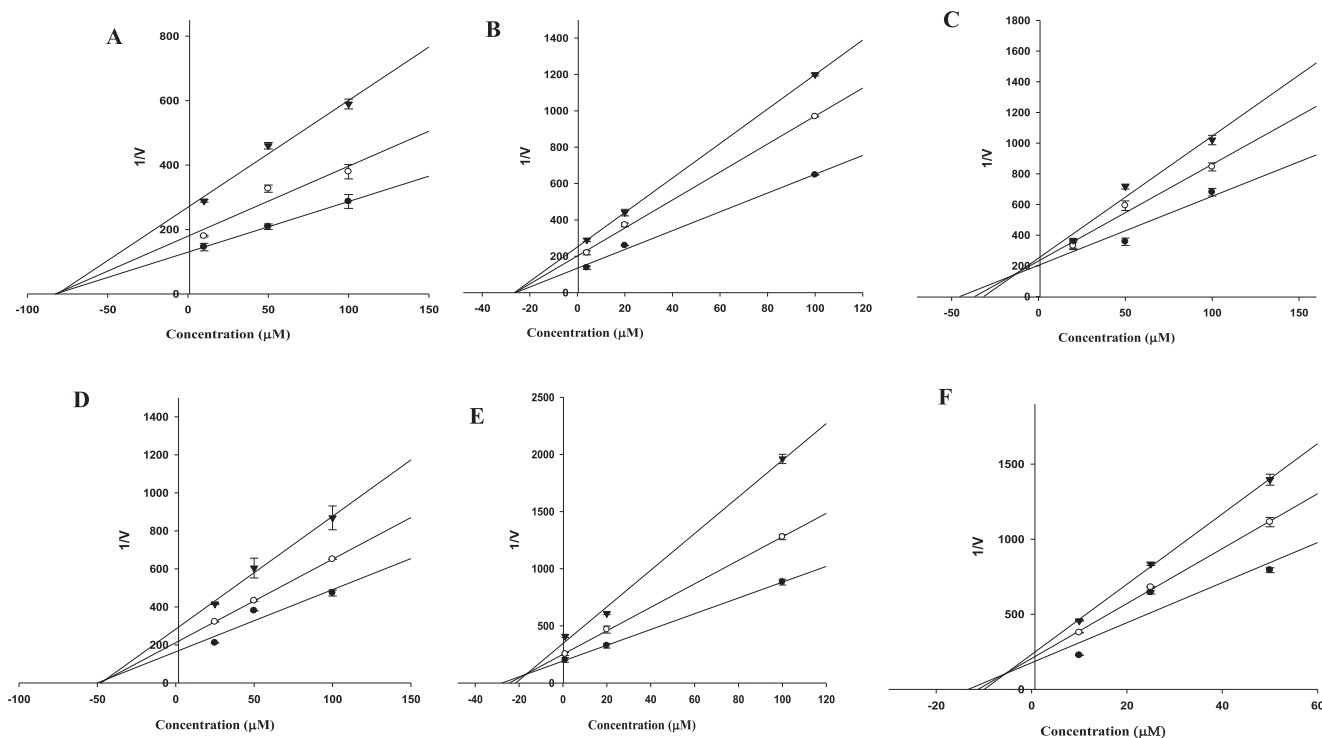


Figure 1. Dixon plot of the inhibition of AChE and BChE by umbelliferone 6-carboxylic acid, esculetin and daphnetin in the presence of different substrate and inhibitor concentrations.

(A) umbelliferone 6-carboxylic acid [0.6 mM (●); 0.4 mM (○); and 0.2 mM (▼) of AChE], (B) esculetin [0.6 mM (●); 0.4 mM (○); and 0.2 mM (▼) of AChE], (C) daphnetin [0.6 mM (●); 0.3 mM (○); and 0.1 mM (▼) of AChE], (D) umbelliferone 6-carboxylic acid [0.6 mM (●); 0.3 mM (○); and 0.1 mM (▼) of BChE], (E) esculetin [0.6 mM (●); 0.3 mM (○); and 0.1 mM (▼) of BChE], and (F) daphnetin [0.6 mM (●); 0.4 mM (○); and 0.2 mM (▼) of BChE].

Table 2Mode of inhibition and dissociation constants (K_i) of coumarins for AChE, BChE and BACE1 based on enzyme kinetic plot.

Test compounds	AChE		BChE		BACE1	
	Inhibition type ^a	K_i (μM) ^b	Inhibition type ^b	K_i (μM) ^a	Inhibition type ^c	K_i (μM) ^d
Umbelliferone 6-carboxylic acid	Noncompetitive	80.99	Noncompetitive	49.78	Mixed	2.08
Esculetin	Noncompetitive	25.09	Mixed e	16.11	Mixed	17.99
Daphnetin	Mixed	19.98	Mixed	7.18	Mixed	16.18
Quercetin ^e	–	–	–	–	Competitive	10.45
Berberine ^f	NA	NA	NA	NA	–	–

a, b Determined by Dixon plots. c,d Determined by Dixon and Lineweaver–Burk plots. e,f Positive controls. NA, not tested.

3.3. Kinetic parameters of coumarins against BACE1 inhibition

To determine the mode of enzyme BACE1 inhibition by the different coumarins we performed kinetics analysis using Lineweaver–Burk plots and Dixon plots [24–26] employing different concentrations of substrate (150 nM, 250 nM, and 375 nM) and inhibitors. K_i values were determined by interpretation of Dixon plots as described above. The test concentrations of the different coumarins were as follows: 10 μM , 1 μM , and 0.1 μM for umbelliferone 6-carboxylic acid; 62.5 μM , 12.5 μM and 2.5 μM for esculetin; and 100 μM , 25 μM , and 2.5 μM for daphnetin. As shown in Figure 2A–F and Table 2, the manner of BACE1 inhibition of umbelliferone 6-carboxylic acid, esculetin and daphnetin was mixed, with K_i values of 2.08 μM , 17.99 μM , and 16.18 μM , respectively. A lower K_i value is indicative of tighter enzyme binding and thus a more effective inhibitor. Thus, these results suggested that umbelliferone 6-carboxylic acid, esculetin and daphnetin might be good BACE1 inhibitors.

3.4. Molecular docking study of the inhibitory activity of the coumarins against BACE1

To follow up our results for the most active BACE1 inhibitory compounds we next performed molecular docking simulations. Specifically, we used Autodock 4.2 to predict and investigate the interactions between umbelliferone 6-carboxylic acid, esculetin, daphnetin and BACE1. Molecular docking models of these three coumarins and 2-Amino-3-[(1*r*)-1-cyclohexyl-2-[(cyclohexylcarbonyl) amino] ethyl]-6-phenoxyquinazolin-3-ium (QUD) are illustrated in Figures 3–5. QUD is the most potent nonpeptic BACE1 inhibitor reported to date according to protein data bank. Ligand–enzyme complexes with umbelliferone 6-carboxylic acid, esculetin, daphnetin and QUD were stably posed in the same pocket of BACE1 by Autodock 4.2. As illustrated in Figures 3–5, the corresponding ligand interactions of umbelliferone 6-carboxylic acid in the active site of BACE1 were facilitated by hydrogen-bonding interactions between the Arg235 residue and one carboxylic group interaction between each of the Tyr71, Arg235 and Thr72 residues with one hydroxyl

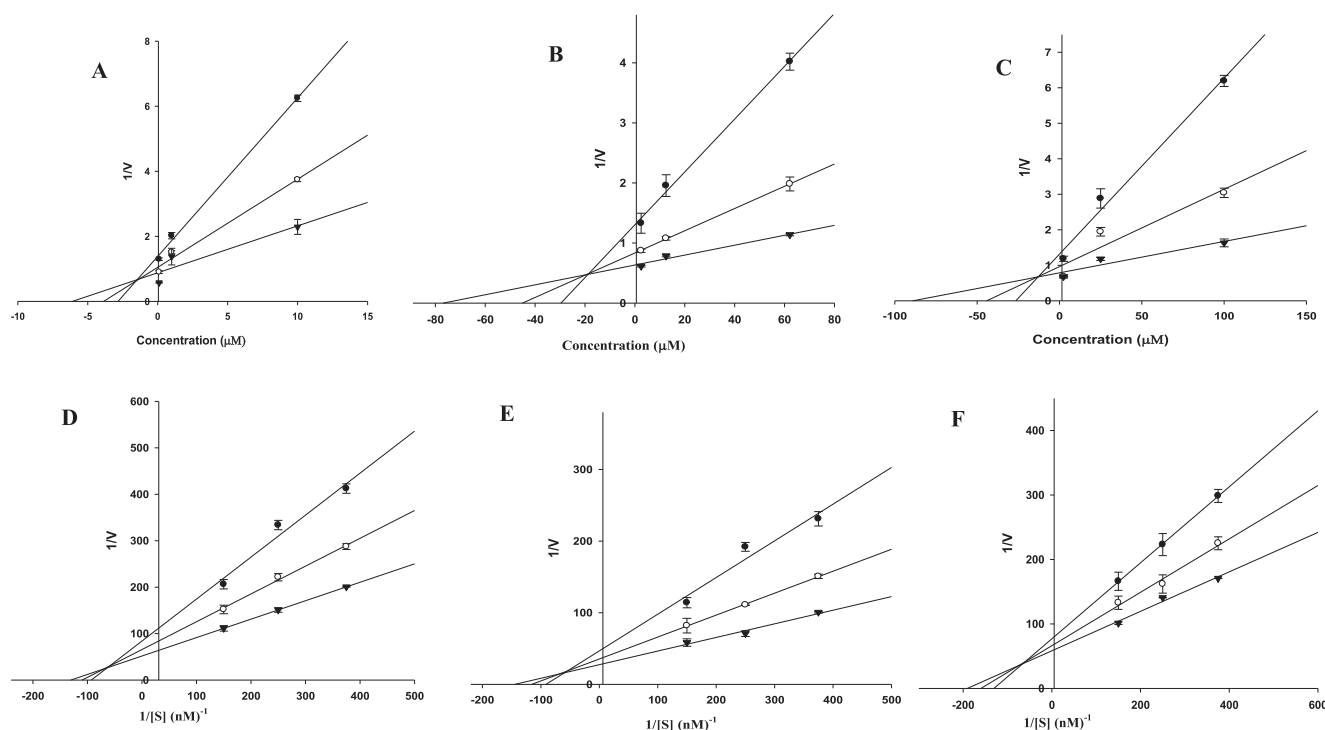


Figure 2. Dixon plots of BACE1 inhibition by coumarins. Umbelliferone 6-carboxylic acid.

(A), esculetin (B), and daphnetin (C) were tested in the presence of different concentrations of substrate: 150 nM (●), 250 nM (○), 375 nM (▼). Lineweaver–Burk plots for BACE1 inhibition by coumarins. BACE1 inhibition was analyzed in the presence of different concentrations of sample as indicated: 0.1 μM (▼), 1 μM (○), and 10 μM (●) for umbelliferone 6-carboxylic acid (D); 2.5 μM (▼), 12.5 μM (○), and 62.5 μM (●) for esculetin (E); and 2.5 μM (▼), 25 μM (○), and 100 μM (●) for daphnetin (F).

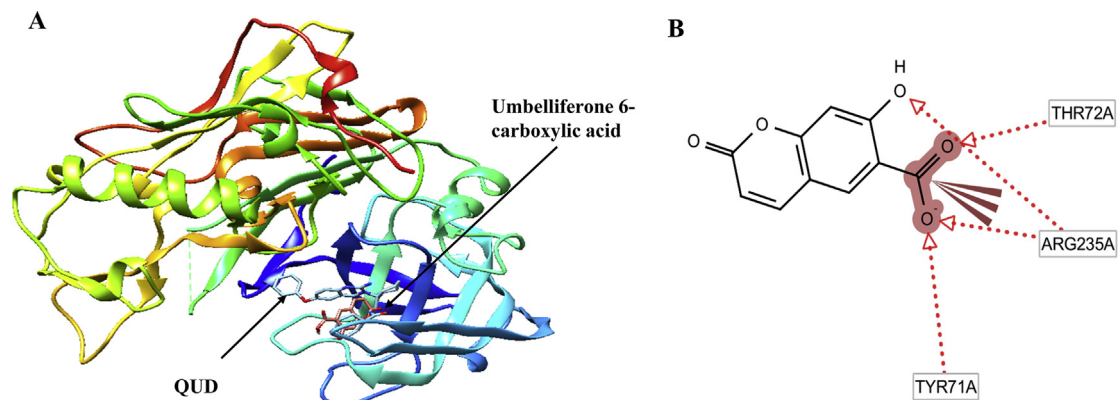


Figure 3. (A) Molecular docking model of BACE1 with umbelliferone 6-carboxylic acid (red color) and QUD (blue color). (B) Diagram of the ligand interaction of umbelliferone 6-carboxylic acid in the active site of BACE1.

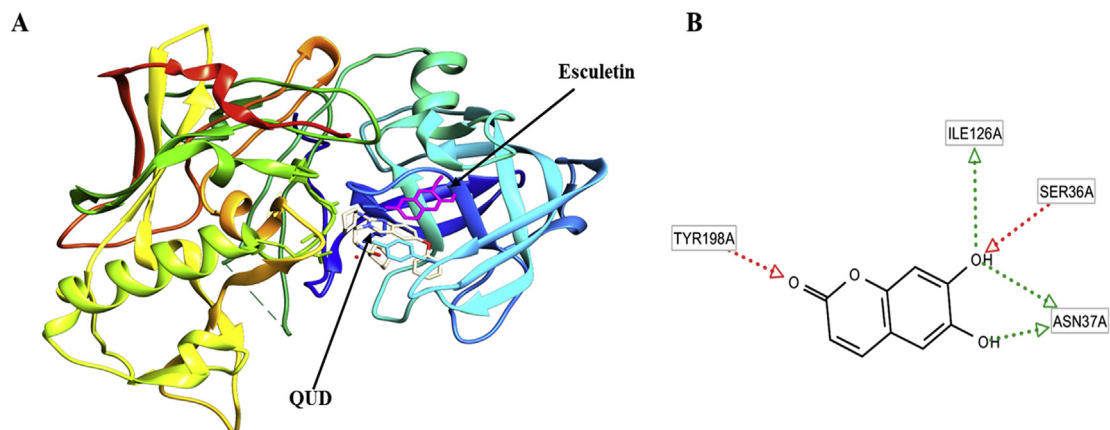


Figure 4. (A) Molecular docking model of BACE1 with esculetin (magenta color) and QUD (white color). (B) Diagram of the ligand interaction of esculetin in the active site of BACE1.

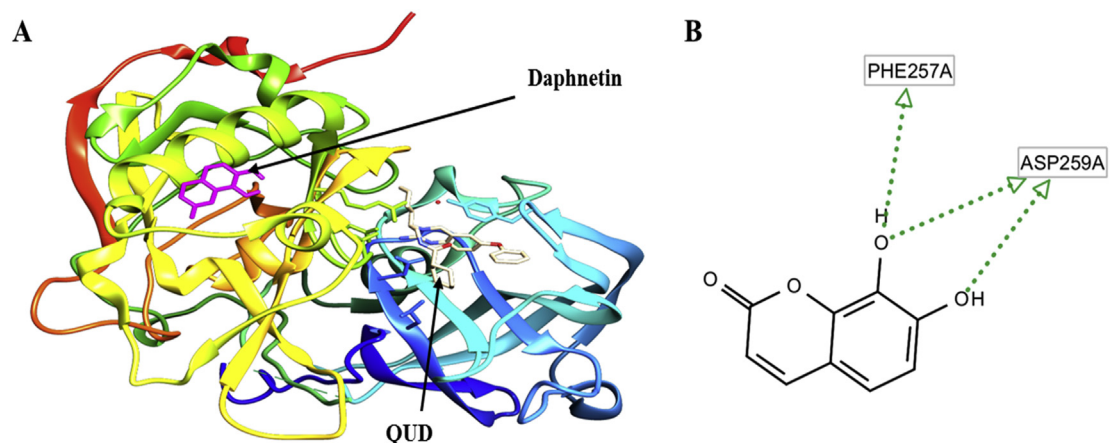


Figure 5. (A) Molecular docking model of BACE1 with daphnetin (magenta color) and QUD (white color). (B) Diagram of the ligand interaction of daphnetin in the active site of BACE1.

group at C-7 and one carboxylic group at C-6. Similarly, the corresponding ligand interaction for esculetin in the active site of BACE1 consisted of two hydrogen-bonding interactions with the Asn37, Ser36, and Ile126 residues of BACE1 and two hydroxyl groups of the compound, while binding of daphnetin in the active site of BACE1 was mediated by two hydrogen-bonding interactions with Asp259 and Phe257 residues of the enzyme and two hydroxyl groups of the compound. The binding energies of these three compounds were -4.58 , -6.25 and -6.37 kcal/mol,

respectively, which compared favorably with that of QUD, which had a binding energy of -10.99 kcal/mol. Taken together, these results indicated that additional hydrogen bonding and carboxylic groups might stabilize the open form of BACE1 and facilitate tighter binding to the active site resulting in more effective enzyme inhibition. Importantly, *in vitro* evaluation and *in silico* molecular docking data both strongly suggest that umbelliferone 6-carboxylic acid, esculetin and daphnetin may inhibit and/or prevent AD by targeting the formation of β -amyloid.

4. Discussion

Recognition of the ability of natural products to positively impact health and well-being continues to grow both culturally and scientifically. As a result, there is a growing interest in demonstrating relationships between consumption of natural products and risk reduction and/or prevention of various disease and health conditions. As such, efforts to study and identify added-value natural products with health-promoting properties have also prompted the development of new drugs. For example, the benefits of natural products have been successfully demonstrated in AD [27]. Although its etiology remains unknown, the neuropathological profile of AD is associated with memory loss and is consistent with the presence of numerous plaques and cholinergic deficiency due to the degeneration or atrophy of cholinergic neurons in the basal forebrain [28]. With respect to the regulation of cognitive functions, the central cholinergic system is considered to be the most important neurotransmitter [29]. Indeed, cholinesterases such as AChE and BChE are considered key enzymes that play significant roles in cholinergic transmission by hydrolyzing the neurotransmitter Ach [30]. Loss of basal forebrain cholinergic cells results in an important reduction of ACh, which is believed to play a defining role in the cognitive impairment associated with AD, senile dementia, ataxia, and myasthenia gravis [29]. Moreover, at the molecular level, patients affected by AD exhibit abnormal deposits of A β and abnormal spiral filaments in neurons, increased oxidative stress, and low levels of Ach [31].

The death of neurons during the progression of AD usually affects the levels of brain neurotransmitters. In addition to ACh, other neurotransmitters such as glutamate and serotonin are also affected during the later stages of AD [32]. There is unfortunately no cure for AD, and treatment strategies are primarily symptomatic. Thus, current efforts to develop new therapeutics are focused on the cholinergic hypothesis, which aim to target AChE inhibition to improve cholinergic neurotransmission in the brain [33]. Interestingly, recent studies have shown that AChE has additional non-cholinergic function by binding to A β [34]. Several of the cholinesterase inhibitors currently in use, namely donepezil, tacrine, rivastigmine, and galantamine, have adverse side-effects such as gastrointestinal disturbances, nausea, vomiting, and diarrhea and also have bioavailability issues [35]. For these reasons, there is growing scientific interest in identifying natural sources of AChE, BChE and BACE1 inhibitors with safer profiles.

To evaluate the potential of coumarins as anti-AD agents, we investigated their ability to inhibit AChE, BChE and BACE1 using a modified version of the assay described by Ellman *et al.* [23]. A total of 10 coumarin derivatives were screened for their ability to inhibit AChE, BChE and BACE1 *in vitro*. The results of the screening showed that esculetin and daphnetin had good AChE and BChE inhibitory activities with IC₅₀ values of 6.13 and 11.57 μ M for AChE, respectively, as well as good BChE inhibitory activities with IC₅₀ values of 8.66 and 9.29 μ M, respectively. With respect to BACE1 inhibition, umbelliferone 6-carboxylic acid, esculetin and daphnetin had strong inhibitory activity with IC₅₀ values of 0.34, 7.67, and 11.19 μ M, respectively.

In order to establish a structure–activity relationship between the coumarins and the target enzyme inhibition, the inhibitory effects of all coumarins against AChE, BChE and BACE1 were investigated. Among the molecules investigated, both esculetin

and daphnetin, which have free hydroxyl groups at the 6, 7, or 8 positions, exhibited the highest inhibitory potential against AChE. A comparison of the inhibitory potential of different coumarin derivatives indicated that the presence of an *o*-dihydroxyl (catechol) group markedly increased inhibitory activity. Umbelliferone has a free hydroxyl group at the 7 position whereas umbelliferone 6-carboxylic acid contains both a free hydroxyl group at the 7 position and a carboxylic group at position 6. Interestingly, despite this difference in structure, the latter compound retained inhibitory activity, although it was reduced compared to esculetin and daphnetin. Substitution with a methoxyl or glycosyl group diminished the inhibitory potential as evidenced by the activity of scopoletin, isoscapoletin, scopolin, scoparone, and 7-methoxy coumarin. 2'-Isopropyl psoralene contains two methyl groups, which appeared to reduce its inhibitory activity towards AChE. Based on the above structure–activity relationship, the AChE inhibitory activity appears to be largely dependent on the presence of the hydroxyl groups at the 6, 7, or 8 positions, with methoxylation and glycosylation at these positions greatly diminishing inhibitor potency.

Regarding BChE inhibitory activity, umbelliferone 6-carboxylic acid, esculetin and daphnetin showed significant inhibitory activity. In particular, daphnetin, esculetin, and umbelliferone-6-carboxylic acid were as good as the positive control berberine. Indeed, both daphnetin and esculetin, which have a free hydroxyl group at the 6, 7, or 8 positions, displayed the highest inhibitory potential against BChE. On the other hand, umbelliferone and umbelliferone 6-carboxylic acid, which both have a free hydroxyl group at the 7 position, retained inhibitory activity but it was reduced compared to esculetin and daphnetin. Similar to the results described above, methoxylation at the 6, 7, or 8 positions (scopoletin, scopolin, scoparone and 7-methoxy coumarin) drastically abolished inhibitory activity, while glycosylation at the 7 position (isoscapoletin) resulted in complete inactivity at the concentrations tested. Based on these observations, it was clear that the presence of a free hydroxyl group is very important for BChE inhibitory activity. Importantly, these results were similar to those described in previous anti-AD studies [12].

In order to elucidate the relationship between structure and activity, we investigated the ability of the different coumarins to inhibit BACE1. Umbelliferone 6-carboxylic acid, esculetin, and daphnetin exhibited promising inhibitory activity against BACE1 compared to the positive control quercetin. Umbelliferone 6-carboxylic acid has a free carboxyl group at the C-6 position while esculetin and daphnetin have free hydroxyl groups at the C-6, 7, or 8 positions. Umbelliferone, isoscapoletin, and 7-methoxy coumarin had moderate inhibitory activity against BACE1. Thus, based on our results, presence of a free carboxyl and free hydroxyl group at the 6, 7 or 8 positions of coumarin appears to be important for BACE1 inhibitory activity.

Various active compounds and extracts obtained from medicinal plants have been assessed for their efficacy against AD [36]. However, synergistic interactions are possible from plant extracts due to the simultaneous presence of dozens of bioactive compounds. Indeed, use of a single molecule for disease treatment and research is often preferred in order to better understand the mechanism of action. Therefore, it is very important to elucidate the active components in plant extracts. In the present study, we screened several pure coumarin derivatives for their ability to inhibit cholinesterase

and BACE1 *in vitro*. There have been relatively few reports concerning the anticholinesterase capacity of coumarins. Previously, Kang *et al.* [37] showed that the methanolic extract of *Angelica gigas* (*A. gigas*) roots (Umbelliferae) has significant anti-AChE activity, which ultimately led to the isolation of twelve coumarin derivatives. Orhan *et al.* [38] has been reported that coumarins have anticholinesterase activity. Relevant to the present study, another furanocoumarin derivative called nodakenin was isolated from *A. gigas* of Korean origin and examined for its effect on learning and memory impairment induced by scopolamine [39]. Decursinol, a coumarin-type compound from *A. gigas* roots, was also shown to have good anti-AChE effects [12]. In another study that employed a structure-based pharmacophore model, the two coumarins scopoletin and scopolin were investigated for their ability to inhibit AChE using a bioautography thin-layer chromatography assay [40], the results of which were consistent with our findings. Specifically, both scopoletin and scopolin had moderate but nevertheless remarkable, dose-dependent, and long-lasting inhibitory effects against AChE. On the other hand, the CH₂Cl₂ extract of *Peucedanum ostruthium* roots was shown to have significant inhibitory effects on AChE [41], and through bioactivity-guided fractionation four coumarin derivatives (ostruthin, imperatorin, ostruthol, and oxypeucedanin hydrate) were identified as the active components.

In an attempt to clarify the manner of AChE inhibition by the active coumarins identified in this study, we performed kinetics analyses using different substrate concentrations (0.6, 0.4, and 0.2 mM for umbelliferone 6-carboxylic acid and esculetin; 0.6, 0.3 and 0.1 mM for daphnetin) and analyzed the data using Dixon plots. As shown in Figure 1A–C, the manner of inhibition of umbelliferone 6-carboxylic acid and esculetin was noncompetitive with K_i values of 80.99 and 25.09 μ M, respectively, while daphnetin showed mixed type inhibition with a K_i value of 19.98 μ M. Likewise, to determine the type of BChE inhibition, we performed kinetics analysis of umbelliferone 6-carboxylic acid, esculetin and daphnetin by monitoring the effects of different concentrations of substrate (0.6–0.1 mM) and analyzed the data by Dixon plots. As shown in our study, umbelliferone 6-carboxylic acid exhibited noncompetitive type inhibition with a K_i value of 49.78 μ M, whereas esculetin and daphnetin showed mixed type inhibition with K_i values of 16.11 and 7.18 μ M, respectively. Lastly, to clarify the mode of enzyme BACE1 inhibition by the most potent coumarins, kinetics analysis was performed and the results were evaluated using Lineweaver–Burk plots and Dixon plots [24–26] at different substrate (150, 250, and 375 nM) and inhibitor concentrations. In analyzing the data, lower K_i values are indicative of tighter binding of inhibitors to the enzyme and thus a more effective inhibitor. Thus, the K_i values indicated that umbelliferone 6-carboxylic acid, esculetin and daphnetin may be excellent candidates as BACE1 inhibitors.

Based on our enzyme kinetic results regarding type of inhibition and K_i of umbelliferone 6-carboxylic acid, esculetin and daphnetin towards BACE1, we next analyzed the molecular structure of BACE1/inhibitor complexes using Autodock 4.2 [42,43]. Specifically, we used this software to simulate binding between BACE1 and inhibitors and evaluate the binding site-directed inhibition of BACE1. The docking results for umbelliferone 6-carboxylic acid, esculetin and daphnetin returned negative binding energies of –4.58, –6.25 and –6.37 kcal/mol, respectively, suggesting that all three coumarins are high affinity

enzyme-inhibitors that are able to tightly bind to the active site of BACE1. Autodock 4.2 is used to simulate inhibitors into the binding sites of enzymes that are located at a distance of 5–6 Å, and in this way molecular docking studies a powerful method to predict substructures that fit into binding pockets of enzyme in order to study inhibition and activation. The ligand–enzyme complexes with these three compounds and QUD were stably posed in the same pocket of BACE1 by Autodock 4.2. The corresponding ligand interactions of umbelliferone 6-carboxylic acid in the active site of BACE1 consisted of one hydrogen-bond between Arg235 and one carboxylic group interaction between the Tyr71, Arg235 and Thr72 residues of BACE1 with one hydroxyl group at C-7 and one carboxylic group. The corresponding ligand interactions of esculetin in the active site of BACE1 comprised two hydrogen-bonding interactions between the enzyme at Asn37, Ser36, and Ile126 with two hydroxyl groups of esculetin, while the binding of daphnetin in the active site of BACE1 consisted of two hydrogen-bonding interactions between enzyme at Asp259 and Phe257 and two hydroxyl groups. Taken together, the *in vitro* results and molecular docking data indicated that umbelliferone 6-carboxylic acid, esculetin and daphnetin have a strong potential to inhibit and prevent AD by targeting β -amyloid formation through BACE1.

In summary, our isolated coumarins have a significant ability to inhibit BACE1, BChE, and AChE. Based on structure–activity relationships of these coumarins, we speculated that the presence of a free hydroxyl group (catechol) at the C-6, 7, and 8 positions plays a predominant role in AChE and BChE inhibition, while the presence of a carboxyl and catechol group plays a crucial role in BACE1 inhibition. Taken together with molecular docking data, the results of the present study suggest that umbelliferone 6-carboxylic acid, esculetin and daphnetin may be good candidates for development as therapeutic agents for the treatment and prevention of AD by targeting β -amyloid formation. Further *in vivo* and cellular based studies are needed to help clarify the detailed mechanism of action of these compounds in the brain membrane and other organs.

Conflict of interest statement

We declare that we have no conflict of interest.

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