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Plant Phenolics as β-Secretase (BACE1) Inhibitors

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Abstract Various plant phenolics were assessed for β-secretase (BACE1) inhibitory activity in order to screen for anti-dementia agents. Among 39 phenolics, eight compounds, 1,2,3-trigalloyl glucopyranoside, acetonyl geraniin, euphorscopin, furosine, helioscopinin A, helioscopinin B, jolkinin, and rugosin E exhibited strong inhibition of BACE1 with IC_{50} values of 5.87×10^{-8}- 54.93×10^{-6} M. Among them, rugosin E was the most potent (IC_{50} 5.87×10^{-8} M). The active compounds were shown to be non-competitive inhibitors by Dixon plot. All the phenolic BACE1 inhibitors except furosin also suppressed prolyl endopeptidase (PEP) activity. However, these phenolic compounds caused less inhibition of α-secretase (tumor necrosis factor a converting enzyme; TACE) and no significant inhibition of other serine proteases such as trypsin, chymotrypsin, and elastase was seen, demonstrating that they are relatively specific to both BACE1 and PEP. No significant structure-activity relationships were found.

Keywords: β-secretase, inhibitor, plant phenolics, Alzheimer's disease

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder primarily marked by the progressive deposition of insoluble amyloid plaque and fibrillary tangles (1), followed by oxidative damage to neurons that ultimately results in dementia (2). The major component of the amyloid plaques is the β-amyloid peptide (Aβ), which is a 40-42 residue internal peptide segment of amyloid precursor protein (APP) (1). The Aβ peptide is liberated by the action of two proteases, β-secretase (BACEl) and γ-secretase (3). BACEl initially cleaves the APP to form the *N*-terminus of Aβ at the Asp+1 residue of the Aβ sequence. Following β-secretase cleavage, C99 is the substrate of a second protease, γ-secretase, which cleaves the APP to generate the *C*-terminus of Aβ, and the mature peptide is secreted from the cell. A third protease, α-secretase (TACE), non-pathologically cleaves the APP in the middle of the Aβ domain; thus precluding the formation of Aβ (4). Aβ forms aggregates that are thought to initiate a pathogenic cascade that ultimately leads to neuronal loss and dementia.

Evidence gathered in recent years clearly implicated the aspartyl protease, BACE1 (β-site APP cleaving enzyme) as the predominant β-secretase (4, 5). There is strong evidence that BACE1 is the major β-secretase in neurons and its absence inhibits production of amyloid and the C99 stubs without any major side-effect (6). The lack of Aβ production in BACE1 deficient mice clearly indicated that BACE1 is the β-secretase and that BACE1 inhibitors should reduce Aβ levels (6). Accordingly, as the key enzyme that initiates Aβ formation *in vivo*, BACE1 is a prime drug target for the inhibition of Aβ production.

Several synthetic inhibitors of BACE1 have been reported. Peptidomimetic BACE1 inhibitors including octapeptide (7), OM99-1 and OM99-2 (8), OM00-3 (9), and aminoethylenes (10) have been synthesized. Although these synthetic peptide-based compounds are strong inhibitors, their molecular size needs to be reduced in order to overcome metabolic instability since enzyme inhibitors with therapeutic potential preferably need to be smaller than 700 Da. Non-peptidomimetic derivatives, such as analogues based on the phenyl-piperazine scaffold with various heterocyclic moieties, have been synthesized to optimize BACE1 inhibition (11). Recently, small sized synthetic inhibitors containing a tetrazole ring and acidic heterocycle bioisosteres such as KMI-570, KMI-684, KMI-420, and KMI-429 were synthesized (12).

In contrast, efforts to discover naturally occurring BACE1 inhibitors have been relatively limited. Several hydroxyl containing inhibitors have been reported (13, 14). Chitosan derivatives from crab shell and latifolin from *Dalbergia sissoo* exhibited weak BACE inhibition (15, 16). Catechins from green tea (17), ellagic acid and punicalagin from pomegranate (18), hispidin from mycelial cultures of *Phellinus linteus* (19) and several compounds isolated from *Sanguisorbae radix* (20) have been studied as BACE1 inhibitors in our previous studies. These natural inhibitors commonly contain a phenolic moiety in their structures. Moreover, many plant phenolics are potential antioxidants against reactive oxygen species (ROS) (21) known as one of the most important causes of AD (22).

To further our research into natural BACE1 inhibitors, we investigated the inhibitory properties of 39 plant-derived phenolic compounds on BACE1 and on additional proteolytic enzymes.

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Materials and Methods

Plant phenolics Thirty-nine plant phenolic compounds isolated and identified by Professor Seung-Ho Lee at College of Pharmacy, Yeungnam University (23) were obtained from the Medicinal Molecules Bank (MEDMOB), Kyungpook National University, Daegu, Korea.

Enzyme assays A BACE1 (recombinant human BACE1) assay kit was purchased from PanVera (Madison, WI, USA). The assay was carried out according to the manufacturer's instructions with minor modifications. Briefly, a mixture of 10 μL of 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 mL of sample dissolved in MeOH or DMSO were incubated for 60 min at 18°C. The mixture was excited at 545 nm and the light emitted at 585 nm was collected. The inhibition ratio was obtained using the following equation:

$$\text{Inhibition (\%)} = [1-\{(S-S_0)/(C-C_0)\}] \times 100$$

where, C was the fluorescence of control (enzyme, assay buffer, and substrate) after 60 min of incubation, C_0 was the fluorescence of control at time zero, S was the fluorescence of tested samples (enzyme, sample solution,

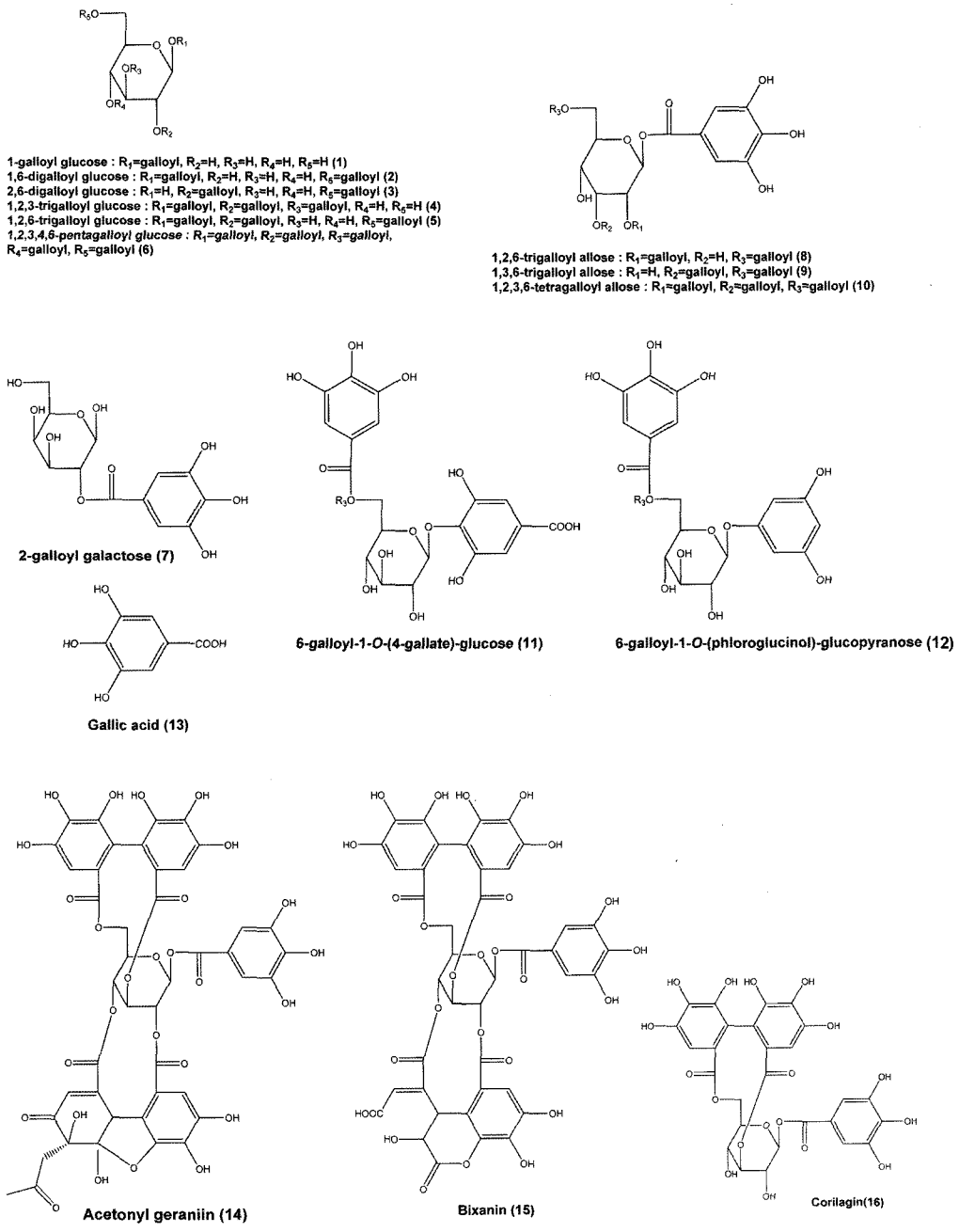
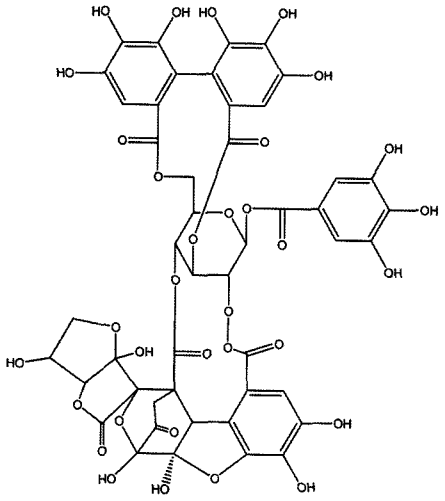
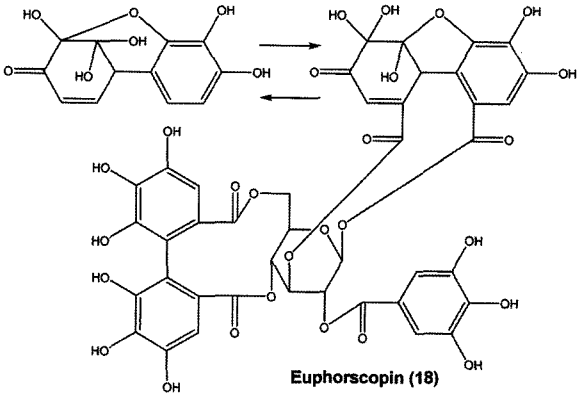


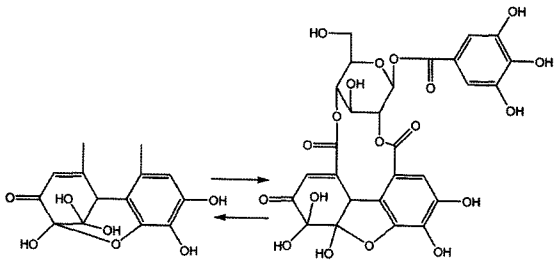
Fig. 1. Structures of plant phenolic compounds (continued).



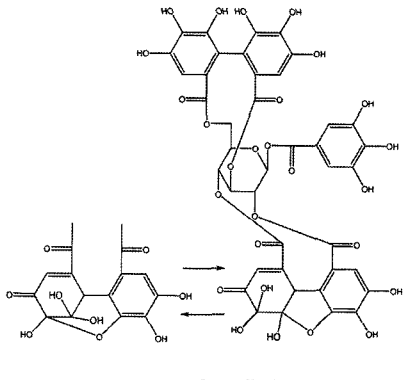
Elaeocarpusin (17)



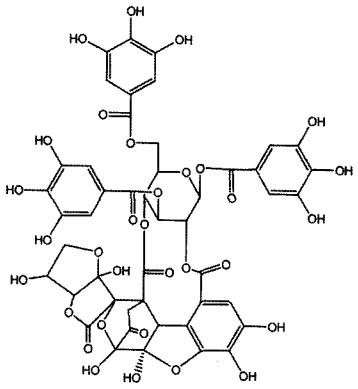
Euphorscopin (18)



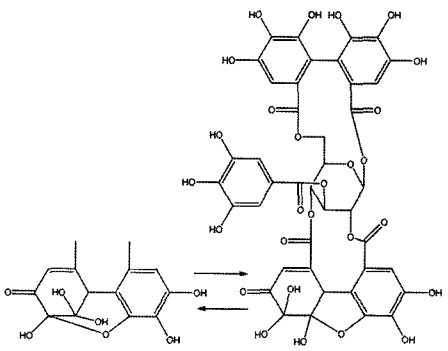
Furosin (19)



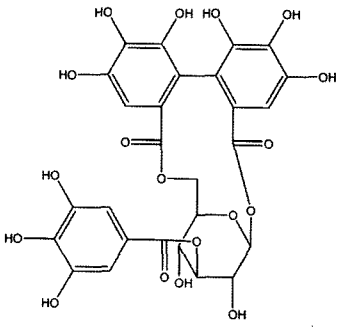
Geraniin (20)



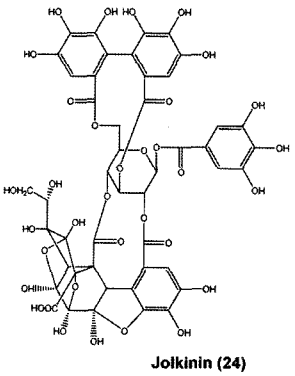
Helioscopin B (21)



Helioscopinin A (22)



Helioscopinin B (23)



Jolkinin (24)

Fig. 1. Structures of plant phenolic compounds (continued).

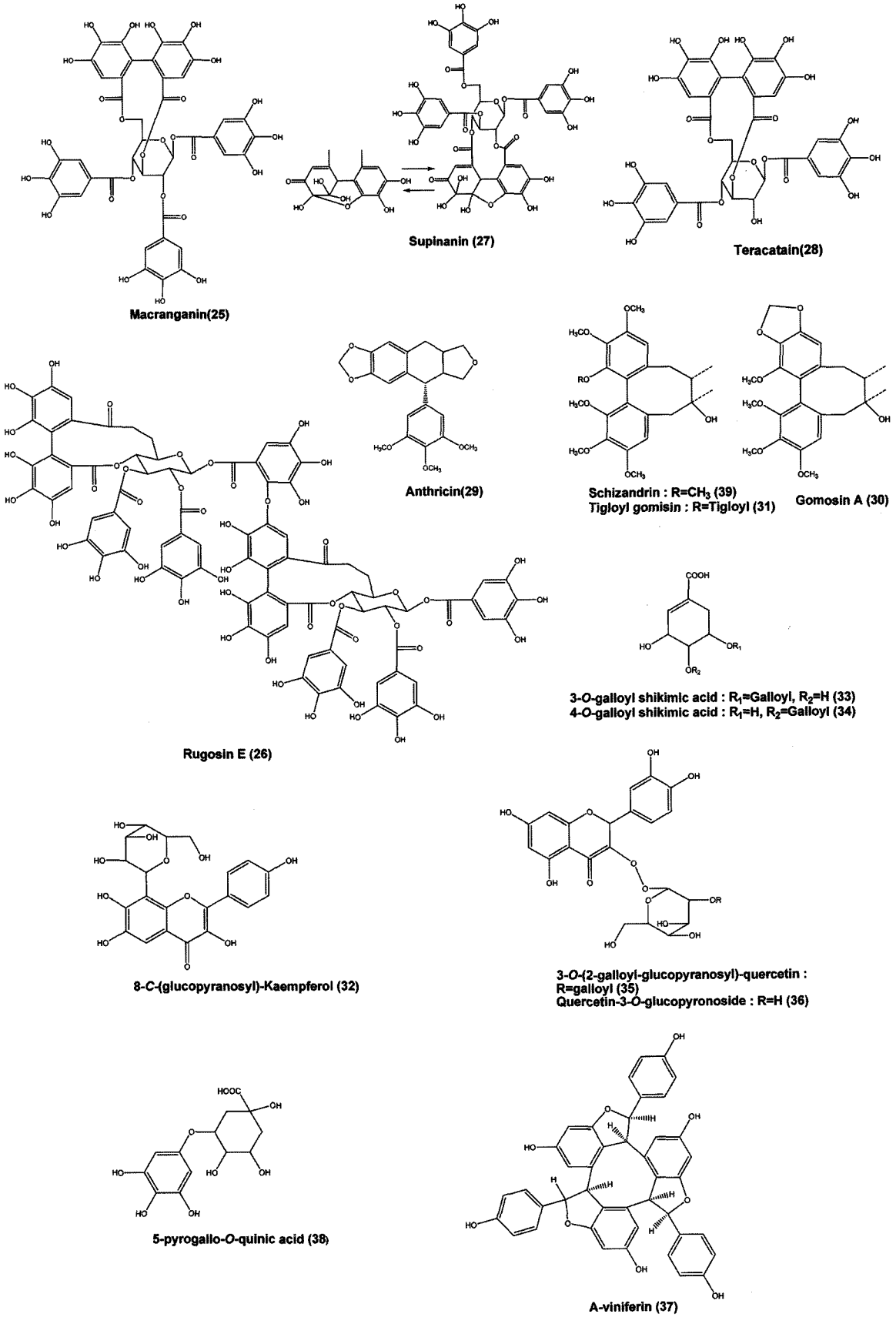


Fig. 1. Structures of plant phenolic compounds.

Table 1. Inhibitory activities of plant phenolics on BACE1

	Compound no.	Compound	IC_{50} (μM)
Gallotannins	1	1-galloyl glucopyranose	400
	2	1,6-digalloyl glucopyranose	400
	3	2,6-digalloyl glucopyranose	400
	4	1,2,3-trigalloyl glucopyranose	9.4324
	5	1,2,6-trigalloyl glucopyranose	400
	6	1,2,3,4,6-pentagalloyl glucopyranose	400
	7	2-galloyl galactose	400
	8	1,2,6-trigalloyl allose	400
	9	1,3,6-trigalloyl allose	400
	10	1,2,3,6-tetragalloyl allose	400
	11	6-galloyl-1-*O*-(4-gallate) -glucopyranose	400
	12	6-galloyl-1-*O*-(phloroglucinol) -glucopyranose	400
	13	Gallic acid	400
Ellagitannins	14	Acetonyl geraniin	0.7137
	15	Bixanin	400
	16	Corilagin	400
	17	Elaeocarpusin	400
	18	Euphorscopin	2.5023
	19	Furosin	17.7723
	20	Geraniin	400
	21	Helioscopin B	400
	22	Helioscopinin A	0.9867
	23	Helioscopinin B	0.4098
	24	Jolkinin	54.9280
	25	Macranganin	400
	26	Rugosin E	0.0587
	27	Suopyranosyl)-kaempferol	400
	33	3-*O*-galloyl shikimic acid	400
	34	4-*O*-galloyl shikimic acid	400
	35	3-*O*-(2-galloyl-glucopyranosyl)-quercetin	400
	36	Quercetin-3-*O*-glucopyranoside	400
	37	A-viniferin	400
	38	5-*O*-galloylquinic acid	400
	39	Schizandrin	400

and substrate) after 60 min of incubation, and S_0 was the fluorescence of the tested samples at time zero.

α-Secretase activity was measured using an α-secretase assay kit with TACE according to the manual from R&D Systems (Minniapolis, MN, USA). Fluorescence was measured with a Bio-TEK ELISA microplate fluorescence reader ELx 800 (Winooski, VT, USA). Chymotrypsin, trypsin, and elastase were assayed according to the protocols described in the Sigma catalog (St. Louis, MO, USA) using *N*-benzoyl-L-Arg-*p*NA, *N*-benzoyl-L-Tyr-*p*NA, and *N*-succinyl-Ala-Ala-Ala-*p*NA respectively as substrates. Optical density (O.D.) was measured by a Bio-TEK ELx 808 spectrophoto-meter (Winooski). All data presented are the mean values of triplicate experiments.

Results and Discussion

Inhibition of BACE1 The structures of plant phenolics are presented in Fig. 1. Out of 39 plant phenolics, eight compounds (**4**, **14**, **18**, **19**, **22-24**, and **26**) exhibited relatively strong inhibition against BACE1. The IC_{50} values of the tested phenolic compounds are presented in Table 1. Compound **26** (rugosin E, IC_{50} 5.87×10^{-8} M) exhibited the strongest BACE1 inhibitory activity followed by **23** (helioscopinin B, IC_{50} 4.10×10^{-7} M), **14** (acetonyl geraniin, IC_{50} 7.14×10^{-7} M), **22** (helioscopinin A, IC_{50} 9.87×10^{-7} M), **18** (euphorscopin, 2.50×10^{-6} M), **4** (1,2,3-trigalloylglucopyranose, 9.43×10^{-6} M), **19** (furosin, 17.77×10^{-6} M), and **24** (jolkinin, 54.93×10^{-6} M). Their IC_{50} values, especially those of **14**, **22**, **23**, and **26**, were remarkably lower than those previously reported for strong natural inhibitors of BACE1. Non-peptide inhibitors from green tea such as (-)-epigallocatechin gallate, (-)-epicatechin gallate, and (-)-gallocatechin gallate had IC_{50} values of 1.6×10^{-6}, 4.5×10^{-6}, and 1.8×10^{-6} M, respectively (17). Ellagic acid (IC_{50} 3.9×10^{-6} M) and punicalagin (IC_{50} 4.1×10^{-7} M) from pomegranate husk (18), tellimagrandin II (IC_{50} 3.1×10^{-6} M) and 1,2,3,4,6-pentagalloylglucopyranoside (IC_{50} 3.76×10^{-6} M) from *Sanguisorbae radix* (20) and chitosan derivatives (15) have been shown to have weaker BACE suppression activities than the phenolic compounds tested in the current study. Peptide BACE1 inhibitors, octapeptide (IC_{50} 0.41×10^{-6} M) and hydroxyethylene dipeptide isosteres (IC_{50} 2×10^{-8}-1.3×10^{-7} M) also exhibited weak or similar inhibition compared to those of the phenolics in the current study (7).

Fundamental studies on the mode of inhibition Dixon plots of the inhibition of eight of the active compounds demonstrated that the inhibition was non-competitive, indicating that they might bind either to the β-secretase subsite or to another regulatory site (Fig. 2). The inhibition constant (*Ki*) values were presented in Fig. 2 (0.823×10^{-6}-13.103×10^{-6} M).

Tannins are water soluble phenolic secondary metabolites of higher plants which can be classified into two major groups, hydrolysable and condensed tannins (24). Hydrolysable tannins contain a central core of polyhydric alcohol such as glucose and hydroxyl groups, esterified either partially, or wholly by gallic acid (gallotannins), or hexahydroxydiphenic (HHDP) acid (ellagitannins) (25). Upon hydrolysis by acids, bases, or certain enzymes, gallotannins yield glucose and gallic acids. The hexahydroxydiphenic acid of ellagitannins undergoes lactonizaiton to produce ellagic acid (26). Both hydrolysable gallotannins and ellagitannins are dietary polyphenols that occur in fruits and nuts and that are implicated to have potent

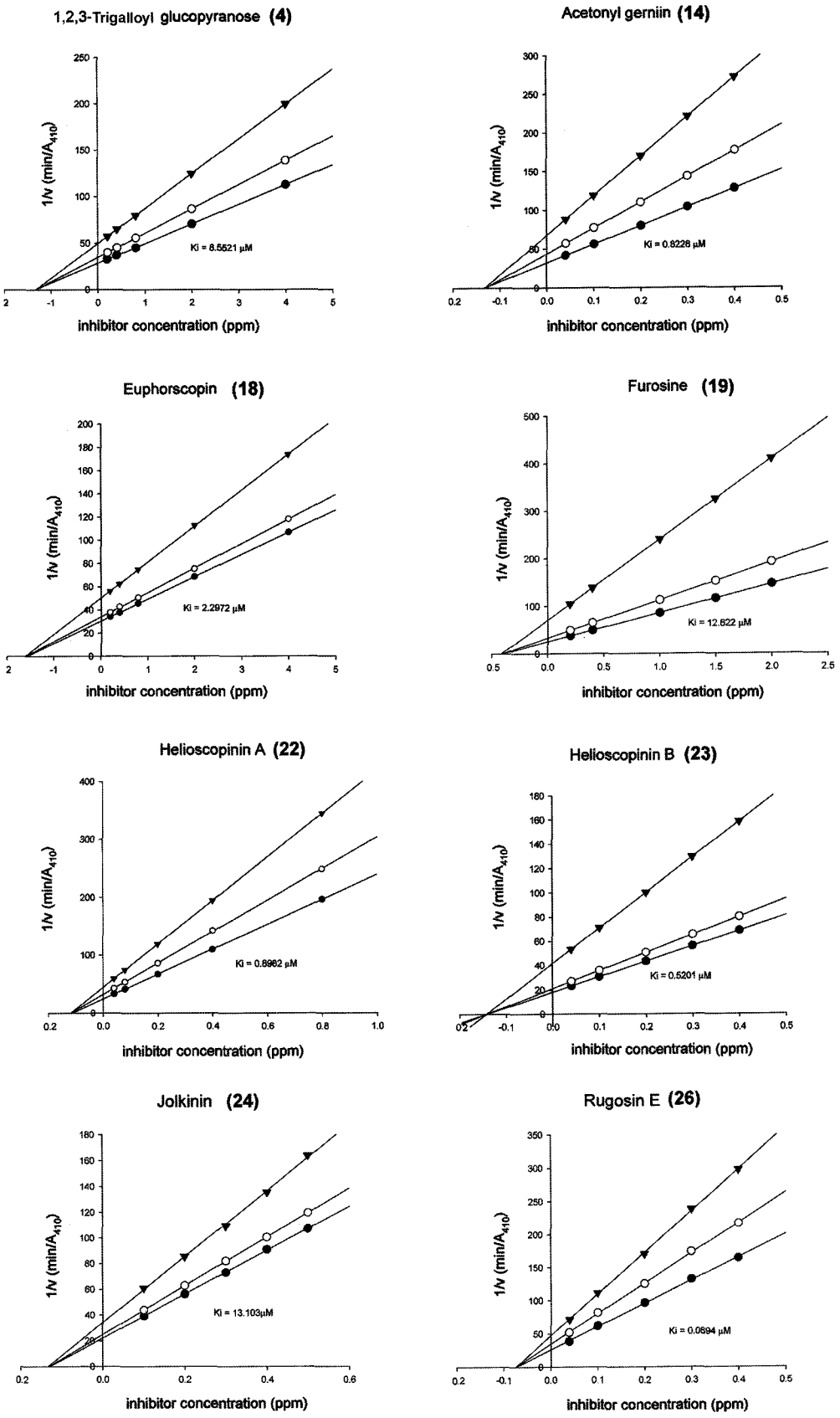


Fig. 2. Dixon plots of BACE1 inhibiting plant phenolics. Substrate concentration: 0.5 mM (−▲−), 0.75 mM (−○−), 1.0 mM (−●−). 1/V was indirectly estimated by taking reciprocal value of the changes in O.D. at 410 nm per min.

Table 2. Inhibitory activities of plant phenolics against TACE

Compound	IC_{50} (μM)
4	> 10
14	> 10
18	> 10
19	> 10
22	> 10
23	> 10
24	> 10
26	> 10

Table 3. Inhibitory activity[1)] of phenolic compounds against other serine proteases

Compound	Chymotrypsin (ppm)		Trypsin (ppm)		Elastase (ppm)	
	5	40	5	40	5	40
4	5.3	19.6	6.6	7.0	31.5	25.2
14	10.7	47.8	2.7	1.6	33.6	44.1
18	1.2	3.7	15.3	9.0	43.7	63.9
19	2.0	3.9	5.6	10.3	12.1	19.5
22	8.5	22.9	5.4	5.8	49.1	72.5
23	3.2	-1.4	1.9	1.8	30.8	39.9
24	4.9	18.1	1.2	-5.3	29.0	18.5
26	-0.8	-1.7	15.5	6.0	59.4	66.9

[1)]The inhibitory activity (%) is the mean±SE of the three independent experiments. For the control, 10 mL of 5% MeOH was added to the reaction mixture instead of a sample solution.

antioxidant, anticancer, antimutagenic, and antiatherosclerotic properties (26). Ellagic acid from the hydrolysis of ellagitannins in humans (27) has been shown to suppress various cancers including those of the colon, esophagus, liver, lung, tongue, and skin in rats and mice in both *in vitro* and *in vivo* studies (28).

On the other hand, polyphenols, especially tannins, are known to have strong affinity for proteins and form tannin-protein complexes leading either to the inactivation of enzymes or to insolubility of proteins. The inhibitory activity of the phenolic compounds against TACE, an α-secretase candidate which is involved in the normal amyloidogenic process, were compared with those of BACE1 to check the enzyme specificity. Eight phenolic BACE1 inhibitors showed weak inhibitory activities against TACE (Table 2). Moreover, the compounds at 5 and 40 ppm showed no significant suppression against other serine proteases such as chymotrypsin and trypsin (Table 3). Although they exhibited mild inhibition of elastase, this was not as significant as that of BACE1. According to our previous work (unpublished result), all BACE1 inhibitors except compound **19** significantly inhibited PEP in a non-competitive manner with a substrate in the Dixon plots (IC_{50} 2.67×10^{-8}-18.91×10^{-8} M, *Ki* 0.0046 $\times10^{-8}$-2.49×10^{-8} M), suggesting that they are relatively specific inhibitors of both proteases. Compound **26** in particular exhibited remarkable inhibition against both PEP (IC_{50} 2.67×10^{-8} M, *Ki* 9.40×10^{-9}) and BACE1 (IC_{50} 5.87×10^{-8} M, *Ki* 6.94×10^{-8} M). It is conceivable that PEP inhibition may be useful in the therapeutic treatment of cognitive disorders since PEP hydrolyzes several proline-containing peptide hormones including vasopressin, oxytocin, substance P, neurotensin, bradykinin, angiotensin II, and thyroptopin-releasing hormone, which have been suggested to play a critical role in memory and learning (29). Remarkable structure-activity relationship (SAR) was not found in BACE1 inhibition.

In order that a compound be considered for treatment of AD, its therapeutic effect must occur *in vivo* especially in the brain, which is protected by the blood-brain barrier and the plasma membrane. For BACE1 inhibitors reported so far, this requirement might be difficult to meet as these BACE inhibitors are synthetic peptidomimetics of the β-cleavage site in APP. The BACE1 inhibition of the phenolic compounds, with hydroxyl groups and high molecular weight, used in this study was weaker than that of a statin-based synthetic peptidomimetic inhibitor and so they might not be directly considered as drug candidates. However, they could act as a starting point for rational non-peptidyl drug design and be useful reagents for studying the enzyme properties of BACE1.

AD is a complex neurodegenerative disorder caused by various factors including β-amyloid aggregation (30), cholinergic synapse degeneration (3), and active oxygen species (22). The immune cells in the brain respond to the plaques and tangles and try to eliminate the debris. In the process of digesting the material within plaques and tangles, microglia release pro-inflammatory proteins and free radicals, which cause secondary damage (31). In addition, the antioxidant defense system in the elderly loses its ability to neutralize oxidative species and the subsequent oxidative stress can act as a risk factor for the initiation and progression of AD (32). Tannins possess strong antioxidative properties (33) and gallate esters were shown to have antioxidative effects *in vivo* and *in vitro* (21). Therefore BACE inhibiting plant phenolics, which are known antioxidants, have the potential to be useful in the prevention and treatment of Alzheimer's disease by inhibiting both BACE and active oxygen species.

Acknowledgments

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