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Plant Phenolics as Prolyl Endopeptidase Inhibitors

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Prolyl endopeptidase (PEP, EC 3.4.21.26), a serine protease, is widely distributed in various organs, particularly in the brains of Alzheimer's disease patients. The expression of PEP in Alzheimer's patients has been found to be significantly higher than that of the normal person, suggesting that a specific PEP inhibitor can be a good candidate for an anti-amnestic drug. In the current study, thirty-nine plant phenolics were investigated to determine their roles as prolyl endopeptidase (PEP) inhibitors. Nineteen compounds such as 1,2,3-trigalloyl glucopyranoside, 1,2,6-trigalloyl glucopyranoside, 1,2,3,4,6-pentagalloyl gluco-pyranoside, 1,2,6-trigalloyl alloside, 1,3,6-trigalloyl alloside, 1,2,3,6-tetragalloyl alloside, acetonyl geraniin, corilagin, elaeocarpusin, euphorscopin, geraniin, helioscopin B, helioscopinin A, helioscopinin B, jolkinin, macranganin, rugosin E, supinanin, and teracatain exhibited strong inhibition against PEP (IC_{50} 26.7 – 443.7×10^{-9} M). Rugosin E (IC_{50} 26.7×10^{-9} M) showed the most effective inhibition followed by 1,2,6-trigalloyl glucopyranoside (IC_{50} 31.4×10^{-9} M) and macranganin (IC_{50} 42.6×10^{-9} M). No significant structure-activity relationship was found; however, at least, three pyrogallol groups seem to be a minimal requirement for stronger activity against PEP. All 19 active compounds inhibited PEP in a non-competitive mode with a substrate in Dixon plots. They did not show significant effects against other serine proteases such as trypsin, chymotrypsin and elastase, indicating that they were relatively specific PEP inhibitors.

Key words: Prolyl endopeptidase (PEP), Inhibitor, Plant phenolics, Alzheimer's disease, Anti-amnesia

INTRODUCTION

Prolyl endopeptidase (PEP; EC 3.4.21.26) is a serine protease, which is known to cleave peptide substrates in the carboxylic sides of prolyl residues. It plays an important role in inactivation of biologically important proline-containing neuropeptides such as oxytocin, arginine vasopressin, substance P, neurotensin, thyrotropin-releasing hormone, bradykinin, and angiotensin I and II (Welches *et al.*, 1993; Toide *et al.*, 1995). Several studies have suggested that PEP plays a role in learning and memory, DNA synthesis, cell differentiation, signal transduction, and sperm motility in reproductive system (Williams *et al.*, 1999; Kimura *et al.*, 1990). The PEP levels of postmortem brains from Alzheimer's disease patients revealed significant increases in the PEP activity, suggesting that PEP plays a functional role in amyloidgenesis in the brain (Cunningham and O'Conor, 1997). It has been postulated that a specific PEP inhibitor could prevent memory loss and increase attention span in patients suffering from senile dementia. In fact, some natural and synthetic PEP inhibitors have been reported to show dose-dependent cognition-enhancing activity in rats with scopolamine-induced amnesia (Portevin *et al.*, 1996; Yoshimoto *et al.*, 1987).

In this context, much effort has been done to search for PEP inhibitors. For example, many pyrrolidine derivatives such as Z-Pro-Prolinal and JTP-4819 have been synthesized as potent cognitive enhancers (Arai *et al.*, 1993). Some plant phenolics including flavonoids had been reported as potent natural inhibitors of PEP (Lee *et al.*, 1998; Fan *et al.*, 1999; Lee *et al.*, 2004; Kim *et al.*, 2000), although their structure-activity relationships remained still unclear due to the limited number and diversity of

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samples introduced.

In the present study, the inhibitory activity of thirty nine plant phenolics were examined in order to find natural anti-amnesia leads and also to add supplementary data for understanding structure-activity relationships among natural phenolics.

MATERIALS AND METHODS

Materials

The plant phenolics, whose purities are more than 95%, were obtained from Medicinal Molecules Bank (MEDMOB), Kyungpook National University, Daegu, Korea, founded by the support of Korea Food and Drug Administration (KFDA). All the penolics had been isolated from Euphorbiaceae plants and identified in the previous work (Lee, 1991) and donated to the MEDMOB. Prolyl endopeptidase and its substrate (*Z*-Gly-Pro-pNA) were purchased from Seikagaku Co. (Tokyo, Japan). *Z*-Pro-Prolinal, synthesized according to the reference (Bakker *et al.*, 1990), was used as a positive control. Chymotrypsin, trypsin, elastase, and their substrates were purchased from Sigma (St. Louis, MO, U.S.A.).

Enzyme assays

PEP activity and inhibition percentage of samples were determined according to the reported method (Song and Raskin, 2002). Chymotrypsin, trypsin, and elastase were assayed according to the protocols described in Sigma catalog (Sigma, MO, USA) using *N*-benzoyl-L-Arg-*p*NA, *N*-benzoyl-L-Tyr-*p*NA, and *N*-succinyl-Ala-Ala-Ala-*p*NA as substrates, respectively. Optical density was measured with a Bio-TEK ELISA autoreader ELX 808 (VT, USA).

RESULTS AND DISCUSSION

The structures of plant phenolics are presented in Fig. 1. Among 39 tested compounds, 19 compounds (**4-6**, **8-10**, **14**, **16-18**, **20-28**) exhibited strong inhibition against PEP (IC_{50} 26.7 – 443.7×10^{-9} M). The PEP inhibitory activities of these compounds except for **5** are reported for the first time in this work. The IC_{50} values of the tested phenolic compounds are presented in Table I. Rugosin E (**26,** IC_{50} 26.7×10^{-9} M) from *Euphorbia supina* showed the most effective inhibition against PEP followed by 1,2,6-trigalloyl glucopyranoside (**5,** IC_{50} 31.4×10^{-9} M) from *Euphorbia helioscopia*, and macranganin (**25,** IC_{50} 42.6×10^{-9} M) from *Euphorbia fisheriana*, whose activities were stronger than that of *Z*-Pro-Prolinal, a positive control (IC_{50} 51.6×10^{-9} M). Their IC_{50} values were remarkably lower than those reported for strong natural inhibitors of PEP such as kinapsin-24 (IC_{50} 1.14 × 10^{-6} M), ginkgolic acid (IC_{50} 0.62 × 10^{-6} M), staurosporine (IC_{50} 0.77×10^{-6} M), 1,2,3,4,6-pentagalloyl-β-D-glucopyranoside (IC_{50} 70 × 10^{-9} M, 170 × 10^{-9} M) and isotamaraxin (IC_{50} 0.25×10^{-6} M) (Kim *et al.*, 2000; Song and Raskin. 2002; Lee *et al.*, 2004; Kimura *et al.*, 1990; Fan *et al.*, 2001; Sultanova *et al.*, 2004). PEP inhibitory activity was not linearly correlated with the number of pyrogallol groups; however, at least, three

Table I. Inhibitory activity of plant phenolics on PEP

	Compound name	IC_{50} (μM)	Ki (μM)	Dixon Plot
1	1-galloyl glucopyranose	>400	-	-
2	1,6-digalloyl glucopyranose	>400	-	-
3	2,6-digalloyl glucopyranose	>400	-	-
4	1,2,3-trigalloyl glucopyranose	0.1729	2.4912	Non
5	1,2,6-trigalloyl glucopyranose	0.0314	0.1936	Non
6	1,2,3,4,6-pentagalloyl glucopyranose	0.0744	0.0272	Non
7	2-galloyl galactose	>400	-	-
8	1,2,6-trigalloyl allose	0.0786	0.2032	Non
9	1,3,6-trigalloyl allose	0.1572	0.0004	Non
10	1,2,3,6-tetragalloyl allose	0.0887	0.4049	Non
11	6-galloyl-1-*O*-(4-gallate)-glucopyranose	>400	-	-
12	6-galloyl-1-*O*-(phloroglucinol)-glucopyranose	>400	-	-
13	Gallic acid	>400	-	-
14	Acetonyl geraniin	0.0991	0.0738	Non
15	Bixanin	23.0937		
16	Corilagin	0.2365	0.2123	Non
17	Elaeocarpusin	0.4437	0.0184	Non
18	Euphorscopin	0.1230	0.0046	Non
19	Furosin	>400	-	-
20	Geraniin	0.0690	0.0171	Non
21	Helioscopin B	0.0718	0.1285	Non
22	Helioscopinin A	0.1283	0.1016	Non
23	Helioscopinin B	0.1891	0.0978	Non
24	Jolkinin	0.0873	0.7895	Non
25	Macranganin	0.0426	0.4414	Non
26	Rugosin E	0.0267	0.0094	Non
27	Supinanin	0.0640	0.0908	Non
28	Teracatain	0.3934	1.7167	Non
29	Anthricin	>400	-	-
30	Gomisin A	>400	-	-
31	Tigloyl gomisin	>400	-	-
32	8-*C*-(glucopyranosyl)-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	Qurecetin-3-*O*-glucopyranoside	>400	-	-
37	α-viniferin	>400	-	-
38	5-*O*-galloylquinic acid	>400	-	-
39	Schizandrin	>400	-	-

pyrogallol groups seem to be a minimal requirement for stronger activity since the compounds with one or two pyrogallol moieties exhibited no significant inhibition. Previous studies demonstrated that flavonoid containing a catechol B-ring has effectively inhibited PEP activity (Fan *et al.*, 2000; Lee *et al.*, 1998). In addition, the presence of a carbonyl group with a catechol or pyrogallol moiety in flavone skeleton has been suggested as the essential structural feature (Kim *et al.*, 2000; Lee *et al.*, 1998). From the data so far, it was impossible to draw clear structure-activity relationships (SAR) among all type of plant phenolics. Further studies including *in silico* molecular

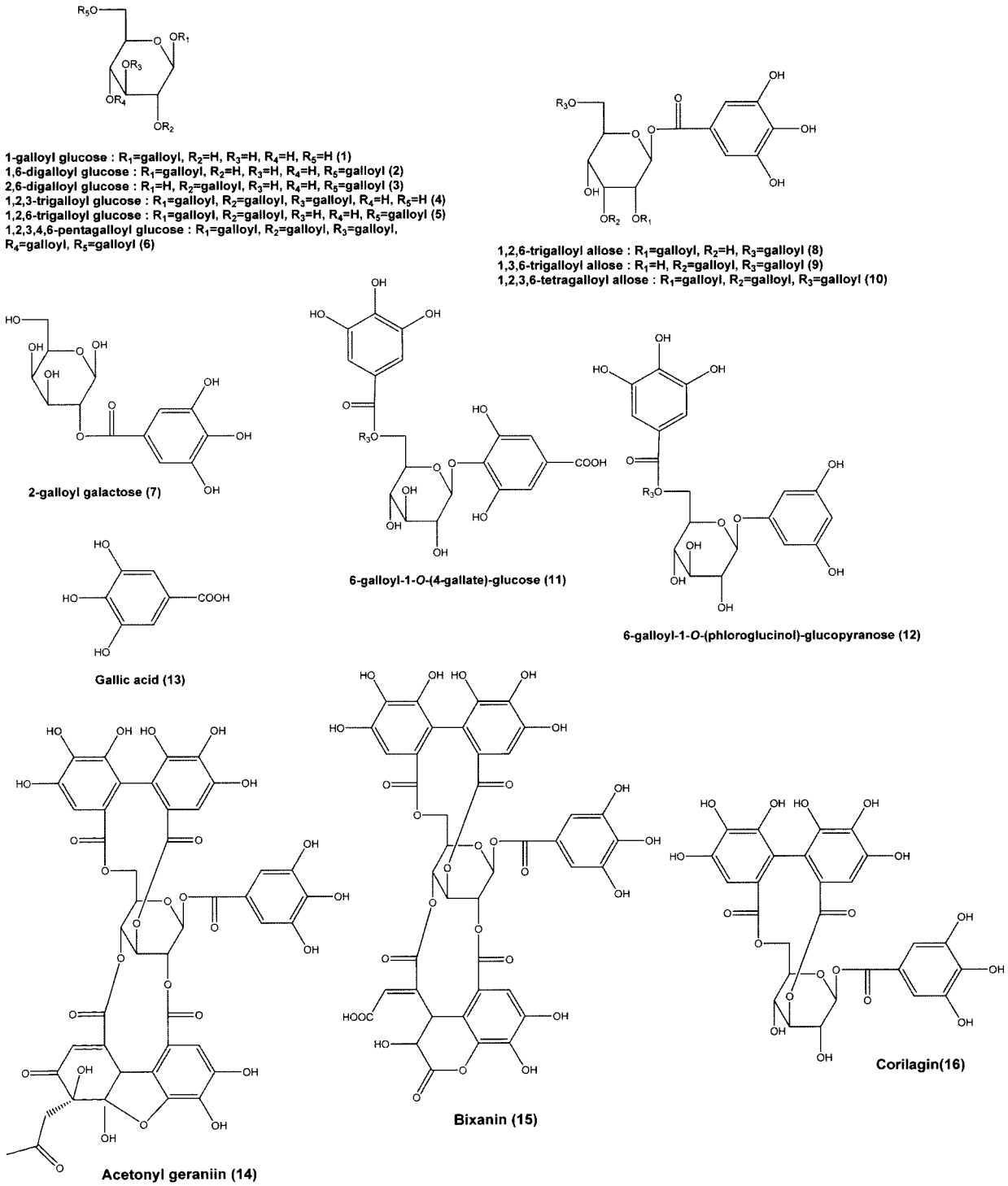
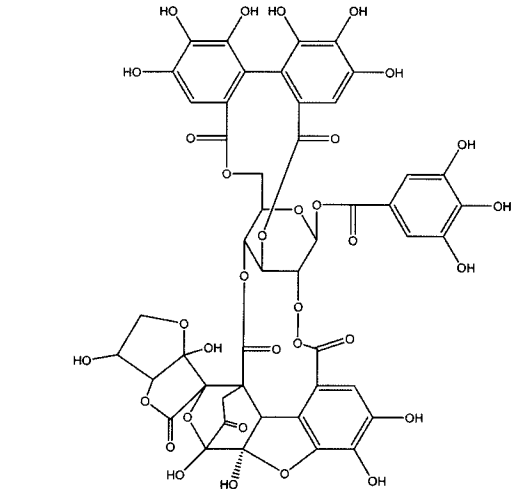
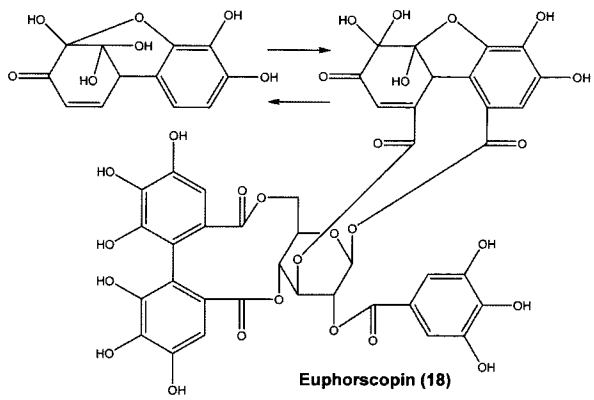


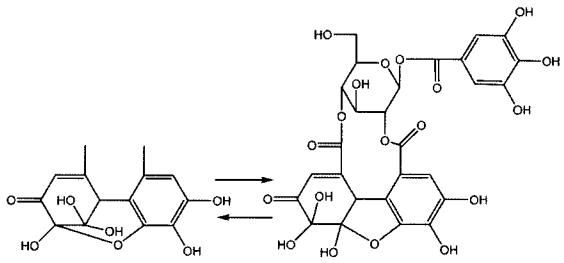
Fig. 1. Structures of tested phenolic compounds



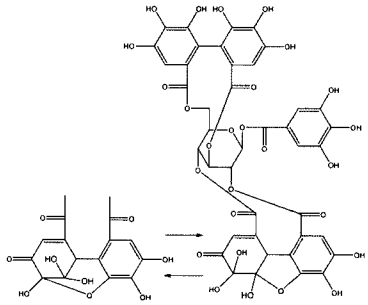
Elaeocarpusin (17)



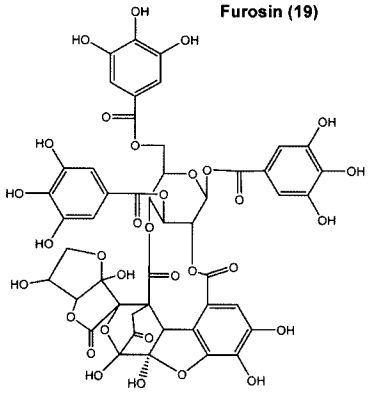
Euphorscopin (18)



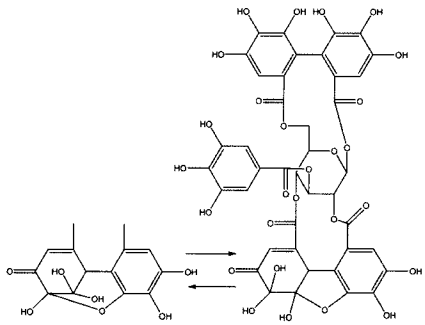
Furosin (19)



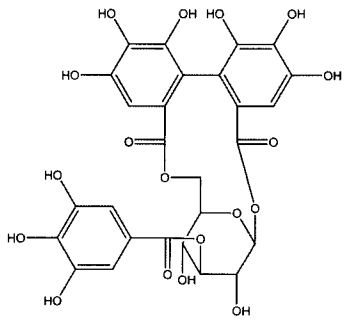
Geraniin (20)



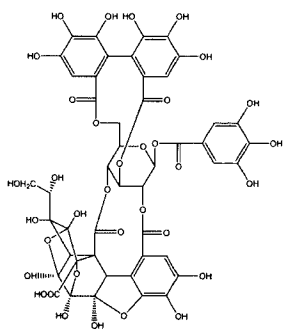
Helioscopin B (21)



Helioscopinin A (22)



Helioscopinin B (23)



Jolkinin (24)

Fig. 1. Continued

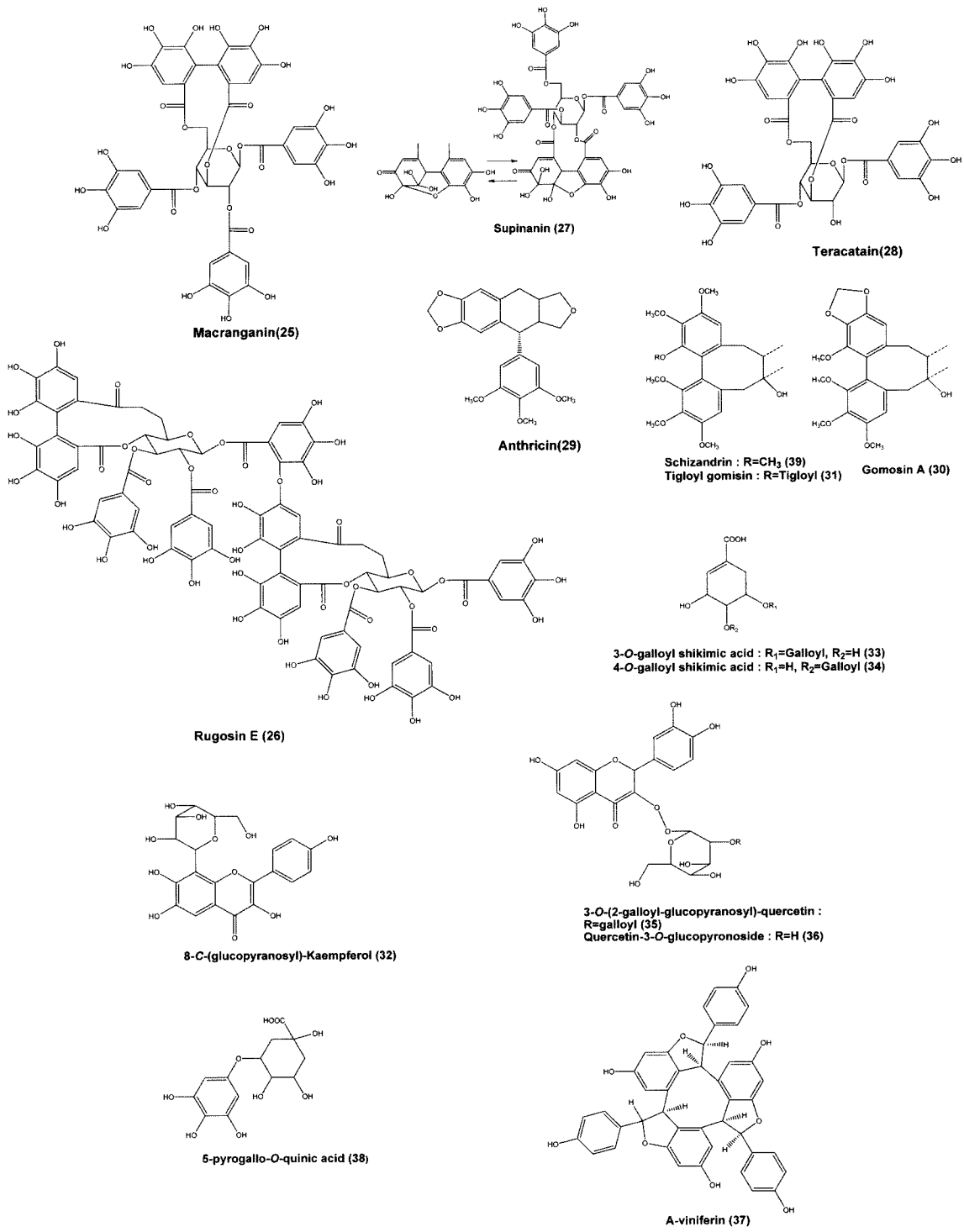


Fig. 1. Continued

modeling study would clarify remaining ambiguity in SAR of plant phenolics.

Tannins are water soluble phenolic secondary metabolites of higher plants, which can be divided into two major groups, hydrolysable tannins and condensed tannins and the former involves two types of tannins such as gallotannins and ellagitannins. Both hydrolyzable gallotannins and ellagitannins are dietary polyphenols occur in fruits and nuts and implicated with potent anti-oxidant, anticancer, antimutagenic and antiatherosclerotic properties (Mingshu *et al.*, 2006). On the other hand, polyphenols, especially tannins are known to have strong

Table II. Inhibitory activity on other serine proteases

	Inhibition (%)[a]					
	Chymotrypsin		Trypsin		Elastase	
	10 μM	100 μM	10 μM	100 μM	10 μM	100 μM
4	3.1±0.1	22.6±0.2	5.3±0.2	8.9±0.5	25.3±2.2	32.0±1.2
5	0.2±0.0	15.3±3.1	5.3±0.1	5.6±3.2	35.3±0.5	42.3±2.2
6	8.6±1.3	20.0±3.3	5.6±0.0	8.3±1.5	40.3±1.2	60.0±0.9
8	5.7±0.8	8.8±0.2	11.3±0.2	9.8±0.8	28.7±0.7	38.2±3.3
9	9.8±1.2	9.9±1.2	15.9±1.2	10.9±1.2	23.2±0.5	30.3±4.2
10	15.7±2.3	10.1±0.5	13.6±1.2	12.3±0.8	40.3±0.2	28.3±2.5
14	9.7±1.5	50.0±3.2	5.6±0.6	4.5±1.2	32.6±0.3	45.5±6.0
16	3.0±0.3	14.2±1.2	3.3±0.3	5.7±0.5	25.3±0.6	39.3±5.0
17	5.6±0.5	15.3±0.3	5.6±1.2	10.2±0.6	60.0±1.2	75.3±2.9
18	3.2±1.1	13.7±0.2	10.3±0.8	8.6±1.2	42.2±1.0	65.2±4.5
20	13.6±3.3	8.9±1.5	15.7±1.3	9.3±0.6	18.0±1.5	19.1±3.9
21	5.5±0.2	7.8±1.2	10.0±1.2	3.8±0.2	28.0±0.6	40.8±5.2
22	8.2±1.5	17.6±1.0	4.3±0.8	4.5±0.6	45.3±5.2	68.3±1.3
23	5.6±0.3	5.6±0.5	2.9±0.9	0.0±0.0	26.3±3.2	40.3±1.1
24	3.2±0.2	20.1±0.8	6.8±1.2	3.1±0.2	21.5±1.8	22.3±2.6
25	25.6±0.6	17.9±3.1	20.3±0.9	12.3±0.6	20.0±0.9	30.2±2.0
26	5.3±1.0	4.3±1.2	17.5±2.2	20.0±0.4	48.3±5.2	70.3±0.9
27	15.6±0.2	20.3±0.5	13.2±1.5	22.7±0.3	10.3±1.1	15.3±1.3
28	10.3±1.2	20.3±3.0	8.8±1.2	5.3±0.1	59.3±1.5	63.2±2.5

[a]The values are mean of duplicated experiments.

affinity for proteins and form tannin-protein complexes leading to either inactivation of enzymes or making proteins insoluble. To check the possibility of false-positive reaction from protein-tannin precipitation, the inhibitory activities of test samples on other serine protease such as elastase, trypsin and chymotrypsin were evaluated (Table II). However, the 19 active phenolics showed no significant inhibition against trypsin and chymotrypsin at 10 and 100 mM, respectively. Although they exhibited mild inhibition against elastase, they were not as significant as PEP, indicating that they are relatively specific inhibitors of PEP as is the case with other natural inhibitors (Lee *et al.*, 1998; Fan *et al.*, 2001).

Dixon plots indicated all 19 active compounds are non-competitive inhibitors with substrates, indicating that phenolic inhibitors might bind to either the enzyme subsite or to another regulatory site (Fig. 2). The inhibition constants (*Ki*) were presented in Table I.

AD is a complex neurodegenerative disorder generated by various factors, such as b-amyloid aggregation, cholinergic synapses and active oxygen species (Behl *et al.*, 1994; Selkoe, 1994; Miranda *et al.*, 2000). It has been demonstrated that oxidative damage precedes senile plaques and neurofibrillary tangles (Perry *et al.*, 2002). The antioxidant defense system in the elderly loses its ability to neutralize oxidative species, and then oxidative stress can act as a risk factor for the initiation and progression of AD (Floyd and Hensley, 2002). All the therapeutic compounds considered for AD must be achievable *in vivo* especially in brain tightly protected by blood-brain barrier (BBB) and plasma membrane. Therefore, the phenolic PEP inhibitors with polar hydroxyl groups and high molecular weight may not be directly considered as drug candidates. However, they could be a starting point for rational drug design from natural sources and be a useful reagent for studying the enzyme properties of PEP since they are not only strong inhibitors of PEP but also tentative *in vivo* and *in vitro* antioxidants (Sroka, 2005).

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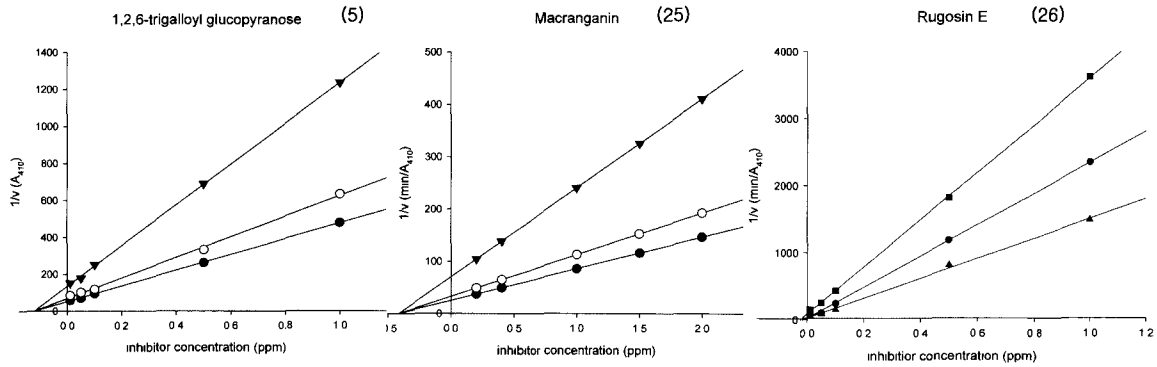

Fig. 2. Three representative Dixon plots of PEP inhibition. Substrate concentration: 0.5 mM (-▲-), 0.75 mM (-○-), 1.0 mM (-●-). 1/V was indirectly estimated by taking reciprocal value of the changes in OD at 410 nm per minute.

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