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Rhamnocitrin isolated from *Prunus padus* var. *seoulensis*: A potent and selective reversible inhibitor of human monoamine oxidase A

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ABSTRACT

Three flavanones and two flavones were isolated from the leaves of *Prunus padus* var. *seoulensis* by the activity-guided screening for new monoamine oxidase (MAO) inhibitors. Among the compounds isolated, rhamnocitrin (**5**) was found to potently and selectively inhibit human MAO-A (hMAO-A, IC_{50} = 0.051 μM) and effectively inhibit hMAO-B (IC_{50} = 2.97 μM). The IC_{50} value of **5** for hMAO-A was the lowest amongst all natural flavonoids reported to date, and the potency was 20.2 times higher than that of toloxatone (1.03 μM), a marketed drug. In addition, **5** reversibly and competitively inhibited hMAO-A and hMAO-B with K_i values of 0.030 and 0.91 μM, respectively. Genkwanin (**4**) was also observed to strongly inhibit hMAO-A and hMAO-B (IC_{50} = 0.14 and 0.35 μM, respectively), and competitively inhibit hMAO-A and hMAO-B (K_i = 0.097 and 0.12 μM, respectively). Molecular docking simulation reveals that the binding affinity of **5** with hMAO-A (−18.49 kcal/mol) is higher than that observed with hMAO-B (0.19 kcal/mol). Compound **5** interacts with hMAO-A at four possible residues (Asn181, Gln215, Thr336, and Tyr444), while hMAO-B forms a single hydrogen bond at Glu84. These findings suggest that compound **5** as well as **4** can be considered as novel potent and reversible hMAO-A and/or hMAO-B inhibitors or useful lead compounds for future development of hMAO inhibitors in neurological disorder therapies.

1. Introduction

Monoamine oxidase (MAO, EC 1.4.3.4), also known as “Neurozyme”, regulates the monoaminergic homeostasis and neurotransmission by oxidative deamination of biogenic amines, such as serotonin, dopamine, and norepinephrine [1]. It mainly exists in the brain, gastrointestinal tract, and platelets as two isoforms: MAO-A and MAO-B [2]. Therapeutic concerns regarding MAO-A and MAO-B are encompassed in two categories: MAO-A inhibitors for the treatment of mental disorders such as depression and anxiety, and MAO-B inhibitors for the treatment of neurodegenerative disorders such as Alzheimer’s and Parkinson’s diseases [1,3]. Although MAO-A and MAO-B show high amino acid identity and similar hydrophobic cavities at their active sites, small differences determine their substrate specificities; MAO-A prefers serotonin, and MAO-B prefers phenylethylamine and benzylamine [4].

MAO inhibitors (MAOIs) are classified into reversible or irreversible; furthermore, they are categorized as MAO-A or MAO-B selective, or MAO-A/B nonselective [1,5]. Nonselective, selective-irreversible, and selective-reversible MAOIs are grouped as first-, second-, and third-generation, respectively [1].

Prunus padus L. (bird cherry) is widely distributed in Europe and Asia including Korea, Japan and China, and is used in traditional medicine for the treatment of numerous diseases, including neuralgia, stroke, edema, and hepatitis [6,7]. This plant has received growing attention for its bioactive compounds that present antioxidant, antimicrobial, and antidiabetic activities [8–10].

In our quest of screening herbal extracts, we found the extract of *P. padus* var. *seoulensis* potently inhibited recombinant human MAO-A (hMAO-A). This study then conducted an activity-guided screening, wherein three flavanones and two flavones were isolated from the leaves of *P. padus* var. *seoulensis*. The two flavones isolated, rhamnocitrin and genkwanin, showed potent inhibitory activities against hMAO-A. Furthermore, two of the three flavanones were found to be new compounds. Although several recent reviews have been published on

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herbal MAO inhibitors and on the inhibitory activities of flavonoids [11–14], inhibitions of MAO enzymes by rhamnocitrin and genkwanin have not been previously described in literature. On the other hand, flavonoids have appealed as acetylcholinesterase inhibitors, as reviewed in the literatures [15,16]. Synthetic flavonoids have also been evaluated as MAO inhibitors [17,18].

The present study reports the isolation of flavonoids from the leaves of *P. padus* var. *seoulensis*, the structures of two new compounds, and the abilities of the compounds to inhibit recombinant hMAO enzymes and AChE.

2. Materials and methods

2.1. General experimental procedures

UV spectra were recorded on a SpectraMax M5 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA, USA). ECD spectra were recorded on a JASCO J-815 CD spectrometer. 1D (1H, 13C, and DEPT) and 2D (COSY, HMQC, and HMBC) nuclear magnetic resonance (NMR) spectra were analyzed by J NM ECZ500R 500 MHz (JEOL, Tokyo, Japan) and Varian UNITY 400 MHz (Varian, Palo Alto, CA, USA). The NMR spectrometer operated at 400 MHz (^{1}H) and 100 MHz (^{13}C), with chemical shifts given in ppm (δ). HRESIMS were measured on an ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometer (UPLC-QTOFMS, Waters, Milford, MA, USA) in the negative-ion mode. Medium-pressure liquid chromatography (MPLC) was performed with a Spot Prep II 250 instrument (Armen, Paris, France). Preparative HPLC was performed using a GX-271 semi-preparative HPLC system (Gilson, Middleton, WI, USA).

2.2. Plant material

Prunus padus var. *seoulensis* (H.Lév.) Nakai leaves were collected at Taebaek-si, Gangwon-do, Republic of Korea (N 37° 12′ 54.4″, E 128° 54′ 39.3″), in May 2003; they were identified by Dr. Tae Jin Kim (Korea Research Institute of Bioscience and Biotechnology, KRIBB). A voucher specimen (KRIB0005525) of this raw material and all compounds are deposited at the Plant Extract Bank of KRIBB in Daejeon, Korea (http://extract.kribb.re.kr/).

2.3. Extraction and isolation

The dried leaves of *P. padus* (84.2 g) were chopped and extracted with methanol (8.0 L, three times) at room temperature for 24 h, filtered, and evaporated using a rotary evaporator below 45 °C to obtain the total extract (24.0 g, 28.5%). The extract (1.0 g) was separated by MPLC using a YMC ODS AQ-HG column (20 × 250 mm, 10 μm, Kyoto, Japan) eluting with MeOH–H_2O (0–10 min, 10–15% MeOH; 10–40 min, 15–40% MeOH; 40–60 min, 40–100% MeOH) to yield ten fractions (PP Frs. 1–10). Before further isolation, this MPLC procedure was repeated 23 times using the same conditions. Each fraction was monitored by UV (254 nm) and corona-charged aerosol detectors (CAD, Chelmsford, MA, USA), and was evaluated for inhibitory activities against MAO enzymes. Among the partitioned fractions, Frs. 8 and 9 exhibited significant inhibitory activities against MAO-A. The constituent profiles of Frs. 8 and 9 were therefore analyzed by UPLC-PDA-QTof-MS, and the major compounds were identified as the major constituents of the extract. Fraction 8 (514.7 mg) was purified by semi-preparative reverse phased HPLC (YMC Triart C18 ExRs, 10 × 250 mm, 5 μm, flow rate: 10 mL/min) using a gradient solvent system (0–5.0 min, 40% MeOH; 5.0–40.0 min, 40–60% MeOH; 40.0–41.0 min, 60–100% MeOH; 41.0–50.0 min, 100% MeOH) to provide compounds **1** (18.3 mg, MS t_R = 6.09 min) and **2** (87.6 mg, MS t_R = 6.11 min). Fraction 9 (14.2 mg) was also purified by the semi-preparative reversed-phase HPLC using a gradient solvent system (0–5.0 min, 65% MeOH; 5.0–30.0 min, 65–85% MeOH; 30.0–31.0 min, 85–100% MeOH; 31.0–40.0 min, 100% MeOH) to provide compounds **3** (8.4 mg, MS t_R = 6.18 min), **4** (10.8 mg, MS t_R = 8.90 min) and **5** (28.1 mg, MS t_R = 9.19 min).

2.4. Chemicals and enzymes

Recombinant hMAO-A and hMAO-B, their respective substrates (kynuramine and benzylamine), and reversible reference inhibitors (toloxatone, quercetin, kaempferol, lazabemide) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Irreversible reference inhibitors (clorgyline and pargyline) were obtained from BioAssay Systems (Hayward, CA, USA) [19]. Acetylcholinesterase (AChE, Type VI-S) from *Electrophorus electricus*, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ACTI), and tacrine as a reference compound were procured from Sigma-Aldrich.

2.5. Enzyme assays

MAO activities were measured in 0.5 mL mixtures by the spectrophotometric continuous method, as described previously [20,21]. The K_m values of kynuramine for hMAO-A and benzylamine for hMAO-B were 0.042 and 0.16 mM, respectively. The concentrations for kynuramine (0.06 mM) and benzylamine (0.3 mM) were 1.4 × and 1.9 × K_m values, respectively. AChE activity was assayed using the method developed by Ellman et al. [22], with slight modifications. The reaction was carried out at 25 °C and monitored for 10 min at 412 nm using AChE (0.2 U/mL) in 0.5 mL mixture of 50 mM sodium phosphate (pH 7.5) in the presence of 0.5 mM DTNB and 0.5 mM ACTI.

2.6. Analysis of inhibitory activities and enzyme kinetics

Totally, 845 herbal extracts procured from the Korea Plant Extract Bank were screened at 25 μg/ml to evaluate their inhibitory activities against hMAO-A or hMAO-B. The experimental plant was then selected based on the inhibitory activity obtained, novelty, and stock availability. Next, we assessed the MAO inhibitory activities of the fractions obtained by column chromatography, and five isolated compounds were further evaluated against hMAO-A and hMAO-B at 10 μM of inhibitors. Thereafter, the IC_{50} values were determined for compounds showing more than 30% inhibitory activity at this concentration. Reversible and irreversible reference inhibitors were included in this screening: Toloxatone and lazabemide as reversible inhibitors for hMAO-A and hMAO-B, respectively; clorgyline and pargyline as irreversible inhibitors for hMAO-A and hMAO-B, respectively. All references were preincubated with MAO enzymes for 30 min prior to activity assays. To compare the potencies under the same conditions, IC_{50} values of quercetin and kaempferol were also determined. The time-dependencies for hMAO-A and hMAO-B inhibitions were investigated for the potent compounds as previously described [23], using 0.06 mM kynuramine and 0.3 mM benzylamine as standards, respectively. Kinetic studies were also performed, and inhibition types and K_i values of the inhibitors were determined by obtaining the Lineweaver-Burk plots and its secondary plot, as described previously [19]. Inhibitions of AChE by the isolated compounds were analyzed by preincubating for 15 min with AChE prior to the measurement.

2.7. Analysis of inhibitor reversibility

Reversibilities of the hMAO-A and hMAO-B inhibitions by the potent inhibitors rhamnocitrin and genkwanin were investigated by the dialysis method using the DiaEasy dialyzers (BioVision Inc., Milpitas, CA, USA), as previously described [21]. The experiments were performed at ~2 × IC_{50} concentrations: 0.25 μM of **4**, 0.1 μM of **5**, 2.0 μM of toloxatone, and 0.014 μM of clorgyline for hMAO-A; 0.7 μM of **4**, 6.0 μM of **5**, 0.08 μM of lazabemide, and 0.16 μM of pargyline for hMAO-B. After preincubation with the MAOs for 30 min, residual activities for undialyzed and dialyzed sets were separately measured; the

relative values for undialyzed (A_U) and dialyzed (A_D) assays were then calculated by comparing with each control set without inhibitor. The reversibility pattern was concluded by comparing the A_U and A_D values of inhibitors with the respective references.

2.8. Docking simulation of MAO enzymes with rhamnocitrin (5)

Docking simulations and visualizations were performed using CDOCKER in Discovery Studio [24]. Predefined active sites obtained from a complex of hMAO-A with 7-methoxy-1-methyl-9H-β-carboline (PDB ID: 2Z5X) and a complex of hMAO-B with pioglitazone (PDB ID: 4A79) were availed of to define the docking pocket of MAO-A and MAO-B [23]. To prepare the docking simulation, the 2D structure of rhamnocitrin (CID: 5320946) was downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and converted into a 3D structure using high temperature molecular dynamics. Then, we checked possible hydrogen bonding interactions between rhamnocitrin and hMAO enzymes using Discovery Studio Visualizer (http://accelrys.com/products/discovery-studio/).

3. Results and discussion

3.1. Bioassay-guided fractionation of the extract from P. Padus using MAOs and isolation of compounds

From the 845 herbal extracts screened, extract of *P. padus* leaves showed the highest inhibitory activity against hMAO-A and was selected for further screening. The extract of *P. padus* leaves was fractionated using the gradient solvent system, then the resultant fractions were evaluated for their inhibitory activities against MAOs (Supplementary material). Our results indicate that fractions 8 and 9 significantly inhibited hMAO-A (Fig. 1 and Supplementary material). The fractions were confirmed and the constituents were separated using semi-preparative HPLC. UPLC-QTof-MS analyses showed the *m/z* values of molecular ions $[M\text{-}H]^-$ of compounds (**1**–**5**) to be 447, 285, 301, 283, and 299, respectively (Table 1 and Supplementary material). The isolated compounds were identified as three flavanones, dihydrowogonin 7-*O*-glucoside (**1**) [25], dihydrowogonin (**2**) [26] and 3,5,7-trihydroxy-8-methoxyflavanone (**3**) [27], and two flavones, genkwanin (**4**) [28] and rhamnocitrin (**5**) [29]. All compounds were structurally elucidated and identified by NMR analyses (Supplementary material) (see Fig. 2).

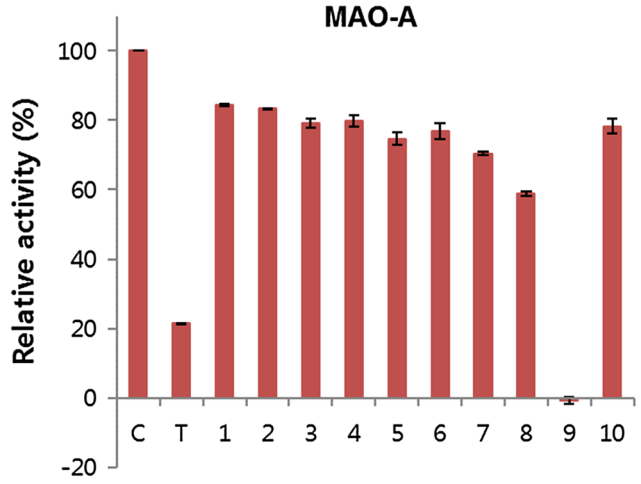

Fig. 1. Inhibitory activities of the methanol extract of *P. padus* var. *seoulensis* and the fractions obtained by YMC ODS AQ-HG chromatography on recombinant hMAO-A. C, control; T, total extract. Relative activities are expressed as residual activities. The numbers on the x axis indicate the fraction numbers.

Table 1
Compounds identified in the total extract and fractions of the leaves of *P. padus* var. *seoulensis* by UPLC-QTof-MS.

Peak	ESI-MS RT(min)	UV (nm)	Detected ion (*m/z*)	Calculated ion (*m/z*)	Fragments	ID	Molecular formula	Fraction
1	6.09	214, 285, 344	447.1320	447.1291	285, 269, 165, 110	Dihydrowogonin 7-*O*-glucoside	$C_{22}H_{24}O_{10}$	Fr. 8
2	6.11	269, 241, 214, 165	285.0802	285.0763	110, 137, 165, 241, 269	Dihydrowogonin	$C_{16}H_{14}O_5$	Fr. 8
3	6.18	218, 293	301.0729	301.0712	286, 258, 194, 165, 137, 110	(2*R*,3*R*)-3,5,7-Trihydroxy-8-methoxyflavanone	$C_{16}H_{14}O_6$	Fr. 8
4	8.90	194, 210, 266, 332	283.0625	283.0606	116, 174, 268	Genkwanin	$C_{16}H_{12}O_5$	Fr. 9
5	9.19	195, 265, 364	299.0590	299.0556	113, 169, 234, 284	Rhamnocitrin	$C_{16}H_{12}O_6$	Fr. 9

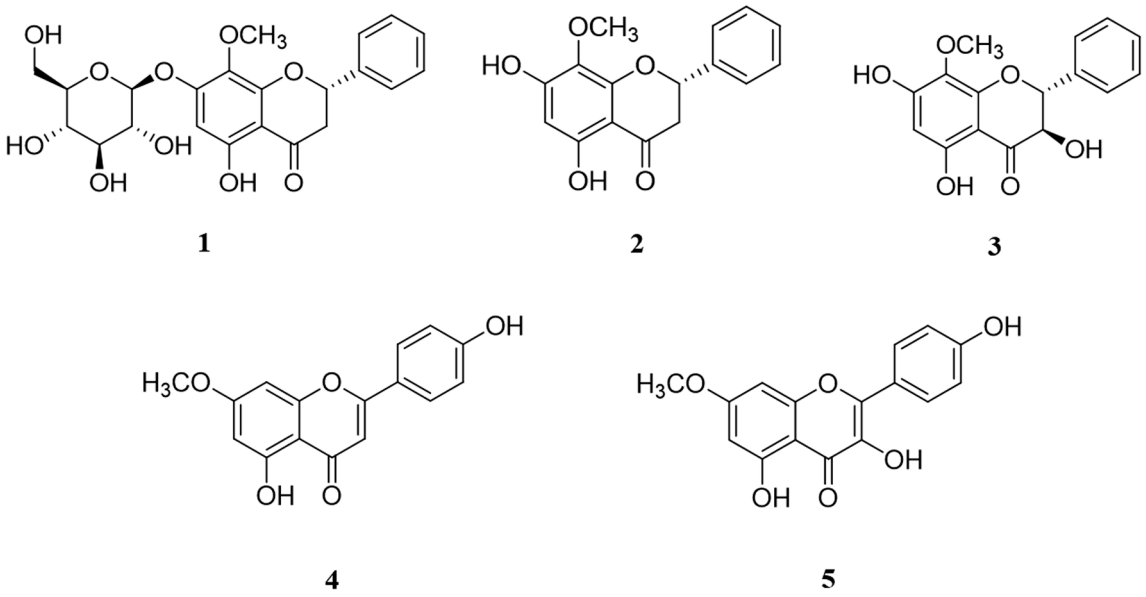

Fig. 2. Structures of compounds **1**–**5** isolated from *Prunus padus* leaves. **1**, dihydrowogonin 7-*O*-glucoside; **2**, dihydrowogonin; **3**, 3,5,7-trihydroxy-8-methoxy flavanone; **4**, genkwanin; **5**, rhamnocitrin.

Table 2
Inhibition of recombinant hMAO enzymes and AChE by compounds isolated from *P. padus* var. *seoulensis*.

Compounds	Residual activity at 10 μM (%)		IC_{50} (μM)			K_i (μM)		SI[a]
	MAO-A	MAO-B	MAO-A	MAO-B	AChE	MAO-A	MAO-B	
1	99.5 ± 0.27	92.2 ± 2.66	> 80	> 80	15.49 ± 0.11	–	–	–
2	90.2 ± 1.21	81.5 ± 0.54	> 80	> 80	21.53 ± 0.32	–	–	–
3	67.6 ± 3.79	81.1 ± 1.73	15.23 ± 0.38	> 80	17.92 ± 0.63	–	–	–
4	0.40 ± 0.01	2.9 ± 0.59	0.14 ± 0.011	0.35 ± 0.027	17.03 ± 0.77	0.097 ± 0.005	0.12 ± 0.012	2.5
5	0.70 ± 0.49	23.6 ± 1.86	0.051 ± 0.0014	2.97 ± 0.39	18.26 ± 0.075	0.030 ± 0.0028	0.91 ± 0.18	58.2
Kaempferol			0.36 ± 0.015	56.5 ± 2.94				
Quercetin			1.51 ± 0.19	> 80				
Toloxatone			1.03 ± 0.014	–				
Lazabemide			–	0.042 ± 0.0010				
Clorgyline			0.0050 ± 0.0001	> 2.0				
Pargyline			> 2.0	0.091 ± 0.005				
Tacrine					0.22 ± 0.001			

Results are expressed as means ± SD of two experiments.
[a] SI was expressed for hMAO-A selectivity by dividing IC_{50} values of hMAO-B by those of hMAO-A.

3.2. MAO inhibitory activities

The five compounds isolated from the extract of *P. padus* var. *seoulensis* were then assayed for inhibitory potential of hMAO-A and hMAO-B; IC_{50} values are shown in Table 2. Compound **5** (rhamnocitrin) is a potent inhibitor of MAO-A with an IC_{50} value of 0.051 μM, and an effective inhibitor of hMAO-B (IC_{50} = 2.97 μM) with a high selectivity index (SI) value of 58.2 (Table 2). Compound **4** (genkwanin) inhibits hMAO-A strongly with an IC_{50} value of 0.14 μM, and hMAO-B effectively (IC_{50} = 0.35 μM) with a low SI of 2.5 (Table 2). The other compounds show insignificant inhibitory activities; compound **3** shows moderate inhibition for hMAO-A (IC_{50} = 15.23 μM).

Compared to compound **4**, compound **5** has an additional 3-hydroxy group. Based on their IC_{50} values, **5** inhibited hMAO-A 2.7 times greater more as compared to **4**, whereas **5** inhibited hMAO-B 8.5 times less than **4** (Table 2). Considering their IC_{50} values, it is suggested that the 3-hydroxy group may be responsible for increased interactions with hMAO-A and decreased interactions with hMAO-B. Compound **3** has an additional 3-hydroxy group, i.e. 3,5,7-trihydroxy-8-methoxy flavanone, compared to **2**. Comparing **3** and **2**, it may also be suggested that the 3-hydroxy group increases the inhibitory activity for hMAO-A and imparts little effect on that for hMAO-B (Table 2). Compound **1**, a glucosyl flavanone, showed weak inhibitory activities for both hMAO-A and hMAO-B.

Amongst the natural compounds isolated from numerous herbal sources, compound **5** was found to be a highly potent inhibitor (IC_{50} = 0.051 μM) for hMAO-A compared to other potent phytochemicals such as acacetin (a flavonoid, 0.19 μM) [30], hispidol (an aurone, 0.26 μM) [19], chelerythrine (an isoquinoline alkaloid, 0.55 μM) [21], 1,5-dihydroxyxanthone (0.73 μM) [31], 7-(6′R-hydroxy-3′,7′-dimethyl-2′E,7′-octadienyloxy) coumarin (1.3 μM) [32], decursin (a coumarin; 1.76 μM) [33], purpurin (an anthraquinone; 2.50 μM) [34], apigenin (a flavonoid, 1.55 μM) [35], demethoxycurcumin (a curcuminoid, 3.09 μM) [20], and genistein (an isoflavonoid, 3.9 μM) [23]. Among the flavonoids, the reported IC_{50} values for kaempferol and quercetin are 0.525 and 3.98 μM, respectively [36]. Another study reports an IC_{50} of 0.01 μM for quercetin for hMAO-A inhibition, which was obtained using beef mitochondrial MAOs [37]. In the current study, we determined the values of kaempferol and quercetin under the same conditions for direct comparisons and obtained values of 0.36 and 1.56 μM, respectively (Table 2). Comparing the literatures and the currently obtained values, we conclude that compound **5** has the lowest IC_{50} value among the natural flavonoids reported to date. Furthermore, it was found to be 20.2 times more potent than toloxatone (1.03 μM), a commercial

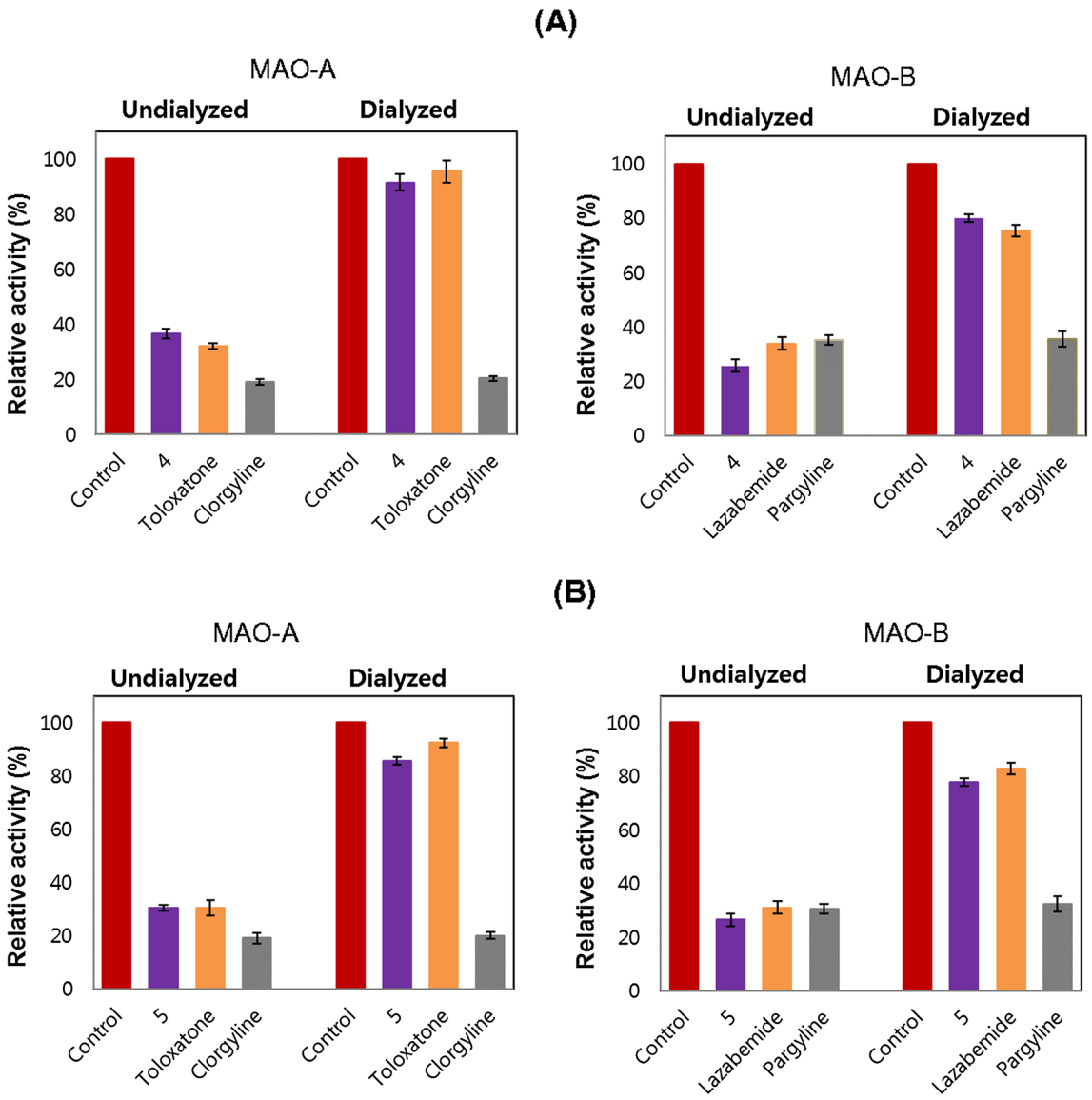

Fig. 3. Recovery of hMAO-A and hMAO-B inhibitions by **4 (A)** and by **5 (B)** using dialysis experiments.

antidepressants [3]. Considering the selectivity, SI of **5** (58.2) is greater than other herbal selective hMAO-A inhibitors such as decursin (40.1) [33], chelerythrine (> 36.4) [21], hispidol (9.4) [19], and acacetin (0.9) [30], and other flavonoids such as genistein (1.1) [23] and apigenin (3.3) [35]; however, the selectivity is lesser than kaempferol (156.9).

Rhamnocitrin has previously been reported to have antibacterial [38,39], antiatherogenic [29], antioxidant [40], anti-inflammatory [41,42], and antitumor [43] activities. Genkwanin is also indicated in various pharmacological effects such as anti-inflammatory [44], antibacterial [38,45], antiplasmodial [46], chemopreventive [47], radical scavenging [48], and anti-proliferative [49–51] activities. However, no study has previously reported on the inhibitory potential of rhamnocitrin or genkwanin on MAO enzymes.

Inhibition of AChE was analyzed as a therapeutic potential for the isolated flavonoids. The five isolated compounds displayed effective inhibitory activities with IC_{50} values ranging from 15.49 to 21.53 μM, and values for compounds **4** and **5** were 17.03 and 18.26 μM, respectively (Table 2). Reports indicate that baicalein is the most potent AChE inhibitor (IC_{50} = 0.61 μM), and IC_{50} values of kaempferol and quercetin were 3.05 and 3.60 μM, respectively [16,52]. Although compounds **4** and **5** are potent inhibitors of hMAO-A and/or hMAO-B, possess other biological activities, and have an AChE inhibitory potency lower than the reference compound tacrine (0.22 μM), it is necessary to further evaluate **4** and **5** for clinical applications including antidepressants or as pharmaceutical agents for the treatment of Alzheimer's and Parkinson's diseases.

On the other hand, flavonoids consist of a 15-carbon skeleton with two phenyl and one heterocyclic ring and are classified into several categories including flavones, isoflavones, flavonols, flavanones, flavanols, and anthocyanidins [53], which are biologically produced by enzymes from chalcones [54]. The flavonoids showed diverse inhibitory activities against hMAO-A or hMAO-B depending on the substituents [23,30,35,36], while most of synthetic flavonoids or chalcones, open chained flavonoids, possessed selective inhibitory activities against hMAO-B [17,18,55].

Time-dependent studies of inhibitors showed that hMAO-A and hMAO-B activities in the presence of compounds **4** and **5** were almost the same during the preincubation times, which indicates that the activities of the two compounds are not time-dependent for both MAO enzymes.

Dialysis experiments to study for recovery of inhibition was performed under constant conditions for all compounds (Fig. 3). The A_U and A_D values for hMAO-A were as follows: compound **4**, 36.5 and

91.4%, respectively; toloxatone (a reversible reference), 32.0 and 95.3%, respectively; and clorgyline (an irreversible reference), 19.0 and 20.3%, respectively (Fig. 3A). Inhibition of hMAO-A by clorgyline was not recovered at all by dialysis, whereas inhibition by toloxatone was substantially recovered. Similarly, the A_U and A_D values for hMAO-B were as follows: compound **4**, 25.7 and 79.8%, respectively; lazabemide (a reversible reference), 33.9 and 75.3%, respectively; and pargyline (an irreversible reference), 35.2 and 35.4%, respectively (Fig. 3A). Inhibition of hMAO-B by pargyline was not recovered, whereas inhibition by lazabemide was substantially recovered. These results indicate that inhibitions by **4** for hMAO-A and hMAO-B were recovered by dialysis and was similar to the control levels. Similar results were obtained for compound **5**, as follows: For hMAO-A, A_U value was recovered to 85.7% from A_D value of 30.4%, similar to the reversible inhibitor (92.5% from 30.4%) (Fig. 3B). For hMAO-B, A_U value was recovered to 77.8% from A_D value of 26.5%, similar to the reversible inhibitor (82.8% from 31.0%). No recoveries were observed for irreversible inhibitors for hMAO-A or hMAO-B (Fig. 3B). These results indicate that both compounds **4** and **5** are reversible inhibitors of hMAO-A or hMAO-B.

We further investigated the modes of hMAO-A and hMAO-B inhibitions by compounds **4** and **5** using Lineweaver-Burk plots. Lineweaver plots for the hMAO-A or hMAO-B inhibitions by compound **4** showed linear lines intersecting the y-axis (Fig. 4A), indicating that **4** is a competitive inhibitor of hMAO-A or hMAO-B. From secondary plots of the slopes against inhibitor concentrations, the K_i values for hMAO-A and hMAO-B inhibitions by **4** were determined to be 0.097 ± 0.005 μM and 0.12 ± 0.012 μM, respectively (Fig. 4A). Compound **5** was also found to be a competitive inhibitor of hMAO-A and hMAO-B with a K_i value of 0.030 ± 0.0028 and 0.91 ± 0.18 μM, respectively (Fig. 4B). The K_i value of **5** for hMAO-A was lower than that of the natural compound harman (0.056 μM), but higher than that of harmine (0.016 μM) [11]. Compared to the synthetic flavonoids, the

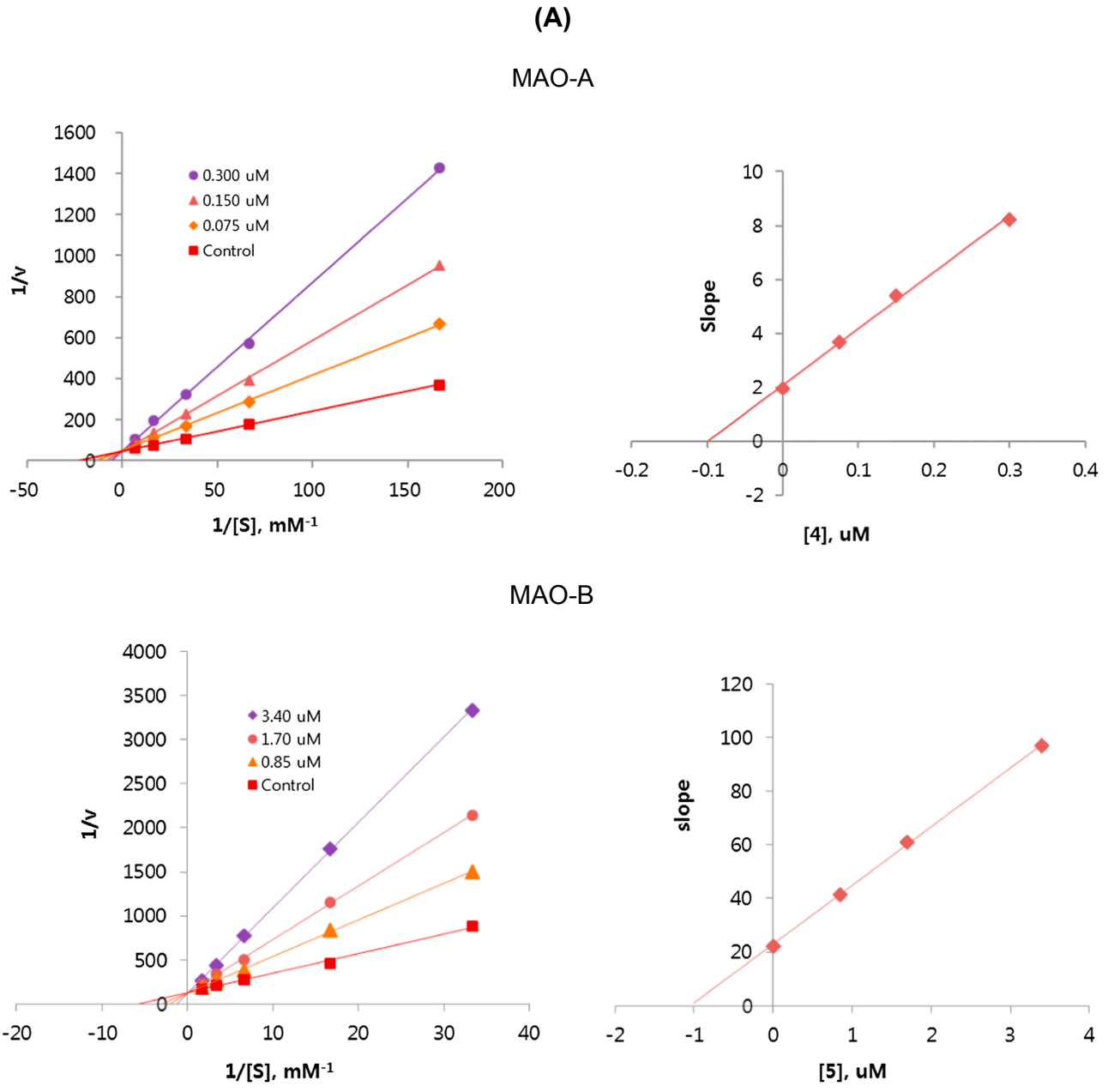

Fig. 4. Lineweaver-Burk plots (left) for hMAO-A and hMAO-B inhibitions by **4 (A)** and **5 (B)** and their secondary plots (right) of the slopes against inhibitor concentrations.

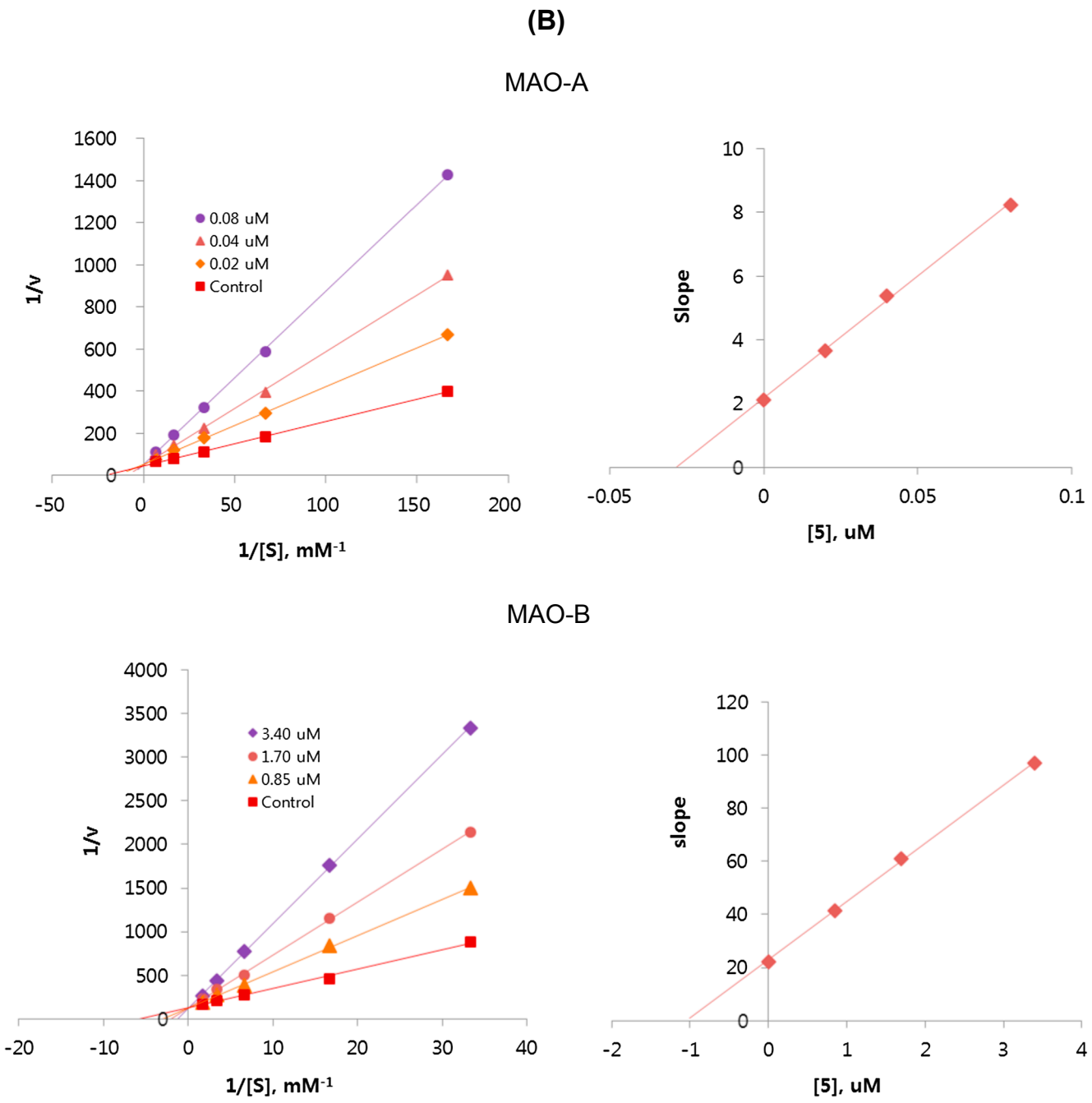

Fig. 4. (*continued*)

K_i value of **5** for hMAO-A was also lower than the most potent compounds **3d** (0.52 μM) of 2-aryl-4H-chromen-4-one (flavone) derivatives, and **18** (0.11 μM) of 2-(arylmethylidene)-2,3-dihydro-1-benzofuran-3-one (aurone) derivatives, most of which showed MAO-B selective inhibitions with K_i values in the range of 0.20–9.48 μM [17,18].

3.3. Molecular docking simulation

Docking simulations showed rhamnocitrin located appropriately at the binding sites of 2Z5X (hMAO-A) or 4A79 (hMAO-B). The binding affinity of rhamnocitrin for hMAO-A (−18.49 kcal/mol) was greater than that of hMAO-B (0.19 kcal/mol) as determined by CDOCKER in Discovery Studio. Docking simulation results revealed rhamnocitrin binds to hMAO-A with four hydrogen-bond interactions of Asn181, Gln215, Thr336, and Tyr444 to fit the pocket (Fig. 5A/B). However, it is predicted that rhamnocitrin would form one hydrogen bond with Glu84 residue of hMAO-B with unfavorable position (Fig. 5C/D). These results might sufficiently explain the selectivity of rhamnocitrin for hMAO-A by identifying hydrogen bonding residues, hydrogen bonding lengths, and location of the active site pocket, and possibly by the size and physiochemical differences of pockets between hMAO-A and hMAO-B, as indicated by IC_{50} values for hMAO-A and hMAO-B of 0.051 ± 0.0014 μM and 2.97 ± 0.39 μM, respectively. Based on the IC_{50} values for hMAO-A by rhamnocitrin and genkwanin, hydroxyl group of 3rd position of chromane ring could provide improved potency towards hMAO-A (~2.7 fold), and as expected, the binding affinity of genkwanin for hMAO-A (−17.52 kcal/mol) was lower than that of rhamnocitrin (Fig. S6). However, docking simulation showed that the

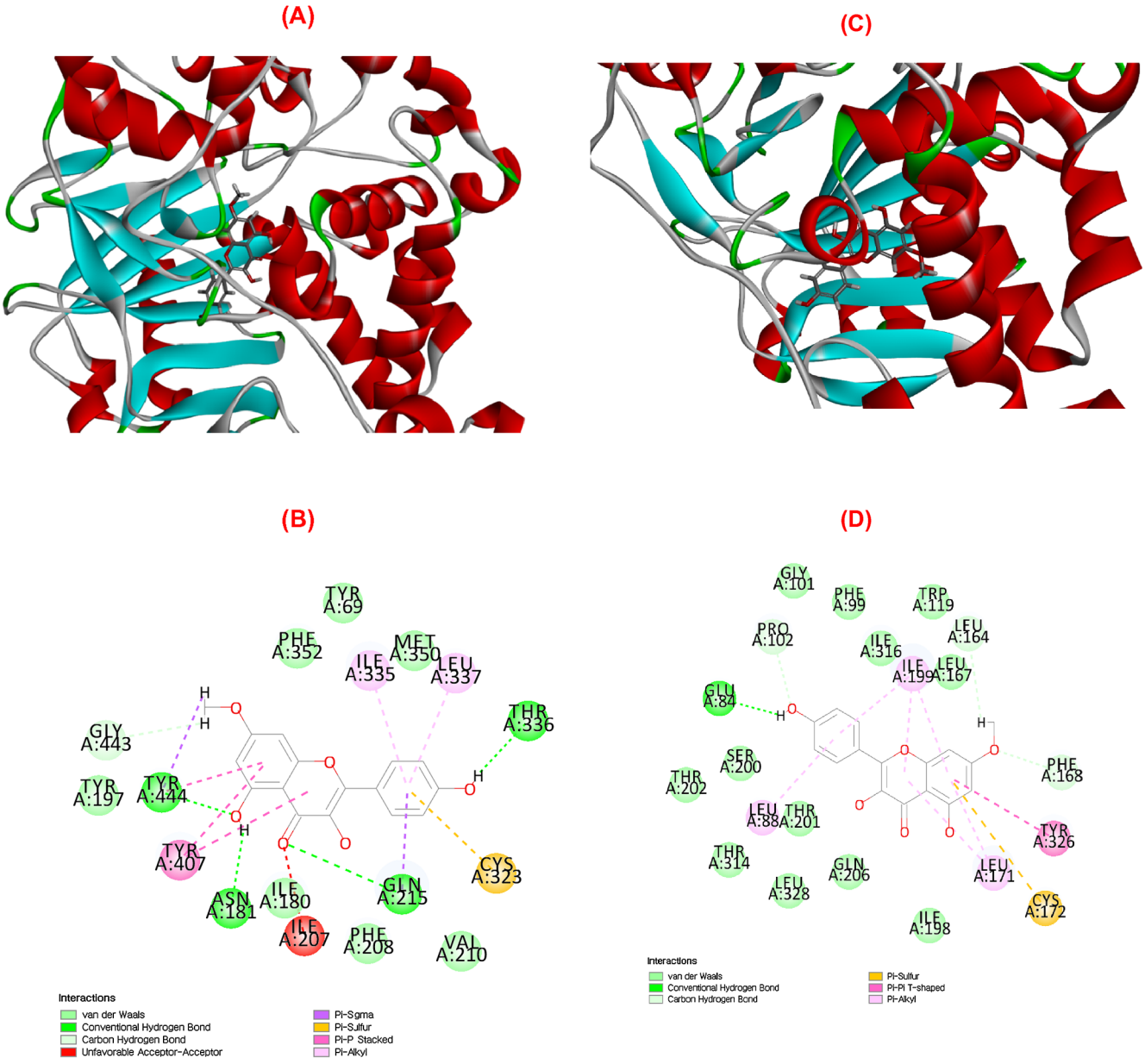

Fig. 5. Docking simulation of rhamnocitrin (**5**) with hMAO-A (2Z5X) **(A)** and possible interactions (**B**), and with hMAO-B (4A79) **(C)** and possible interactions (**D**). The rhamnocitrin molecule is represented by cyan color. Hydrogen bonding is depicted in green-colored line. The binding affinities to hMAO-A and hMAO-B were −18.49 and 0.19 kcal/mol, respectively, by CDOCKER in Discovery Studio.

hydroxyl group did not interact any amino acid residues of hMAO-A and hMAO-B (Fig. 5B/D), and could not find any difference in binding poses by the presence and the absence of the hydroxyl group.

4. Conclusion

Three flavanones and two flavones were isolated from the leaves of *P. padus* var. *seoulensis* with bioassay-guided fractionation. Of these, compound **5** (rhamnocitrin) was found to be a highly potent, highly selective, reversible, and competitive inhibitor of hMAO-A, and to be the most potent (IC_{50} = 0.051 μM) amongst the natural flavonoids reported so far. Compound **4** (genkwanin) was a potent, near non-selective, reversible and competitive inhibitor of hMAO-A, and an effective inhibitor of hMAO-B. The IC_{50} value of **5** for the inhibition of hMAO-A was 20.2 times more potent than that of toloxatone, a commercial drug. Docking analysis showed compound **5** had a greater binding affinity for hMAO-A than for hMAO-B, interacting with hMAO-A at four different residues (Asn181, Gln215, Thr336, and Tyr444), while hMAO-B forms only one hydrogen bond at Glu84. The findings indicate that both compound **5** and **4** can be considered as novel potent and reversible hMAO-A and/or hMAO-B inhibitors for the development of MAO inhibitors in neurological disorder therapies.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2018.10.051.

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