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BIOLOGICAL ACTIVITY OF SINAPIC ACID DERIVATIVES ISOLATED FROM *RAPHANUS SATIVUS*

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Abstract – Radish sprout (*Raphanus sativus*) is a vegetable sprout that contains various sinapic acid derivatives. In this study, three sinapic acid derivatives **1**-**3** isolated from *R. sativus*, sinapic acid (**4**), and compounds **5**-**7**, derived from the methylation of compounds **2**-**4**, respectively, were used to investigate the structure-activity relationship for various bioactivities. Compounds with a number of sinapoyl and phenolic hydroxyl groups were found to have antioxidant and angiotensin converting enzyme (ACE) inhibitory activities. In addition, the sugar domains and the phenolic hydroxyl group in sinapoyl group is related to α-glucosidase inhibitory activity. Therefore, these sinapic acid derivatives are promising candidates to move forward to *in vivo* studies for the treatment of lifestyle-related diseases such as diabetes and hypertension.

INTRODUCTION

Lifestyle-related diseases are a leading cause of death, and currently accounts for 60% of deaths in Japan. Lifestyle-related diseases is a general term used to describe health conditions caused by poor lifestyle choices such as lack of exercise, stress, alcohol abuse, and smoking, and includes obesity, diabetes, hypertension and hyperlipidemia. It is important to make better daily lifestyle choices in order to prevent and treat the symptoms of lifestyle-related diseases. Therefore, maintaining proper dietary habits is essential for health.

Vegetables supply vitamins and minerals that function to regulate and maintain the body's homeostasis and health. Here, we focus on vegetable sprouts. There are many kinds of vegetable sprouts, such as radish sprout, broccoli sprout, and alfalfa that can be easily purchased or home-grown. It usually takes a few months to cultivate vegetables, however vegetable sprouts are ready to be harvested within one to

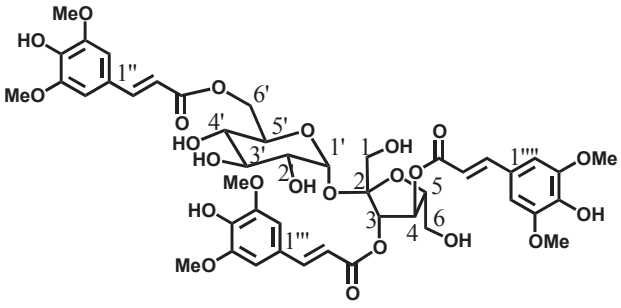
two weeks. In this study, we focused on radish sprout (*Raphanus sativus*).

R. sativus is a member of the Brassicaceae family of flowering plants including vegetable sprouts and is a popular plant-based food. Broccoli sprout, another member of the Brassicaceae vegetable sprout family, contains sulforaphane that possesses strong antioxidant[1] and antibacterial activities.[2] Therefore, it is likely that other Brassicaceae vegetable sprouts may also be beneficial. Two sinapic acid derivatives β-D-(3-sinapoyl)-fructofuranosyl-α-D-(6-sinapoyl)glucopyranoside (**2**) and 1-sinapoyl-β-D-glucopyranoside (**3**) have been isolated from *R. sativus*, and are known to possess antioxidant activity and growth inhibition.[3-7] In this study, we isolated several sinapic acid derivatives from *R. sativus*, and investigated their biological activity.

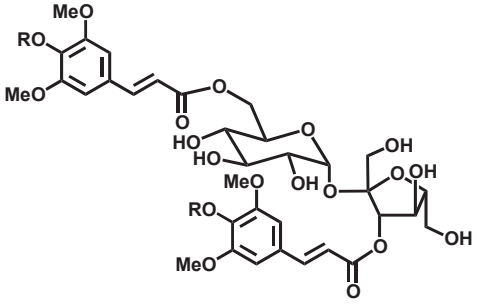
RESULTS AND DISCUSSION

Isolation of sinapic acid derivatives from *R. sativus*

R. sativus sprouts (fresh weight: 1.1 kg) were extracted with MeOH, and the MeOH extract (RS, 28.9 g) was partitioned with hexane and H_2O. The H_2O layer was successively partitioned with EtOAc and BuOH to afford hexane (RS-H, 3.0 g), EtOAc (RS-EA, 0.8 g), BuOH (RS-Bu, 4.1 g), and H_2O (RS-W, 20.5 g)-soluble materials. RS-EA and RS-Bu were separated by SiO_2 column and preparative SiO_2 TLC to yield β-D-(3,4-disinapoyl)fructofuranosyl-α-D-(6-sinapoyl)glucopyranoside (**1**; 7.9 mg), β-D-(3-sinapoyl)-fructofuranosyl-α-D-(6-sinapoyl)glucopyranoside (**2**; 304 mg), and 1-sinapoyl-β-D-glucopyranoside (**3**; 540 mg). The structure of compounds **1**-**3** were confirmed by NMR analysis, and the findings were compared to those reported.[7]



1



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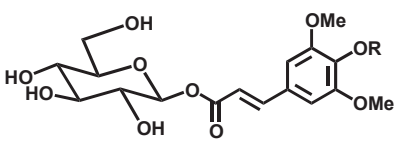
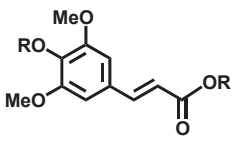
5: R=Me



3: R=H
6: R=Me



4: R=H
7: R=Me

Figure 1. Structure of compounds **1**-**7**

Methylation of compounds 2-4

Compounds **2** and **3** and sinapic acid (**4**) were subjected to methylation with MeI and K_2CO_3 in DMF to obtain compounds **5**-**7**.

Antioxidant activity

The antioxidant activity of compounds **1**-**7** (Figure 1) was evaluated using the 1,1-dipheny-2-picrylhydrazyl (DPPH) radical scavenging assay and superoxide dismutase (SOD) assay, with epigallocatechin gallate (EGCG) as a positive control. The analyses of compounds **1**-**7** are depicted in Figure 2. Compounds **1** and **2** exhibited stronger antioxidant activity compared to the other compounds, indicating that the number of sinapoyl groups is a determinant of antioxidant potency. The antioxidant activity of compounds **5**-**7** was strongly decreased compared to compounds **2**-**4**, suggesting that the phenolic hydroxyl group in sinapoyl group is related to the antioxidant activity.

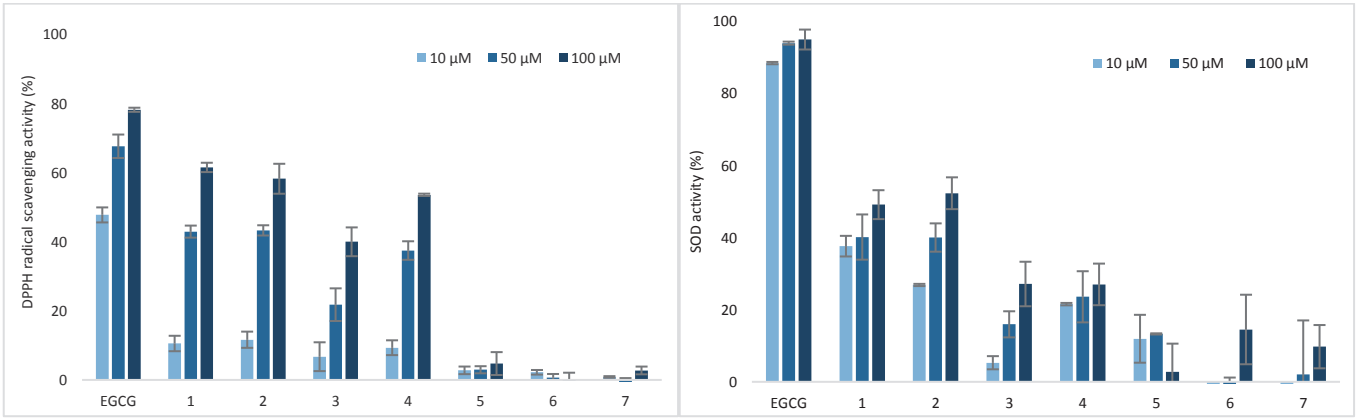


Figure 2. Antioxidant activity of compounds **1**-**7** evaluated using DPPH radical scavenging assay (left) and SOD assay (right)

α-Glucosidase inhibitory assay

α-Glucosidase inhibitory assay was performed to evaluate the inhibitory effect of compounds **1**-**7**. Acarbose was used as a positive control. The inhibitory activity of compounds **1**-**7** is shown in Figure 3. Compounds **1**-**3** exhibited stronger α-glucosidase inhibitory activity than compound **4**, suggesting that the sugar component of these compounds is essential for their activity. The α-glucosidase inhibitory activity of compounds **5** and **6** decreased compared to compounds **2** and **3**, suggesting that the phenolic hydroxyl group in sinapoyl group is also related to α-glucosidase inhibition.

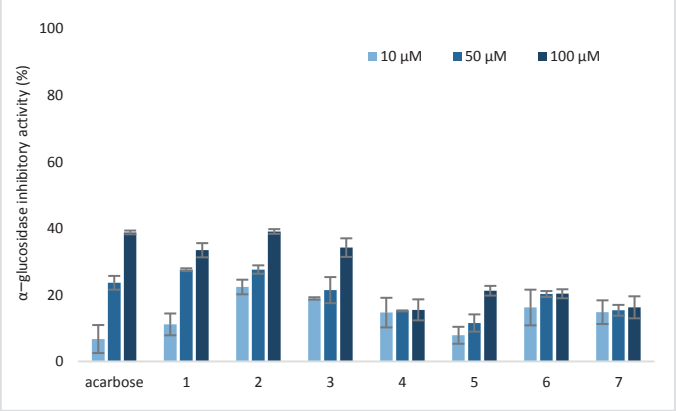


Figure 3. α-Glucosidase inhibitory activity of compounds **1**-**7**

ACE inhibitory assay

An ACE inhibitory assay was performed to evaluate the inhibitory effect of compounds **1**-**7**, with captopril as a positive control. The ACE inhibitory activity of compounds **1**-**7** is shown in Figure 4. When comparing compounds **1**-**3**, ACE inhibition was highest in compound **1**, followed by compounds **2** and **3**. Indicating that as the number of sinapoyl groups increased, ACE inhibition also increased. The ACE inhibitory activity of compounds **5** and **6** strongly decreased compared to compounds **2** and **3**, suggesting that the phenolic hydroxyl group is also important for ACE inhibition. However, compound **4**, with a sinapoyl group, had the lowest activity. Additional studies are required to verify these findings.

In conclusion, the sinapic acid derivatives isolated from radish sprout have potential utility as antidiabetic and antihypertensive agents through inhibition of α-glucosidase and ACE. Therefore, these sinapic acid derivatives are promising candidates to move forward to *in vivo* studies for the treatment of lifestyle-related diseases such as diabetes and hypertension.

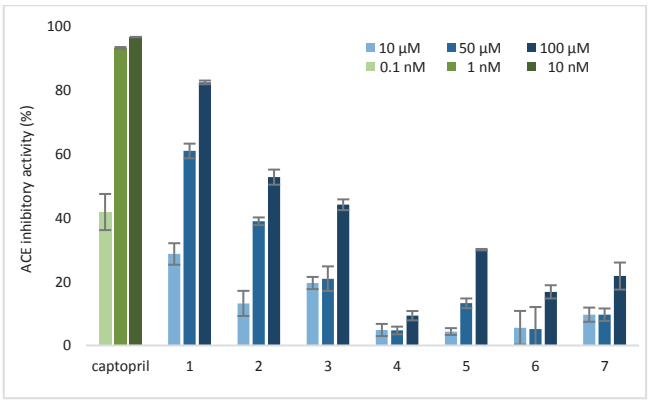


Figure 4. ACE inhibitory activity of compounds **1**-**7**

EXPERIMENTAL

General Procedures

Optical rotations were recorded on a Jasco P-2200 and UV spectra were recorded on a Shimadzu UV-3100PC spectrometer. IR spectra were recorded on a JASCO FT/IR-300 spectrometer, while ^{1}H and ^{13}C NMR spectra were measured and recorded on Bruker Avance 500 and 600 spectrometers, respectively. The resonances of CD_3OD at δ_H 3.35 and δ_C 49.8 were used as internal references for the ^{1}H and ^{13}C NMR spectra, respectively, while ESIMS was performed on a Waters SYNAPT G2 mass spectrometer. Syntheses were conducted under a nitrogen atmosphere. *R. sativus* was purchased from Higashifuzi-kaihatsunojyo and sinapic acid (**4**) was purchased from Sigma-Aldrich, Inc.

Isolation of the sinapic acid derivatives 1-3 from *R. sativus*

R. sativus (fresh weight: 1.1 kg) was extracted with MeOH (1 L × 3), and the MeOH extract (RS, 28.9 g) was partitioned with hexane (1 L × 3) and H_2O (1 L). The H_2O layer was partitioned with EtOAc (1 L × 3) and BuOH (1 L × 3) successively, to afford hexane (RS-H, 3.0 g), EtOAc (RS-EA, 0.8 g), BuOH (RS-Bu, 4.1 g), and H_2O (RS-W, 20.5 g)-soluble materials. RS-EA was divided into 7 fractions (RS-EA-1~7) by SiO_2 column (*Φ*4.6×31 cm, $CHCl_3$/MeOH, 3:7→4:6→5:5→6:4→7:3→10:0), and RS-EA-6 was subsequently separated into 13 fractions (RS-EA-6-1~13) by SiO_2 PTLC (200×200×0.5 mm, $CHCl_3$/MeOH, 5:1), to yield β-D-(3,4-disinapoyl)fructofuranosyl-α-D-(6-sinapoyl)glucopyranoside (**1**, RS-EA-6-7, 7.9 mg). RS-Bu was divided into 11 fractions (RS-Bu-1~11) by SiO_2 column (*Φ*4.6×32 cm, $CHCl_3$/MeOH, 3:7→4:6→5:5→6:4→10:0), to yield β-D-(3-sinapoyl)-fructofuranosyl-α-D-(6-sinapoyl)glucopyranoside (**2**, RS-Bu-9, 304 mg) and 1-sinapoyl-β-D-glucopyranoside (**3**, RS-Bu-4, 540 mg).

Methylation of compounds 2-4

Methylation of compound **2**

Compound **2** (14.8 mg, 0.02 mM) was dissolved in DMF (3 mL), and K_2CO_3 (27.6 mg, 0.2 mM) and iodomethane (MeI) (12.5 μL, 0.2 mM) was added to the solution. After stirring at room temperature for 24 h, the mixture was neutralized with 10% HCl, poured into H_2O (30 mL), extracted with EtOAc (30 mL×3), dried over $MgSO_4$, filtered, and the solvent was removed *in vacuo*. The residue was purified by SiO_2 PTLC (200×100×0.5 mm, $CHCl_3$/MeOH, 85:15) to afford compound **5** (7.9 mg, 52%) as a colorless powder; $[\alpha]_D^{24}$ -36.1° (MeOH); UV λ_{max} (MeOH) nm (log ε) 230 (4.5) and 307 (4.5); IR (KBr) ν_{max} cm^{-1} 3410, 1712, 1635, 1581, and 1504; ^{1}H NMR (CD_3OD) δ: 7.68 (1H, d, *J* = 15.9 Hz, H-7’’), 7.60 (1H, d, *J* = 15.9 Hz, H-7’’’), 6.95 (2H, s, H-2’’’,6’’’), 6.90 (2H, s, H-2’’,6’’), 6.55 (1H, d, *J* = 15.9 Hz, H-8’’), 6.54 (1H, d, *J* = 15.9 Hz, H-8’’’), 5.51 (1H, d, *J* = 8.1 Hz, H-3), 5.51 (1H, d, *J* = 3.8 Hz, H-1’), 4.70 (1H, d, *J* =

11.8 Hz, H-6'), 4.51 (1H, t, *J* = 8.1 Hz, H-4), 4.28 (1H, m, H-5'), 4.20 (1H, dd, *J* = 7.5, 11.8 Hz, H-6'), 3.97 (1H, m, H-5), 3.89 (1H, m, H-6), 3.86 (6H, s, 3'',5''-OMe), 3.84 (6H, s, 3''',5'''-OMe), 3.81 (1H, m, H-6), 3.78 (3H, s, 4''-OMe), 3.77 (3H, s, 4'''-OMe), 3.65 (1H, t, *J* = 9.5 Hz, H-3'), 3.60 (1H, d, *J* = 11.4 Hz, H-1), 3.47 (1H, dd, *J* = 3.8, 9.5 Hz, H-2'), and 3.28 (1H, m, H-4'). ^{13}C NMR (CD_3OD) δ: 169.5 (C, C-9'''), 168.6 (C, C-9''), 155.6 (2C, C-3'',5''), 155.6 (2C, C-3''',5'''), 148.0 (CH, C-7''), 147.4 (CH, C-7'''), 142.3 (C, C-4''), 142.3 (C, C-4'''), 132.3 (C, C-1'''), 132.3 (C, C-1''), 119.0 (CH, C-8''), 118.6 (CH, C-8'''), 107.7 (2CH, C-2''',6'''), 107.5 (2CH, C-2'',6''), 105.6 (C, C-2), 93.4 (CH, C-1'), 85.1 (CH, C-5), 80.1 (CH, C-3), 75.9 (CH, C-3'), 75.0 (CH, C-4), 73.9 (CH, C-2'), 73.3 (CH, C-5'), 72.8 (CH, C-4'), 66.6 (CH_2, C-1), 66.5 (CH_2, C-6'), 64.6 (CH_2, C-6), 62.0 ($2CH_3$, 4'',4'''-OMe), 57.6 ($2CH_3$, 3'',5''-OMe), and 57.5 ($2CH_3$, 3''',5'''-OMe); ESI-MS (negative ion) *m/z* 781 $[M-H]^-$ HRESI-MS (negative ion) *m/z* 781.2526 $[M-H]^-$, (calcd for $C_{36}H_{45}O_{19}$: 781.2555).

Methylation of compound **3**

Compound **3** (20.1 mg, 0.052 mM) was dissolved in DMF (3 mL), and K_2CO_3 (71.9 mg, 0.52 mM) and MeI (32 μL, 0.52 mM) was added to the solution. After stirring at room temperature for 24 h, the mixture was neutralized with 10% HCl, poured into H_2O (30 mL), extracted with EtOAc (30 mL×3), dried over $MgSO_4$, filtered, and the solvent was removed *in vacuo*. The residue was purified by SiO_2 PTLC (200×100×0.5 mm, $CHCl_3$/MeOH, 9:1) to afford compound **6** (4.0 mg, 19%) as a colorless powder; $[\alpha]_D^{24}$ +12.8° (MeOH); UV λ_{max} (MeOH) nm (log ε) 230 (4.3) and 307 (4.3); IR (KBr) ν_{max} cm^{-1} 3402, 1712, 1635, 1581, and 1504; ^{1}H NMR (CD_3OD) δ: 7.74 (1H, d, *J* = 16.0 Hz, H-7'), 6.95 (2H, s, H-2',6'), 6.53 (1H, d, *J* = 16.0 Hz, H-8'), 5.58 (1H, d, *J* = 7.9 Hz, H-1), 3.87 (6H, s, 3',5'-OMe), 3.84 (1H, d, *J* = 2.2 Hz, H-6) 3.79 (3H, s, 4'-OMe), 3.69 (1H, dd, *J* = 5.0, 12.1 Hz, H-6), and 3.3-3.5 (overlapped, H-2-5). ^{13}C NMR (CD_3OD) δ: 168.0 (C, C-9'), 155.7 (2C, C-3',5'), 148.5 (CH, C-7'), 142.4 (C, C-4'), 132.2 (C, C-1'), 118.4 (CH, C-8'), 107.7 (2CH, C-2',6'), 96.7 (CH, C-1), 79.7 (CH, C-5), 78.8 (CH, C-3), 74.9 (CH, C-2), 71.9 (CH, C-4), 63.1 (CH_2, C-6), 62.0 (CH_3, 4'-OMe), and 57.5 ($2CH_3$, 3',5'-OMe); ESI-MS (positive ion) *m/z* 423 $[M+Na]^+$, HRESI-MS (positive ion) *m/z* 423.1254 $[M+Na]^+$ (calcd for $C_{18}H_{24}O_{10}Na$: 423.1267).

Methylation of compound **4**

Compound **4** (10 mg, 0.045 mM) was dissolved in DMF (3 mL), and K_2CO_3 (62.0 mg, 0.45 mM) and MeI (28 μL, 0.45 mM) was added to the solution. After stirring at room temperature for 24 h, the mixture was neutralized with 10% HCl, poured into H_2O (30 mL), extracted with EtOAc (30 mL×3), dried over $MgSO_4$, filtered, the solvent was removed *in vacuo* and afforded compound **7** (10.8 mg, 95%) as a pale yellow powder: UV λ_{max} (MeOH) nm (log ε) 229 (4.3) and 304 (4.3); IR (KBr) ν_{max} cm^{-1} 1697, 1635,

1581, and 1504; ^{1}H NMR (CD_3OD) δ: 7.61 (1H, d, *J* = 16.0 Hz, H-7), 6.91 (2H, s, H-2,6), 6.48 (1H, d, *J* = 16.0 Hz, H-8), 3.86 (6H, s, 3,5-OMe), 3.78 (3H, s, 4-OMe), and 3.77 (3H, s, 9-OMe). ^{13}C NMR (CD_3OD) δ: 170.0 (C, C-9), 155.6 (2C, C-3,5), 147.2 (CH, C-7), 142.1 (C, C-4), 132.4 (C, C-1), 118.8 (CH, C-8), 107.5 (2CH, C-2,6), 62.0 (CH_3, 4-OMe), 57.5 ($2CH_3$, 3,5-OMe), 52.9 (CH_3, 9-OMe), ESI-MS (positive ion) *m/z* 275 $[M+Na]^+$, HRESI-MS (positive ion) *m/z* 275.0897 $[M+Na]^+$ (calcd for $C_{13}H_{16}O_5Na$: 275.0895).

DPPH radical scavenging assay

DPPH solution [dissolved 1 mM in EtOH-0.4 mM 2-morpholinoethanesulfonic acid (MES) buffer (pH 6.1)-Milli Q, 4:1:3] (190 μL) was mixed with each sample (10 μL) in MeOH at different concentrations (10, 50, and 100 μM) and incubated in the dark at room temperature for 15 min, and the absorbance at a wavelength of 490 nm (A_{sample}) was measured. The absorbance of a negative control ($A_{control}$) composed of only solvent and blank (A_{blank}) without DPPH was also determined. The DPPH radical scavenging activity was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [1-(A_{sample}-A_{blank})/A_{control}]\times 100$$

SOD assay

SOD activity was measured using a SOD Assay Kit-WST purchased from Dojindo Molecular Technologies, Inc. WST working solution [diluted WST solution (1.0 mL) was prepared with Buffer solution (19 mL)], and Enzyme working solution [(diluted Enzyme solution (15 μL) with Dilution buffer (2.5 mL)]. WST working solution (200 μL) was mixed with each sample (20 μL) in MeOH at different concentrations (10, 50, and 100 μM) and Enzyme working solution (20 μL) was added. The samples were incubated in the dark at 37 °C for 20 min and an absorbance at a wavelength of 450 nm (A_{sample}) was measured. The absorbance of a coloring without inhibitor (A_{blank1}), sample blank (A_{blank2}) and reagent blank (A_{blank3}) was also determined. The SOD activity was calculated using the following equation:

$$\text{SOD activity (\%)} = [(A_{blank1}-A_{blank3})-(A_{sample}-A_{blank2})]/(A_{blank1}-A_{blank3})\times 100$$

α-Glucosidase inhibitory assay

The reaction mixture comprising of 0.1 M phosphate buffer (pH 6.8) (50 μL), 1 mM 4-nitrophenyl α-D-galactopyranoside (PNPG) (10 μL), and each sample (10 μL) in EtOH at different concentrations (10, 50, and 100 μM) was pre-incubated at 37 °C for 5 min. Yeast α-glucosidase (0.07 U/mL) (10 μL) was added to each sample as a substrate, and incubated at 37 °C for 30 min. The reaction was stopped by

adding Na_2CO_3 (50 μL), and absorbance at a wavelength of 410 nm (A_{sample}) was measured. The absorbance of a negative control ($A_{control}$) composed of only solvent and blank (A_{blank}) without α-glucosidase was also determined. The inhibitory activity was calculated using the following equation:

α-Glucosidase inhibitory activity (%) = $[1-(A_{sample}-A_{blank})/A_{control}]\times 100$

ACE inhibitory assay

ACE inhibitory activity was measured using ACE Kit-WST purchased from Dojindo Molecular Technologies, Inc. The Enzyme working solution [dissolved Enzyme B with Milli Q (2.0 mL) and Enzyme B solution (1.5 mL) added to Enzyme A] and the Indicator working solution [dissolved Enzyme C and Coenzyme with Milli Q (3.0 mL) with Enzyme C solution (2.8 mL) and Coenzyme solution (2.8 mL) added to Indicator solution (5.0 mL)], were prepared. The Indicator working solution (200 μL) was mixed with each sample in MeOH (20 μL) at different concentrations (10, 50, and 100 μM). Substrate buffer (20 μL) and Enzyme working solution (20 μL) was added incubated in the dark at 37 °C for 60 min and absorbance at a wavelength 450 nm (A_{sample}) was measured. The absorbance of a positive control (without ACE inhibition) (A_{blank1}) and reagent blank (A_{blank2}) was also determined. The ACE inhibitory activity was calculated using the following equation:

ACE inhibitory activity (%) = $[(A_{blank1}-A_{sample})/(A_{blank1}-A_{blank2})]\times 100$

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