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Isolation of Angiotensin Converting Enzyme (ACE) Inhibitory Activity Quercetin from *Peperomia pellucida*

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Abstract : *Peperomia pellucida* (L.) Kunth. (Piperaceae) is empirically used in traditional medicine to control blood pressure. The objective of this study was to isolate ACE inhibitor from aerial part of the plant. Using chromatography technique was isolated flavonoid as ACE inhibitor. The chemical structure was determined as 3',4', Dihydroxy-3-5-dimethoxy flavone-7-O-β-rhamnose based on the spectral data (UV, IR, LC-MS, ^{1}H-NMR, ^{13}C-NMR, and 2D-NMR) and compared with that reference. *In vitro* assay of ACE inhibitory activity that showed IC_{50} of the compound was 7.72 µg/mL. The result of kinetic suggested that the compound inhibit the enzyme activity by competing with the substrate for the active site. The results supported the traditional use of the plant antihypertensive.
Key words : *Peperomia pellucida*, ACE, isolation, antihypertensive, flavonoid.

Introduction

Angiotensin converting enzyme (ACE) is a zinc metallopeptidase that converts the angiotensin I to angiotensin II[1]. ACE inhibitors contribute in the treatment of hypertension, heart failure, myocardial infarction, diabetes mellitus, chronic renal disorders, and stroke [2-4].

P.pellucida (L.) Kunth is native to tropical Central and South America. It is widely distributed throughout the tropics and is often naturally as a weed and occasionally cultivated. It is widely used medicinally throughout the tropics, Nigeria, Brazil, China, Phillipines, also in tropical Africa. The areal parts are applied againts abdominal pain, abscesses, acne, rheumatic pain, gout, headache, kidney problems, cardiac arrhythmia, fatigue, prostate problems, and also againts high blood pressure [5]. The plant is empirically used as antihypertensive by Esan people of Edo State, Nigeria [6] and even noted in traditional Ayurvedic Medicinal Plant[7]. Based on the research of Saputri, et al. (2015) that methanol extract of the aerial part of this plant has shown a potent ACE inhibitory activity[8].

The aerial part of this plant was reported contain : tannins, saponins, phenols, steroids, terpenoids, amino acids and alkaloids[9-11]. Some compounds were isolated from this plant, including: apiole, tetrahydrofuran lignans, dihydronaphthalene, dillapiole, pellucidin A, peperomins, xanthone patuloside A, dillapiole, and

chromene [12-18]. However, ACE inhibitor from *P. pellucida* have not yet been identified. Therefore, the objective of study was to isolate ACE inhibitor from the aerial part of *P. pellucida*.

Material and Method

Plant Materials

The aerial of *Peperomia pellucida* were collected in Center for Plant Conservation, Bogor Botanical Garden, Indonesian Institute of Science. The voucher specimens were identified and deposited at the Herbarium Bogoriense, West Java, Indonesia.

Chemical and Buffer

The chemicals used in this study were angiotensin converting enzyme (ACE) and hippuryl-L-histidyl-L-leucine (HHL) substrate were puchased from Sigma Aldrich, USA. Captopril was obtained from Kimia Farma, Indonesia. Na_2CO_3, hydrochloric acid, potassium dihydrogen phosphate, aceton, ethyl acetate, n-hexane, methanol, chloroform, NaOH, silica gel TLC plates GF_{254}, preparative TLC plates were purchased from Merck, Jerman.

General

Micropippet 100-1000 µL (Eppendorf, Germany), mass spectra were obtained with a Water, UPLC-Qtof HR-MS XEV^{otm} mass spectrometer (Waters, Milford, MA, USA), The UV-Visible spectra were obtained on Shimadzu series 1800 spectrophotometer (Kyoto, Japan). The IR spectra were recorded on a Perkin-Elmer spectrum-100 FT-IR (Waltham, MA, USA) in KBr. ^{1}H- and ^{13}C-NMR spectra were obtained with a JEOL JNM A-500 spectrometer using TMS as internal standard (Tokyo, Japan). Chromatographic separations were carried out on silica gel 60 (Merck, Darmstadt, Germany). Preparative TLC glass plates were precoated with silica gel GF_{254} 2 mm (Merck, Germany).

Extraction and Isolation

The plants materials were dried at room temperature and ground to powders. The samples (4,0 kg) were defatted with n-hexane, and then macerated with methanol. The ornganic layer was concentrated under pressure using rotary vacuum evaporator, and dried using oven vaccum at 40^{o}C to give methanolic extract (574 g). The methanolic extract was partitioned succesively by with n-hexane, dichlormetane, ethyl acetate and methanol. The organic layer were evaporated using rotary vacuum evaporator to give hexane, dichlormetane, ethyl acetate and metnaol extracts of 54 g, 6.53 g, 6.74 g and 168 g, respectively. A portion of the EtOAc extract (6.74 g) was subjected to coloumn chromatography using gradient elution of n-hexane/EtOAc/MeOH to afford 225 fractions (F1-F225). Fraction F84 – F88 (920 mg) were combined and was subjected to silica gel column chromatography using gradient elution of n-hexane/EtOAc/MeOH as eluting solvents to afford 131 fractions (SF1 – SF131). Fraction SF33 – F78 (68 mg) were combined and was was preparative TLC on silica gel GF_{254}, eluted with EtOAc : MeOH (85 : 15) to give compound 1 (6.8 mg).

ACE Inhibitory Activity Assay

ACE inhibitor activity was determined according to Meyer, et.al. (2009) with some modification[19]. Briefly, 20 µL of the sample solution was added to 50 µL of 8 mM HHL as substrate and 10 µL of ACE solution (0.25 U/mL). The mixture were mixed well and incubated for 1 hour in 37^o C. The reaction was stopped by adding 62.5 µL HCl 1M. The hippuric acid formed was extracted with 375 µL of ethyl acetate. Finally, ethyl acetate layer was dried in vacuum oven and 4 mL of water was added. The absorbance of hippuric acid was measured by using UV-Visible spectrophotometer at 228 nm. Blanks were measured by replacing ACE with water while 100% activity value was determined by replacing sample with 20 µL of water.

The percentage inhibition of ACE activity was calculated as follows:

$$\text{ACE Inhibitory Activity (\%)} = \frac{(A - B) - (C - D)}{(A - B)} \times 100$$

where A represents absorbance in the presence of ACE, B absorbance of the reaction blank, C absorbance in the presence of ACE and inhibitor, and D absorbance of the sample blank. The IC_{50} value was defined as the concentration of sample in mg/mL required to reduce 50% of ACE activity, which was determined by regression analysis of ACE inhibition (%) versus sample concentration.

Determination of the Kinetic Properties

Kinetics test of ACE inhibition activity was measured by increasing the substrate concentration HHL (2, 4, 8, 16, 32 mM). Samples to be used as an ACE inhibitory activity is the most active compounds that have morethan 50% inhibitory activity. The kinetics parameters can be determined by analysis of the data using Lineweaver-Burk to get Michaelis-Menten kinetics constant which is calculated based on the regression equation: $V_i = (V_{maxA} \times S)/(K_{MA} + S)$ where V_i = initial rate; V_{maxA}= apparent maximum rate; K_{MA}= apparent Michaelis constant, and S= substrate concentration. K_{MA} and V_{maxA} were plotted vs. concentration of inhibitor (I). K_i values were calculated adjusting the curves to the equation: $K_{MA} = K_M (1+I/K_i)$

Results and Discussion

Structure elucidation of compound 1

Compound 1 was obtained as amorphous yellow crystal and soluble in methanol. UV-Vis spectrum showed a strong absorption peak at the initial peak of the wavelength (λ) 212 nm, the wavelength (λ) with an adjacent peak is 255 nm and 269 nm, and peaks with a weaker absorption at 343 nm λ. Peak at a wavelength of 255 nm and 343 nm is a typical characteristic of the spectrum of flavonoid compounds [20].

Data from the infrared spectrum is known for some of the functional groups to observe area the wavelength absorption (cm^{-1}) the emergence of the peak. In the wave absorption in 1436, 1496, and in 1571 there are peaks indicating the presence of C-C bonds in a ring, wavelength absorption in 1656 that there is a sharp peak indicates the group C = O. In the wavelength absorption of 2852-2924 there were peaks indicate the presence of CH (stretching), the wavelengths absorption of 1024 to 1261 there were peaks indicate the CO group, and the wavelengths absorption of 3427 and 3203 indicate a hydroxyl group (OH). LC-MS data indicate the molecular ion peak $[M + H]^+$ at 477.14 so that the compound has a molecular weight $[M]^+ = 476.14$

The NMR analysis results can be viewed as shown in Table 1. ^{1}H-NMR spectrum (500 MHz, using CD_3OD solvent) showed tree aromatic proton signals at 7.47 ppm (1H, *dd*, *J*=2.6 Hz & 8.15 Hz, H-2'), 6.96 ppm (1H, *d*, *J*=8.45 Hz, H-3'), 7.36 ppm (1H, *d*, *J*= 2,6 Hz, H-6') which indicates subsitusion C-3' and C-4' on B flavonol ring and two meta-coupling proton signal at 6.48 ppm, (1H, *s*, H-6) and 6.58 ppm (1H, *s*, H-8) on the ring A (the determination is supported by the HMQC spectrum of two-dimensional or (Heteronuclear Multiple Quantum Correlation). At the chemical shift of 3.89 ppm (3H, *s*) and 3.92 ppm (3H, *t*) is methoxy group (-OCH_3). Chemical shift at 3.48 ppm (2H, *t*, *J* = 4.5 Hz), 3,68 ppm (2H, *m*), 3.98 ppm (1H, *d*, *J* = 2,6 Hz), 4.17 ppm (1H, *t*, *J* = 9.7 Hz), dan 4.97 ppm (1H, *d*, *J* = 9.75 Hz) showed the presence of sugar moeity of mannose type. Whereas, wavelength absoption at 0.90 ppm (3H, *d*, *J*=7.5 Hz) showed that there methil group (-CH_3) that is bound to the carbonyl C glucose group. ^{13}C-NMR spectrum (500 MHz, using CD_3OD solvent) showed 23 signals consist of C quarternery = 9, C Tertiary (CH) = 11, and C secondary (CH_3) = 3. The chemical shifts at 170.4 (C-5), 157.5 (C-3 '), and 165.5 (C-4') indicates that the C at that position O. The types of carbon atoms can be determined also by DEPT spectrum analysis (Distortion Enhancement by Polarization Transfer), which indicates that at δ C 96.1, δ 103.9, 118.3 δ, δ 115.5, 75.1 δ, δ 72 , 9, δ 67.8, δ 80.5 and 72.5 δ is a C atom that binds one atom of hydrogen. While the C atom at δ 184.4, 175.3 δ, δ 170.4, 167.5 δ, δ 165.5, 157.5 δ, and δ 91.1 is a C quaternary atom.

Based on the UV, FT-IR, MS and NMR (1D and 2D) spectral data compound 1 was determined as 3',4', dihydroxy-3-5-dimethoxy flavone-7-*O*-β-rhamnose

Table 1. NMR data (500 MHz for ^{1}H and 125 MHz for ^{13}C, in $CDCl_3$), HMBC for compound 1

Isolate		
^{1}H-NMR	**^{13}C-NMR**	**HMBC**
-	165.5	
-	154.6	
-	184.4	
-	175.3	
6.48 (1H, *s*)	96.1	180.7, 175.3, 103.9
-	180.7	
6.58 (1H, *s*)	103.9	180.7, 96.1, 170.4
-	170.4	
-	115.5	
-	125.2	
7.47 (1H, *dd*, *J* = 2.6 Hz & 8.15 Hz)	118.4	157.5, 167.5, 115.4
-	157.5	
-	167.5	
6.96 (1H, *d*, *J* = 8.15 Hz)	112.1	125.2
7.36 (1H, *d*, *J* = 2.6 Hz	115.4	167.5
4.97 (1H, *d*, *J* = 9.75	75.1	72.9
4.17 (1H, *t*, *J* = 9.7)	72.9	80.5, 75.1
3.98 (1H, *d*, *J* = 2.6)	67.8	
3.48 (2H, *t*, *J* = 4.5 Hz)	80.5	72.9
3,68 (2H, *m*)	72.5	80.5
0.90 (3H, *d*, *J* = 7.15 Hz)	14.6	

This correlation can be drawn on the structure of compound in Fig 1

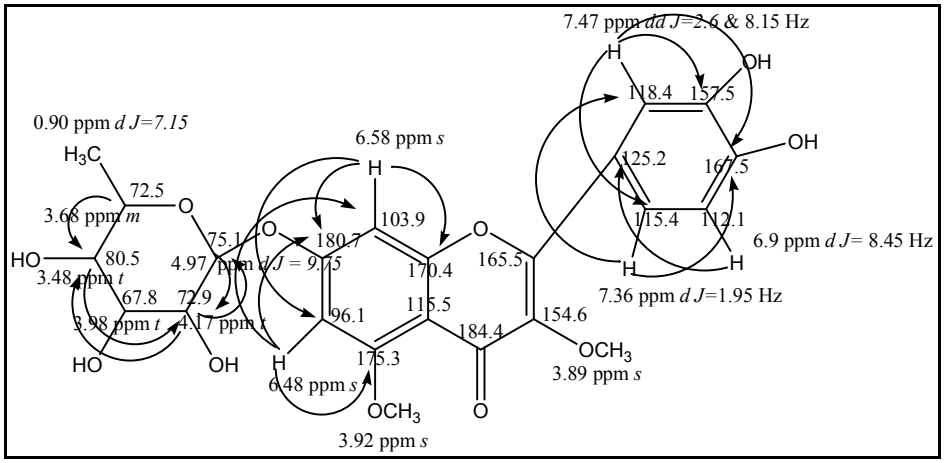

Figure 1. Selected HMBC and COSY correlations for compound 1

Activity on ACE Inhibition

The effect of ethyl acetate fraction and isolate 1 compound on ACE activity were evaluated. Effect of ACE inhibitory activity was performed by in vitro method using HHL as a substrate. ACE converts HHL into hipuric acid and histidil-leusin. The activity on ACE inhibition was evaluated based on the level of hipuric acid by measuring its absorbance using spectrophotometer. The activity was measured quantitatively in the presence or absence of the extract. Captopril was used as the positive control.

Flavonoid compounds from several plants has been widely reported have activity against ACE inhibition by in vitro assay to HHL substrate degradation [21-31].

The results demonstrated that captopril, ethyl acetate fraction, and isolate 1 showed IC_{50} values are 3.59 µg/mL, 3.44 µg/mL, and 7.72 µg/mL respectively. Compound 1 have half of the ability of captopril to ACE inhibitory activity. It means that potential to be developed as an antihypertensive drug from natural product. Compound 1 has a hydroxyl group able of binding to the enzyme's active site. Therefore, it is possible to inhibit of ACE activity.

Captopril is used as a standard of bioassay to inhibit ACE activity because it is a drug commonly used as first-line therapy for patients with hypertension and congestive heart failure. In addition, captopril has a high affinity to ACE which is a natural substrate that can inhibit the formation of angiotensin II and prevent the increase of blood pressure[32]

Table 2. Percentage inhibition and IC_{50} values of the ethyl acetate fraction, captopril, and compound 1

Sample	Concentration (µg/mL)	Persen Inhibition (%)	IC_{50} (µg/mL)
Ethyl acetate fraction	25.000	91.026	3.44
	12.500	73.077	
	6.250	58.974	
	3.125	55.128	
	1.563	35.897	
Kaptopril	25.000	90.08	3.59
	12.500	69.70	
	6.250	57.58	
	3.125	49.31	
	1.563	42.70	
Compound 1	25.000	70.25	7.72
	12.500	61.43	
	6.250	49.86	
	3.125	43.25	
	1.563	38.29	

Activity on Enzyme Kinetics

The results of enzyme kinetics activity showed that there was intersection at the *X* and *Y* axes coordinate. The intersection at the X axis can be interpreted that the kinetic test has conducted similar with competitive inhibition type, it means that compound 1 and HHL was competed to bind to the active site of the enzyme[31].

Type of competitive inhibition has been widely reported previously on the compounds of other plants to inhibit of ACE activity such as flavan-3-ol, prosianidin[28] and anthocyanin delfinidin and sianidin-3-*O*-sambubiosida from *Hibiscus sabdariffa*[31].

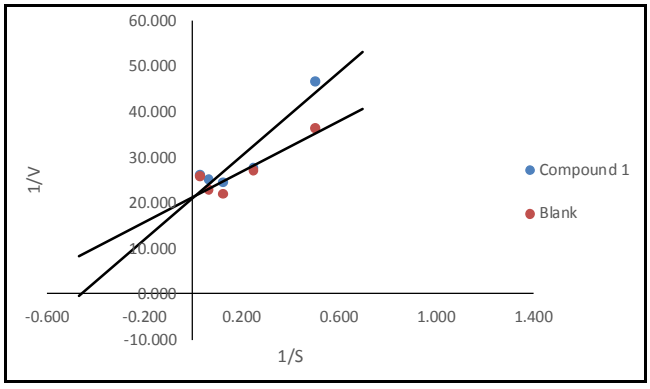

Figure 2. Lineweaver-Burk chart of kinetics tests using HHL substrate on ACE inhibitory activity by compound 1

Conclusion

The present investigation revealed that 3',4', dihydroxy-3-5-dimethoxy flavone-7-O-β-rhamnose was isolated from *Peperomia pellucida* (L.) Kunth. showed inhibition on ACE activity and potent as a folk medicinal of antihypertensive.

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