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With warm personal regards,
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A new angiotensin-converting enzyme inhibitor from *Peperomia pellucida* (L.) Kunth

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ABSTRACT

Objective: To isolate, identify, and evaluate a new angiotensin-converting enzyme (ACE) inhibitor from *Peperomia pellucida* (L) Kunth herbs.

Methods: A dried sample of *Peperomia pellucida*~~*P. pellucida*~~ herb was successively macerated with n-hexane and ethyl acetate. The ethyl acetate extract solution was evaporated to obtain the crude extract. ~~The crude extract was subjected to v~~Vacuum liquid column

chromatography and thin layer chromatography were performed to obtain two pure compounds. Then, both compounds were elucidated and identified using the spectroscopic method. ~~For ACE inhibitor assay,~~ Angiotensin-converting enzyme~~ACE~~ inhibitory activity studies of both compounds were ~~conducted~~ determined using angiotensin-converting enzyme~~ACE~~ kit WST-1 with spectrophotometer microplate reader 96-well at 450 nm wavelength.

Results: ~~According to the study results, t~~Two bioactive compounds were successfully isolated from *Peperomia pellucida*~~*P. pellucida*~~ herb, including a new compound of 2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene (**1**) and pellucidin A (**2**). Both compounds demonstrated angiotensin-converting enzyme~~ACE~~ inhibitory activity, with IC_{50} values of 72 µM (equivalent to 27.95 µg/mL) and 11 µM (equivalent to 4.4 µg/mL), respectively.

Conclusion~~s~~: In the present study, two active angiotensin-converting enzyme~~ACE~~ inhibitors compounds ~~was~~ are successfully isolated and purified from *Peperomia pellucida*~~*P. pellucida*~~ which is used as an antihypertensive in traditional medicine, and ~~warrant further study~~support its use as an angiotensin-converting enzyme~~ACE~~-inhibiting drug~~s~~.

Keywords:

2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene

~~;~~ Angiotensin-converting enzyme~~ACE~~ inhibitor

~~; p~~Pellucidin A

~~;~~ *Peperomia pellucida* (L) Kunth.

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1. Introduction

Angiotensin-converting enzyme (ACE) is an essential enzyme that has a role in the regulation of blood pressure, as well as fluid and electrolyte balance in the human body, as it

modulates the renin-angiotensin-aldosterone system ~~[1]~~[1,~~[~~2]. ACE (a Zn^{2+} -binding metalloenzyme) increases the blood pressure when it is converted from angiotensin ~~I~~ into the angiotensin ~~II~~, which acts a vasoconstrictor, thus contributing to hypertension~~–~~[3]. Hypertension is a disease with reasonably high prevalence worldwide, causing blood pressure disorders and heart failure~~–~~[4]. ACE is an ideal target for hypertension-controlling drugs ~~[5]~~[5,~~[~~6], and several ACE inhibitors are widely available for the treatment of hypertension, including zofenopril, fosinopril, enalapril, ramipril, lisinopril, and captopril. ~~All ACE inhibitors, however~~However, all ACE inhibitors produce unpleasant side effects including fatigue, dizziness, and headaches~~–~~[2].

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Natural products have been the primary subjects of recent drug discovery. These studies have examined natural active compounds in an effort to discover new ACE inhibitors that are economical, safe to use, and ~~produce~~ of minimal side effects~~–~~[5~~]~~ ~~[~~7]. Since the development of an *in vitro* ACE inhibitory activity assay by Cushman and Cheung in 1971 [2], drug discovery studies ~~examining~~ on ACE inhibitors from natural products have been more effective ~~[8]~~[8,~~[~~9].

Peperomia pellucida (L) Kunth herbs are one of the plant species that are traditionally used to lower blood pressure. *P. pellucida* herb extract has ACE inhibitor activity with an IC_{50} of 7.17 µg/mL~~–~~[10] and the fraction and isolates (quercetin) have activity (IC_{50}) of 3.44 and 7.22 µg/mL~~–~~[11], respectively~~, which is in agreement with the use of *P. pellucida* herb as a traditional herbal medicine~~. This herb contains secondary metabolites such as alkaloid, saponin, terpenoid, and polyphenol~~–~~[12]. Several polyphenolic compounds have been successfully isolated ~~among others;~~including dillapiole~~–~~[13], ~~Peperomins~~ peperomins[14], pellucidin A~~–~~[15], chromene~~–~~[16], and quercetin~~–~~[11]. ~~Until now, h~~However, until now, only quercetin has been successfully demonstrated to have ACE inhibitory activity~~–~~[11]. *P. pellucida* herb has enormous potential as a herbal medicine, but so far it has not been ~~able to~~

~~be~~ commercially used as herbal medicine and is still considered as a weed mainly ~~for~~ by farmers in oil palm plantations. Also, it has a poor yield value ~~from this herb~~ (mainly in the form of simplicial and extract). On the other hands, ~~to be recognized or used as herbal medicine or traditional medicine,~~ it still needs further scientific data to confirm its use as herbal medicine or traditional medicine.

The present study aimed to isolate and identify new bioactive compounds from *P. pellucida* as potential ACE inhibitors. We report the successful isolation of two compounds with ACE inhibitory activity: pellucidin A (which it was first identified ~~in~~ from *P. pellucida* herb extract by Bayma and his colleague ~~[~~15]) and a new compound of 2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene (~~as can be seen in~~ **Figure 1**). To our knowledge, the *in vitro* ACE inhibition activity of both compounds has not previously reported.

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2. Materials and methods

2.1. ~~General~~Reagents and apparatus

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The reagents ~~used in the present study,~~ including n-hexane, ethyl acetate, chloroform, and methanol~~,~~ were purchased from PT. SmartLab Indonesia (West Java, Indonesia). Silica gel 60H (Merck), silica gel $GF_{254+366}$ (Merck), silica gel GF_{254} analytical (Merck) and preparative thin-layer chromatography (TLC)~~TLC~~ plates were purchased from Sigma-Aldrich (*via* PT. Elo Karsa, Indonesia). Captopril was obtained from Kimia Farma, Indonesia. An ACE Kit-WST1 was purchased from Doijindo Laboratories, Japan. The apparatus ~~used in the present study~~ included 1-100 and 100-1000 µL micropipettes (Eppendorf, Germany), 96-well Microplate reader (VersaMax™ ELISA Microplate Reader, USA), Perkin-Elmer spectrum-100 FT-IR (Waltham, MA, USA), Shimadzu series 1800 spectrophotometer (Kyoto, Japan) UPLC-QToF-HR-MS XEV^{otm} mass spectrophotometer (Water, Milford, MA, USA), and an Agilent DD2 500 MHz NMR (^{1}H and ^{13}C; New Haven, USA).

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2.2. Plants ~~Materials~~ materials and ~~Sample~~ sample ~~Preparation~~ preparation

P. pellucida herb materials were collected (March to May 2016) from the oil palm plantation at North Mamuju in West Sulawesi, Indonesia. The sample was identified at the Herbarium Bogoriense, Bogor, West Java, Indonesia. The voucher specimen was prepared as a dried powder sample and stored at a cool temperature (0-5℃~~°C~~) until ~~ready to~~ use. The sample specimen was deposited at the Laboratory of Pharmacognosy–Phytochemistry, Faculty of Pharmacy, Universitas Indonesia, Depok, West Java, Indonesia.

2.3. Extraction, ~~Isolation~~isolation, and ~~Structure~~ structure ~~Elucidation~~elucidation

A dried sample of *P. pellucida* herbs (3 ~~K~~gkg) was successively macerated with n-hexane and ethyl acetate for 24 hours. The ethyl acetate extract solution was evaporated using a rotary vacuum evaporator to obtain the crude extract. The ethyl acetate extract (8 g) was subjected to vacuum liquid column chromatography ~~(VLCC:~~ (170 mm ~~x~~× 70 mm) using stationary phase of silica gel 60H (80 g) and 150 mL gradient elution of n-hexane: ethyl acetate (100:0, 80:20, 60:40, 40:60, 20:80, 0:100) and ethyl acetate: methanol (80:20, 60:40, 40:60, 20:80, 0:100), respectively to produce 11 fractions (A1-A11). The combined fraction of A2 and A3 (3.06 g) was subjected to vacuum liquid column chromatography~~VLCC~~ using different gradient elution of n-hexane: ethyl acetate (80:20, 70:30, 60:40, 40:60, 0:100) and ethyl acetate: methanol (50:50), respectively to ~~afford~~ obtain 6 sub-fractions (B1-B6), and then each fraction was tested for ACE inhibitor activity. The most active sub-fraction of B1 (1.28 g) was recrystallized using 50% chloroform (in methanol) to obtain~~give~~ crystal powder compounds. Based on the TLC profile, the crystal powder compound contains two spots and then was separated using preparative TLC with eluent n-hexane/ethyl acetate (2:1) to obtain both pure compounds ~~include~~ including compound 1 (11.33 mg) and compound 2 (6.24 mg), respectively. Structure elucidation was performed using spectroscopy method such as spectrophotometer UV-VIS, FT-IR, UPLC-QToF-MS/MS at Pusat Penelitian Kimia,

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Lembaga Ilmu Pengetahuan Indonesia ~~(LIPI)~~ Serpong, Tangerang, Banten, Indonesia, and NMR (^{1}H and ^{13}C) at Department of Chemistry, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung ~~(ITB)~~, Bandung, West Java, Indonesia and Chemistry Division University of Malaya.

2.4. *In ~~Vitro~~ vitro* A~~ngiotensin Converting Enzyme~~CE Inhibitory ~~Activity~~ activity ~~Assay~~assay

The ACE inhibitory activity assay of 4,6,7-trimethoxy-1-(2,4,5-trimethoxybenzyl)-1H-indene (**1**) and Pellucidin A (**2**) was performed using an ACE Kit-WST1 (Doijindo Laboratories, Japan) according to the manufacturer's instructions and some literature [17~~] [,~~19~~],[~~20]. The assay used 3-hydroxybutyryglycyl-glycyl-glycine ~~(3HB-GGG)~~ as the substrate, and the absorbance was measured at 450 nm using a VersaMax™ ELISA Microplate Reader. Captopril was used as a positive control.

3. Results

3.1. Identification of 2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene (1)

2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene: pale yellowish white amorphous powder, m.p. 153-155 ℃~~°C~~ (n-hexane/EtOAc); UV λ_{max} (log ε) 203.0 (3.211), 230.0 (0.915), and 292.0 (0.484) nm; IR ν^{film} cm^{-1} 2838, 2928, 2959, 2998, 1596, 1198, 1172, 1154, 1124, and 1106; ^{1}H-NMR and ^{13}C-NMR (~~shown in~~ Table 1); ESI-TOFMS [M + Na^{+}] *m/z* 409.16247 (calc. $C_{22}H_{26}O_6$ [M^{+}] *m/z* 386.1727). Based on the spectroscopic analyses, the structure of the compound isolated was determined~~,~~ as shown in Figure 2.

Table 1. Chemical shift data of proton (500 MHz, $CDCl_3$), carbon (125 MHz, $CDCl_3$), and HMBC of 2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene (**1**).

Position	^{13}C-NMR (δ_C, *J*)	^{1}H-NMR (δ_H, *J*)	HMBC
1	132.4	-	-

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2	140.4	-	-
3	152.5	-	-
4	96.1	6.44 (1H, s)	C-2, C-3, C-5, C-6
5	151.6	-	-
6	117.1		
7	121.2	6.81 (1H, d, *J*=6.60 Hz)	-
8	123.6	5.67 (1H, m, *J*= 5.85 Hz)	C-6, C-7
9	28.4	2.65 (1H, dd, *J*= 8.28 Hz) & 2.42 (1H, m, *J*= 1.35 Hz)	C-1, C-2, C-6, C-7, C-8
10	29.8	4.88 (1H, d, *J*= 8.1 Hz)	C-1, C-8, C-9, C-11
11	124.7	-	-
12	150.7	-	-
13	98.0	6.53 (1H, s)	C-11, C-12, C-14, C-15
14	142.5	-	-
15	148.0	-	-
16	114.4	6.30 (1H, s)	C-11, C-12, C-14, C-15
(-OCH_3)-2	60.5	3.39 (3H, s)	C-2
(-OCH_3)-3	56.0	3.86 (3H, s)	C-3
(-OCH_3)-5	56.4	3.86 (3H, s)	C-5
(-OCH_3)-12	56.2	3.83 (3H, s)	C-12
(-OCH_3)-14	56.9	3.57 (3H, s)	C-14
(-OCH_3)-15	56.8	3.90 (3H, s)	C-15

HMBC: heteronuclear multiple bond correlation.

3.2. Identification of pellucidin A (2)

Pellucidin A: $C_{22}H_{28}O_6$ with ESI-TOF-MS [M + Na] *m/z* 411.1786 (calcd. $[M]^+$ *m/z* 388.1888), showed FT-IR peaks at 1611, 1521, and 1489 cm^{-1}, revealing its aromatic ring; and at 2989 and 2936 cm^{-1}, showing the vibration of the C-H group. ^{1}H-NMR ($CDCl_3$, 500 MHz) and ^{13}C-NMR ($CDCl_3$, 500 MHz) ~~showed in~~ (**Table 2**). Based on the spectroscopic

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analyses, the structure of ~~Pellucidin~~ pellucidin A was determined~~,~~ as shown in **Figure 3** according to the literature~~-~~[15].

Table 2. Chemical shift of proton (500 MHz, $CDCl_3$), carbon (125 MHz, $CDCl_3$), and HMBC of pellucidin A (**2**).

Position	^{13}C-NMR (δ_C, *J*)	^{1}H-NMR (δ_H, *J*)	HMBC
1/1’	124.8	-	-
2/2’	147.8	-	-
3/3’	97.9	6.48 (1H, *s*)	C-1/1’, C-2/2’, C-4/4’, C-5/5’
4/4’	151.3	-	-
5/5’	143.3	-	-
6/6’	112.1	6.98 (1H, *s*)	C-1/1’, C-2/2’, C-4/4’, C-5/5’, C-7/7’
7/7’	40.6	3.86 (1H, *d*, *J*= 4,15 Hz)	C-1/1’, C-2/2’, C-6/6’, C-8/8’
8/8’	27.2	2.32 (1H, *d*, *J*=5.05 Hz) & 1.94 (1H, *m*, J=5.35 Hz)	C-7/7’
(-OCH_3)-2/2’	56.8	3.75 (3H, *s*)	C-2/2’
(-OCH_3)-5/5’	56.7	3.85 (3H, *s*)	C-5/5’
(-OCH_3)-4/4’	56.4	3.85 (3H, *s*)	C-4/4’

HMBC: heteronuclear multiple bond correlation.

3.3. ~~Angiotensin-converting enzyme (~~ACE~~)~~ inhibitory activity

The *in vitro* ACE-inhibitory activity assay was performed using an ACE analysis Kit-WST1 (Doijindo, Jepang). This assay was conducted on both isolated compounds using

captopril as a positive control (to compare the amount of 3HB formed by ACE activity) and a blank containing no ACE for method validation.

Table 3 shows that captopril ~~has~~ had an IC_{50} value of 1.08×10^{-7} µM, equivalent to 2.35 ×~~×~~ 10^{-11} µg/mL. This result was similar to the results reported by Ibadallah and his colleagues [20] using the 3HB method (with an IC_{50} value of 1.0 ×~~×~~ 10^{-11} µg/mL).[24] Both isolated compounds (pellucidin A and the novel polyphenol) from *P. pellucida* had IC_{50} values of 72 µM (equivalent to 27.95 µg/mL) and 11 µM (equivalent to 4.4 µg/mL), respectively.

Table 3. Results of ACE inhibitor assay.

Samples	Concentration (µM)	Percentage inhibitory (%)	Regression formulas (R^2)	IC_{50} (µM)
Captopril (positive control)	4.6×10^{-5}	88.73	Y=12.601X+183.94 (R^2 = 0.958 9)	1.08×10^{-7}
	2.3×10^{-5}	72.99		
	2.3×10^{-7}	52.50		
	4.6×10^{-8}	48.88		
	3.2×10^{-9}	27.78		
2.3.5-trimethoxy-9-(12.14.15-trimethoxybenzyl)-1H-indene	26	81.34	Y=81.233X–2.1908 (R^2 = 0.972 2)	11
	21	68.87		
	16	64.07		
	10.3	41.14		
	5.2	24.79		
Pellucidin A	260	74.55	Y=46.824X–17.724 (R^2 = 0.995 5)	72
	130	62.08		
	64	49.81		
	32	34.23		
	16	17.99		

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4. Discussion

This study ~~was~~ successfully ~~performed~~ isolat~~ion~~ed, ~~identification~~identified, and elucidated structure~~l~~y ~~elucidation~~ two active compounds as an ACE inhibitor from *P. pellucida*. Both compounds were separated and purified using column and preparative ~~thin layer chromatography~~TLC. Moreover, the determination of structure the compounds were conducted using the spectroscopic method. ~~For~~ ACE inhibitor activity~~,~~ was also analyzed using an ACE analysis Kit-WST1. Based on the best of our knowledge, one of them is a novel compound (compound **1**).

The molecular formula of novel compound **(1)**: $C_{22}H_{26}O_6$ ([M^+] *m/z* 386.1727), showed FT-IR peaks at 2838, 2928, 2959, and 2998 cm^{-1}, revealing its C-H group (in the ranges of 2900 – 3100 cm^{-1}); peaks at 1198, 1172, 1154, 1124, and 1106 cm^{-1} showed the C-O group vibration (1085–1150 cm^{-1}); and a peak at 1596 cm^{-1} revealing its C=O aromatic group. ^{1}H-NMR spectra ($CDCl_3$, 500 MHz), showed three singlet protons (1H each, s) at δ_H 6.53, 6.43, and 6.30 ppm (aromatic proton) and five singlet protons (3H, s) at δ_H 3.90, 3.86, 3.86, 3.83, 3.57, and 3.39 ppm (methoxy proton). A doublet proton at δ_H 4.88 (1H, d, J = 8.1 Hz), a doublet of doublet proton at 6.81 (1H, d, J = 6.60 Hz), two multiplet protons at δ_H 5.67 (1H, m), 2.65 (1H, m) and 2.42 (1H, m). ^{13}C-NMR spectra ($CDCl_3$, 125 MHz)~~, in Table 1~~ showed 22 carbon signals at δ_C 28.4, 29.8, 56.0, 56.2, 56.4, 56.8, 57.0, 60.5, 96.1, 98.1, 114.4, 117.1, 121.2, 123.6, 124.7, 132.4, 140.4, 142.5, 148.0, 150.7, 151.6, and 152.5 ppm. The distortionless enhangcement by polarization transfer~~DEPT~~ spectrum showed a methylene (δ_C 29.8) group, six methine (δ_C 28.4, 98.0, 96.1, 114.5, 121.2, and 123.6) groups, and six methoxyl (δ_C 56.0, 56.2, 56.4, 56.8, 57.0, and 60.5) groups. The homonuclear correlation spectroscopy~~HH-COSY~~ spectrum showed correlations of the proton 4.88 (1H, d, J = 8.1 Hz) with a methylene group (δ_C 29.8) and the proton 2.65 (1H, m) with C

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atom (δ_C 123.6) bond proton 5.67 (1H, m). The heteronuclear single quantum coherence~~HSQC~~ spectrum showed correlations between proton and carbon at 6.43 (1H, s) with C (δ_C 96.1), 6.30 (1H, s) with C (δ_C 114.5), 6.53 (1H, s) with C (δ_C 98.0), 6.81 (1H, dd, J = 3.20, 3.15 Hz) with C (δ_C 121.2), 5.67 (1H, m) with C (δ_C 123.6), 4.88 (1H, d, J = 8.1 Hz) with C (δ_C 28.4), and 2.65 (1H, m) and 2.42 (1H, m) with C (δ_C 29.8). The heteronuclear multiple bond correlation ~~HMBC~~ spectrum revealed the correlation of signals in the aromatic carbon region at 6.53 (1H, s) and 6.30 (1H, s) with each other (δ_C 124.7, 142.5, 148.0, and 150.7), and 6.43 (1H, s) with each C atom (δ_C 117.1, 140.4, 151.6, and 152.5). Furthermore, the heteronuclear multiple bond correlation~~HMBC~~ spectrum showed correlation of the signal proton at 5.67 (1H, m) with C atoms (δ_C 121.2 and 123.6), 4.88 (1H, d, J = 8.1 Hz) with C atoms (δ_C 117.1, 121.2, 123.6, 132.4, and 140.4), and 2.65 (1H, m) and 2.42 (1H, m) with C atoms (δ_C 28.4, 123.6, 124.7, and 132.4). Thus, the structure of **1** was elucidated as 2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene (a novel compound structure). Furthermore, compound **2** was also isolated and identified as pellucidin A by comparison of its spectroscopic data with this reported in the literature~~-~~[15].

Since Skeggs *et al.* succeeded in isolating ACE from horse plasma between 1954 and 1957, and in the development of the *in vitro* ACE inhibitor assay method~~-~~[2], the research and discovery of new ACE inhibitor drugs have become more productive. Medicinal plant biodiversity is a valuable resource for drug discovery, and medicinal plant-based products may be explored ~~for solutions~~ to combat hypertension. In the present study, we successfully isolated two compounds from the ACE-inhibitory fraction of *P. pellucida* herb extract, namely pellucidin A and a new compound. Pellucidin A is a dimeric ArC_2 that was initially reported by Bayma and his colleague~~—~~[15] and 2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene is a novel compound, the structure of which was established in the present study.

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Both compounds show inhibitory activity against ACE, with IC_{50} values of 72 µM (equivalent to 27.95 µg/mL) and 11 µM (equivalent to 4.4 µg/mL), respectively. According to the inhibitory activity of these compounds (less than 50 µg/mL), both have the potential ~~to be further developed into~~as pharmaceutical ACE inhibitors. However, ~~Both~~ two isolates~~, however,~~ show reduced activity compared to current ACE inhibitor drugs, although activity assays showed that 2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene had stronger ACE-inhibitory activity than quercetin compounds ~~obtained~~ in the previous study [11]. Some studies have reported that other compounds have activities similar to those ~~of both~~ compounds ~~obtained~~ in the present study, including compounds belonging to the phenolic group (gallic acid, vanillic acid, catechol pyrogallol), flavonoid group (quercetin, kaempferol, rutin, apigenin, epicatechin), and stilbene groups [21], and also flavonoid-rich extract from *Actinidia macrosperma* [22], and *Onopordon acanthium* [8].

Until present, three bioactive compounds as ACE inhibitors have been found from *P. pellucida* herbs, including quercetin [11], pellucidin A and a new compound of 2,3,5-trimethoxy-9- (12,14,15-trimethoxybenzyl) -1H-indene ~~which became a novelty~~ in this study. These compounds can be further developed ~~as antihypertensive or marker of this plant. In addition, it can be developed~~ into a pharmaceutical product as an antihypertensive herbal medicine with a green extraction approach as reported in a previous study [23].

5. Conclusion

In the present study, two active compounds showing ACE inhibitor activity were successfully extracted and purified from *P. pellucida*, include a new compound of (2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene) and pellucidin A. Both compounds are responsible for the ACE inhibitor activity of *P. pellucida*, which is used as an antihypertensive in traditional medicine~~, and warrant further study as ACE inhibiting drugs~~.

Both these compounds may be used as markers for the active ACE inhibitor extract/fraction of *P. pellucida*.

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Conflict of Interest

No potential conflict of interest was reported by the authors

Acknowledgments

All author is grateful to the Directorate of Research and Humanity Engagement Universitas Indonesia for funding this study *via* grant "Hibah TADOK 2018" Directorate of Research and Humanity Engagement Universitas Indonesia ~~(~~grant number: 1234/UN2.R3.1/HKP.05.00/2018~~)~~.

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Funding

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References

[1] Neves MF, Cunha AR, Cunha MR, Gismondi RA, Oigman W. The role of renin-angiotensin-aldosterone system and its new component in arterial stiffness and vascular aging. *High Blood Press Cardiovasc Prev*~~.~~ 2018; **25**(2): 137–145.

[2] Ahmad I, Yanuar A, Mulia K, ~~and~~ Mun'im A. Review of angiotensin~~-~~-converting enzyme inhibitory assay: Rapid method in drug discovery of herbal plants. *Pharmacogn Rev*~~.~~ 2017; **11**(21): 1–7.

[3] Muñoz-Durango N, Fuentes CA, Castillo AE, González-Gómez LM, Vecchiola A, Fardella CE, ~~Kalergis AM~~et al. Role of the renin-angiotensin-aldosterone system

beyond blood regulation; Molecular and cellular mechanism involved in end-organ damage during arterial hypertension. *Int J Mol Sci*. 2016; **17**(7): 797-814.

[4] Mendis S, Puska P, and Norrving B. *Global atlas on cardiovascular disease prevention and control*. Geneva: World Health Organization.; 2011.

[5] Andrade PB, Valentao P, Pereira DM. *Natural products targeting clinically relevant enzymes*. Germany: Wiley-VCH Verlag GmbH & Co. KGaA Boschstr.; 2017:, p. 45-58.

[6] Bai RR, Wu XM, Xu JY. Current natural products with antihypertensive activity. *Chin J Nat Med*. 2015; **13**(10): 721-729.

[7] Deo P, Hewawasam E, Karakoulakis A, Claudie DJ, Nelson R, Simpson BS, Smith NM, Semple SJet al. *In vitro* inhibitory activities of selected Australian medicinal plant extracts against protein glycation, angiotensin-converting enzyme (ACE) and digestive enzymes linked to type II diabetes. *BMC Complem Altern M*. 2016; **16**: 435-446.

[8] Sharifi N, Souri E, Ziai SASA, Amin G, Amini M, and Amanlou M. Isolation, identification and molecular docking studies of a new isolated compound, from *Onopordon acanthium*: A novel angiotensin converting enzyme (ACE) inhibitor. *J. Ethnopharmacol*. 2013; **148**(3): 934–939.

[9] Muhammad SA, and Fatima N. *In silico* analysis and molecular docking studies of potential angiotensin-converting enzyme inhibitor using quercetin glycosides. *Pharmacogn Mag*. 2015; **11**(Suppl 1): S123-126.

[10] Saputri FC, Mun'im A, Lukmanto D, Aisyah S, and Rinandy J. Inhibition of angiotensin-converting enzyme (ACE) activity by some Indonesia edible plants. *Int J Pharm Sci Res.* 2015; **6**(3): 1054–1059.

[11] Kurniawan A, Saputri FC, Rissyelly, Ahmad I, and Mun'im A. Isolation of angiotensin-converting enzyme (ACE) inhibitory activity quercetin from *Peperomia*

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pellucida. *Int J PharmTech Res*. 2016; **9**(7): 115–121.

[12] Verma RS, Padalia RC, Goswani P, and Chauhan A. Essential oil composition of *Peperomia pellucida* (L.) from India. *J Essent Oil Res*. 2014; **26**(1): 29–33.

[13] Rojas-Martínez R, Arrieta J, Cruz-Antonio L, Arrieta-Baez D, Velázquez-Méndez AM, and Sánchez-Mendoza ME. Dillapiole, isolated from *Peperomia pellucida*, shows gastroprotector activity against ethanol-induced gastric lesions in Wistar rats. *Molecules* 2013; **18**(9): 11327–11337.

[14] Xu S, Li N, Ning MM, Zhou CH, Yang QR, and Wang MW. Bioactive compounds from *Peperomia pellucida*. *J Nat Prod*. 2006; **69**(2): 247–250.

[15] Bayma JDC, Arruda MSP, Müller AH, Arruda AC, and Canto WC. A dimeric ArC_2 compound from *Peperomia pellucida*. *Phytochemistry* 2000; **55**(7): 779–782.

[16] Susilawati Y, Nugraha R, Muhtadi A, Soetardjo S, and Supratman U. (S)-2-Methyl-2-(4-methylpent-3-enyl)-6-(propane-2-ylidene)-3,4,6,7-tetrahydropyrano[4,3-g]chromen-9(2H)-one. *Molbank* 2015; **2015** (2): M855.

[17] Lam LH, Shimamura T, Manabe S, Ishiyama M, and Ukeda H. Assay of angiotensin I-converting enzyme-inhibiting activity based on the detection of 3-hydroxybutyrate with water-soluble tetrazolium salt. *Anal Sci*. 2008; **24**(8): 1057–1060.

[18] Lam LH, Shimamura T, Ishiyama M, and Ukeda H. Flow injection analysis of angiotensin I-converting enzyme inhibitory activity with enzymatic reactors. *Talanta* 2009; **79**(4): 1130–1134.

[19] Lam LH, Shimamura T, Sakaguchi K, Noguchi K, Ishiyama M, Fujimura Y, Ukeda H et al. Assay of angiotensin I-converting enzyme-inhibiting activity based on the detection of 3-hydroxybutyric acid. *Anal Biochem*. 2007; **364**(2): 104–111.

[20] Ibadallah BX, Abdullah N, and Shuib AS. Identification of angiotensin-converting enzyme inhibitory proteins from mycelium of *Pleurotus pulmonarius* (Oyster

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Mushroom). *Planta Med*. 2015; **81**(2): 123–129.

[21] Al Shukor N, Van Camp J, Gonzales GB, Staljanssens D, Struijs K, Zotti MJ, Raes K, Smagghe G et al. Angiotensin-converting enzyme inhibitory effects by plant phenolic compounds: A study of structure-activity relationships. *J Agric Food Chem*. 2013; **61**(48): 11832–11839.

[22] Hettihewa SK, Hemar Y, Rupasinghe HPV. Flavonoid-rich extract of *Actinidia macrosperma* (a wild Kiwifruit) inhibits angiotensin-converting enzyme *in vitro*. *Foods*. 2018; **7**(9): 146–154.

[23] Ahmad I, Yanuar A, Mulia K, Mun'im A. Optimization of ionic liquid-based microwave-assisted extraction of polyphenolic content from *Peperomia pellucida* (L) Kunth using response surface methodology. *Asian Pac J Trop Biomed*. 2017; **7**(7): 660-665.

Figure and Table Legend

Figure 1. A new compound of 2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene (**1**) and Pellucidin A (**2**) from *P. pellucida*

Figure 2. Two–dimensional NMR spectrum of new compound of 2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)- 1H-indene (**1**).

HSQC: heteronuclear single quantum coherence; HMBC: heteronuclear multiple bond correlation; **HH-COSY:** homonuclear correlation spectroscopy.

Figure 3. Two–dimensional NMR spectrum of pellucidin A (**2**).

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Table 1. Chemical shift data of proton (500 MHz, $CDCl_3$), carbon (125 MHz, $CDCl_3$), and HMBC of a new compound of 2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene (**1**) isolated from *P. pellucida*.

Table 2. ~~The c~~Chemical shift of proton (500 MHz, $CDCl_3$), carbon (125 MHz, $CDCl_3$), and HMBC of pellucidin A (**2**) isolated from *P. pellucida*

Table 3. Results of ACE inhibitor assay of captopril, and both isolated compounds