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Potential Use of Some Indonesian Plants to Inhibits Angiotensin-converting Enzyme *In Vitro*

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Abstract

BACKGROUND: Some Indonesian plants, such as *Vaccinium varingiaefolium* Miq., *Plectranthus scutellarioides* (L.) R.Br., *Syzygium myrtifolium* Walp., and *Eclipta prostrata* (L.) L., are rich of flavonoid and anthocyanin. Flavonoid, flavan-3-ol, quercetin, anthocyanin, and tannin compounds can reduce systemic vascular resistance because they cause vasodilation and are thought to be able to influence the function of angiotensin-converting enzyme (ACE) and inhibit ACE activity, which plays an important role in the process of hypertension.

AIM: This study aims to determine the potential of some Indonesian plants to inhibit ACE activity.

METHODS: Testing of ACE inhibitory activity is carried out by the hippuric acid compounds formed as a result of the reaction between the substrate and the enzyme, then measured spectrophotometrically. The inhibitory and IC_{50} values of each test sample were compared with the positive control of Captopril.

RESULTS: The four plant extracts contained secondary metabolites, such as flavonoids, tannins, saponins, quinones, steroids, triterpenoids, and essential oils. Ethanol extract of *V. varingiaefolium* Miq., *P. scutellarioides* (L.) R.Br., *S. myrtifolium* Walp., and *E. prostrata* (L.) L. each had an IC_{50} value of ACE inhibition activity of 131.4 ppm, 206. 7 ppm, 151.2 ppm, and 196.0 ppm. The IC_{50} value of the Captopril with inhibition of ACE activity is 11.1 ppm.

CONCLUSION: This study shows that some Indonesian plants have the activity to inhibit the ACE and potential antihypertensive drug candidates with ACE inhibitory activity.

Introduction

Hypertension is a risk factor that may promote cardiovascular and kidney diseases. This pathological condition is asymptomatic or symptomatic, it can occur for years and is often associated with a heart attack or stroke, so it is often referred to as a “silent killer” [1], [2]. Data showed that there were 26.4% of the world’s population suffered from hypertension in 2000 and it was predicted that this number would increase to 60% by 2025. Because hypertension sufferers will increase rapidly, new therapeutic approaches for managing hypertension are very important to develop [3], [4]. The renin-angiotensin-aldosterone (RAA) system plays a key role in the mechanism of regulating blood pressure. An enzyme that has an important role in the RAA system is angiotensin-converting enzyme (ACE). ACE is a zinc metallopeptidase that converts angiotensin I (inactive decapeptide) to angiotensin II (strong vasoconstrictor), and bradykinin (hypotensive peptide) into an inactive component [3], [5]. ACE is a peptidyl dipeptide hydrolase enzyme that contains zinc and has an active side consisting of three parts, namely, a carboxylic binding section such as the guanidinium arginine group, a bag that holds the lipophilic side chains of amino acid residues in terminal C and Zn ions [6], [7]. ACE inhibitors such as Captopril and Lisinopril play key roles in treating hypertension and maintaining the electrolyte balance. They are commonly used as they are safe and well tolerated with few side effects [3]. Natural products could be important sources of ACE inhibitors such as Captopril. Active substances derived from plants can also be a source of new ACE inhibitors.

Nowadays, in addition to synthetic drugs, plants have also been widely studied as having compounds that are thought to affect ACE activity such as flavonoids and tannins [3], [8], [9], [10], [11], [12], [13]. Many researchers reported that flavonoid compounds that can inhibit ACE activity are quercetin, flavan-3-ol, and anthocyanin [9], [14]. *Vaccinium varingiaefolium* Miq., *Plectranthus scutellarioides* (L). R.Br., *Syzygium myrtifolium* Walp., and *Eclipta prostrata* (L.) L. are plants that grow a lot scattered in Indonesia and have been studied to have a variety of benefits, especially in the field of health and treatment. The four plants contain flavonoid compounds that are thought to have antihypertensive activity through inhibition of ACE, *V. varingiaefolium* Miq. and *S. myrtifolium* Walp. contain anthocyanin flavonoids which are effective in inhibiting ACE activity [14], [15], [16]. Besides *P. scutellarioides* (L). R.Br. and *E. prostrata* (L.) L. plants, besides having flavonoid content, they have also been used by the local community as an empirical treatment

for hypertension [17], [18]. Therefore, this study was conducted to assess the ability of the four plants and their potential in inhibiting ACE activity and discover a possible new ACE inhibition activity using an *in vitro* ACE inhibition assay.

Materials and Methods

V. varingiaefolium Miq., *P. scutellarioides* (L.) R.Br., *S. myrtifolium* Walp., and *E. prostrata* (L.) L. leaves were obtained from the Research Institute for Spice and Medicinal Crops (BALITTRO) in Bogor, Indonesia, and determined in the Research Center for Biology, Indonesian Institute of Sciences (LIPI), Cibinong, Indonesia; Captopril (Dexa Medika, Indonesia); ACE, Hippuryl-L-Histidyl-L-Leusin (HHL), 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid (HEPES) buffer, hippuric acid (HA) purchased from Sigma-Aldrich, St. Louis, Missouri, United States; NaCl 0,9% (Otsuka), amyl alcohol, chloroform, ammonia, anhydrous acetic acid, sulfuric acid, NaOH, ferric (III) chloride, gelatine, Steasny reagent, Sodium acetate, Dragendroff reagent, Mayer reagent, and petroleum ether were purchased from Merck Co. and aquadest.

Samples extraction

Each ±300 g of Simplicia powder from the sample was macerated using 70% (v/v) ethanol as a solvent. Samples were macerated for the first 6 h and then allowed to stand for the next 18 h. Remacerated ±10 times. Then, the filtrate obtained was concentrated using a rotary evaporator until a thick extract was obtained.

Phytochemical screening

Phytochemical screening was performed on ethanol extracts of *V. varingiaefolium* Miq. leaves, *P. scutellarioides* (L.) R.Br. leaves, *S. myrtifolium* Walp. leaves, and *E. prostrata* (L.) L. leaves. Phytochemical screening was carried out to determine the content of secondary metabolites of the four plant extracts. This screening includes a qualitative examination of alkaloids, flavonoids, quinones, saponins, tannins, steroids/triterpenoids, essential oils, and coumarin [19].

Determination of total flavonoid content

The determination of flavonoids was carried out using the aluminum chloride method. This method uses quercetin as the standard and the flavonoid content is measured to be equivalent to quercetin. A number of extracts containing 200 mg of powdered simplicia were placed in an erlenmeyer flask, along with 1 mL of HMT solution, 20 mL of acetone P, and 2 mL of 25% HCl solution, and then refluxed for 30 min is filtered using cotton, and the filtrate is put into a 100 mL flask. The residue is refluxed with 20 mL acetone for 30 min, filtered, and mixed with the filtrate obtained into a 100 mL flask. Added acetone to the mark. Pipetted 20 mL into a separating funnel, added 20 mL of water and extracted 3 times using ethyl acetate entered the ethyl acetate phase into a flask of 50 mL, and added ethyl acetate to the mark. Prepare 10 mL of each extract test solution and 10 mg of quercetin raw solution, put them into a 25 mL flask, add 5% v/v glacial acetic acid solution to the methanol P until the mark and do the same for the test solution and standard solution with the addition of $AlCl_3$ [19].

ACE inhibitory activity test

The Cushman and Cheung methods were used to test ACE inhibitor activity. The assay method is based on the hydrolysis of the substrate HHL by ACE, and measuring the amount of HA using a spectrophotometer. The sample solution at concentration of 40 ppm to 120 ppm (50 μL) and 50 μL substrate (5 mM HHL in a 100 mM phosphate buffer containing 300 mM NaCl at pH 8.3) was incubated at 37°C for 15 min, then a 50 μL solution was added. ACE (4 mU/mL) then incubated for 30 min at the same temperature. The reaction was stopped by the addition of 200 μL HCl 1 M. The resulting HA was extracted with 1.5 mL ethyl acetate, and centrifuged (4000 rpm) for 15 min. 1 mL of the supernatant is transferred to another test tube and evaporated until it becomes a residue. Then, the residue is dissolved in 3.0 mL of water. Absorbance was measured at 228 nm using a UV-Vis spectrophotometer (UV-Mini 1240 Shimadzu). The uptake of each extract is then used to calculate ACE inhibitory activity which is expressed as the percentage of ACE inhibition. ACE inhibition (%) was calculated by the equation:

$$\text{ACE Inhibition (\%)} = [(A_{blank} - A_{inhibitor})/(A_{blank} - A_{control})] \times 100\%$$

The IC_{50} was defined as the concentration of extract in ppm required 50% of ACE activity, which was determined by regression analysis of ACE inhibition (%) versus extract concentration [1], [10], [20].

Results

V. varingiaefolium Miq., *P. scutellarioides* (L.) R.Br., *S. myrtifolium* Walp., and *E. prostrata* (L.) L have been determined at the Research Center for Biology, Indonesian Institute of Sciences (LIPI), Cibinong, Indonesia. In this study, extraction was done

by maceration using 70% ethanol. The yield of extracts from the four test samples is shown in Table 1.

Table 1: The yield of crude extract

Sample	Part of plant	The yield of extract (%)
Vaccinium varingiaefolium Miq.	Leaves	17.50
Plectranthus scutellarioides (L.) R.Br.	Leaves	16.30
Syzygium myrtifolium Walp.	Leaves	30.80
Eclipta prostrata (L.) L.	Leaves	45.50

Phytochemical screening extract

The results of phytochemical screening showed the presence of several secondary metabolites in the extracts such as flavonoids, saponins, steroids, triterpenoids, tannins, and essential oils. Followings are the results of phytochemical screening conducted on an ethanol extract of *V. varingiaefolium* Miq. leaves, *P. scutellarioides* (L.) R.Br. leaves, *S. myrtifolium* Walp. leaves, and *E. prostrata* (L.) L. leaves in Table 2.

Table 2: Phytochemical screening of the active extract

Phytochemical constituents	Leaf of Plant			
	Vaccinium varingiaefolium Miq	*Plectranthus scutellarioides* (L.) R.Br.)	*Syzygium myrtifolium* Walp.	*Eclipta prostrata* (L.) L.
Alkaloids	-	-	-	-
Flavonoids	+	+	+	+
Saponins	+	+	+	+
Tannins galat	+	-	+	+
Tannins catechins	+	-	+	-
Steroids	-	+	-	+
Triterpenoids	+	+	+	+
Essential oils	+	-	+	-
Coumarin	-	-	-	-

Total of flavonoid content

Total of flavonoid content from ethanol extract of *V. varingiaefolium* Miq. leaves, *P. scutellarioides* (L.) R.Br. leaves, *S. myrtifolium* Walp. leaves, and *E. prostrata* (L.) L. leaves is shown in Table 3.

Table 3: Determination of total flavonoid levels

Sample	Flavonoid level (%)
Vaccinium varingiaefolium Miq	2.84
Plectranthus scutellarioides (L.) R.Br.	2.51
Syzygium myrtifolium Walp.	2.29
Eclipta prostrata (L.) L.	2.74

ACE inhibitory activity

The activities of four ethanol extract from Indonesia edible plants on the inhibition of ACE were evaluated. ACE inhibitor activity of ethanol extract of *V. varingiaefolium* Miq. leaves, *P. scutellarioides* (L.) R.Br. leaves, *S. myrtifolium* Walp. leaves, and *E. prostrata* (L.) L. leaves are shown in Table 4, and Figure 1 shows the comparison of IC_{50} values of extracts.

Table 4: Angiotensin-converting Enzyme (ACE) inhibition activity of the four studied plants

Sample	Percentage Inhibition of ACE by sample (%)				
	40 ppm	60 ppm	80 ppm	100 ppm	120 ppm
Vaccinium varingiaefolium Miq.	30.51	34.34	39.49	44.43	49.69
Plectranthus scutellarioides (L.) R.Br.	19.17	22.01	25.91	30.10	34.17
Syzygium myrtifolium Walp.	23.34	26.82	32.81	38.89	42.59
Eclipta prostrata (L.) L.	23.43	27.30	30.70	34.12	37.25

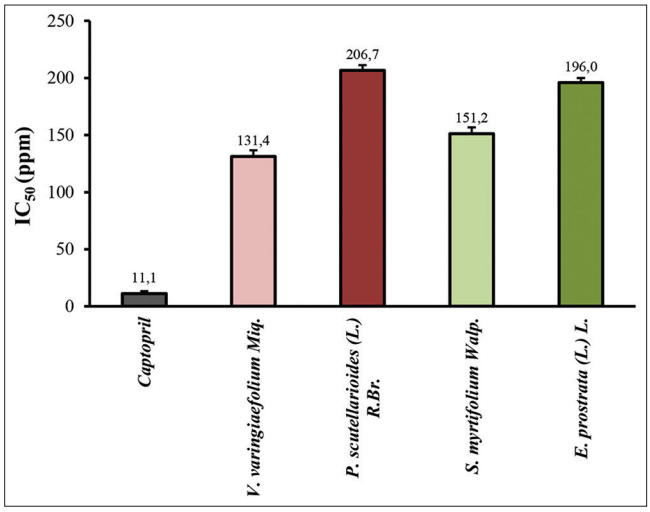

Figure 1: IC_{50} (ppm) the Captopril and four studied plants (Vaccinium varingiaefolium Miq., Plectranthus scutellarioides (L.) R.Br., Syzygium myrtifolium Walp. and Eclipta prostrata (L.) L. leaves extract (mean±SD)

Discussion

One of the effective treatments for the hypertension is ACE inhibitors. Therefore, Indonesia plants can be important resources to develop new natural drug candidates. In this study, the extraction of four selected plants was carried out by maceration using 70% (v/v) ethanol. This maceration method is suitable to attract compounds with unknown properties, because it can maintain compounds in samples that are not heat-resistant as to prevent compound damage.

Based on the present research results, the four studied plants extracts contain flavonoid compounds, saponins, tannins, and triterpenoids. Ethanol extract of *V. varingiaefolium* Miq. leaves and *S. myrtifolium* Walp. leaves has essential oils not found in the ethanol extract of *E. prostrata* (L.) L. leaves and ethanol extract of *P. scutellarioides* (L.) R.Br. leaves. These compounds are thought to have an important role in providing pharmacological activities such as anti-oxidant, anti-aging, antimicrobial, anti-inflammation, hepatoprotective, and hypolipidemic effects. The compounds that have been studied that can inhibit the activity of ACE are flavonoids and tannins and these compounds were found in all four studied extracts. Most of the recent studies revealed that the inhibitory activity of herbal extracts on ACE is due to the flavonoid content in plants. [8], [14], [21], [22], [23].

The total flavonoid content of four studied plants extracted in this research was determined (Table 3). Most of these four extracts had a large flavonoid total. Therefore, they can be important resources to develop new natural drug candidates. Flavonoids, flavanols, flavonols, anthocyanins, isoflavones, flavons, and other phenolic compounds have proved to be effective in inhibiting ACE activity [3]. Therefore, the secondary metabolites in these four studied plants'

extracts could be responsible for ACE inhibition activity. *V. varingiaefolium* Miq. leaves extract had the highest ACE inhibition activity (IC_{50} of 131.4 ppm). It is clear that the highest flavonoid content in *V. varingiaefolium* Miq. leaves extract interfered in enzymatic activity.

In this study, the samples used were several Indonesian plants that were empirically known to have antihypertensive activities, such as E. prostrata (L.) L. and *P. scutellarioides* (L.) R.Br. [24], [25], [26]. Furthermore, *V. varingiaefolium* Miq. and *S. myrtifolium* Walp. were evaluated because they contain anthocyanin, a flavonoid chemical compound thought to effectively inhibit the activity of ACE [14], [15], [16], [27], [28]. In this research, Captopril is used as a positive control that has ACE inhibitory activity. Captopril acts as a powerful and specific inhibitor of ACE, which will compete with angiotensin I, as a natural substrate, to prevent conversion to angiotensin II. In this research, an angiotensin I substitute HHL substrate was used. HHL reacted with ACE will form Histidyl-L-Leusin and HA, where the formed HA will be measured to determine the inhibitory activity of ACE. This test was carried out under optimum conditions, at 37°C incubation temperature, pH 8.3 and 30 min incubation time. The test results obtained are absorbance data. The lower the absorbance value produced, the greater the inhibitory power of ACE activity. The absorbance measures the residual HA that comes from the reaction between the substrate and ACE which is not inhibited by plant extracts [10], [29].

Captopril and other test materials which have inhibitory activity against, when reacted with ACE and the substrate will form hypuric acid which is the result of the breakdown of HHL substrate that reacts with the active site of the enzyme. Tests were carried out on all four samples with the same concentration of 40 ppm, 60 ppm, 80 ppm, 100 ppm, and 120 ppm to identify the effect of adding a concentration to the inhibition of ACE activity. Captopril as a synthetic drug commonly used for hypertension patients. It has the best IC_{50} value which shows that Captopril has a strong potential in inhibiting the activity of ACE. IC_{50} values of the four plant extracts were greater than those of Captopril. According to Balasuriya *et al*., compounds act as inhibitors of ACE in plants, one of which is a flavonoid compound. Flavonoids such as flavan-3- ol and anthocyanin have been studied *in vitro* and the results are relevant to other studies using experimental animals [6], [14], [30].

The results of this study indicated that all four extracts have inhibitory activity against ACE but require greater concentrations when compared to Captopril. Research by Balasuriya *et al*. showed that a quercetin class of flavonoid compounds found in apple peel extract has an IC_{50} value of 49 ppm and is the most effective ACE inhibitor compared with other plant extracts [31]. Research by Sakaida *et al*. showed that the ethanol extract of blueberry leaves had an IC_{50} value of 46 ppm [29], [32]. Another study conducted by Rinayanti *et al.* showed that the *Hibiscus rosasinensis* leaves methanol extract had an IC_{50} value of 271 ppm [33]. Therefore, ethanol extract of *V. varingiaefolium* Miq. leaves and ethanol extract of *S. myrtifolium* Walp, which had IC_{50} values of 131.4 ppm and 151.2 ppm, were recognized to be more effective than *H. rosasinensis* leaves methanol extract but were less effective when compared with ethanol extracts of blueberry leaves and ethanol extract of apple peel. Meanwhile, ethanol extract of *E. prostrata* (L.) L leaves and ethanol extract of *P. scutellarioides* (L.) R.Br. leaves which have IC_{50} values 196.0 ppm and 206.7 ppm are said to be less effective in inhibiting ACE when compared with ethanol extracts of *V. varingiaefolium* Miq. and *S. myrtifolium* Walp leaves. The ethanol extract of *V. varingiaefolium* Miq. leaves have the strongest potential in inhibiting ACE activity compared to the other three extracts because it has the smallest IC_{50} value. Therefore, further research is needed to determine the content of active compounds from *V. varingiaefolium* Miq. leaves which are thought to inhibit ACE activity. IC_{50} values of plant extracts that are thought to originate from flavonoid compounds are greater than the antihypertensive drug that is often prescribed namely Captopril, so flavonoids or plant extracts can only be used as “preventative nutraceutical” for hypertensive patients [6]. The active site of ACE is known to consist of three moieties. The first moiety is a carboxylate binding functionality such as the guanidinium group of arginine. The second is a pocket that accommodates a hydrophobic side chain of C-terminal amino acid residues and the third is a zinc ion. The zinc ion coordinates to the carbonyl of the penultimate peptide bond of the substrate, whereby the carbonyl group becomes polarized and is subjected to a nucleophilic attack. Flavonoids were suggested to show *in vitro* activity through the generation of chelate complexes within the active center of ACE [14]. Free hydroxyl groups of phenolic compounds are also suggested to be important structural moieties to chelate the zinc ions. This chelating process will inactivate the ACE [1]. Furthermore, *V. varingiaefolium* Miq. leaves that have high total flavonoids and ACE inhibitors activity are valuable plants for the *in silico* mechanism of chelate complexes within the active center of ACE and isolation of active compounds in further studies.

Conclusion

Ethanol extract of *V. varingiaefolium* Miq. leaves, *P. scutellarioides* (L.) R.Br., *S. myrtifolium* Walp., and *E. prostrata* (L.) L has inhibitory activity against ACE. The ethanol extract of *V. varingiaefolium* Miq. leaves has the strongest potential compared to the other three plants.

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