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ORIGINAL ARTICLE

Angiotensin-Converting Enzyme Inhibitory Activity of *Senna garrettiana* Active Compounds: Potential Markers for Standardized Herbal Medicines

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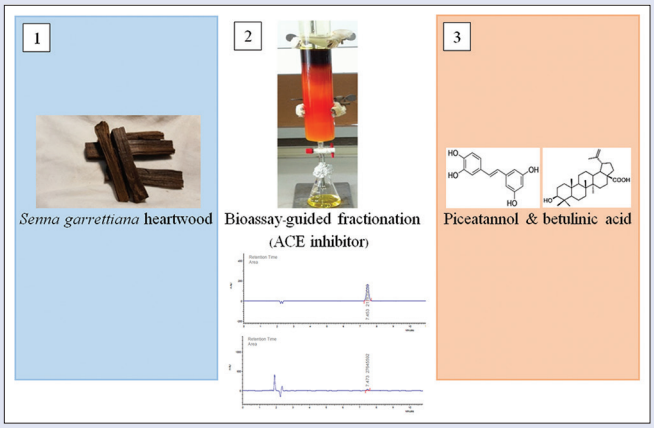
ABSTRACT

Background: Fifteen medicinal plants used in traditional Thai herbal medicine (HM) for hypertension treatment were previously tested for their inhibitory activity against an angiotensin-converting enzyme (ACE). The ethyl acetate extract from one of these medicinal plants, *Senna garrettiana,* possessed satisfactory ACE inhibitory (ACEi) activity. **Objective:** For this study, *S. garrettiana* extract was further subjected to an isolation process to uncover the active compounds with ACEi activity. **Materials and Methods:** The ethyl acetate fraction was subjected to isolate and purify by column chromatography accompany with the ACEi assay. The structures of all compounds were elucidated using nuclear magnetic resonance spectroscopy. **Results:** It was found that *S. garrettiana* extract possessed two active compounds, piceatannol and betulinic acid, which both had good ACEi activity displaying IC_{50} values of 8.44 μM and 26.77 μM, respectively. **Conclusions:** These findings show that both compounds can be used as markers for quality control of any standardized HM for hypertension treatment and not just for traditional Thai herbal remedies. Moreover, to the best of our knowledge, this is the first report investigating both compounds for their ACEi activity.

Keywords: Angiotensin-converting enzyme inhibitory activity, betulinic acid, hypertension, piceatannol, *Senna garrettiana*

SUMMARY

- In Thailand, there is one of the renowned traditional Thai herbal medicines (HMs) for hypertension treatment, which contains *Senna garrettiana* as its major constituents. According to the World Health Organization, since the analysis of marker compounds is required for evaluating the quality of standardized HMs, regrettably, there is no report of marker compounds from *S. garrettiana* for antihypertensive activity. Thus, the main purpose of this article is to find potential marker compounds from *S. garrettiana* for a standardized HM for hypertension treatment using an *in vitro* ACE inhibitor model. Bioassay-guided fractionation of *S. garrettiana* fractions led to isolation of piceatannol and betulinic acid which possessed satisfactory ACE inhibitory activity. These compounds can be used as markers for a standardized HM aimed at controlling blood pressure. Moreover, both compounds can be used as candidates for finding new ACE inhibitors with fewer undesirable side effects.



Abbreviations used: ACE: Angiotensin-converting enzyme; ACEi: Angiotensin-converting enzyme inhibitory; CH_2Cl_2: Dichloromethane; DMSO: Dimethyl sulfoxide; HA: Hippuric acid; HCl: Hydrochloric acid; HHL: Hippuryl-L-histidyl-L-leucine; HM: Herbal medicine; HMs: Herbal medicines; HPLC: High-performance liquid chromatography; IC_{50}: The half maximal inhibitory concentration; L-NAME: N (ω)-nitro-L-arginine methyl ester; MeOH: Methanol; NaCl: Sodium chloride; NADPH: Nicotinamide adenine dinucleotide phosphate; NMR: Nuclear magnetic resonance spectroscopy; NO: Nitric oxide; TDR: The Special Programme for Research and Training in Tropical Disease; μL: Microliter; μM: Micromolar.

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INTRODUCTION

Although herbal medicines (HMs) have been used for hundreds of years around the world, their practice has not been officially recognized in many countries. This is because the safety, efficacy, and quality control data of HMs are insufficient to meet the standards needed to support their use globally. The main characteristic of a HM is that it, either presenting as single herb or as mixture of herbs, contains many chemical constituents. This may be the reason why quality control of HMs is more problematic than that of conventional medicine. Therefore, a standardized HM is needed.[1,2] In general, according to the Special Programme for Research and Training in Tropical Disease, analysis of marker compounds coupling with chemical fingerprint is required for evaluating the quality of standardized HMs.[3] For marker compounds,

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it is indicated that using related pharmacologically active compounds as markers is more favorable than the other compounds with unknown pharmacological activity. For example, one standardized HM which shows promising results is *Ginkgo biloba* leaf extract: its markers, flavone glycosides, have neuromodulatory effects relevant to Alzheimer's disease treatment.[4]

A recent survey has revealed a high prevalence of hypertension in Thai people.[5] Since current health-care service is inadequate, the use of HMs has attained a reputable status in rural areas of Thailand. One of the renowned traditional Thai HMs, recorded in the official classic medical book by the former Department of Health Service Support, is recommended for hypertension treatment. This HM is comprised several medicinal plants, including *Senna garrettiana* as one of its main constituents.[6] The heartwood of *S. garrettiana* has also been reported as a traditional medicine for diseases involving blood circulation.[7] In addition, among the screening of 15 medicinal plants, the extract of *S. garrettiana* was reported to have high angiotensin-converting enzyme inhibitory activity (ACEi).[8] This evidence guided us to observe the possibility of investigating the existing marker compounds in this plant which could possess antihypertensive activity.

To find the related pharmacological marker compounds, appropriate experiment models for lowering blood pressure were reviewed. According to an Act for the Prevention of Cruelty to Animals, which was formed in the UK, the minimal use of animals in experiments must be considered.[9] While the diuretic, calcium channel, beta-adrenergic, and alpha-adrenergic blocker models all require the use of animals, an ACE inhibitor provides an *in vitro* assay.[10,11] Moreover, the *in vitro* ACEi model advantages include lower costs and less time consumption, compared to those for animal experiments. In this study, the main purpose is to find potential marker compounds from *S. garrettiana* for a standardized HM for hypertension treatment using an *in vitro* ACEi model. Moreover, these marker compounds could be considered for developing an ACE inhibitor from a natural source.

MATERIALS AND METHODS

Plant material and chemicals

S. garrettiana heartwood was purchased from Charoensuk herbal drugstore and authenticated by the Department of Pharmacognosy at the Faculty of Pharmacy, Rangsit University (voucher specimen # 10101). Contaminants were removed, and the heartwood was ground using a blender. The heartwood powder was stored at room temperature and protected from light. Hippuric acid (HA), ACE from rabbit lung, hippuryl-L-histidyl-L-leucine (HHL), betulinic acid, chrysophanol, and piceatannol were purchased from Sigma-Aldrich (USA). High-performance liquid chromatography (HPLC) grade acetonitrile, analytical grade acetic acid, dichloromethane (CH_2Cl_2), ethyl acetate, ethanol, hexane, and methanol (MeOH) were all purchased from B and J (Korea). Analytical grade formic acid and dimethyl sulfoxide (DMSO) were purchased from Merck (Germany). Distilled water was purchased from Puris, Expe-CB Ele10 Water System (South Korea).

Preparation of plant extract

The air-dried heartwood of *S. garrettiana* powder (1.58 kg) was extracted with ethyl acetate (11 L four times) by sonication for 1 h, and the residue was dissolved in 2 L of 95% ethanol. Each fraction was evaporated to dryness to give 35.8 g of ethyl acetate and 56.8 g of 95% ethanolic fractions.

Isolation of compounds from the extract

The ethyl acetate fraction (30.0 g) which possessed high ACEi was chromatographed on silica gel (800 g), using hexane/ethyl acetate (90:10 to ethyl acetate 100%) and ethyl acetate/methanol (100:0 to methanol 20%), to afford six fractions (F1-F6). Fraction F4 (8.5 g) was subjected to column chromatography on 150 g of silica gel eluted with CH_2Cl_2/MeOH (90:10 to methanol 40%) to give six subfractions (F4.1–F4.6). Subfraction F4.4 (100.0 mg) was purified by column chromatography (Sephadex LH-20) using 100% MeOH to give chrysophanol (1) (golden yellow plates, 33 mg). The purity of 1 was detected by spraying with potassium hydroxide solution, followed by heating at 120°C. Subfraction F4.5 (2.5 g) was subjected to column chromatography on 100 g of silica gel eluted with hexane/ethyl acetate (70:30), which finally afforded betulinic acid (2) (white crystals, 20 mg). The purity of 2 was detected by spraying with anisaldehyde/vanillin followed by heating at 120°C. Subfraction F4.6 (2.5 g) was purified by column chromatography on 100 g of silica gel using hexane/CH_2Cl_2 (50:50 to CH_2Cl_2 100%) and CH_2Cl_2/MeOH (100:0 to MeOH 40%) to give seven subfractions (F1b-F8b). Further column chromatography of the subfraction F4b was on 50 g of Sephadex LH-20 using MeOH to obtain piceatannol (3) (white powder, 20 mg).

Identification of compounds

The structures of compounds 1–3 were elucidated using spectroscopic techniques and compared with reported spectral data. The nuclear magnetic resonance (NMR) spectroscopy (^{1}H and ^{13}C NMR) spectra were recorded at AVANCE III 500 MHz Digital NMR Spectrometer 1 (Bruker Biospin; AV-500). The δ values were reported as ppm relative to TMS in DMSO-*d*6 and MeOH-*d*4, respectively.

Angiotensin-converting enzyme inhibitory assay

The assay was performed according to a previously reported method with some modification.[12] The buffer, 50 mM Tris buffer pH 8.3 containing 300 mM NaCl, was used to dilute the enzyme and substrate HHL, while 10% DMSO in buffer was used to dilute the extract. An antihypertensive agent, captopril, was used as a positive control, and 10% DMSO was used as a negative control. The total reaction volume was 70 µL. The solution (10 µL) of extract fractions or isolated compounds was added to the substrate solution (50 µL) and incubated at 37°C for 30 min. Then, 2 mU ACE solution (10 µL) in 50 mM Tris buffer pH 8.3 containing 300 mM NaCl was added, and the mixture was incubated at 37°C for 30 min. The enzyme reaction was stopped by addition of 1 M HCl (85 µL). The samples were prepared in duplicate.

Determination of hippuric acid

HA was yielded by HHL hydrolysis catalyzed by purified rabbit ACE. HA from the reaction was analyzed by analytical reverse-phase HPLC. All solutions were filtered through 0.45 µm nylon filter before analysis. The column was Agilent 5 TC-C18 (2), 150 mm × 4.6 mm, 5 µm. The mobile phase comprised 0.05 M aqueous acetic acid and acetonitrile using a step gradient mode at a ratio of 87.5:12.5 (0–8 min) and 40:60 (9–14 min). The system was equilibrated at 87.5:12.5 for 5 min. The injection volume was 10 µL; flow rate was 1 mL/min. HA was detected at 228 nm. The samples were injected in triplicate. The values of percentage inhibition were calculated using the equation (×100 [peak area of HA in negative control-peak area of HA in sample]/[peak area of HA in negative control]). Finally, the IC_{50} value, the concentration of inhibitor required to inhibit 50% of the ACE activity, was determined by regression analysis of the percentage inhibition versus the log of the inhibitor concentration.

RESULTS AND DISCUSSION

ACE is a zinc-containing metalloenzyme which, in the body, regulates blood pressure by catalyzing the conversion of angiotensin I into angiotensin II.[13] In the *in vitro* ACEi assay, ACE catalyzed the degradation

of HHL to form HA so that ACEi was detected from the decrease in the peak area of HA using HPLC. In this study, an appropriate HPLC condition was achieved with good linearity (correlation coefficients of 0.9996) and selectivity. The retention time of HA was 7.45 min which separated from the other peaks in the reaction.

Since the ethyl acetate extract of *S. garrettiana* showed high ACE percentage inhibition, with 90.64 at the screened concentration of 1 mg/mL, it was selected for the purification of the active compounds by chromatography using ACEi-guided fractionation. The most active fraction (fraction 4) that gave the highest ACE percentage inhibition at 80.72 was further subjected to column chromatography to give six subfractions. These subfractions were used to determine their percentage inhibition. The result indicated that fractions 4.5 and 4.6 showed high ACE percentage inhibition with 94.69 and 97.08, respectively. Since the screened concentration of 1 mg/mL was a high concentration, a false-positive result can be occurred. Before further purification step, the IC_{50} values were observed by diluting the fractions 4.5 and 4.6 into many concentrations. Satisfactory IC_{50} values ensured us to continue the experiment. The ACE percentage inhibition and the IC_{50} of *S. garrettiana* are shown in Table 1.

Bioassay-guided investigation of *S. garrettiana* subfractions 4.4 and 4.6 led to isolation of three pure known compounds: one anthraquinone, one triterpenoid, and one stilbene, identified as chrysophanol (1), betulinic acid (2), and piceatannol (3), respectively [Figure 1]. All the structures were confirmed from NMR spectral data in Tables 2-4. The isolated compounds 1–3 were investigated for their ACEi. Although the ACEi effects of all active compounds were significantly less than the captopril (IC_{50} = 0.02 μM), a well-known ACE inhibitor, the IC_{50} values of compounds 2 and 3 in Table 5 suggested that they still had satisfactory ACEi activity.[14] Only compound 1 showed weak inhibitory activity.

Betulinic acid, a naturally occurrence pentacyclic triterpenoid, is presented in many plant species, including *Betula pubescens*, *Melaleuca leucadendron*, and *Ziziphus joazeiro*.[15-17] It is well known for its anticancer activity.[18,19] However, in this study, it was shown that this compound may also have a benefit as an antihypertensive agent, through ACEi. The evidence from other publications showed that betulinic acid has a potential in cardiovascular disease treatment. For example, first, it can reduce nicotinamide adenine dinucleotide phosphate (NADPH) oxidase expression in human endothelial cells through the protein kinase C-independent mechanism.[20] Since NADPH oxidase expression can produce reactive oxygen species which lead to endothelial dysfunction, reducing its expression means reducing cardiovascular risk factors. Second, it was found that betulinic acid can attenuate endothelial dysfunction in diabetic apolipoprotein-E gene knockout mice and may, therefore, be useful for atherosclerosis treatment.[21] Third, Zizyphi Spinosi Semen, a traditional Chinese herb containing betulinic acid as a main component, can decrease blood pressure in L-NAME-induced hypertensive rats.[22] Moreover, ursolic acid, which is also pentacyclic triterpenoid, has been reported to possess some ACEi, but the activity is weaker than that of betulinic acid in this study.[23] Ursolic acid and oleanolic acid were reported to contribute to the antihypertensive effect of genetic hypertension in rats.[24] Investigation on ACEi of ursolic and oleanolic acids using an *in silico* model suggested that both compounds may possess this activity.[25] This evidence suggests that pentacyclic triterpenoid possesses some ACEi in which the differences in carbon number in ring-E may affect its potency.

Piceatannol is a stilbene naturally available from fruits such as grapes and from medicinal plants such as *Euphorbia lagascae* and *Mezoneuron cucullatum*.[26-28] It possesses anti-HIV-1 integrase activity.[29] Moreover, it has been reported to have a beneficial effect on cardiovascular disease, acting as an antiarrhythmic agent, and preventing endothelial dysfunction.[30,31] Piceatannol also exhibits vasorelaxation effects, in which can decrease blood pressure. Research shows that piceatannol is a potent vascular relaxant in rat-isolated aorta, and the mechanism may be mediated through the endothelium-dependent nitric oxide signaling pathway.[32,33] Although there have been no reports about the ACEi of piceatannol, resveratrol was reported to possess this activity with a IC_{50} reading of 0.97 mM.[34] Resveratrol, a well-known stilbene

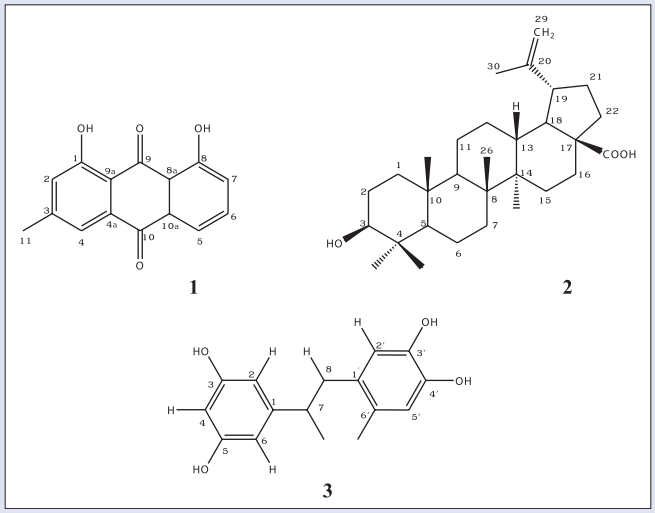

Figure 1: Structures of isolated compounds 1–3 from the heartwood of *Senna garrettiana*

Table 1: Angiotensin-converting enzyme percentage inhibition of *Senna garrettiana* fractions

S. garrettiana crude extract	*S. garrettiana* fractions	*S. garrettiana* subfractions	ACE percentage inhibition	IC_{50} (μg/ml)
Ethyl acetate			90.64±0.84	
	Fraction 1		-	
	Fraction 2		-	
	Fraction 3		-	
	Fraction 4		80.72±0.80	
	Fraction 5		43.75±5.13	
	Fraction 6		77.08±1.55	
		Fraction 4.1	6.40±0.84	
		Fraction 4.2	-	
		Fraction 4.3	9.65±3.09	
		Fraction 4.4	57.92±3.01	
		Fraction 4.5	94.69±0.00	4.56±0.80
		Fraction 4.6	97.08±0.00	8.24±0.77

Mean±SD; (n=3). SD: Standard deviation; ACE: Angiotensin-converting enzyme; *S. garrettiana: Senna garrettiana*

Table 2: ^{1}H nuclear magnetic resonance spectroscopy spectral data of compound 1 ($CDCl_3$; 500 MHz) and reference

Positions	^{1}H (compound 1)	^{1}H (Zhang *et al.*, 2012)
1		
2	7.09 (1H, *d*)	7.11 (1H, *d*)
3		
4	7.65 (1H, *d*)	7.66 (1H, *d*)
5	7.82 (1H, *dd*)	7.83 (1H, *dd*)
6	7.67 (1H, *t*)	7.67 (1H, *t*)
7	7.29 (1H, *t*)	7.30 (1H, *dd*)
8		
9		
10	2.45 (3H, *s*)	2.47 (3H, *s*)
11		
4a		
8a		
9a		
10a		
1-OH	12.03	12.01
8-OH	12.12	12.13

Table 3: ^{1}H nuclear magnetic resonance spectroscopy spectral data of compound 2 (dimethyl sulfoxide-d_6; 500 MHz) and reference

Positions	^{1}H (compound 2)	^{1}H (Zhang *et al.*, 2011)
1		
2		
3	4.66 (1H, *d*)	4.69 (1H, *d*)
4		
5		
6		
8		
9		
10		
11		
12		
13		
14		
15		
16		
17		
18		
19	2.94 (1H, *m*)	3.00 (1H, *m*)
20		
21		
22		
23	0.84 (3H, *s*)	0.88 (3H, *s*)
24	0.62 (3H, *s*)	0.66 (3H, *s*)
25	0.74 (3H, *s*)	0.78 (3H, *s*)
26	0.90 (3H, *s*)	0.89 (3H, *s*)
27	0.95 (3H, *s*)	0.94 (3H, *s*)
28		
29	4.54 (1H, *s*)	4.56 (1H, *s*)
30	1.62 (3H, *s*)	1.65 (3H, *s*)

in grapes, has been suggested as an intermediate in piceatannol biosynthesis.[35] From a study of structure–activity relationships, it was found that resveratrol created hydrogen bonds through its hydroxyl groups with amino acids in the active site of ACE, leading to a blockage of catalytic activity of ACE.[34] This evidence can be used to support the ACEi of piceatannol. Moreover, considering the IC_{50} value of piceatannol from this study, the inhibition activity was much more than that of resveratrol even if both are stilbenes. This may be because of the presence of a catechol group in piceatannol structure, which is reported to be the important catechol group in some phenolic compounds for ACEi.[36]

Table 4: ^{1}H nuclear magnetic resonance spectroscopy spectral data of compound 3 (methanol-d_4; 500 MHz) and reference

Positions	^{1}H (compound 3)	^{1}H (Brinker and Seigler, 1991)
1		
2, 6	6.42 (2H, *d*)	6.43 (2H, *d*)
3, 5		
4	6.14 (1H, *t*)	6.15 (1H, *t*)
7	6.75 (1H, *d*)	6.73 (1H, *d*)
8	6.89 (1H, *d*)	6.89 (1H, *d*)
1’		
2’	6.73 (1H, *d*)	
3’		
4’		
5’	6.83 (1H, *dd*)	6.83 (1H, *dd*)
6’	6.96 (1H, *d*)	6.97 (1H, *d*)

Table 5: Angiotensin-converting enzyme inhibitory of compounds from *Senna garrettiana*

Compound	IC_{50} (μM)
Chrysophanol	>100
Betulinic acid	26.77±0.8
Piceatannol	8.44±1.1

Mean±SD (n=3). SD: Standard deviation

The use of pharmacological-related compounds as markers for quality control of standardized HMs is predicted to be widespread in the upcoming years, according to the World Health Organization Traditional Medicine Strategy 2014–2023.[37] Moreover, betulinic acid and piceatannol are promising candidates in therapeutic development due to their IC_{50} value, which was not too strong or too weak. The side effects of synthetic ACE inhibitors, such as coughing and skin rashes, are suggested to be caused by the increase of bradykinin.[38] ACE activates the degradation of bradykinin; thus, the bradykinin is elevated because ACE activity is completely blocked by synthetic ACE inhibitors, which normally have IC_{50} values in nM units. Since both piceatannol and betulinic acid have IC_{50} of 8.44 μM and 26.77 μM, respectively, they may cause fewer secondary effects.

CONCLUSIONS

From ethnobotany through bioassay-guided investigations of *S. garrettiana*, piceatannol and betulinic acid were found to possess ACEi. These compounds can be used as markers for a standardized HM aimed at controlling blood pressure. Moreover, both compounds can be used as candidates for finding new ACE inhibitors with fewer undesirable side effects.

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Conflicts of interest

There are no conflicts of interest.

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