

# Angiotensin-converting enzyme inhibitory activity of some Vietnamese medicinal plants

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## **Abstract:**

This study investigated the angiotensin 1-converting enzyme (ACE) inhibitory activity of certain medicinal plants that have been traditionally used as an anti-hypertensive, diuretic, or related diseases treatment in Vietnam. Ten different medicinal plants were selected and screened for *in vitro* ACE inhibitory activity. A spectrophotometric assay was developed for the determination of ACE activity in the presence of ACE inhibitors using hippuryl-L-histidyl-L-leucine as the ACE-specific substrate. When the *in vitro* activities of ACE were assessed in the presence and absence of aqueous or ethanol extracts from each medicinal plant, 2 out of the 22 medicinal plant extracts inhibited the ACE activity by more than 90% at a concentration of 50 µg/ml. The estimated IC<sub>50</sub> value of the *L. rubra* and *U. sessilifructus* were 1.31±0.44 and 12.86 µg/ml, respectively. These results suggest that the *L. rubra* rhizome and *U. sessilifructus* extract are potential anti-hypertensive drug-like candidates with ACE inhibitory activity.

**Keywords:** angiotensin 1-converting enzyme, antihypertensive, *Leea rubra*, *Uncaria sessilifructus*.

**Classification number:** 3.3

## **Introduction**

Hypertension is a leading cause of death worldwide. About 7.6 million mortalities are annually recorded due to hypertension and this number accounts for approximately 13.5% of total mortality [1]. Currently, many types of synthetic anti-hypertension drugs have been used for the treatment of patients with hypertension. Unfortunately, the proportion of treated and managed cases with such antihypertensive drugs is relatively low in developing countries, probably because of high medical care costs combined with low annual incomes [2]. Therefore, the development of alternative therapeutics for hypertension remains a critical issue in those countries [3].

Various medicinal plants have been investigated to explore natural resources with therapeutic potentials for the patients with hypertension by focusing on calcium channels, β-receptors, diuretic activity, and the renin-angiotensin system (RAS). Particularly, the angiotensin-converting enzyme (ACE) in the RAS has been targeted to develop anti-hypertensive drugs since ACE plays a vital role in blood pressure regulation. This enzyme catalyses the conversion of angiotensin I to angiotensin II and thereby causes vasoconstriction. At the moment, ACE inhibitors such as captopril and lisinopril are clinically used as first-line drugs for the treatment of patients with hypertension. However, the use of these drugs is, in some cases, limited because of their various side effects such as cough, numbness, and mild skin itching, etc. Therefore, the natural source-based development of ACE inhibitors with fewer side effects has been attracting much interest.

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These backgrounds have prompted us to investigate the ACE inhibitory of certain medicinal plants that are traditionally used as anti-hypertensives, diuretics, and as treatments of related diseases in Vietnam by using an *in vitro* screening system of ACE inhibitors. Numerous methods for the measurement of ACE activity have been developed and reported, including ultraviolet spectrophotometric (USP), visible spectrophotometric (VSP), fluorometric, radiochemical, high-performance liquid chromatography (HPLC), and capillary electrophoresis [4-6]. In this study, using the spectrophotometric method based on the detection of the production of Hippuric acid (HA) from Hip-His-Leu (HHL) by the action of ACE in the presence of ACE inhibitors, we determine the ACE inhibitory activities of 22 different extracts from 10 medicinal plants. This study suggests that the *L. rubra* rhizome and *U. sessilifructus* extract are potential anti-hypertensive drug-like candidates with ACE inhibitory activity.

## Materials and methods

### Plant materials

Ten medicinal plants were used for the *in vitro* screening study: *Eucommi aulmoides*, *Apium graveolens*, *Calliasia fragrans*, *Catharanthus roseus*, *Artocarpus altilis*, *Diospyros kaki*, *Annona muricata*, *Uncaria sessilifructus*, *Leea rubra*, and *Hibiscus sabdariffa*. The plants were collected from several locations in Vietnam in 2019, identified by Dr. Pham Thanh Huyen [Department of Medicinal Plant Resources, National Institute of Medicinal Materials (NIMM), Vietnam] and deposited at NIMM with voucher specimen numbers as shown in Table 1.

### Extract preparation

The plant materials were cleaned, dried at 60°C in a hot air oven, and then cut into small pieces. For the *in vitro* study, each plant material (20 g) was extracted with water or ethanol 50% (1:7 w/v) at reflux for 2 h and filtered using a filter paper (Whatnam® qualitative filter paper, Grade 93, 580×580 cm, Merck, Darmstadt, Germany). This step was repeated 3 times. The filtered samples were combined and then concentrated under reduced pressure at 60°C. The obtained extract was then dried in a vacuum oven at 60°C until the moisture content of the extract was below 5%. The yield of the extraction from each plant is described in Table 1. These plant extracts samples were stored at 4°C and

deposited at NIMM.

### ACE inhibition assay

The plant extracts were dissolved in DMSO to make a stock solution with a concentration of 100 mg/ml and stored at -20°C until use. For the *in vitro* ACE inhibition assay, the stock solutions were diluted by a borate buffer of pH 8.3. The concentration of each plant extract in the ACE enzyme reaction was 50 µg/ml. To estimate the IC<sub>50</sub> values of the herbal extracts, a dilution of the stock solutions were made with the borate buffer to achieve test concentrations of 0.156, 0.3125, 0.625, 1.25, and 2.5 µg/ml. Captopril (Across organics, Belgium), an ACE inhibitor, was used as a positive control, which was diluted in water at a concentration of 1 mM, then the stock was diluted in the borate buffer to reach the test concentration range.

The ACE inhibitory activity was assayed according to the previous method [5] with minor modifications. The composition and reaction procedure are as follows: 12.5 µl of the inhibitor samples (plant extracts or captopril), 27.5 µl borate buffer, and 10 µl ACE 0.02 U/ml were mixed. After incubation for 5 min at 37°C, the mixture was gently blended with 75 µl HHL solution (N-Hippuryl-His-Leu hydrate powder, Sigma, Germany) by using a vortex and subsequently incubated at 37°C for 30 min. The reaction was terminated by adding 100 µl HCl 1 M and mixing. The control sample was prepared by replacing the inhibitor solution with the borate buffer. The blank sample (of both control sample and tested samples) was prepared by replacing the ACE solution by the borate buffer. Each sample was mixed with 150 µl quinoline (Across organics, Belgium). Subsequently, 75 µl of benzene sulfonyl chloride (Sigma, Germany) was added into mixture. After 45 min incubation in dark at 25°C, 300 µl of ethanol was introduced into the mixture. The absorbance of 200 µl of the mixture was determined at 490 nm using an ELISA system 96 well - EL x808 Biotek. Each reaction was done in replicas of two. In parallel with each reaction, the samples and controls had a blank to draw a comparison. The controls were similar to the test tubes but the samples were replaced with buffer.

ACE inhibitory activity was determined using the formula:

$$I (\%) = \frac{(OD_{control} - OD_{test})}{OD_{control}} \times 100 (\%)$$

where OD control is the absorbance value (optical density)

of the control tubes, OD test is the absorbance (optical density) value of test tubes, and the IC<sub>50</sub> value is the concentration of herbal extract or captopril that inhibited 50% of ACE activity under experimental conditions using regression analysis.

#### Total phenolic content of aqueous *L. rubra* rhizome extract

Total phenolic content of the *L. rubra* rhizome extract was performed using the Folin-Ciocalteu method as previous described [7, 8]. Briefly, 20 µl of the sample (5 mg/ml) and 930 µl of 2% Na<sub>2</sub>CO<sub>3</sub> were seeded in a tube to which 50 µl of Folin-Ciocalteu reagent was added. The reaction mixture was incubated at 40° C for 60 min and the absorption of the mixtures was read at 760 nm. The sample was tested at a final concentration of 100 µg/ml in triplicate and a calibration graph with four data points for gallic acid was obtained. The total phenolic content of the sample was expressed as mg of gallic acid equivalents (GAE) per gram of extract. On the basis of this analysis, the total phenolic content of the aqueous *L. rubra* rhizome extract amounts to 65.1 mg GAE/g.

## Results

Ten medicinal plants were obtained from different locations in Vietnam. Aqueous and ethanolic extract from these medicinal materials were screened for their *in vitro* activities to inhibit ACE. As shown in Table 1, the plant extracts showed more than 70% ACE inhibition at a concentration of 50 µg/ml, including the *L. rubra* rhizome (aqueous extract) and *U. sessilifructus* (ethanol extracts), *C. fragran* (aqueous and ethanolic extract), *C. roseus* (ethanolic extract), *A. graveolens* (aqueous extract), and *A. muricata* (aqueous extract). Especially, the *L. rubra* rhizome aqueous and *U. sessilifructus* ethanol extracts showed more than 90% inhibition at a concentration of 50 µg/ml.

The ACE inhibition effects of the aqueous *L. rubra* rhizome and *U. sessilifructus* ethanol extracts were further analysed. As shown in Fig. 1, the inhibitory effect of the *L. rubra* rhizome aqueous extract and *U. sessilifructus* ethanol extracts on the *in vitro* ACE activity were concentration dependent. The estimated IC<sub>50</sub> values of the *L. rubra* rhizome aqueous extract and *U. sessilifructus* ethanol extract on the *in vitro* ACE activity were 1.3±0.5 µg/ml and 12.86±3.08 µg/ml, respectively. On the other hand, captopril, an ACE inhibitor used as a positive control had an IC<sub>50</sub> value of 18.0±2.0 nM.

**Table 1. Plant materials used for ACE inhibition *in vitro* assay, part of plant, extraction solvent and the percentage of ACE inhibition.**

Plants	Family name	Collection data			Plant parts	Extraction solvent	Yield of extraction (%)	% ACE inhibition (50 µg/ml)
		Date	Site	Voucher no.				
<i>Callisia fragran</i> (Lindl.) Woodson	Commelinaceae	3/2019	Thai Nguyen province	DL-260219	Leaves	Water	29.2	82.8±1.7
						Ethanol	36.2	71.9±6.4
<i>Leea rubra</i> Blume	Leeaceae	4/2019	Yen Bai province	DL-230219	Rhizome	Water	20.9	100±0.0
						Ethanol	23.9	63.5±9.8
					Aerial part	Water	8.2	50.0±7.1
						Ethanol	9.8	12.0±6.8
<i>Eucommia ulmoides</i> Oliv.	Eucommiaceae	6/2018	Lao Cai province	DL-240219	Stem bark	Water	17.7	67.5±14.2
						Ethanol	18.3	16.0±14.2
<i>Catharanthus roseus</i> G. Don	Apocynaceae	4/2018	Thai Binh province	DL-220219	Aerialpart	Water	31.1	58.6±12.2
						Ethanol	29.9	79.7±4.2
<i>Apium graveolens</i> Linn.	Apiaceae	4/2018	Thai Binh province	DL-210219	Aerialpart	Water	34.2	79.5±7.6
						Ethanol	35.1	37.5±2.1
<i>Artocarpus altilis</i> Fosberg	Moraceae	4/2018	Ho Chi Minh city	DL-200219	Leaves	Water	15.0	48.6±6.6
						Ethanol	14.7	67.8±10.7
<i>Diospyros kaki</i> Thunb.	Ebenaceae	8/2018	Lang Son province	DL-250219	Leaves	Water	15.9	22.5±7.5
						Ethanol	16.90	25.6±9.38
<i>Annona muricata</i> Linn.	Annonaceae	3/2019	Thai Nguyen province	DL-270219	Leaves	Water	30.7	73.1±18.8
						Ethanol	32.7	19.4±4.3
<i>Uncaria sessilifructus</i> Roxb.	Rubiaceae	3/2018	Bac Can province	DL-190219	Stem with hook	Water	18.3	67.0±7.0
						Ethanol	12.1	93.5±4.0
<i>Hibiscus sabdariffa</i> Linn.	Malvaceae	4/2019	Phu Yen province	DL-280219	Calyxes	Water	35.1	65.8±4.5
						Ethanol	45.1	39.1±7.4

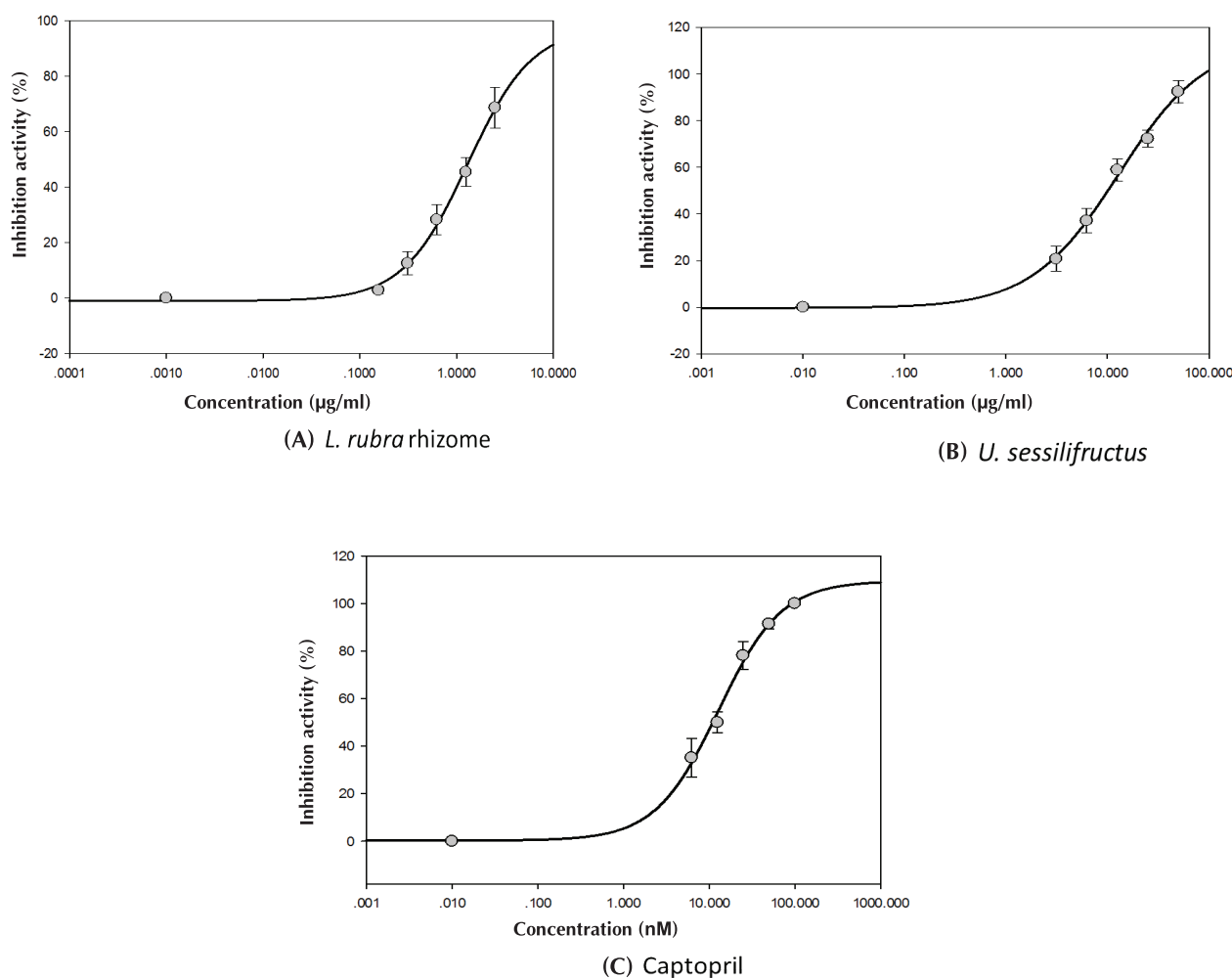


Fig 1. Inhibition activity of *L. rubra* rhizome extract (A), *U. sessilifructus* ethanol extract (B) and captopril (C) on ACE *in vitro*.

**Discussion**

In this study, we used the VSP method to screen 22 extracts from 10 traditional herbs for their *in vitro* ACE inhibitory potentials using captopril as the positive control. The IC<sub>50</sub> values of captopril determined in this study using this VSP method (18.0 nM) are in agreement with the range of 5-23 nM reported previously [4, 5, 9]. USP/VSP and HPLC are commonly used in the assay for determining ACE activity and inhibition *in vitro*. The USP method was developed by Cushman and Cheung in 1970 based on the hydrolysis of hippuryl -L-histidy where Hip-His-Leu converts into Hippuric acid (HA) and His-Leu (HL) by ACE. HA then was extracted by ethyl acetate and measured by USP at a wavelength 228 nm. However, this method has weak points such as a complicated process, wasted time,

and instability during separation of HHL from HA because both absorb wavelengths of 228 nm. Therefore, HA may be overestimated [5]. The VSP method was developed by modifying the USP method by using spectrophotometry in the visible light wavelength range. For the modified VSP methods, the ethyl acetate extraction step was replaced by adding a colorimetric agent that binds with HA such as benzene sulfonyl chloride in the presence of quinoline. The VSP method process has the advantages of simplicity, high sensitivity, and cost effectiveness because of the absence of the procedure that separates HA from the reaction mixture [5, 6]. The HPLC method was employed to assay ACE activity with reaction principles similar to the USP method. Although the HPLC method exhibits higher precision and reproducibility than VSP method, the use of HPLC requires costly instrumentation requiring proper preventative



maintenance, the highest grade of purity solvents, and a complex and time consuming procedure. In contrast, the VSP provides a simple and rapid means for chemical analysis [5] that is suitable for screening proposes with a large number of samples.

The present study demonstrated that the *L. rubra* rhizome aqueous extract exhibited the highest *in vitro* ACE inhibitory potency of all the plant materials screened with an  $IC_{50}$  of  $1.31 \pm 0.44 \mu\text{g/ml}$ . *L. rubra* belongs to the family Vitaceae. *L. rubra*, which is widely distributed in Vietnam, Thailand, and Cambodia. *L. rubra* is an herb with dusty red fruits that ripens to a black, purple red stem with an average height of about 2.5 m. Traditionally, this plant is used for the treatment of inflammation and pain [10]. Surprisingly, there is another species of *Leea* genus that is often used in traditional remedies for hypertension [11]. According to the previous study demonstrating the *in vitro* ACE inhibitory effect of some Brazilian medicinal herbs, the ethanolic extract of aerial part of *L. rubra* inhibited the activity of ACE by  $57.0 \pm 12.5\%$  at a concentration of  $100 \mu\text{g/ml}$  [11]. Consistent with Braga's study, our *in vitro* results show that aqueous and ethanolic extracts of *L. rubra*'s aerial part inhibited ACE activity by 50.0 and 12.0%, respectively, at a concentration  $50 \mu\text{g/ml}$ . Moreover, we found that aqueous and ethanolic *L. rubra* rhizome extracts inhibited the ACE activity by 100 and 63.5%, respectively. This finding demonstrates that the *L. rubra* rhizome aqueous extract exhibits a higher ACE inhibitory activity than the aerial part. Moreover, with an  $IC_{50}$  as low as  $1.31 \pm 0.44 \mu\text{g/ml}$ , the *L. rubra* rhizome extract is considered as a crude plant extract that possesses potent ACE inhibition. In our dedication to discover effective anti-hypertension therapies from natural products, the effect of this plant extract in hypertensive rat models is under investigation in our laboratory.

The main chemical compositions of the *L. rubra* rhizome extract that causes the angiotensin-converting enzyme inhibitory activity are unclear. As far as we know, no specific work on *L. rubra* rhizome isolate has been reported to date. However, based on a chemical analysis, we demonstrated that this crude extract consists of high phenolic content with the total phenolic content was estimated as 65.1 mg GAE/g. Moreover, Phuong, et al. (2015) [12] isolated and identified 5 phenolic compounds including gallic acid, proto-catechuic acid, 4-hydroxybenzoic acid, arctiin, and kaempferol-3-O- $\alpha$ -L-rhamnopyranosyl(1 $\rightarrow$ 2)- $\alpha$ -L-arabinofuranoside from *L. rubra* leaves. From the *L. rubra*

leaves, this group also isolated and identified 4 flavonoids including myricitrin, 7-methoxymyricitrin, rhamnetin-3-O- $\alpha$ -L-rhamnopyranoside, and juglanin [13]. In addition, there was a study demonstrating the hypotensive effect of gallic acid suspected by acting on the renin-angiotensin system [14]. Thus, there is a possibility that gallic acid as well as flavonoids constituents of *L. rubra* may be the active ingredients contributing to the ACE inhibition effect of the *L. rubra* rhizome extract, however, further study is needed to demonstrate this possibility.

Besides the *L. rubra* rhizome extract, we found that *U. sessilifructus* extracts exhibit significant ACE inhibition activity. As far as we know, the ACE inhibition effect of *U. sessilifructus* has not been reported previously. However, Hansen, et al. (1995) [15] reported that *U. rhynchophylla* is one of 7 species that inhibit ACE by more than 50% among the 31 species investigated. Notably, five *Uncaria* species, namely *U. rhynchophylla*, *U. macrophylla*, *U. hirsuta*, *U. sinensis*, and *U. sessilifructus* are documented in Chinese Pharmacopoeia as the raw materials of *Uncariae Ramulus Cum Uncis*. This *Uncaria* species has been traditionally used for the treatment of hypertension, headache, and fever. The indole alkaloid content of *U. sessilifructus* is considered as the antihypertensive active ingredient [16, 17]. Feng, et al. (2019) [18] reported that the *Uncaria* species including *U. sessilifructus* reduced the systolic blood pressure in spontaneously hypertensive rats (SHRs). Thus, these results allow us to speculate the ACE inhibition activity contributing to the anti-hypertensive action of this plant.

A negative result of the ACE inhibition screening does not always mean that this plant species does not work as an anti-hypertensive drug as compounds may influence other hypotensive mechanisms. It also should be noted that the presence of potent ACE inhibition does not mean that the species are potent anti-hypertensive drugs. Therefore, further studies on pure compounds isolated from the active extracts are necessary. Also, the effect of a potential candidate should be evaluated using an animal model of hypertension.

## Conclusions

In conclusion, our results showed that the *L. rubra* rhizome and *U. sessilifructus* extracts exhibit significant ACE inhibitory activity. This study suggests that the *L. rubra* rhizome and *U. sessilifructus* may be beneficial for the treatment of hypertension.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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