

## INHIBITION OF ANGIOTENSIN CONVERTING ENZYME (ACE) BY *VIOLA MANDSHURICA* EXTRACTION

Man Kyu Huh

Department of Molecular Biology/Dong-eui University  
KOREA

### ABSTRACT

Angiotensin-converting enzyme (ACE, EC 3.4.15.1) inhibitor plays a critical role in treating hypertension by causing blood vessels to constrict. The purpose of this study was to estimate ACE inhibition activity by *Viola mandshurica* using an in vitro assay. The assay method is based on the hydrolysis of the substrate HHL by ACE, and measuring the amount of HA using RP-HPLC. ACE inhibitor activity was evaluated by determining the degree of hydrolysis rate of substrate, hippuryl-L-histidyl-L-leucine (HHL). At 240 nm, the absorbance of treatment groups was the highest in *V. mandshurica* and HHL. More exactly, the absorbance ratio of HHL and extracts from *V. mandshurica* at 228 nm was high than that at 240 nm. The standard control reagent, captopril was used as a positive control for ACE inhibition. The extraction of *V. mandshurica* leaves showed inhibition activity more than 39.1%. The extraction of *V. mandshurica* petioles showed inhibition activity more than 28.7%. The roots of *V. mandshurica* demonstrated ACE inhibitory activity at a concentration of 20%, showing an inhibition greater than 46%. The roots extraction for ACE inhibitor was more effective than leaf and petiole extractions. The results of this study suggest that the root extraction of *V. mandshurica* can be utilized in further studies as one of high ACE inhibitory effectors.

**Keywords:** Angiotensin-converting enzyme (ACE), ACE inhibitor, *Viola mandshurica*.

### INTRODUCTION

One in three adults worldwide has raised blood pressure – a condition that causes around half of all deaths from stroke and heart disease (WHO, 2013). It is predicted that this rate would increase by 60% in 2025 (Kearney et al., 2005). Thus, it is not surprising events that hypertension is a silent killer that rarely causes symptoms.

Angiotensin converting enzyme (ACE, EC 3.4.15.1) is a dipeptidyl-liberating exopeptidase classically associated with the renin-angiotensin system regulation peptidyl blood pressure (Mullary et al., 1996; Lee et al., 2003). Its physiological function is to catalyze the cleavage of histidyl-leucine from angiotensin I to yield the potent vasopressor octapeptide angiotensin II (Groff et al., 1993). Although ACE conversion of angiotensin I to angiotensin II is a normal regulatory process in the body, high ACE activity leads to increased concentration of angiotensin II and hypertension. Therefore, development of agents that inhibit the conversion of angiotensin I to angiotensin II, and bradykinin to inactive components began as a therapeutic strategy to treat hypertension. ACE inhibitors such as captopril and lisinopril play key roles in treating hypertension and maintaining the electrolyte balance (Carretero and Oparil, 2000). This process has been targeted by the development of drugs called ACE inhibitors that are commonly used in treating hypertension and diabetes. Some endogenous

peptides, such as enkephalines and beta-endorphin were developed to be competitive substrates and inhibitors of ACE (Lee et al., 2003).

*Viola mandshurica* W. Becker is a perennial herb and found throughout the East Asian region, China including Taiwan, Korea, Mongolia, Japan including Okinawa and the Russian Far East Korea (Lee, 2007). *V. mandshurica* grows up in a variety of habitats, from undisturbed woodlands to urban areas, and from low-lying plains to mountainous regions. A number of varieties have been developed by horticulturalists and are popular as garden plants. Many researchers have reported that *V. mandshurica* have potential antioxidant and anti-diabetic properties (Lee et al., 2008) and can be used to treat asthma by inhibiting inflammatory response (Lee et al., 2010). Also, *V. mandshurica* could lead to development of anti-asthma pharmaceuticals using the same principles (Jeon et al., 2009) and protect nerve cells from oxidative stress and even reduce apoptosis, which may lead to treatments for neuronal diseases (Kwak et al., 2011).

The aim of the current study was to isolate and characterize the constituents responsible of the ACE activity of the aqueous extractions of *V. mandshuric*.

## MATERIALS AND METHODS

### Preparation of Crude Extracts

Each of Leaves (100 g), petioles (100 g), and roots (100 g) of *V. mandshurica*, was ground with pestles and liquid nitrogen at  $-70^{\circ}\text{C}$  and homogenized prior to beginning extraction experiments. These pulverized methods using liquid nitrogen gas were studied which methods are effective for material extraction. The extraction solvent was hot water. Extracted sample was filtered and the filtrate diluted to 5000 mL with hot water in a volumetric flask. The ultrasound extraction was carried out using an ultrasonic bath (5510, Branson, USA). The sample was treated with ultrasound at  $65^{\circ}\text{C}$  for a given duration. The sample was evaporated to dryness under reduced pressure and controlled temperature by using rotary vacuum evaporator (N-1001S-W, Eyela, Tokyo, Japan).

### ACE Inhibition Assay

Angiotensin converting enzyme (ACE) from rabbit lung, hippuryl-L-histidyl-leucine (HHL), sodium borate buffer, hippuric acid (HA) and captopril were purchased from Sigma-Aldrich Co. (England).

The assay method is based on the hydrolysis of the substrate HHL by ACE, and measuring the amount of HA using RP-HPLC (Horiuchi et al., 1982). Sodium borate buffer solution was prepared by dissolving 50 mM sodium borate buffer and 300 mM NaCl in 1000 ml water and adjusting the solution to pH 8.3 by 1 M NaOH solution.

The substrate solution (9 mM) was prepared by dissolving HHL (19.74 mg) in 5 ml of sodium borate buffer. 0.0625%, 0.125%, 0.25%, 0.5%, 1.0%, and 2.0% concentrations of crude extracts were prepared as the concentration of standard criterion for the linearity analysis. First, ACE solution (25  $\mu\text{l}$ ) (80 mU/ml) was added to 25  $\mu\text{l}$  of inhibitor solution (or solvent as negative control). After 3 min preincubation at  $37^{\circ}\text{C}$ , 25  $\mu\text{l}$  substrate solution was added and the mixture was incubated at  $37^{\circ}\text{C}$  for 30 min with shaking at 300 rpm in an Eppendorf thermomixer. The reaction was stopped by addition of 50  $\mu\text{l}$  of 1 M HCl and then the reaction mixture was subjected to reversed-phase chromatography (RP-HPLC, cLC,

Gilson, Wisconsin, USA). The mobile phase was an isocratic system consisting of a mixture of 10 mM  $\text{KH}_2\text{PO}_4$  (adjusted to pH 3 with  $\text{H}_3\text{PO}_4$ ) and methanol (50:50, v/v). The flow rate was 1 ml/min and the injection volume was 20  $\mu\text{l}$ . Analyses were detected by a PDA detector at the wavelength of 228 nm.

ACE inhibition was calculated on the ratio of the area under curve (AUC) of HA peak in an inhibitor sample to that of negative control sample as follows:

ACE inhibition (%) =  $[1 - (\text{AUC inhibitor} / \text{AUC control})] \times 100$ .

AUC inhibitor : AUC of the HA peak with inhibitor.

AUC control : AUC of the HA peak of control sample without inhibitor.

The substrate and crude extractions were analyzed at a wider absorbance from 220 nm to 380 nm.

Control and repeat tests were analyzed by a one sample t test with values above the 95% confidence interval considered significant ( $P < 0.05$ ). The difference in group mean values among in vivo treated groups were analyzed by one way analysis of variance followed by Student Newman Keuls (SNK) multiple comparisons test (Zar, 1984). In some cases the paired t-test was used for comparisons.

## RESULTS

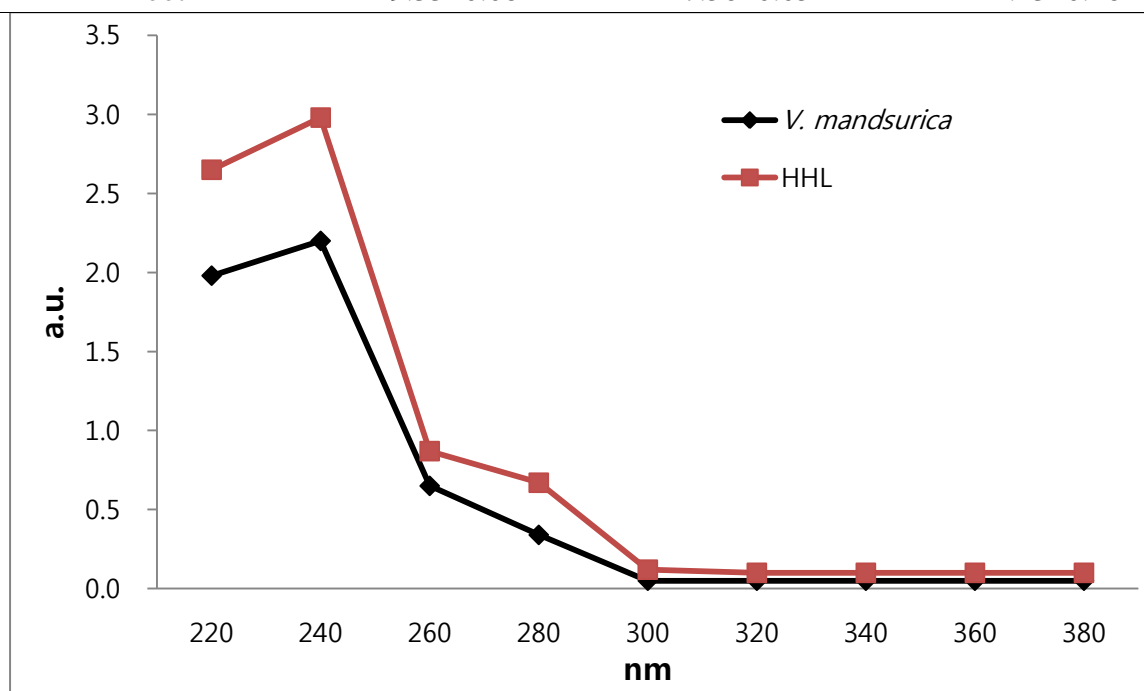
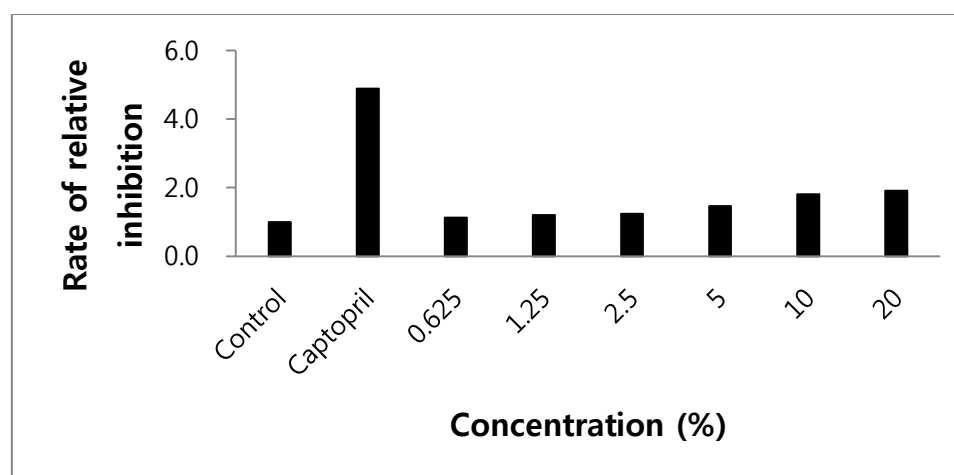
The mean contents of crude extracts from *V. mandshurica* leaves were 6.1 mg in heat treatment, 4.5 mg in ultrasonic treatment, and 7.3 mg in combined with heat and ultrasonic (Table 1). The mean contents of crude extracts from *V. mandshurica* petioles were lower than those of leaf extractions. The mean contents of crude extracts from *V. mandshurica* roots were 9.3 mg in heat treatment, 7.6 mg in ultrasonic treatment, and 12.2 mg in combined with heat and ultrasonic.

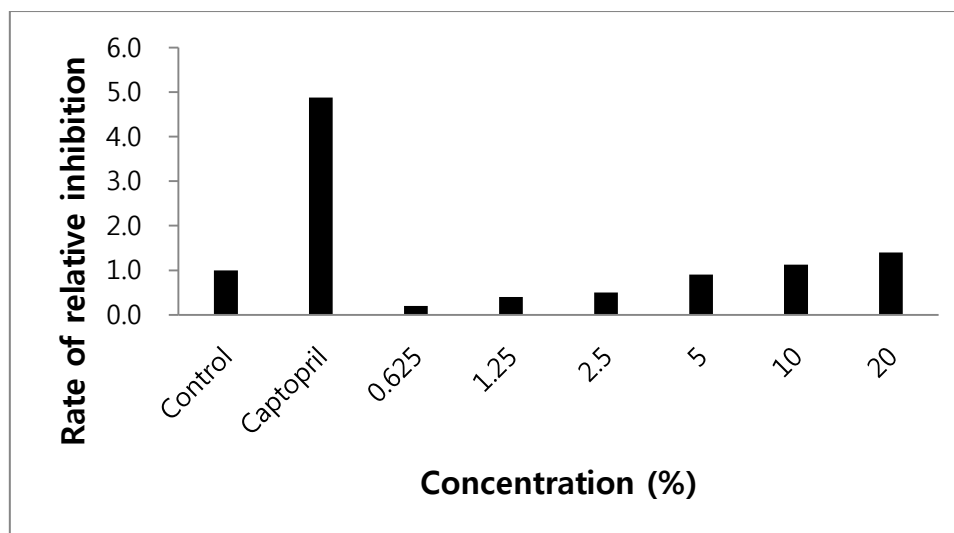
At 240 nm, the absorbance of treatment groups was the highest in *V. mandshurica* and HHL (Fig. 1). More exactly, the absorbance ratio of HHL and extracts from *V. mandshurica* at 228 nm was high than that at 240 nm (data not showed).

In order to verify the popularized potential in practice, I prepared *V. mandshurica* as ACE inhibitor and compared its ACE inhibitory activity with captopril. The result showed that ACE inhibitory rate of *V. mandshurica* leaf was  $1.913 \pm 0.32$  in the concentration of 20%, and inhibitory rate of captopril was  $4.489 \pm 0.44$  in the concentration of 5 mM (Fig. 2). Thus, the extraction of *V. mandshurica* leaves showed inhibition activity more than 39.1%. ACE inhibitory rate of *V. mandshurica* petioles was  $1.402 \pm 0.55$  in the concentration of 20%, and inhibitory rate of captopril was  $4.489 \pm 0.44$  in the concentration of 5 mM (Fig. 3). Thus, the extraction of *V. mandshurica* petioles showed inhibition activity more than 28.7%. ACE inhibitory rate of *V. mandshurica* root was  $2.237 \pm 0.35$  in the concentration of 45.8% (Fig. 4). The values of absorbance were increased as increasing density of crude extracts for *V. mandshurica*. The extractions of *V. mandshurica* roots showed inhibition activity less than 46%. Therefore, roots of *V. mandshurica* possessed more ACE inhibition activity, compared to those of leaves and petioles.

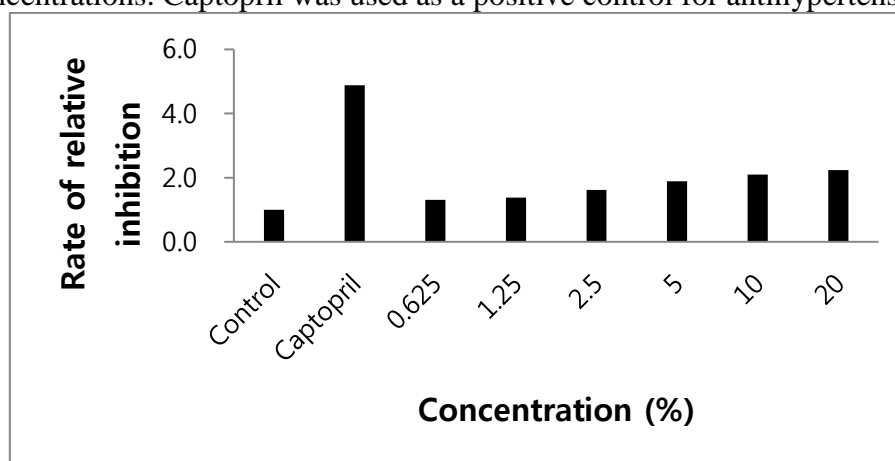
**Table 1.** The extracted dry weight (mg) from 100 g samples of *Viola mandshurica*

Sample	Treatment		
	Heat	Ultrasonic	Heat + ultrasonic
Leaf	6.13±0.02	4.45±0.02	7.30±0.06
Petiole	4.07±0.05	3.68±0.04	6.26±0.10
Root	9.33±0.08	7.56±0.05	12.23±0.16

**Fig. 1.** The ultraviolet absorbance spectra of *Viola mandshurica* (mixtures of leaves, petioles, and roots) and HHL.**Fig. 2.** ACE inhibition activity of *Viola mandshurica* leaves of derived from rabbit lung at various concentrations. Captopril was used as a positive control for antihypertensive effect.



**Fig. 3.** ACE inhibition activity of *Viola mandshurica* petioles of derived from rabbit lung at various concentrations. Captopril was used as a positive control for antihypertensive effect.



**Fig. 4.** ACE inhibition activity of *Viola mandshurica* roots of derived from rabbit lung at various concentrations. Captopril was used as a positive control for antihypertensive effect.

## DISCUSSION

Hypertension is responsible for at least 45% of deaths in the world due to heart disease, and 51% of deaths due to stroke (WHO, 2013). The prevalence of hypertension is highest in the African Region at 46% of adults aged 25 and above, while the lowest prevalence at 35% is found in the Americas (WHO, 2013).

To access to good quality medicines for hypertension is effective, inexpensive, and vital at the primary care level. As with other noncommunicable diseases, awareness aids early detection while self-care helps ensure regular intake of medication, healthy behaviours and better control of the condition. Hypertension puts strain on the heart, leading to hypertensive heart disease and coronary artery disease if not treated. Hypertension is also a major risk factor for stroke, aneurysms of the arteries, peripheral arterial disease and is a cause of chronic kidney disease.

There are a number of choices for the treatment of hypertension. Some treatments include diuretics,  $\beta$ -blockers, calcium channel blockers and angiotensin II receptor blockers, the most common of which is angiotensin converting enzyme inhibitors. One of the most effective

medications for the treatment of hypertension is angiotensin converting enzyme inhibitors (Sørensen et al., 1998). Meanwhile, medicinal plants have been used for treating illnesses. Therefore, they can be important resources to develop new drug candidates (Lipp, 1996).

The extractions of *V. mandshurica* leaves and petioles showed 39% inhibition activity and 29%, respectively. The extractions of *V. mandshurica* roots showed much inhibition activity (46%). Among these active medicinal plants, *V. mandshurica* roots could be utilized for the treatment of hypertension in traditional medicine and this research revealed that the mechanism of action of the mentioned plants in treatment of hypertension could be done through ACE inhibition. In addition, Antihypertensive activity is reported in traditional medicine for similar species of the above mentioned plants including *Crataegus oxyacantha* (Lacaille-Dubois et al., 2001), *Onopordon leptolepis* and *Onopordon carmanicum* (Esmaili and, Saremnia, 2012).

## CONCLUSIONS

*V. mandshurica* leaves showed inhibition activity more than 39%. The extractions of *V. mandshurica* roots showed inhibition activity less than 46%. The roots extraction of *V. mandshurica* for ACE inhibitor was more effective than leaf and petiole extractions. The results of this study suggest that the roots extract of *V. mandshurica* has significant ACE inhibitory effect.

## REFERENCES

- Carretero, Carretero, O.A. & Oparil, S. (2000) Essential hypertension. Part I: definition and etiology. *Circulation*, 21, 329-335.
- Esmaili, A. & Saremnia, B. (2012) Preparation of extract-loaded nanocapsules from *Onopordon leptolepis* DC. *Industrial Crops and Products*, 21, 259-263.
- Groff, J.L., Harp, J.B. & DiGirolamo, M. (1993) Simplified enzymatic assay of angiotensin-converting enzyme in serum. *Clinical Chemistry*, 36, 400-404.
- Horiuchi, M., Fujimura, K., Terashima, T. & Iso, T. (1982) Method for determination of angiotensin converting enzyme activity in blood and tissue by high performance liquid chromatography. *Journal of Chromatography*, 233, 123-130.
- Jeon, G.I., Yoon, M.Y., Park, H.R., Lee, S.C. & Park, E. (2009) Neuroprotective activity of *Viola mandshurica* extracts on hydrogen peroxide-Induced DNA damage and cell death in PC12 Cells. *Annals of the New York Academy of Sciences*, 1171, 576-582.
- Kearney, P.M., Whelton, M., Reynolds, K., Muntner, P., Whelton, P.K. & He J. (2005) Global burden of hypertension: analysis of worldwide data. *Lancet*, 365, 217-223.
- Kwak, Y.J., Kim, K.S., Kim, K.M., Yu, H.Y., Chung, E., Kim, S.J., Cha, J.Y., Lee, Y.C. & Lee, J.H. (2011) Fermented *Viola mandshurica* inhibits melanogenesis in B16 melanoma cells. *Bioscience, Biotechnology, and Biochemistry*, 75, 841-847.
- Lacaille-Dubois, M.A., Franck, U. & Wagner, H. (2001) Search for potential Angiotensin Converting Enzyme (ACE)-inhibitors from plants. *Phytomedicine*, 21, 47-52.
- Lee, B.B., Park, S.R., Han, C.S., Han, D.Y., Park, E.J., Park, H.R. & Lee, S.C. (2008) Antioxidant activity and inhibition activity against  $\alpha$ -Amylase and  $\alpha$ -Glucosidase of *Viola mandshurica* extracts. *Journal of The Korean Society of Food Science and Nutrition*, 37, 405-409.
- Lee, M.H., Lin, Y.S., Lin, Y.H., Hsu, F.L. & Hou, W.C. (2003) The mucliage of yam (*Dioscorea batatas* Decne) tuber exhibited angiotensin converting enzyme inhibitory activities. *Botanical Bulletin - Acadmia Sinica*, 44, 267-273.
- Lee, M.Y., Yuk, J.E., Kwon, O.K., Kim, H.S., Oh, S.R., Lee, H.K. & Ahn, K.S. (2010) Anti-inflammatory and anti-asthmatic effects of *Viola mandshurica* W. Becker (VM)

- ethanolic (EtOH) extract on airway inflammation in a mouse model of allergic asthma. *Journal of Ethnopharmacology*, 127, 159-164.
- Lee, Y.N., *New Flora of Korea*, Kyo-Hak Publishing Co., Seoul, Korea, 2007.
- Lipp, F.J. (1996) The efficacy, history, and politics of medicinal plants. *Alternative Therrapies in Health and Medicine*, 21, 36-41.
- Mullally, M.M., Meisel, H. & FitzGerald, R.J. (1996) Synthetic peptides corresponding to a-lactalbumin and b-lactoglobulin sequences with angiotension-I-converting enzyme inhibitory activity. *Biological Chemistry*, 377, 259-260.
- Sørensen, A.M., Christensen, S., Jonassen, T.E., Andersen, D. & Petersen, J.S. (1998) Teratogenic effects of ACE-inhibitors and angiotensin II receptor antagonists. *Ugeskrift for Laeger*, 160, 1460-1464.
- World Health Organization (2013) A global brief on hypertension. Silent killer, global public health crisis. World Health Day 2013, 40.
- Zar, J.H. (1984) *Biostatistical analysis*; 2nd edition, Pretice-Hall, Inc. New Jersey.