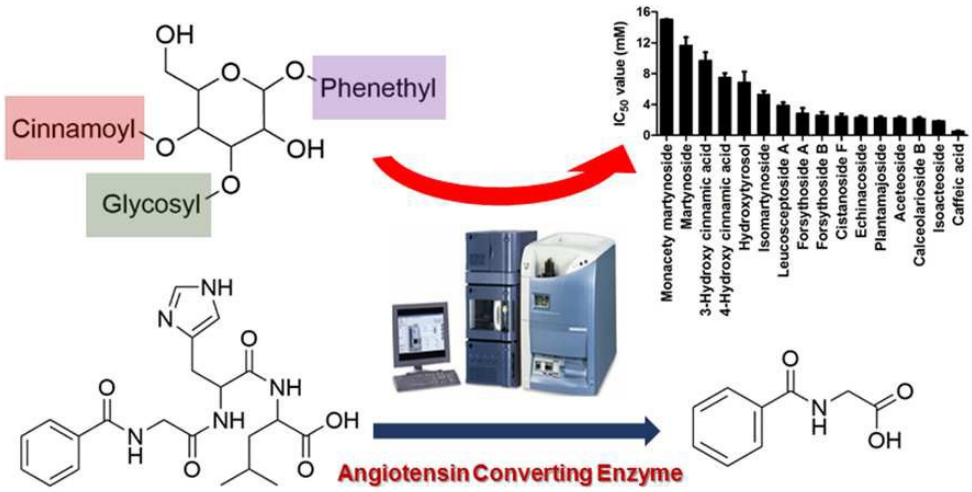
RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Graphic Abstract

Structure-inhibition relationship of phenylethanoid glycosides against ACE were investigated based on an improved UPLC-MS/MS technique. The results demonstrated that more hydroxyl groups and less structural steric hindrance bring about increasing ACEI of phenylethanoid glycosides.



Structure-Inhibition Relationship of Phenylethanoid Glycosides on Angiotensin Converting Enzyme Using Ultra-Performance Liquid Chromatography-Tandem Quadrupole Mass Spectrometry[§]

Pengfei Li,[†a] Meng Qi,[†a] Haijun Hu,[a] Qing Liu,[ab] Qiming Yang,[a] Dandan Wang,[a] Fujiang Gu,[a] S.W. Annie Bligh,[c] Zhengtao Wang[‡ab] and Li Yang[‡ab]

[a] *The MOE Key Laboratory for Standardization of Chinese Medicines and the SATCM Key Laboratory for New Resources and Quality Evaluation of Chinese Medicines, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai 201210, China.* ***E-mail:*** *ztwang@shutcm.edu.cn (Zhengtao Wang); yangli7951@hotmail.com (Li Yang);* ***Fax:*** *+86-21-51322519;* ***Tel:*** *+86-21-51322507*

[b] *Shanghai R&D Centre for Standardization of Chinese Medicines, Shanghai 201203, China*

[c] *Department of Life Sciences, Faculty of Science and Technology, University of Westminster, London W1W 6UW, UK*

[§] Electronic supplementary information (ESI) available.

[†] Both authors contributed equally to this work.

Abstract

Angiotensin-converting enzyme (ACE) plays a critical role in rennin-angiotensin system. Recently, natural products isolated from herbal medicines revealed inhibitory effects against ACE which suggested their potential activities in regulating blood pressure. In this study, ACE inhibition (ACEI) of 21 phenylethanoid glycosides and related phenolic compounds were investigated by measuring the production of HA a rapid, sensitive, accurate and specific ultra-performance liquid chromatography-tandem quadrupole mass spectrometry (UPLC-MS/MS) method. The test compounds showed different inhibitory potencies on ACE ranging from 5.29 to 95.01% at 50 mM, and the compounds with ACEI higher than 50% were selected for further IC_{50} determination. The IC_{50} values were from 0.53 ± 0.04 to 15.035 ± 0.036 mM. The structure-inhibition relationship were then explored and the result showed that cinnamoyl groups played an essential role in ACEI of phenylethanoid glycosides. Furthermore, the sub-structures of increasing ACEI for phenylethanoid glycosides is more hydroxyls and less steric hindrance to chelate the active site Zn^{2+} of ACE. In summary, our results suggested that phenylethanoid glycosides are a widely available source of anti-hypertensive natural products and the information provided from structure-inhibition relationship study could aid the design of structurally modified phenylethanoid glycosides as anti-hypertensive drugs.

Keywords: phenylethanoid glycosides; angiotensin-converting enzyme; structure-inhibition relationship; UPLC-MS/MS

1. Introduction

Hypertension is a common chronic disease and has been recognized as a public health problem throughout the world. It can lead to heart, brain, kidney failure, and other complications. Statistics in 2000 suggest that more than 25% of the world's adult population (about one billion) suffered from hypertension, and the proportion will increase to 29% (1.56 billion) by 2025 [1]. For its poorly diagnosed and controlled, prevention and treatment of hypertension has become a difficult task for global medicine.

Renin-angiotensin system (RAS) plays crucial roles in regulations of blood pressure and electrolyte homeostasis [2,3]. Angiotensin-converting enzyme (ACE; peptidyl dipeptide hydrolase, EC 3.4.15.1), a dipeptidyl carboxy peptidase widely distributed in the body, serves as a key factor to convert inactive deca-peptide angiotensin I (Ang I) into potent vasoconstrictor octa-peptide angiotensin II (Ang II) [4]. Therefore, ACE inhibition (ACEI) is considered as a therapeutic approach for hypertension. Synthetic ACE inhibitors, such as captopril, benazapril and fosinopril, have been widely used in clinic to treat hypertension, congestive heart failure, and hypertension-related organ damages [5-7]. These activities are mainly attributed to the binding of polyphenols to Zn^{2+} at the active center of ACE [8,9]. However, undesirable side effects such as skin rashes, cough, renal impairment, and angioneurotic edema have placed the use of available synthetic ACE inhibitors in a dilemma [10,11]. Development of new ACE inhibitors from natural products with less side effects has become a global focus. Phenylethanoid glycosides are a type of natural glycosides

commonly connected with substituted phenethyl and cinnamoyl groups. Recent reports on some medicinal plants containing phenylethanoid glycosides showed significant therapeutic effects in hypertension mainly due to their significant ACE inhibitory activity [12,13].

ACE activity *in vitro* was usually evaluated by monitoring the transformation from a substrate to the product catalyzed by ACE. Hippuryl-histidyl-leucine (HHL) as the substrate can be converted to hippuric acid (HA) by the action of ACE. Thus, measuring the production of HA can reflect the activity of ACE. Many techniques such as UV spectrophotometry, fluorospectrophotometry, CE, HPLC and UPLC-MS have been reported to quantify HA , while have shortcomings of poor efficiency, accuracy or selectivity [14-18]. The UPLC-tandem quadrupole mass spectrometry (UPLC-MS/MS) inherits the rapid and sensitive properties of UPLC-MS and shows much better specificity than UPLC-MS besides. Therefore, in the present study, a validated UPLC-MS/MS method was established for screening ACEI of twenty-one phenylethanoid glycosides and related phenolic compounds. The IC_{50} values and structure-inhibition relationships of the test compounds were then investigated.

2. Materials and methods

2.1 Chemicals and regents

ACE (from rabbit lung, EC 3.4.15.1), hippuryl-histidyl-leucine (HHL), Tris base and caffeic acid were purchased from Sigma chemical (St. Louis, USA). Hippuric acid (HA) was obtained from Sinopharm Chemcial Reagent Co. Ltd (Shanghai, China).

Cinnamic acid, 3-hydroxycinnamic acid, 4-hydroxycinnamic acid, ethyl cinnamate, 3-methoxycinnamic acid, 3,4-dimethoxycinnamic acid and hydroxytyrosol were from J&K Scientific Ltd. (Shanghai, China). Echinacoside, forsythoside A, forsythoside B, angoroside C, calceolarioside B were purchased from Meryer Chemical Technology Co. Ltd. (Shanghai, China). Aceteoside, isoacteoside, plantanmajoside, leucosceptoside A, clerodenoside A, isomartynoside, monoacetyl martyonside, darendoside B, martyonside, cistanoside F were isolated from the roots of *Clerodendrum bungei* in our laboratory and characterised by NMR and MS methods, with purities more than 95% (dried, HPLC-UV). HPLC-grade acetic acid was obtained from Tedia Inc. (Fairfield, USA). HPLC-grade acetonitrile was from Fisher Co. (Geel, Belgium). Water was purified using a Milli-Q Academic System (Millipore, Billerica, USA).

2.2 Sample preparation

The 75 mM of Tris buffer solution containing 200 mM of NaCl (pH=8.3) were freshly prepared. The ACE was dissolved in the Tris buffer to make a working solution of 0.05 U/mL and stored at -80 °C before use. The substrate HHL was also dissolved in the Tris buffer to obtain a 2.91 mM solution. Test compounds were dissolved into a series of concentrations with 0.5% DMSO.

2.3 Incubation procedure

First, 20 μL of enzyme solution and 10 μL of test compound solution were

pre-incubated for 5 min at 37 °C, then 35 μL of Tris buffer and 10 of μL substrate solution HHL were added and incubated for 50 min at 37 °C. The reaction was terminated immediately by the addition of 100 μL of acetonitrile (0 °C). The mixture was centrifuged (20000 rpm, 15 min, 4 °C) and the supernatant was used for analysis.

2.4 UPLC-MS/MS analysis

The separation was achieved on a Waters Acquity UPLC system (Waters Corp., Milford, USA) with an Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm). The column temperature was maintained at 45 °C. The mobile phase was consisted of 0.5% acetic acid in water (A), and acetonitrile (B) at a flow rate of 0.4 mL/min. The gradient elution was as follows: 0-0.4 min, 5% B; 0.4-1.2 min, linear from 5% to 35% B; 1.2-2.5 min, linear from 35% to 90% B; 2.5-4 min, held at 90% B for 1.5 min; 4-5 min, 5% B for equilibration of the column. The inject volume was 2 μL under a partial loop with needle overfill mode.

A Micromass Quattro Premier XE tandem quadruple mass spectrometer (Waters Corp., Manchester, UK) with electrospray ionization (ESI) source was applied for quantification. The precursor-product ion transition for HA (*m/z* 177.9→76.7) and HHL (*m/z* 428.3→175.9) were applied using the multiple reaction monitoring (MRM) in negative ionization mode (**Fig. 1**). The mass spectrometer parameters were set as follows: capillary voltage, 3.50 kV; extractor voltage, 2 kV; source temperature, 120 °C; desolvation temperature, 350 °C; desolvation gas flow, 800 L/h; cone gas flow, 50 L/h. Nitrogen (99.9% purity) was used as cone gas and argon (99.999%

purity) as collision gas. The inter-channel delay and the inter-scan delay were 0.005 s and 0.05 s, respectively. Instrumental control and date acquisition were performed using Masslynx 4.1 software.

2.5 Measurement of ACEI in vitro

The ACEI was calculated by following the production of HA using the following equation:

$$ACEI\ (\%) = \frac{C0-C}{C0} \times 100\%$$

Here C0 is the HA concentration without the test compound and C is the HA concentration with test compound. The test compounds in the concentration of 50 mM were measured for their ACEI. Compounds with ACEI higher than 50% was selected for the IC_{50} investigation. The IC_{50} value of each selected compound was recorded in triplicate, and was expressed as mean ± SD by SPSS 16.0 software (SPSS Corp., USA). Differences between groups were defined as significant (*) when $P < 0.05$.

3. Results and discussion

3.1 Optimization of reaction conditions

We have improved the reaction system based on the method from Geng F [18] with respect to the incubation and pre-incubation time, the concentration of chloride ion, and dissolving agent for test compounds, as shown in **Fig. 2**. Different incubation times at 5, 10, 20, 30, 40, 50, 60, 90, and 120 min were investigated. More stable production of HA was displayed after incubated for 50 min, which was chosen as the

optimal incubating time (Fig. 2A). As the pre-incubation can affect the combination between the enzyme and the test compounds, pre-incubated ACE with captopril (positive control, 20 nM) and acteoside solution (25 mM) for 0, 5, 10, 15, 30, and 60 min were tested. The result indicated that after pre-incubation for 5 min the calculated inhibition for captopril and acteoside were both obviously increased, but no significant difference at the time points of 5, 10, 15, 30, and 60 min (Fig. 2B). Meanwhile, previous study indicated the activity of ACE is highly dependent on the catalysis of chloride ion [19]. The production of HA with different concentrtions of NaCl (0, 100, 200, 300, and 400 mM) added in the Tris buffer were tested and ACE showed highest activity in the system with Tris buffer containing 200 mM of NaCl (Fig. 2C). In addition, due to the insolubility of some test compounds in water, solvents of methanol, acetonitrile, 0.5% DMSO, DMSO, dimethyl formamide, tetrahydrofuran and pyridine were investigated. In the result, methanol and 0.5% DMSO showed little inhibition on ACE activity. We further compared the ACEI of captopril (20 nM) and acteoside (25 mM) dissolved in water, methanol and 0.5% DMSO. The result confirmed no significant influence on ACE activity between these three solvents (Fig. 2D and 2E). Finally, the optimal reaction conditions were well selected as shown in *Materials and methods* section.

3.2 Validation of assay method

Numerous methods have been reported to quantify HA for ACE activity. In the spectrophotometry method, HA was first extracted with ethyl acetate before analysis.

The process was very complicated and time-consuming and considerable interferences were brought in because the un-hydrolyzed HHL was also extracted. HPLC method was established to provide acceptable detection but limited sensitivity. Meanwhile, the liquid conditions was easily interfered by different inhibitors during the quantification. Currently, rapid, sensitive and selective UPLC-MS technique was involved in ACE activity evaluation. However, the response of HA was still affected by caffeic acid due to the similar retention time and molecular weight between them, suggesting that the selectivity of the selected ion monitoring (SIM) scan of UPLC-MS was not sufficient for our experiment (**Supplementary Fig 1 and Suppl Table 1**[§]). Therefore, an improved UPLC-MS/MS method with higher specific MRM scan was established to avoid interference in HA detection. The UPLC-MS/MS chromatographs were shown in **Fig. 3**.

The calibration displayed a good linear behavior over the HA concentration range from 0.056 μM to 28.07 μM ($y=125.97x+3.74$, $r^2=0.9998$). The limit of detection (S/N=3) of HA was 0.017 μM and the limit of quantification (S/N=10) suitable for quantitative detection was 0.056 μM. Low, medium, and high concentrations (0.056, 1.143, 28.07 μM) of HA were added into the incubation system without ACE to generate three quality control (QC) samples. The accuracy of this method was validated by recovery of the QC samples at three concentration levels. The average accuracies of HA at three concentration levels were 96.43%, 101.8%, 99.68% with RSDs of 0.935%, 1.176%, 1.264%, respectively, shown in **Table 1**. The intra-day and inter-day precisions were respectively measured by repeating analysis of each QC

199 samples five times for one day and three consecutive days. The stability was
200 evaluated by occasionally analysis of each QC samples placed at room temperature
201 for 24 h and 4 °C for 72 h. The results in **Table 1** showed that the RSDs of intra-day
202 precisions at three concentration levels were 4.342%, 3.993%, 1.030%, and inter-day
203 precisions were 7.343%, 7.921%, 1.834%. The RSDs of stabilities at three
204 concentration levels were 7.350%, 5.148%, 2.269% at room temperature, and 8.433%,
205 4.753%, 6.043% at 4 °C.

206 Furthermore, the developed UPLC-MS/MS method has been compared with the
207 UPLC-MS method according to the IC50 values of the positive control captopril and
208 several representative compounds (**Suppl Table 2**[§]). There is good correlation
209 between these two methods (R=0.996955) and it shows no significant differences
210 (p=0.6127) based on pearson correlation analysis and two-tailed unpaired Student's
211 t-tests, respectively. These results demonstrated that the improved assay method are
212 well established and can fully meet the requirements of ACEI screening.

213

214 *3.3 ACEI screening and IC_{50} measurement*

215 Twenty-one phenylethanoid glycosides and related phenolic compounds were
216 screened for their ACEI activity *in vitro* (**Table 2**). The compounds showed different
217 potencies on ACE with the inhibition ranged from 5.29 to 95.01% at 50 mM. The
218 compounds exhibiting ACE inhibitory potencies higher than 50% were in sequence
219 caffeic acid, isoacteoside, calceolarioside B, acteoside, plantamajoside, echinacoside,
220 cistanoside F, martynoside, forsythoside B, forsythoside A, leycosceptoside A,

monacety marynoside, isomartynoside, hydroxytyrosol, 4-hydroxy cinnamic acid and 3-hydroxy cinnamic acid, which were carried out for further IC_{50} investigations. The rest of test compounds angoroside C, cinnamic acid, 3,4-dimethoxy cinnamic acid, clerodenoside A and 3-methoxy cinnamic acid showed ACEI lower than 50% (**Fig. 4**). The IC_{50} values were measured from 0.53 ± 0.10 to 15.04 ± 0.04 mM, shown in **Table 3**. Captopril (100 nM) was used as positive control in ACEI screening and the IC_{50} value was 2.11 ± 0.57 nM, which was closed to the literature results.

3.4 The possible active group of phenylethanoid glycosides

Phenylethanoid glycosides are structurally composed of the glycosyl, phenethyl and cinnamoyl groups substituted with hydroxyls, methoxyls, or acetyls. Recent report also revealed that phenylethanoid glycosides displayed as prodrugs and degraded into phenolic products for further metabolism *in vivo* [20]. Therefore, we evaluated the ACEI of structurally related phenolic compounds as well as phenylethanoid glycosides with different glycosyl groups for finding the possible active groups of phenylethanoid glycosides binding to ACE *in vitro*. Caffeoyl-containing acteoside, cistanoside F and caffeic acid showed similar ACEI with IC_{50} values of 2.22 ± 0.21, 2.46 ± 0.35 and 0.53 ± 0.10 mM, respectively. Furthermore, as another phenolic group of acteoside, hydroxytyrosol displayed ACEI with the IC_{50} of 6.87 ± 1.39 mM, weaker than caffeic acid. However, to observe the influence of different glycosyls of phenylethanoid glycosides on ACEI, acteoside, plantamajoside, forsythoside A, forsythoside B, echinacoside, isoacteoside and calceolarioside B were involved into the measurements

but showed no significant differences in IC_{50} values. The results indicated that phenolic groups played more important roles in inhibition of ACE than glycosyl groups. In addition, cinnamoyls might be more essential than phenethyls in ACEI, mainly because the cinnamoyl groups had greater conjugate system and could helped to maintain a planar structure of phenylethanoid glycosides [21].

3.5 The significance of the hydroxyls in phenylethanoid glycosides

Previous study suggested the presence of hydroxyl groups might be important for the inhibition of the zinc metalloproteinases [22,23]. In this study, the methylation of hydroxyls significantly reduced the ACEI potencies of phenylethanoid glycosides. When acteoside was transformed to leucosceptoside A by methylation of one hydroxyl group, a 73.70% increase of IC_{50} value was generated. And methylation of two hydroxyl groups of acteoside and isoacteoside produced 5.24 and 2.89 times of IC_{50} increase, respectively. A similar reduction of activity occurs in cinnamic acid derivatives when hydroxyl groups were methylated. Meanwhile, the acetylation of hydroxyls on glycosyl represented great reduction in ACEI activity when comparing the activities of martynoside, monacety martynoside and clerodenoside A. As mentioned, the number of hydroxyls seems to be closely related to the ACEI capacity of phenylethanoid glycosides.

3.6 The importance of the esterification position between cinnamoyls and glycosyls in phenylethanoid glycosides

In the ACEI measurements of the test compounds, the data exhibited that isomartynoside with cinnamoyl groups linked to C-6 position of central glycosyls were significantly stronger than martynoside linked to C-4 position, indicating that the esterification of cinnamoyls and glycosyls at C-4 positions might produce steric hindrance from binding to ACE. However, isoacteoside and acteoside did not display similar reduction, which could be related to the absence of substituting on hydroxyls, altering the significance of the esterification at C-4 position.

4. Conclusion

An improved UPLC-MS/MS method was established to measure ACEI potencies of phenylethanoid glycosides by quantifying the production of HA from HHL. The established method was suitable for high-throughput screening of potential ACE inhibitors isolated from herbal medicines, with obvious advantages of short analysis time (2.5 min), favorable sensitivity (LOD 0.017 and LOQ 0.056 μM for HA), high selectivity (MRM mode) and excellent reliability (validated accuracies, precisions and stabilities). By this method, *in vitro* ACEI of 21 phenylethanoid glycosides and related phenolic compounds were tested and the structure-inhibition relationships were investigated. In our study, phenolic groups especially cinnamoyl groups of phenylethanoid glycoside played important roles in the inhibition of ACE, and more hydroxyl groups and less structural steric hindrance had great influence on increasing ACEI. The result suggested that phenylethanoid glycosides exerted their ACE inhibition by chelating hydroxyl groups with Zn^{2+}. This work provided valuable

287 methodologies for screening of potential ACE inhibitors and demonstrated that
288 hydroxylation of phenylethanoids can improve the potential of these compounds as
289 antihypertensive drugs.
290
291 **Acknowledgements**
292 This work was supported by the National S&T Major Special Project
293 (2012ZX09103201-045), the National Natural Science Foundation of China
294 (81222053 and 81403070), the Program for New Century Excellent Talents in
295 University (NCET-12-1056), the "Shu-Guang Scholar" Project of Shanghai
296 Municipal Education Commission (11SG41), the Program for S&T Achievements
297 Transformation and Industrialization Project (12401900403), and China Postdoctoral
298 Science Foundation (2014M551438).

References

1 Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J, *Lancet*, 2005, 365, 217-223.

2 Guang C, Phillips RD, Jiang B, Milani F, *Arch. Cardiovasc. Dis.*, 2012, 105, 373-385.

3 Jimsheena VK, Gowda LR, *Peptides*, 2010, 31, 1165-1176.

4 Skeggs LT Jr, Marsh WH, Kahn JR, Shumway NP, *J. Exp. Med.*, 1954, 99, 275-282.

5 Ismail MA, Nabil Aboul-Enein M, Abouzid KA, Abou El Ella DA, Ismail NS, *Bioorg. Med. Chem.*, 2009, 17, 3739-3746.

6 Lin L, Lv S, Li B, *Food Chem.*, 2012, 131, 225-230.

7 Natesh R, Schwager SL, Sturrock ED, Acharya KR, *Nature*, 2003, 421, 551-554.

8 Williams TA, Corvol P, Soubrier F, *J. Biol. Chem.*, 1994, 269, 29430-29434.

9 Natesh R, Schwager SL, Evans HR, Sturrock ED, Acharya KR, *Biochemistry*, 2004, 43, 8718-8724.

10 Jao CL, Huang SL, Hsu KC, *BioMedicine*, 2012, 2, 130-136.

11 Wijesekara I, Kim SK, *Mar. Drugs*, 2010, 8, 1080-1093.

12 Kang DG, Lee YS, Kim HJ, Lee YM, Lee HS, *J. Ethnopharmacol.*, 2003, 89, 151-154.

13 Geng F, Yang L, Chou GX, Wang Z, *J. Phytother. Res.*, 2010, 24, 1088-1094.

14 Holmquist B, Bünning P, Riordan JF, *Anal. Biochem.*, 1979, 95, 540-548.

15 Wu J, Aluko RE, Muir AD, *J. Chromatogr. A*, 2002, 950, 125-130.

16 Watanabe T, Mazumder TK, Nagai S, Tsuji K, Terabe S, *Anal. Sci.*, 2003, 19, 159-161.

17 Xiao X, Luo X, Chen B, Yao S, *J. Chromatogr. B*, 2006, 834, 48-54.

18 Geng F, He YQ, Yang L, Wang Z, *Biomed. Chromatogr.*, 2010, 24, 312-317.

19 Zhang C, Wu S, Xu D, *J. Phys. Chem. B*, 2013, 117, 6635-6645.

20 Qi M, Xiong A, Li P, Yang Q, Yang L, Wang Z, *J. Chromatogr. B*, 2013, 940, 77-85.

21 Guerrero L, Castillo J, Quiñones M, Garcia-Vallvé S, Arola L, Pujadas G, Muguerza B, *PLoS One*, 2012, 7, e49493.

22 Parellada J, Suárez G, Guinea M, *J. Enzyme Inhib.*, 1998, 13, 347-359.

23 Loizzo MR, Said A, Tundis R, Rashed K, Statti GA, Hufner A, Menichini F, *Phytother. Res.*, 2007, 21, 32-36.

Figure legends

Fig. 1 Chemical structures and product ion mass spectra of (A) HA and (B) HHL.

Fig. 2 Optimization of reaction conditions. (A) incubation time, (B) pre-incubation time, (C) the concentration of NaCl, and (D, E) dissolving agent of test compounds.

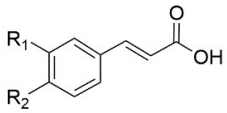
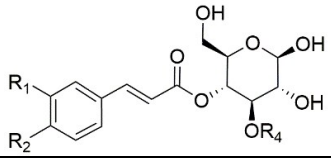
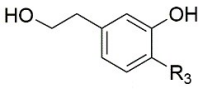
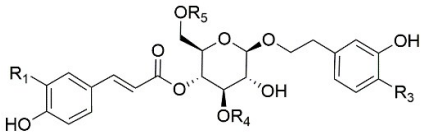
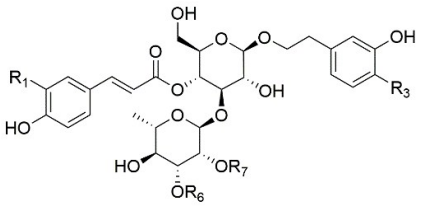
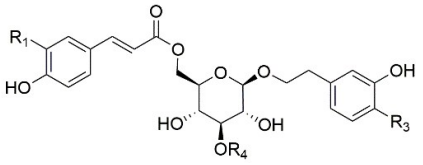
Fig. 3 UPLC-MS/MS chromatograms of HA and HHL in ACE reaction system: (A) total ionization chromatogram of reaction solution; (B) MRM chromatogram of HHL; (C) MRM chromatogram of HA.

Fig. 4 Effect of the test compounds on ACE inhibitory activities at 50 mM.

Table 1 Accuracy, precision and stability for HA quantification in UPLC-MS/MS analysis

QC samples	Added conc. (mM)	Measured conc. (mM)	Mean accuracy (%)	Precision RSD (%)		Stability RSD (%)	
				RSD (n=5)	Intra-day (n=5)	Inter-day (n=15)	Room temp. (n=6)	4 ℃ (n=6)
Low	0.056	0.054	96.43	0.953	4.342	7.343	7.350	8.433
Medium	1.143	1.123	101.8	1.176	3.993	7.921	5.148	4.753
High	28.07	27.98	99.68	1.264	1.030	1.834	2.269	6.043

Table 2 Chemical structures of the compounds used in this study

Structure	R_1	R_2	R_3	R_4	R_5	R_6	R_7	Compound
	H	H						Cinnamic acid
	OH	H						3-Hydroxy cinnamic acid*
	OH	OH						Caffeic acid*
	H	OH						4-Hydroxy cinnamic acid*
	OMet[a]	H						3-Methoxy cinnamic acid
	OMet	OMet						3,4-Dimethoxy cinnamic acid
	OH	OH		Rha[b]				Cistanoside F*
			OH					Hydroxytyrosol*
	OH		OH	Rha	H			Acteoside*
	OMet		OH	Rha	H			Leucosceptoside A*
	OMet		OMet	Rha	H			Martynoside*
	OH		OH	Glu[c]	H			Plantamajoside*
	OMet		OMet	Rha	Ara[d]			Angoroside C
	OH		OH	H	Rha			Forsythoside A*
	OH		OH	Rha	Api[e]			Forsythoside B*
	OH		OH	Rha	Glu			Echinacoside*
	OMet		OMet			Ac[f]	Ac	Clerodenoside A
	OMet		OMet			Ac	H	Monacety martynoside*
	OH		OH	Rha				Isoacteoside*
	OMet		OMet	Rha				Isomartynoside*
	OH		OH	H				Calceolarioside B*

Notes: [a] Methoxyl. [b] Rhamnose. [c] Glucose. [d] Arabinose. [e] Apiose. [f] Acetyl. * Compounds for IC_{50} measurements.

Table 3 IC_{50} values of the compounds on ACE inhibitory activities

Compound	IC_{50} values (mM)
Monacety martynoside	15.04 ± 0.04
Martynoside	11.66 ± 1.07
3-Hydroxy cinnamic acid	9.70 ± 1.08
4-Hydroxy cinnamic acid	7.53 ± 0.51
Hydroxytyrosol	6.87 ± 1.39
Isomartynoside	5.31 ± 0.43
Leucosceptoside A	3.86 ± 0.40
Forsythoside A	2.85 ± 0.71
Forsythoside B	2.61 ± 0.40
Cistanoside F	2.46 ± 0.35
Echinacoside	2.33 ± 0.20
Plantamajoside	2.28 ± 0.19
Acteoside	2.22 ± 0.21
Calceolarioside B	2.15 ± 0.20
Isoacteoside	1.85 ± 0.02
Caffeic acid	0.53 ± 0.10

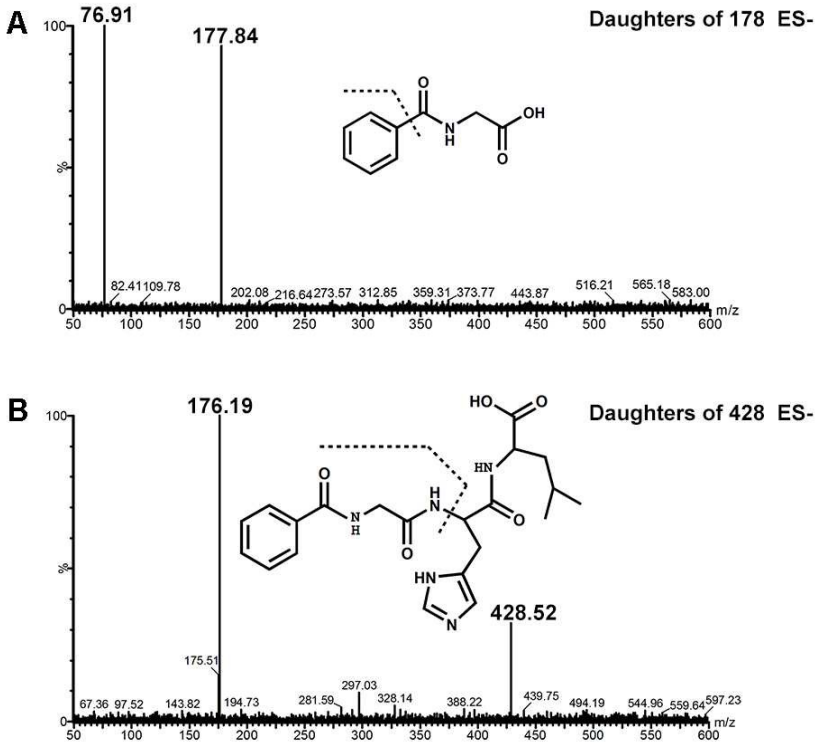


Fig. 1 Chemical structures and product ion mass spectra of (A) HA and (B) HHL.

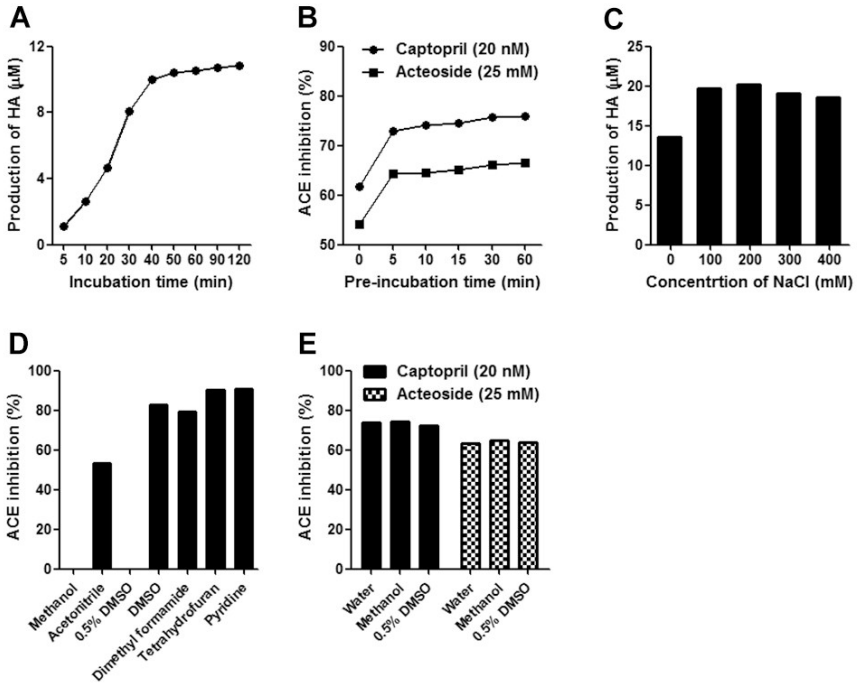


Fig. 2 Optimization of reaction conditions. (A) incubation time, (B) pre-incubation time, (C) the concentration of NaCl, and (D, E) dissolving agent of test compounds.

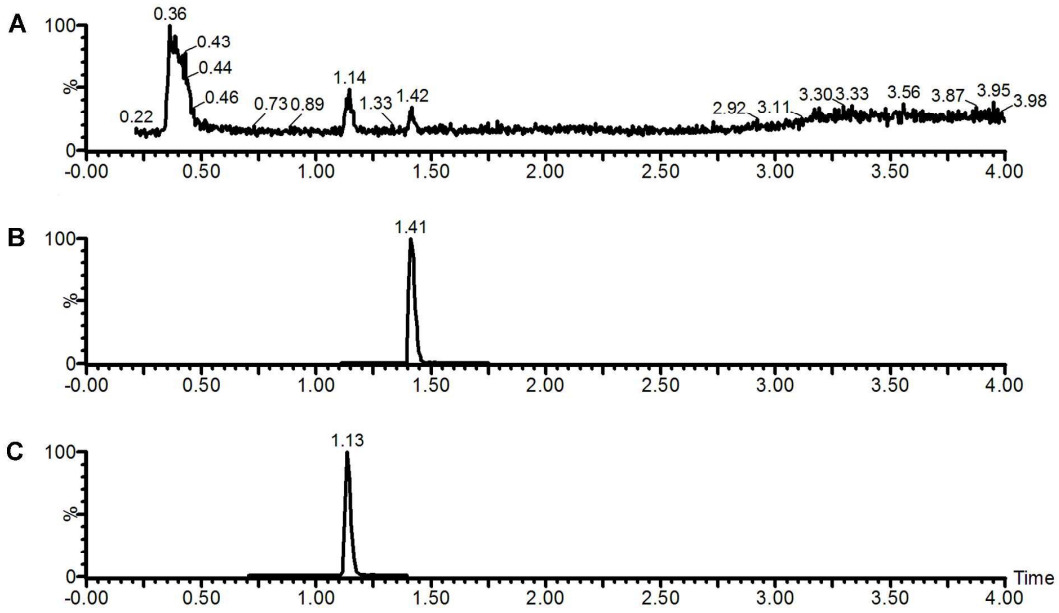


Fig. 3 UPLC-MS/MS chromatograms of HA and HHL in ACE reaction system: (A) total ionization chromatogram of reaction solution; (B) MRM chromatogram of HHL; (C) MRM chromatogram of HA.

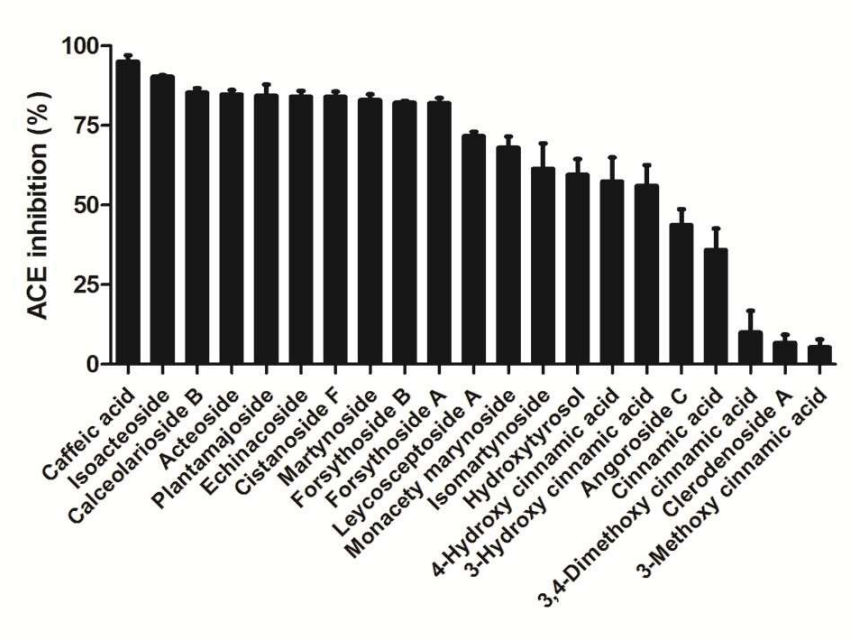


Fig. 4 Effect of the test compounds on ACE inhibitory activities at 50 mM.