Note

Angiotensin I-Converting Enzyme (ACE) Inhibitory Activity of Ursolic Acid Isolated from *Thymus vulgaris*, L.

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Received December 19, 2013 ; Accepted January 28, 2014

Thymus vulgaris L. (Thyme) is an aromatic medicinal herb widely used in folk medicine, as a food seasoning and herbal tea, and as an essential oil. A 70% EtOH extract from the leaves of thyme was prepared and a component was purified and characterized using an *in vitro* bio-assay based on the inhibition of angiotensin I-converting enzyme (ACE). Ursolic acid (1), a known pentacyclic triterpene acid, was isolated from the leaves of thyme and its structure was characterized by ¹H, ¹³C-NMR and 2D NMR spectroscopy, and by mass spectroscopy. Compound 1 showed an IC₅₀ value of 3.05 mM. This is the first study to report the ACE inhibitory activity of 1.

Keywords: ACE inhibitor, ursolic acid, *thymus vulgaris*, L.

Introduction

Hypertension is one of the most common worldwide diseases and is considered to be a central factor in strokes and heart attacks attributed to untreated high blood pressure. The inhibition of angiotensin I-converting enzyme (ACE) (EC 3.4.15.1) is an important therapeutic target in the treatment of hypertension and cardiac failure. ACE plays an important role in the regulation of blood pressure. This regulation is mediated by the enzyme cascade of the renin-angiotensin system (RAS). ACE removes histidyl-leucine from angiotensin I to form the potent vasoconstricting octapeptide, angiotensin II, which is responsible for triggering vasoconstrictive effects. Angiotensin II also stimulates the secretion of aldosterone, which increases blood pressure by promoting the retention of sodium and water. In addition, ACE degrades the nonapeptide, bradykinin, which is a potent vasodilator (Hansen *et al.*, 1996a and 1996b). ACE inhibitors could be valuable for the prevention of hypertension, and these inhibitors are in fact frequently used in therapy to reduce the morbidity and

mortality of patients with hypertension (Hou *et al.*, 2003; Tundis *et al.*, 2013). The inhibition of ACE is an effective screening method for identifying new antihypertensive agents (Lacaille-Dubois *et al.*, 2001).

Thymus vulgaris L. (Thyme) belongs to the Lamiaceae family, which is composed primarily of thyme, basil, oregano, rosemary, and sage. Thyme is an aromatic medicinal herb native to the Mediterranean region and is widely used in folk medicine, as a food seasoning, as a herbal tea, and as an essential oil. Thyme shows many activities, including antioxidant, antibacterial, antifungal, antileishmanial, anti-inflammatory, antispasmodic, immunomodulatory, and anticancer activity against the Hela cell line (Cerdeira *et al.*, 2013; Durgadevi and Kalava, 2013). Our investigation of the metabolites of this herb led to the isolation of ursolic acid (1) as an ACE inhibitor. In this study, we describe the isolation of 1 from hydroethanolic extracts of thyme leaves, and the structural identification and the ACE inhibitory activity of 1.

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Material and Methods

General experimental procedures Melting points were determined using a Yanaco MP-S3 micromelting point apparatus (Kyoto, Japan). Optical rotation data were determined with a Horiba SEPA-200 polarimeter (Kyoto, Japan). The ^1H -NMR and ^{13}C NMR spectra were recorded using a Bruker AVANCETM III 600 (Karlsruhe, Germany). EI-MS spectra were recorded using a Shimadzu GCMS-QP2010 Plus mass spectrometer (Kyoto, Japan). Silica gel [Wako Pure Chemical Industries, Ltd (Osaka, Japan); 75–150 μm] was used for column chromatography.

Chemicals Air-dried leaves of *T. vulgaris* L. were purchased from Jewel Colours Japan PT Lab. Co., Ltd (Tokyo, Japan). Apigenin, dimethylsulfoxide, hippuryl-L-histidyl-L-leucine (Hip-His-Leu), rosmarinic acid, and 2,4,6-trinitrobenzene sulfonate (TNBS) were purchased from Wako Pure Chemical Industries, Ltd. Rabbit lung angiotensin I-converting enzyme (EC 3.4.15.1) was purchased from Sigma-Aldrich Co. (St. Louis, USA). Apigenin and rosmarinic acid were used as positive controls as they had previously been reported to show ACE inhibitory activity (Li *et al.*, 2008; Shukor *et al.*, 2013).

Extraction and isolation The leaves of *T. vulgaris* L. (300 g) were extracted twice with 70% EtOH. The combined extracts were concentrated *in vacuo* and then partitioned repeatedly with a sequence of *n*-hexane, EtOAc, *n*-butanol, and distilled water. A concentrated *n*-hexane fraction (6.14 g), EtOAc fraction (7.99 g), *n*-butanol fraction (0.50 g), and distilled water fraction (4.13 g) were obtained. The EtOAc fraction was loaded onto a silica gel column and eluted with $\text{CHCl}_3/\text{MeOH}$. The fraction (2.10 g) obtained by eluting with 3% MeOH was fractionated for a second time by column chromatography on silica gel (*n*-hexane/acetone). The fraction (460 mg) obtained by eluting with 50% acetone was further fractionated for a third time by column chromatography on silica gel (*n*-hexane/EtOAc). The fraction (155 mg) obtained by eluting with 30% EtOAc was recrystallized from acetone to yield 26.1 mg of compound **1** as a colorless amorphous solid.

Compound 1 (ursolic acid): Colorless amorphous solid. mp 238–240°C. $[\alpha]_D^{25} = +72.1^\circ$ ($c = 0.1$, EtOH). ^1H NMR (600 MHz, $\text{DMSO}-d_6$): δ 0.67 (1H, m, 5-H), 0.69 (3H, s, 25-H), 0.73 (3H, s, 24-H), 0.76 (3H, s, 26-H), 0.82 (3H, d, $J = 6.5$ Hz, 29-H), 0.85 (2H, m, 7-H), 0.86 (2H, m, 16-H), 0.88 (3H, d, $J = 6.3$ Hz, 30-H), 0.90 (3H, s, 23-H), 0.92 (1H, m, 19-H), 0.95 (1H, m, 20-H), 1.00 (2H, ddd, $J = 13.0, 4.9, 2.4$ Hz, 2-H), 1.05 (3H, s, 27-H), 1.29 (1H, m, 21-H), 1.49 (8H, m, 1-H, 6-H, 9-H, 15-H, 21-H), 1.61 (2H, m, 22-H), 1.88 (2H, m, 11-H), 2.12 (1H, d, $J = 5.8$ Hz, 18-H), 3.00 (1H, m, 3-H), 5.15 (1H, d, $J = 4.9$ Hz, 12-H). ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$): δ 15.2 (q, C-25), 16.0 (q, C-26), 16.9 (q, C-24), 17.0 (q, C-29), 18.0 (t, C-6), 21.0 (q, C-23), 22.8 (t, C-11), 23.2 (q, C-27), 23.8 (t, C-16), 27.0 (t, C-15), 27.5 (t, C-2), 28.2 (q, C-30), 32.7 (t, C-21), 32.8 (t, C-7), 36.3 (s, C-10), 36.5 (t, C-22), 38.2 (s, C-8), 38.3 (t, C-1), 38.4 (d, C-19), 38.5 (d, C-20), 38.8 (s, C-4), 41.6 (s, C-14), 46.8 (d, C-9), 47.0 (s, C-17), 52.3 (d, C-18), 54.7 (d, C-5),

76.8 (d, C-3), 124.5 (d, C-12), 138.2 (s, C-13), 178.3 (s, C-28). MS (EI): m/z (%) = 456 (2) [M^+], 248 (92), 203 (40), 133 (33), 57 (88), 43 (100).

ACE inhibitory assay ACE inhibitory activity was assayed according to the method of Matsui *et al.* (1992). A mixture containing 0.03 mL of sample solution in 5% dimethylsulfoxide, 0.1 mL of 0.06 U/mL ACE, and 0.25 mL of 7.6 mM Hip-His-Leu in borate buffer (pH 8.3) containing 200 mM NaCl was incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.25 mL of 0.5 M HCl, then the solution was adjusted to pH 9.12 by adding 0.2 mL of Kolthoff buffer [0.1 M Na_2HPO_4 –1.0 M NaOH (1 : 2)], followed by 0.025 mL of 0.1 M TNBS in 0.1 M Na_2HPO_4 . After the mixture was incubated at 37°C for 20 min, 4.5 mL of 4 mM Na_2SO_3 in 0.2 M NaH_2PO_4 was added and the absorbance was measured at 416 nm with a Hitachi (Tokyo, Japan) U-1800 spectrophotometer. The percent inhibition of ACE activity was calculated according to the following equation: the inhibitory activity (%) = [(Control Abs – Control_{blank} Abs) – (Sample Abs – Sample_{blank} Abs)] / (Control Abs – Control_{blank} Abs) $\times$ 100, where control is the activity of the enzyme with added distilled water instead of sample solution and blank is the activity without the enzyme. The ACE inhibitor concentration required to inhibit 50% of the ACE activity under the assayed conditions was defined as the IC_{50} value. All experiments were performed in triplicate.

Results and Discussion

The hydroethanolic extracts of the leaves of *T. vulgaris* L. inhibited ACE activity by 36% at a concentration of 10 mg/mL. A component in the extract was purified by solvent partition, silica gel column chromatography and recrystallization to afford compound **1**.

Structural characterization was carried out using a combination of 1D ^1H , ^{13}C , DEPT, and HSQC experiments. Compound **1** was obtained as a colorless amorphous solid with mp of 238–240°C and an optical rotation of $+72.1^\circ$ ($c = 0.1$, EtOH). The ^{13}C - and ^1H -NMR spectra of **1** indicated the presence of seven methyl carbons, nine aliphatic methylene carbons, six aliphatic methine carbons, five quaternary carbons, two sp^2 carbons, and one carbonyl carbon. In the mass spectrum, fragments were observed at m/z 248 ($\text{C}_{16}\text{H}_{24}\text{O}_2$), 203 ($\text{C}_{15}\text{H}_{23}$), and 133 ($\text{C}_{10}\text{H}_{13}$), which are derived from the retro-Diels-Alder cleavages of the amyrin skeleton (Budzikiewicz *et al.*, 1963). All these data showed that **1** belongs to the ursane series of triterpenoids (Siddiqui *et al.*, 1986). Two aliphatic methine signals at δ 38.4 (C-19) and 38.5 (C-20) in the ^{13}C -NMR spectrum, and two coupling constants of 6.5 Hz (29-H) and 6.3 Hz (30-H) in the ^1H -NMR spectrum, indicated the presence of a $-\text{CH}(\text{CH}_3)-\text{CH}(\text{CH}_3)-$ system. Compound **1** was identified as ursolic acid (3 β -hydroxy-urs-12-en-28-oic-acid) by comparing its physicochemical properties with those reported earlier (Seebacher *et al.*, 2003; Taketa *et al.*, 2004; Gnoatto *et al.*, 2008) (Fig. 1).

Fig. 1. Structure of **1**

Compound **1** is a natural pentacyclic triterpene acid widely present in many plant species such as *Calluna vulgaris*, *Eribotrya japonica* L., *Eucalyptus* hybrid, *Glechoma hederacea* L., *Melaleuca leucadendron* L., *Ocimum canctum* L., *Rosmarinus officinalis* L., *Pyrola rotundifolia*, *Psychotria serpens* L., *Sambucus chinensis* L., *Solanum incanum* L., and *Tripterospermum taiwanense* (Liu, 1995), as well as *Thymus* species (Ismaili *et al.*, 2004; Janicsak *et al.*, 2006; Özgen *et al.*, 2011; Gordo *et al.*, 2012). It has been reported that fairly high concentrations of **1** are found in *T. vulgaris* L. (0.643% dry weight) (Janicsák *et al.*, 2006) and dried cranberry (0.659%) (Zhang *et al.*, 2013). The concentration of **1** ranged from trace amounts to 4.019% in 88 taxa of the Lamiaceae family (Janicsák *et al.*, 2006). Among natural products, the terpenoids represent a unique and most important class of bioactive compounds. Compound **1** exhibits biological and pharmacological properties, including antioxidant, anti-inflammatory, antihyperlipidemia, hepatoprotective, anticancer, and antimicrobial activities combined with low toxicity, and is often used in the folk medicines of many Asian countries (Liu, 1995; Zhang *et al.*, 2006; Gnoatto *et al.*, 2008; Jäger *et al.*, 2009). Additionally, **1** has been recommended for use in cancer therapy in Japan, and cosmetic preparations containing **1** have been patented in Japan for the prevention of skin cancer (Muto *et al.*, 1990; Ishida *et al.*, 1990). However, there is no information regarding the ACE inhibitory activity of **1**.

Compound **1**, apigenin, and rosmarinic acid used as a positive control were examined for their effects on rabbit lung ACE. The IC₅₀ values of **1**, apigenin, and rosmarinic acid were 3.05 mM, 2.93 mM, and 1.67 mM, respectively

(Table 1). The ACE inhibitory activity of **1** was weaker than that of rosmarinic acid, but similar to that of apigenin. ACE is a dipeptidyl carboxypeptidase in which the catalytic zinc ion is bound to the protein by a His₂Glu motif. ACE removes histidyl-leucine from angiotensin I to form the physiologically active octapeptide, angiotensin II, a potent vasoconstrictor that inactivates the vasodilating nonapeptide, bradykinin. The hydroxy groups of apigenin and the hydroxy groups and/or the carboxy group of

Table 1. IC₅₀ value of **1**, apigenin and rosmarinic acid

Compound	IC ₅₀ (mM)
1	3.05 ^b
Apigenin	2.93 ^b
Rosmarinic acid	1.67 ^a

Means with same letter are not significantly different ($P < 0.05$, Duncan's multiple-range test).

rosmarinic acid might bind to the catalytic zinc ion present at the active centre of ACE. These functional groups appear to increase the potency to inhibit ACE (Li *et al.*, 2008; Shukor *et al.*, 2013; Day and Cohen, 2013). From our results and the inhibitory activities of **1** against aromatase and protein tyrosine phosphatase 1B, it appears that the carboxy group at C-17 and/or the hydroxy group at C-3 of **1** is required for ACE inhibition, since these functional groups might bind to the catalytically functional zinc ion in the active centre of ACE (Zhang *et al.*, 2006; Gnoatto *et al.*, 2008; Tundis *et al.*, 2013). However, the ursane-type triterpenoid skeleton of **1** might contribute to reduce ACE inhibitory activity. This is the first report of the ACE inhibitory activity of **1**.

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