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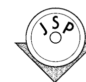
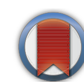

ORIGINAL PAPER

Phytochemical profile and angiotensin I converting enzyme (ACE) inhibitory activity of *Limonium michelsonii* Lincz

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Abstract Members of the genus *Limonium* are widely used as medicinal herbs due to their health-promoting effects, such as an ability to improve blood circulation by inhibiting angiotensin I converting enzyme (ACE). While the potential of *L. michelsonii* Lincz. (a medicinal plant endemic to Kazakhstan) to inhibit ACE has been demonstrated, the inhibitory activities of its secondary metabolites have not been explored. In this work, the principal phenolic compounds (**1–20**) among these metabolites were isolated to determine the components responsible for ACE inhibition. The natural abundances of the active constituents within the target plant were characterized by UPLC-Q-TOF/MS analysis. All of the isolated compounds except for gallates **10–12** were found to significantly inhibit ACE, with IC_{50} values of between 7.1 and 138.4 μM. Unexpectedly, the flavonol glycosides **16–20** were observed to be more potent than the corresponding aglycones **4** and **5**. For example, quercetin (**4**) had $IC_{50} = 30.3$ μM, whereas its glycosides (**16**, **17**) had $IC_{50} = 10.2$ and 14.5 μM, respectively. A similar trend was observed for myricetin (**5**) and its glycosides (**18–20**). In a kinetic study, the flavonols **3–5** and **16–20** and the dihydroflavonols **8** and **9** behaved as competitive inhibitors, whereas other flavones (**1**, **2**, **13–15**) and flavanones (**6**, **7**) performed noncompetitive inhibition.

Keywords *Limonium michelsonii* Lincz. · Angiotensin I converting enzyme · Phenolic metabolites · Competitive inhibitors

Abbreviations

ACE Angiotensin I converting enzyme
IC_{50} Inhibitor concentration that produces a 50% decrease in activity
K_i Inhibition constant
V_{max} Maximum velocity
K_m Michaelis–Menten constant

Introduction

Limonium michelsonii Lincz. is a plant that is native to the Almaty region of Kazakhstan and belongs to the Plumbaginaceae family. Species of the genus *Limonium*, which are distributed worldwide and total around 300, are salt-tolerant halophytes that are known as sea-lavenders or marsh-rosemaries. Among them, 18 species are found in Kazakhstan [1]. *Limonium* species are widely used in Chinese and Kazakh folk medicines to treat poor blood circulation, hemorrhage, menstrual disorders, fever, arthritis, hepatitis, alopecia, gastric ulcers, and bronchitis [2–4]. Phytochemical studies have found that members of this genus also contain complex bioactive constituents such as flavonoids, sulfated phenolics, aliphatic compounds, polysaccharides, tannins, alkaloids, terpenes, lignans, and minerals [5–10]. Plants of this genus have been reported to show various pharmacological activities, such as

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antifungal, antimalarial, antitumor, antibacterial, anti-inflammatory, and antiviral effects [11–13]. However, there is no report of a detailed phytochemical and pharmaceutical study of *L. michelsonii*.

Angiotensin I converting enzyme (ACE; EC 3.4.15.1) is a zinc metallopeptidase that plays a critical role in blood pressure control through its participation in the renin–angiotensin system (RAS) [14]. It catalyzes the conversion of the precursor angiotensin I into the potent vasoconstrictor angiotensin II, leading to increased blood pressure and a high risk of hypertension-related complications, and it lowers bradykinin production because it is a strong vasodilator [15]. Angiotensin-converting enzyme (ACE) inhibitors are among the main therapeutic agents used to treat hypertension and other cardiovascular diseases. On the other hand, ACE inhibitors also affect fibrinolysis by blocking the production of angiotensin II, decreasing the level of plasminogen activator inhibitor-1 (PAI-1) and hence improving blood circulation [16]. Indeed, the species *L. michelsonii* has traditionally been used to invigorate poor blood circulation, and its inhibitory effects on ACE have been demonstrated by assessing an *L. michelsonii* extract and compounds within it.

The work reported in the present paper was performed to elucidate the phenolic secondary metabolites of *L. michelsonii* and their ACE-inhibitory activities, and to understand the chemotaxonomy of *L. michelsonii*, all of which had not previously been explored. The work included isolating each metabolite, identifying its structure, and assigning it via UPLC-Q-TOF/MS analysis. We also tried to examine the ACE-inhibitory potential of each compound isolated and to study the kinetics of ACE under the influence of each of the compounds.

Materials and methods

Apparatus and chemicals

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM 500 (500 MHz in ^{1}H and 125 MHz in ^{13}C NMR) spectrometer (Bruker, Karlsruhe, Germany) in CD_3OD or DMSO-D_6 with TMS used as the internal standard. EIMS and HREIMS were obtained on a JEOL JMS-700 mass spectrometer (JEOL, Tokyo, Japan). Analytical-grade methanol, acetonitrile, and acetic acid for HPLC were purchased from JT Baker (Phillipsburg, NJ, USA). Column chromatography was performed over silica gel (230–400 mesh, Merck, Darmstadt, Germany), MCI GEL CHP20P (63–150 μM, Supelco, Sigma–Aldrich, St. Louis, MO, USA), and Sephadex LH-20 (Amersham Biosciences, Little Chalfont, UK). Enzymatic assays were carried out on a SpectraMax M3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA, USA). Captopril and angiotensin I converting enzyme from rabbit lung were purchased from Sigma–Aldrich. Abz-Gly-Phe(NO_2)-Pro was purchased from Bachem Feinchemikalien (Bubendorf, Switzerland). Qualitative analyses were carried out using an Agilent 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA). An UPLC system coupled with a Q-TOF/MS (from Waters Corp., Milford, MA, USA) was used for ESIMS and HRESIMS analysis. Reagent-grade chemicals were from Sigma Chemical Co. (St. Louis, MO, USA).

Plant material

Flowering whole *Limonium michelsonii* Lincz. plants were collected in June 2016 from the piedmont steppe of the Toraigir Mountains of the Almaty region and identified by Dr. Alibek Ydyrys. Specimen (No.6351) was deposited in the Herbarium of Laboratory Plant Biomorphology, Faculty of Biology and Biotechnology, Al-Farabi Kazakh National University, Almaty, Kazakhstan.

Extraction and isolation

Finely powdered whole *L. michelsonii* plant (1.1 kg) was extracted (10 L × 3) for two weeks in total at room temperature. The combined extract was evaporated under reduced pressure to give a residue (153 g) which was dissolved in water and partitioned with solvents of increasing polarity to give *n*-hexane (33.2 g), $CHCl_3$ (4.11 g), and EtOAc (17.0 g) extracts. The EtOAc extract was then separated stepwise from water to methanol using column chromatography with MCI gel to yield nine fractions (A–I). Fraction A, enriched with **10**, was further chromatographed over a SiO_2 column to yield 17.6 mg of **10**. Fraction B, enriched with **18** and **20**, was chromatographed over a Sephadex LH-20 column with methanol to afford 49.2 mg of **18** and 196.6 mg of **20**. Fraction C was repeatedly separated on a Sephadex LH-20 column with methanol and further rechromatographed over a SiO_2 column to yield compounds **9** (12.3 mg), **11** (12.0 mg), and **19** (99.6 mg). Fraction D gave compounds **13** (17.8 mg) and **16** (31.0 mg) after repeated separation on a Sephadex LH-20 column with methanol. Fractions E–F were repeatedly separated on a Sephadex LH-20 column with methanol and further purified over a SiO_2 column to yield compounds **5** (78.0 mg), **8** (79.8 mg), **12** (7.20 mg), **14** (28.0 mg), and **17** (19.1 mg). Fraction G was rechromatographed using a Sephadex LH-20 column with methanol to afford compound **7** (16.0 mg) and a precipitate enriched in **15**. The precipitate was recrystallized from methanol to give 8.5 mg of **15**. Fraction H was repeatedly separated on a Sephadex LH-20 column with methanol to

yield compounds **1** (1.8 g), **2** (9.7 mg), **3** (10.0 mg), **4** (31.5 mg), and **6** (29.3 mg). The identified structures of all the isolated compounds were confirmed by comparing them with data in the literature (see the Electronic supplementary material, ESM).

Assay of angiotensin I converting enzyme inhibitory activity

The angiotensin I converting enzyme (ACE) from rabbit lung (EC 3.4.15.1) inhibition assay performed in this work was a modified version of previous methods [28]. The ACE inhibitory activity was monitored by quantifying the reaction product Abz-Gly using fluorescence, based on the reaction between ACE and Abz-Gly-Phe(NO_2)-Pro. Initially, all of the phenolic compounds were dissolved in DMSO at 8.0 mM and then diluted to different levels. The enzymatic reaction mixture was composed of 140 µL of 150 mM Tris–HCl buffer containing 1.125 M NaCl (pH 8.3), 10 µL of the test sample or DMSO as a control, 40 µL of 0.4 mM Abz-Gly-Phe(NO_2)-Pro, and 10 µL of 0.1 U/mL angiotensin I converting enzyme in an opaque flat-bottomed 96-well plate. The reaction was incubated at 37 °C for 1 h and monitored using a spectrophotometer (excitation wavelength:320 nm; emission wavelength: 420 nm). Captopril was used as positive control. All assays were repeated more than three times. The inhibitory activity of each isolated compound was gauged by determining the concentration of it that inhibited enzymatic activity by 50%. IC_{50} values were calculated using the following equation: activity (%) = 100 [1/(1 + ([*I*]/IC_{50}))]. Kinetic parameters were determined using a Lineweaver–Burk double-reciprocal plot and a Dixon plot for increasing concentrations of substrates and inhibitors.

UPLC-Q-TOF/MS analysis

Quantification of the relative abundances of the compounds assayed in this manuscript within the ethyl acetate extract was carried out using an UPLC system (Waters Corp.) that employed a two-phase solvent system, a photodiode array detector (PDA), and an autosampler. A Q-TOP mass spectrometer was used to identify compounds in positive ESI mode. The capillary voltage of the ionization source was 3.0 kV, a temperature of 300 °C was maintained for desolvation, while a temperature of 150 °C was maintained for the ionization source. The sample and extraction cone voltages were 20 V and 0.8 V, respectively, the trap collision energy was 6.0, the trap gas flow rate was 1.5 mL/min, the ion energy was 1.0, and the collision energy was 4.0 V. Mass spectrometry data were obtained in the range *m/z* 100–650. An Acquity UPLC BEH C18 column (2.1 × 150 mm, 1.7 µm; Waters Corp.) was used. The absorbance of the eluent was scanned from 200 to 400 nm by a PDA detector and analyzed by Q-TOP MS. The solvent system was composed of A (0.1% aqueous formic acid, v/v) and B (0.1% acetonitrile formic acid, v/v) with the following gradient elution applied: 0–1 min, B: 0–5%; 1–30 min, B: 5–13%; 30–40 min, B: 13–13%; 40–50 min, B: 13–20%; 50–60 min, B: 20–23% 60–65 min, B: 23–30%. The solvent flow rate was maintained at 0.3 mL/min, and 40 °C was set as the optimum temperature for the operations.

Statistical analysis

All measurements were performed at least in triplicate. The results were subjected to variance analysis using SigmaPlot. Differences were considered significant when $p < 0.05$.

Results and discussion

Constituent characterization

The secondary metabolites of several *Limonium* species have been examined in detail previously, but, before we carried out the work reported here, the secondary metabolites of the species *L. michelsonii* had not been explored. The purpose of this study was to identify the ACE-inhibitory compounds in the target plant. The methanol extract was fractionated with different polar solvents. The high potency of the ethylacetate extract (76% inhibition, 50 µg/ml) encouraged us to identify the compounds responsible for ACE inhibition (Fig. 3a). Compounds **1–20** were isolated from the ethylacetate extract of *L. michelsonii* by silica gel, reversed-phase silica gel, and Sephadex LH-20, as described above. By analyzing spectroscopic data (including 2D NMR spectra and HRESIMS) and comparing with them with literature data, the isolated compounds were identified as apigenin (**1**), luteolin (**2**), kaempferol (**3**), quercetin (**4**), myricetin (**5**), naringenin (**6**), eriodictyol (**7**), ampelopsin (**8**), taxifolin (**9**), gallic acid (**10**), methylgallate (**11**), vanillic acid (**12**), apigenin-7-*O*-β-D-glucopyranoside (**13**), apigenin-7-*O*-β-D-glucuronide (**14**), apigenin-7-*O*-β-D-(6″-methylglucuronide) (**15**), quercetin-3-*O*-β-D-galactopyranoside (**16**), quercetin-3-*O*-α-L-arabinofuranoside (**17**), myricetin-3-*O*-β-D-galactopyranoside (**18**), myricetin-3-*O*-α-L-arabinofuranoside (**19**), and myricetin-3-*O*-(6″-*O*-galloyl)-β-D-glucopyranoside (**20**), as shown in Fig. 1 and the ESM [17–27]. Among the isolated compounds, the two glycosides **13** and **14** were found to occur in a *Limonium* species for the first time. These compounds could therefore be used to distinguish *L. michelsonii* from other *Limonium* species.

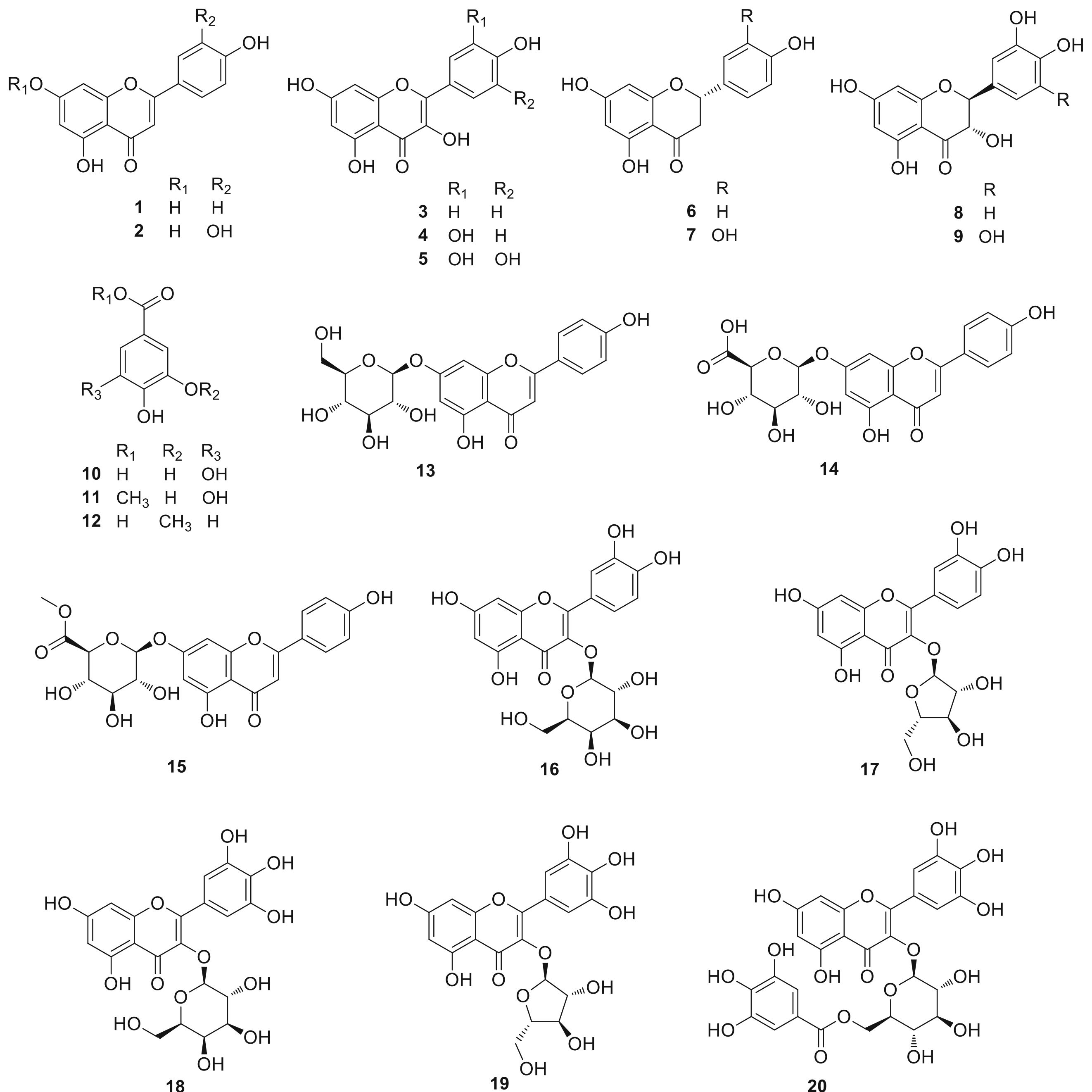

Fig. 1 Structures of the phenolic metabolites **1**–**20** from *Limonium michelsonii* Lincz

Analysis by UPLC-Q-TOF/MS

It is well known that mass spectrometry is one of the most widely used and important analytical methods for determining metabolites in complex mixtures. Specially, UPLC coupled with mass spectrometry is an excellent strategy for simultaneously identifying complex compounds in mixtures from natural sources. Therefore, phenolic compounds in the *L. michelsonii* extract were tentatively determined using UPLC-Q-TOF/MS analysis. As presented in Fig. 2, complete chromatographic separation of the various phenolic metabolites, including major and minor peaks, was achieved within 60 min at a wavelength of 254 nm. First, we obtained fifteen peaks from the EtOAc extract using ESI in positive ion mode. MS analysis of the peaks indicated exactly identical molecular ions [M^+] and elementary compositions to those displayed by the isolated compounds **1**–**7** and **13**–**20**.

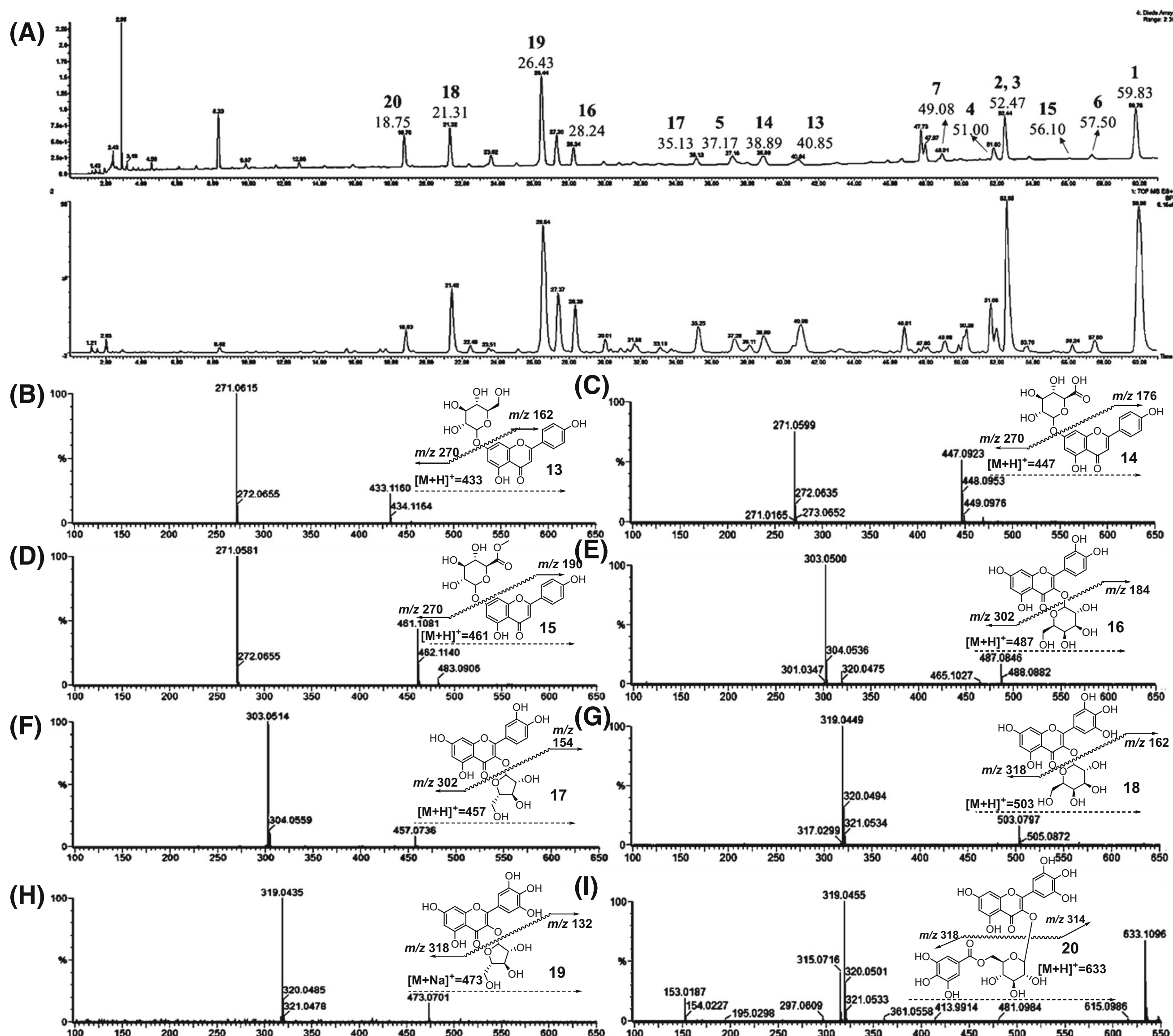

Fig. 2 **a** Typical UPLC-PDA and BPI chromatograms of the ethylacetate extract of *L. michelsonii*. **b–i** Mass fragmentation patterns for the flavonoid glycosides **13**–**20** obtained by positive-mode UPLC-Q-TOF/MS analysis

Using their elemental compositions and mass fragmentation patterns, the individual metabolites present in the extract were identified. The MS spectra of the eight glycosides **13**–**20** are displayed in Fig. 2b–i. Peaks for **13**–**15** (t_R = 40.9, 38.9, and 56.1 min) corresponded to molecular ions $[M + H]^+$ with *m/z* 433.1160 (calc. *m/z* 433.1135), *m/z* 447.0923 (calc. *m/z* 447.0927), and *m/z* 461.1081 (calc. *m/z* 461.1084). These peaks (for **13**–**15**) also presented an identical fragment ion peak at *m/z* 271.0, assigned to apigenin. Hence, the three peaks were assigned to apigenin-7-*O*-β-D-glucopyranoside (**13**), apigenin-7-*O*-β-D-glucuronide (**14**), and apigenin-7-*O*-β-D-(6″-methylglucuronide) (**15**), respectively. Similarly, the peaks for **16** and **17** (t_R = 28.2 min and 35.1 min, respectively) exhibited molecular ions $[M + Na]^+$ at *m/z* 487.0846 (calc. *m/z* 487.0852) and $[M + Na]^+$ at *m/z* 457.0736 (calc. *m/z* 457.0747). Both of these peaks presented the same ion peak at *m/z* 303.0, which typically corresponds to a quercetin fragment. The peaks were therefore assigned to quercetin-3-*O*-β-D-galactopyranoside (**16**) and quercetin-3-*O*-α-L-arabinofuranoside (**17**). Finally, three compounds (**18**–**20**; t_R = 21.3, 26.4, and 18.7 min, respectively) with molecular ions $[M + Na]^+$ at *m/z* 503.0797 (calc. for $C_{21}H_{20}O_{13}Na$ 503.0802), *m/z* 473.0701 (calc. for $C_{20}H_{18}O_{12}Na$ 473.0696), and $[M + H]^+$ at *m/z* 633.1096 (calc. for $C_{28}H_{25}O_{17}$ 633.1092) gave an identical fragment ion corresponding to the aglycone myricetin (*m/z* 319.0). Accordingly, these compounds were assigned to myricetin-

3-O-β-D-galactopyranoside (**18**), myricetin-3-O-α-L-arabinofuranoside (**19**), and myricetin-3-O-(6″-O-galloyl)-β-D-glucopyranoside (**20**), respectively. Thus, the present study provided chemotaxonomic information by exploring the UPLC and TIC patterns of the phenolic metabolites in the target plant and assigning each peak observed.

Angiotensin I converting enzyme (ACE)-inhibitory activity

The compounds isolated from the ethylacetate extract were tested for their inhibitory activities against ACE. The enzyme was assayed according to a standard procedure described in the literature by following the hydrolysis of Abz-Gly-Phe(NO_2)-Pro fluorometrically. The inhibitory profiles of all the compounds (**1**–**20**) against ACE are displayed in Table 1. Apart from the three gallates **10**–**12**, the compounds showed dose-dependent inhibitory effects against ACE (Fig. 3b). The most active compound was found to be luteolin **2**, with an IC_{50} of 7.1 μM. Interestingly, the glycosides showed much greater inhibitory activities than their aglycone parent compounds. The quercetin glycosides **16** and **17** were around threefold more potent than their parent compound, quercetin (**4**; IC_{50} = 35.3 μM): these glycoside compounds (**16**, **17**) had IC_{50} values of 10.2 and 14.5 μM, respectively. Similarly, the myricetin glycosides **18**–**20** (IC_{50} = 14.9–23.1 μM) showed more than double the potency of the aglycone myricetin **5** (IC_{50} = 40.9 μM). The higher activities of glycosides **16**–**20** can be attributed to the presence of sugar moieties on the C ring. However, enhanced enzyme inhibitory activities were not observed for the apigenin glycosides **13**–**15**, in which the sugar moieties are attached to the A ring (Fig. 1).

Table 1 Inhibitory effects of phenolic metabolites on angiotensin I converting enzyme (ACE)

Compound	IC_{50}^{a} (μM) for ACE	Inhibition mode (K_i^b, μM)
1	21.2 ± 0.2	Noncompetitive (24.8 ± 0.4)
2	7.1 ± 0.2	Noncompetitive (6.3 ± 0.1)
3	35.3 ± 0.7	Competitive (16.4 ± 0.3)
4	30.4 ± 0.2	Competitive (17.5 ± 0.5)
5	40.9 ± 0.8	Competitive (27.7 ± 0.7)
6	83.6 ± 0.6	Noncompetitive (92.7 ± 0.8)
7	62.3 ± 0.5	Noncompetitive (52.7 ± 0.9)
8	114.8 ± 0.9	Competitive (52.1 ± 0.6)
9	138.4 ± 0.8	Competitive (70.4 ± 0.7)
10	>200	NT[c]
11	>200	NT[c]
12	>200	NT[c]
13	37.5 ± 0.4	Noncompetitive (39.7 ± 0.2)
14	27.6 ± 0.8	Noncompetitive (26.2 ± 0.7)
15	54.7 ± 0.4	Noncompetitive (54.2 ± 0.1)
16	10.2 ± 0.6	Competitive (6.2 ± 0.2)
17	14.8 ± 0.5	Competitive (6.9 ± 0.7)
18	20.3 ± 0.4	Competitive (10.4 ± 0.2)
19	23.1 ± 0.7	Competitive (13.5 ± 0.5)
20	14.9 ± 0.3	Competitive (5.9 ± 0.1)
Captopril[d]	0.2 ± 0.05	NT[c]

[a] All compounds were examined in a set of experiments repeated three times; the IC_{50} value of each compound represents the concentration of it that caused a 50% drop in enzyme activity

[b] Inhibition constant

[c] Not tested

[d] Positive control

Analysis of enzyme kinetics

Studies of the kinetics of the enzyme under the influence of the inhibitors were performed. A similar relationship of inhibitor concentration to enzyme activity was seen regardless of the inhibitor considered. Relevant data for the most potent glycoside, compound **16**, are illustrated in Fig. 4a–c. Plotting the residual enzyme activity versus the enzyme concentration for compound **16** gave a family of straight lines with the same y-axis intercept, indicating that **16** is a reversible inhibitor (Fig. 4a). The inhibition mode was analyzed using a Lineweaver–Burk plot, which showed that compound **16** behaved as a competitive inhibitor because increasing the concentration of the inhibitor resulted in a family of lines which declined and intercepted the y-axis at the same point (Fig. 4b). This analysis showed that the inhibitor performed typical competitive inhibition in which V_{max} remained constant while K_m increased. Interestingly, all of the flavonols **3**–**5** and **16**–**20** and the dihydroflavonols **8** and **9** showed competitive inhibitory modes (Table 1; Fig. 5d–f), whereas the flavones **1**, **2**, and **13**–**15** and the flavanones **6** and **7** were found to be noncompetitive inhibitors (Fig. 5a–c). Analysis showed that, in the presence of increasing concentrations of those compounds, V_{max} decreased but K_m did not change: as can be seen in the graph, $1/K_m$ (the x-intercept) was unaffected by inhibitor concentration, whereas $1/V_{max}$ became more positive with increasing inhibitor concentration (Fig. 5). The inhibition kinetics were explored by analyzing Dixon plots, which were obtained by plotting $1/V$ versus [I] for various substrate concentrations. The Dixon plots gave a family of straight lines that passed through the same point in the second quadrant of the graph, yielding the inhibition constant (K_i). The K_i value for compound **16** was measured from Fig. 4c as 6.2 μM. The values of the inhibition constant (K_i) for the other inhibitors were determined using a similar analysis; these values are displayed in Table 1.

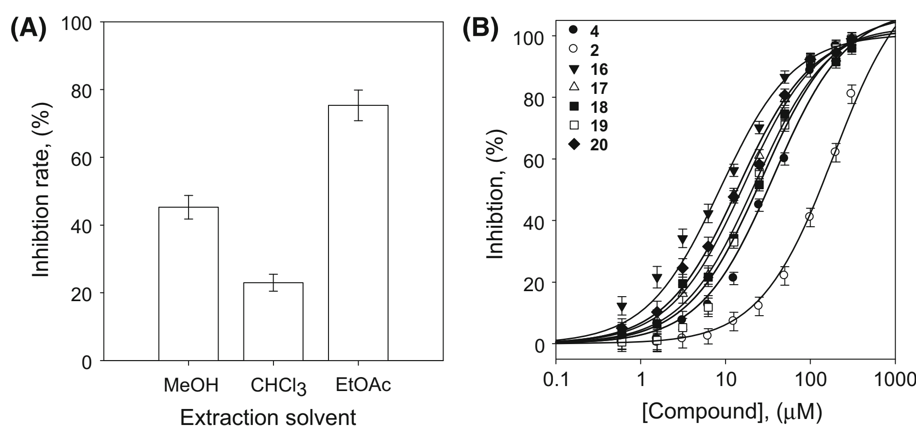

Fig. 3 **a** Inhibition of ACE activity caused by different extracts from *L. michelsonii*. **b** Inhibitory effects of compounds **4**, **9**, and **16–20** on ACE activity

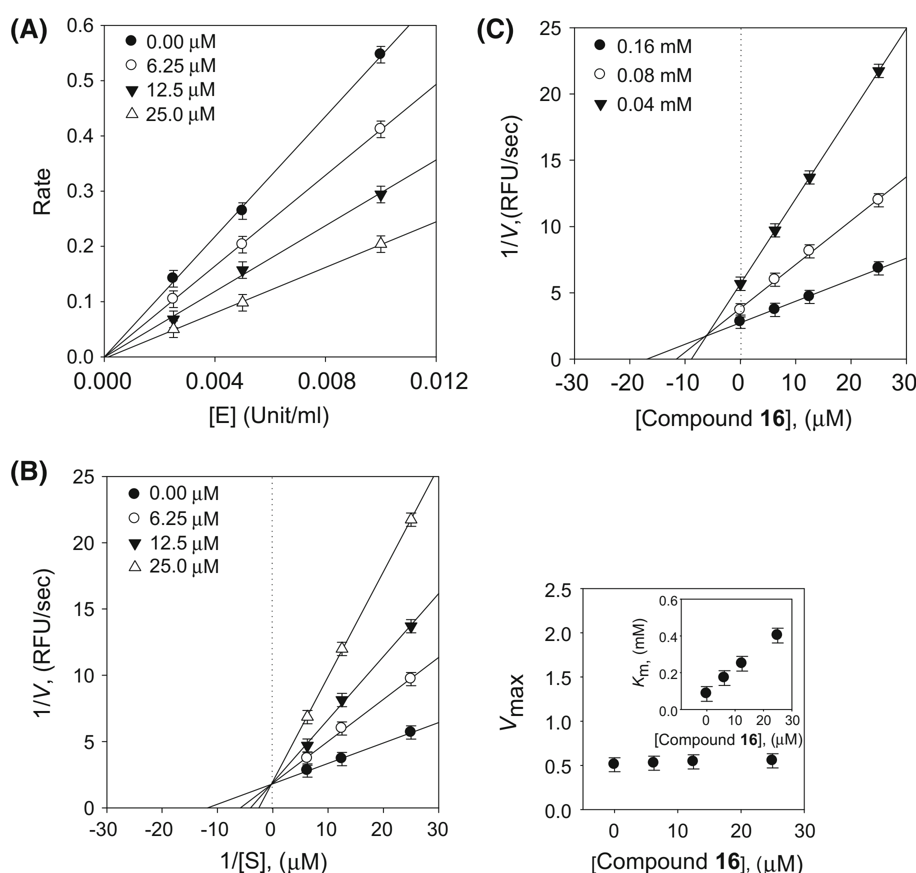

Fig. 4 Graphical presentation of the kinetic study. **a** Reversible ACE inhibition by compound **16** at various concentrations. **b** Lineweaver–Burk plot for ACE inhibition by **16**. *Inset* replots of the concentration of inhibitor **16** versus V_{max} and K_m. **c** Dixon plot for ACE inhibition by **16**

Conclusions

We have established that the whole *L. michelsonii* plant is a rich source of ACE inhibitors. Twenty phenolic compounds (**1–20**) were isolated from the EtOAc extract of this plant, and most of those compounds showed significant inhibitory activity against ACE ($IC_{50} = 7.1–138.4$ μM). Interestingly, the glycoside compounds **16–20** presented better ACE inhibition than the corresponding aglycones **4** and **5**. In a kinetic study, flavonols and dihydroflavonols behaved as competitive inhibitors, whereas other flavones and flavanones performed noncompetitive inhibition.

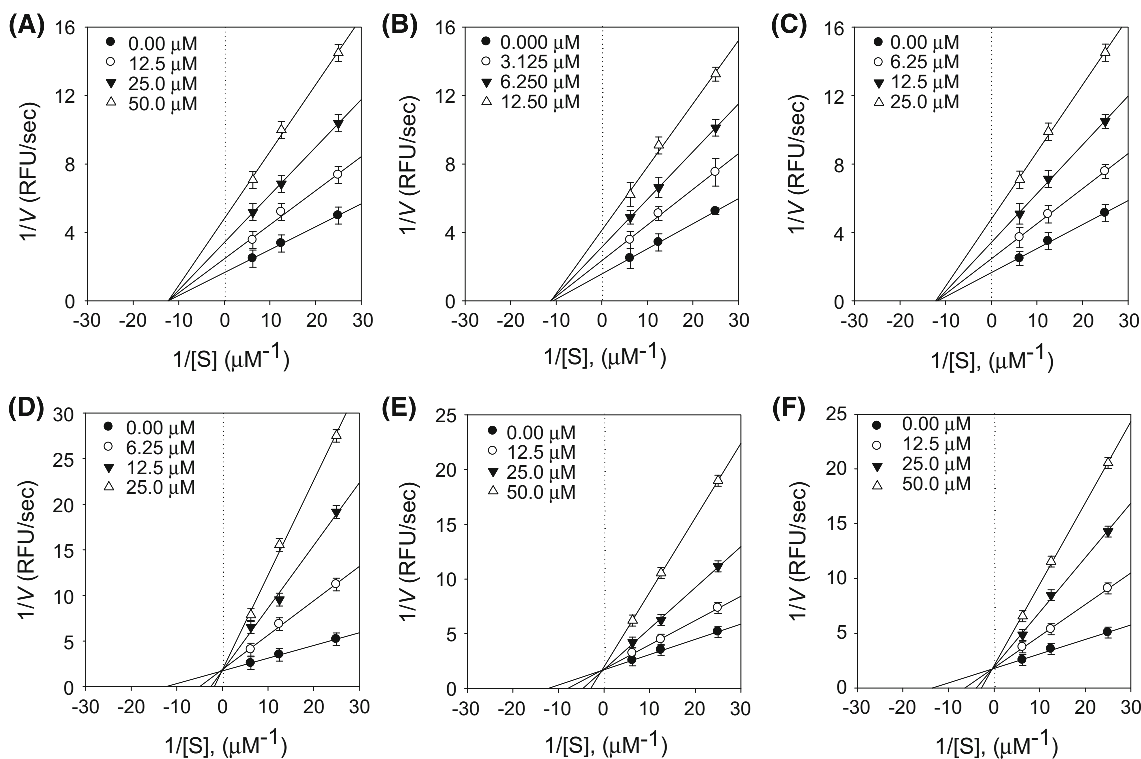

Fig. 5 Lineweaver–Burk plots for ACE inhibition by compounds **1** (**a**), **2** (**b**), **14** (**c**), **17** (**d**), **19** (**e**), and **20** (**f**), respectively

Importantly, a detailed analysis of the phenolic metabolites of *L. michelsonii* is reported here for the first time. Individual compounds in the EtOAc extract of *L. michelsonii* were assigned using UPLC-Q-TOF/MS.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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