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Original Article

Chemical investigation of *Hyptis suaveolens* seed, a potential antihyperuricemic nutraceutical, with assistance of HPLC-SPE-NMR



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ABSTRACT

The seed of *Hyptis suaveolens*, commonly known as wild flour ball (san fen yuan) in Taiwan, serves as a main refreshing drink substance in several regions. This study investigated firstly its secondary metabolites, leading to the isolation of five major caffeoylquinic acid derivatives (1–5) from the ethanol extract. In addition, ten minors, including three caffeoylquinic acid derivatives (12–14), were characterized via assistance of HPLC-SPE-NMR. Of these isolates, sodium 4,5-dicaffeoylquininate (2) and methyl 3,5-dicaffeoylquininate (4) showed moderate inhibitory activity against xanthine oxidase with the respective IC_{50} values of 69.4 μ M and 92.1 μ M (c.f. allopurinol IC_{50} 28.4 μ M). Quantitative HPLC analysis of the EtOH extract indicates the content of sodium 3,5-dicaffeoylquininate (1) and sodium 4,5-dicaffeoylquininate (2) to be 0.1% and 0.08% (w/w, dry seed), respectively. This study not only discloses the bioactive constituents, but also demonstrates the potential of *H. suaveolens* seed as an antihyperuricemic nutraceutical.

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1. Introduction

Hyptis suaveolens (L.) Poir. is distributed worldwide in the tropical regions [1], and its seed, known as pignut or chan, has been used for preparation of refreshing drinks in several countries such as Mexico and Taiwan. Like psyllium seed, it will swell while immersing in water and appear like flour ball. Thus, it is called “wild flour ball” in Taiwan. The ethanol extract of *H. suaveolens* aerial part has been demonstrated to possess gastro-protective activity *in vivo* [2], and the essential oils from its leaf displayed antimicrobial [3] and insecticidal

activities [4,5]. Past chemical studies on *H. suaveolens* seed mainly focused on the protein [6], and oligo- and polysaccharides [7,8]. The bioactive constituents of small molecules in this material, however, required investigation.

Gout is a metabolic disorder associated with hyperuricemia caused by overproduction or underexcretion of uric acid. As uric acid is produced via oxidation of xanthine/hypoxanthine by xanthine oxidase, mainly in intestine and liver, the inhibition of this key enzyme can lower uric acid level, leading to the prevention of recurrent gout [9]. Natural products have been considered as a potential source of healthy supplement.

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Recently, our lab has isolated several unusual phenylpropanoids from aerial part of *Hyptis rhomboids* and these isolates, e.g. hyprhombins B & C, epihyprhombin B, and netpetoidin B, showed good inhibitory activity against xanthine oxidase [10]. This current study was aimed to disclose whether the seed of a related plant, *H. suaveolens* (L.) Poir., contained similar constituents, hence beneficial for hyperuricemic persons.

The high performance liquid chromatography-solid phase extraction-nuclear magnetic resonance (HPLC-SPE-NMR) hyphenation has been applied in natural product investigation for years [11–13]. This hyphenation is powerful to screen the chemical constituents of the plants because of its high efficiency in separation and analysis (HPLC), provision of informative data for structural characterization (NMR), and cumulative compound trapping, removal of eluent interference (SPE) to enhance the sensitivity for NMR measurement.

Herein we report our effort in isolation and identification of quinic acid derivatives possessing xanthine oxidase inhibitory activity from *H. suaveolens* seeds via the assistance of HPLC-SPE-NMR.

2. Material and methods

2.1. Instrumentation

Optical rotations were obtained on a JASCO P-2000 polarimeter (Hachioji, Tokyo). UV spectra (MeOH) were measured on a Hitachi U-2900 double-beam spectrophotometer (Hitachi, Japan). Electron circular dichroic (CD) spectra (MeOH) were measured on a JASCO J-720 spectropolarimeter (Hachioji, Tokyo). NMR spectra were recorded by Bruker AV-400, and AV III-600 (CD_3OD , δ_{H} 3.30 and δ_{C} 49.0 ppm). HPLC-SPE-NMR (600 MHz), composed of an Agilent 1100 liquid chromatograph (Waldbronn, Germany), a Phenomenex Prodigy ODS3 (C-18) 100 Å (250 × 4.6 mm, 5 μm) column, coupled with a diode array detector (DAD, G1315A) and a Knauer K120 HPLC pump (makeup pump), a Prospekt 2 automated solid-phase extraction unit (Spark Holland, Emmen, Holland), containing 192 HySphere resin GP cartridges (10 × 2 mm, 10–12 μm), connecting to a Gilson Liquid Handler 215 automated tube transfer (TT) system (Gilson, Inc., Middleton, WI, USA), and a Bruker AV III-600 spectrometer. HPLC-ESIMS (electrospray ionization mass spectrometry) was performed on an Agilent 1100 series liquid chromatograph, followed by an Esquire 2000 mass spectrometer (Bruker Daltonics, Germany). TLC analysis was carried out on silica gel plates (KG60-F254, Merck).

2.2. Plant material

The seeds of *H. suaveolens* (L.) Poir. were purchased from Yuan-Fong grain store in Zhongzheng District, Taipei, Taiwan in December, 2017. The voucher specimen (NTUSP10612A) was authenticated by the author (S.S.L.).

2.3. Extraction and isolation

The dry milled seeds (1.2 kg) of *H. suaveolens* immersed in 95% EtOH (3 × 3 L) were stirred at room temperature for 12 h, and at

50 °C for another 12 h, then filtered. The EtOH extract (30.9 g) was yielded after evaporation of the combined filtrate under reduced pressure at 40 °C. The suspension of the ethanol extract (30.4 g) in distilled water (150 mL) was partitioned against dichloromethane, ethyl acetate, and *n*-butanol sequentially, each 150 mL for three times, to give the corresponding fraction soluble in CH_2Cl_2 (14.6 g), EtOAc (1.4 g), *n*-BuOH (3.1 g), and H_2O (11.2 g) after evaporation. Both EtOAc- (1.2 g) and *n*-BuOH- (1.3 g) soluble fractions were separated on a Sephadex LH-20 column (2.5 cm × 75.0 cm; MeOH) to give 11 subfractions (frs. E1–11) and nine subfractions (frs. B1–9), respectively, combined on the basis of TLC analysis. Frs. E6 (162.7 mg) and B6 (73.7 mg) were 1, E8 (56.5 mg) was 2, and B8 (18.9 mg) was 3.

Separation of an aliquot of fr. E5 (135.8 mg out of 308.4 mg) on a Lobar Lichrospher RP-18 Type A column (240 × 10 mm, 40–63 μm ; Merck, Germany), eluted by CH_3CN -0.1% $\text{HOAc}_{(\text{aq})}$ 23:77 with a flow rate of 2.2 mL min⁻¹, yielded five fractions (frs. E5-1–5). Frs. E5-2 (6.7 mg), E5-3 (78.8 mg), and E5-5 (22.4 mg) were compounds 3, 1, and 4, respectively. Separation of fr. E5-4 (5.9 mg) on a semi-preparative RP-18 HPLC column (Phenomenex Prodigy ODS3 100 Å, 250 × 10 mm, 5 μm) (10 × 50 μL ; 5.9 mg/0.5 mL), delivered by CH_3CN -0.1% $\text{HCO}_2\text{H}_{(\text{aq})}$ 24:76 with a flow rate of 2.2 mL/min and monitored at 280 nm, gave 5 (1.6 mg, t_{R} : 34.1 min).

2.4. HPLC conditions used in HPLC-SPE and HPLC-ESIMS for frs. E3, E4, and B5

Chemical constituents in the minor fractions, frs. E3 (1.2 mg), E4 (3.3 mg), and B5 (3.1 mg), were characterized by HPLC-SPE-TT-NMR and HPLC-ESIMS. HPLC separation was carried out on an analytical RP-18 column as indicated above. The HPLC conditions were as follows: delivery system, CH_3CN -0.1% $\text{HCO}_2\text{H}_{(\text{aq})}$ 5% to 21% in 40 min (linear gradient), 21% for 2 min, to 95% in 1 min (linear gradient), and 95% for 12 min, for separation of fr. E3; CH_3CN -0.1% $\text{HCO}_2\text{H}_{(\text{aq})}$ 5% to 23% in 20 min (linear gradient), 23% for 50 min, for separation of fr. E4; CH_3CN -0.1% $\text{HCO}_2\text{H}_{(\text{aq})}$ 5% to 22% in 20 min (linear gradient), 22% for 20 min, to 28% in 1 min (linear gradient), 28% for 7 min, for separation of fr. B5; all with a flow rate of 0.5 mL min⁻¹ and detection at 280 nm. ESIMS data were acquired under the following settings: negative mode, nebulizer pressure 15 psi, drying gas 10 L min⁻¹ at 250 °C.

2.5. SPE-TT-NMR procedures

After the HPLC separation, the eluate was added water by a make-up pump with a flow rate of 1.2 mL min⁻¹, and each compound peak was passed through an individual HySphere-Resin GP cartridge (10 × 2 mm, 10–12 μm) in the Prospekt 2 automated solid-phase extraction unit. This HPLC-SPE process was repeated six to seven times. The concentration of each sample and the volume of injection per HPLC run are shown in supplementary data (Figs. S1–3). Each compound loaded cartridge was flushed with dry nitrogen stream for 30 min to remove the eluent residue and the trapped compound in the cartridge was transferred into a 2-mm NMR tube with CD_3OD by a Gilson Liquid Handler 215 automated tube transfer (TT) system.

The NMR tubes were then placed on an automatic NMR tube exchanging system to record NMR spectra by a Bruker AV III-600 spectrometer using a multiple solvent suppression pulse program at 298 K.

2.6. Measurement of circular dichroic spectroscopy

After the measurement of NMR spectra as indicated above, each sample solution was evaporated under reduced pressure at 40 °C to give a dry residue which was redissolved in MeOH (5 mL) for CD measurement. The settings for CD measurement were as follows: step resolution 0.1 nm, scan speed 50 nm min⁻¹, response 0.25 s, and sensitivity 200 mdeg.

2.7. Quantitative analysis of dicaffeoylquinic acids (1–3) in EtOH extract

The reference compounds 1–3 were isolated as described above (Section 2.3). Each reference compound was weighed accurately, dissolved in MeOH, and diluted to five concentrations (1000, 500, 100, 50, 10 µg mL⁻¹) to establish linear regression curve. The EtOH extract of *H. suaveolens* seed, obtained as described in Section 2.3, was weighed accurately, and prepared as the 5.0 mg mL⁻¹ solution (MeOH). The quantitative RP-18 HPLC analysis was carried out on the same HPLC equipment as described above. The HPLC conditions were as follows: delivery system, CH₃CN-0.1% HCO₂H_(aq) 24% for 25 min, to 95% in 1 min (linear gradient), and 95% for 9 min; flow rate, 0.5 mL min⁻¹; detection 280 nm. Each concentration of the reference compounds and the EtOH extract of *H. suaveolens* seed were analyzed in triplicate, and the injection volume of each analysis was 10 µL. The regression curve was established by mean peak areas (mAu × s) versus the amounts (µg) of reference compound, and the linearity was evaluated by coefficient of correlation (R²).

2.8. Xanthine oxidase inhibitory bioassay

The bioassay of fractions and compounds against xanthine oxidase was carried out by a method modified from a report [14]. To the corresponding well in a 96-well plate was added vehicle (10 µL, MeOH–H₂O 1:9, v/v) or sample in vehicle (10 µL), and 0.5 mM xanthine (Sigma) solution (60 µL), then xanthine oxidase (30 µL, 0.05 U mL⁻¹) (EC 1.2.3.2, bovine milk, Sigma) was added to start the reaction. The produced uric acid was determined by measuring the absorbance at 290 nm on a Microplate spectrophotometer SPECTRAMax[®] PLUS (Molecular Devices) at 2 min intervals. The inhibitory percentage against xanthine oxidase (%) was calculated by the following equation: inhibition (%) = 100 × [1 – (A_{sample} – A_{blank})/ (A_{control} – A_{blank})]. Compounds 2 and 4 with better inhibitory activity were selected to determine the IC₅₀ value. The IC₅₀ value was determined by the dose–response curve (Sigmoid regression) of six concentrations (10, 20, 50, 100, 200, and 500 µM) of each test sample in triplicate, using log value of concentration as x-axis and inhibition percentage as y-axis. Allopurinol (Synmosa Biopharma Corporation) was used as a positive control whose IC₅₀ values were found to be 28.4 ± 1.1 µM.

3. Results and discussion

3.1. Isolation and structural characterization of major dicaffeoylquinic acid derivatives

The 95% ethanol extract of *H. suaveolens* seeds was divided into fractions soluble in CH₂Cl₂, EtOAc, *n*-BuOH, and H₂O via liquid–liquid partitioning process. Through xanthine oxidase inhibition assay, the EtOAc soluble fraction showed the best inhibitory activity, up to 52.90% at 100 µg mL⁻¹ (Fig. 1). Separation of this bioactive fraction on Sephadex LH-20 and C-18 columns afforded five compounds (1–5) (Fig. 2).

These five compounds contained a dicaffeoylquinic acid moiety in common, as reflected by the ¹H NMR spectra, displaying two sets of AMX system in the aromatic region, and two sets of *trans*-coupling system for olefinic protons (1: H-7', δ 7.61, d, *J* = 15.9 Hz; H-7'', δ 7.57, d, *J* = 15.8 Hz; H-8', δ 6.34, d, *J* = 15.9 Hz; H-8'', δ 6.26, d, *J* = 16.0 Hz), designating to two *trans*-caffeoyl residues; two double triplets and a double doublet for three oxymethine protons (1: H-3, δ 5.42, dt, *J* = 6.8, 3.7 Hz; H-5, δ 5.38, dt, *J* = 4.7, 7.2 Hz; H-4, δ 3.96, dd, *J* = 7.4, 4.7 Hz), and four double doublets belonging to four protons of two methylenes (1: H-2a, δ 2.31, dd, *J* = 13.9, 3.8 Hz; H-6a, δ 2.24, dd, *J* = 13.6, 7.6 Hz; H-6b, δ 2.20, dd, *J* = 14.0, 4.1 Hz; H-2b, δ 2.15, dd, *J* = 13.9, 6.9 Hz), characteristic for a quinic acid residue (Table 1). The coupling relationship of these protons was verified by analysis of the COSY spectra (Figs. S4–6, Supplementary data). Compound 1 was identified as sodium 3,5-dicaffeoylquinic acid [15], supported by the ¹H NMR spectrum, showing downfield shifted H-3 and H-5 in the quinic acid moiety, δ 5.42 and 5.38 ppm, and the ESI-MS data ([M+Na]⁺ at *m/z* 561.3, [M+H]⁺ at *m/z* 539.1, [M–H][–] at *m/z* 536.9, and [M–Na][–] at *m/z* 514.9). Compound 2 was identified as sodium 4,5-dicaffeoylquinic acid [16], supported by the ¹H NMR spectrum, showing downfield shifted H-4 and H-5 in the quinic acid moiety, δ 5.11 (dd, *J* = 8.9, 3.0 Hz) and 5.61 (dt, *J* = 5.0, 9.1 Hz) (Supplementary data). Compound 3 was identified as sodium 3,4-dicaffeoylquinic acid [16], supported by the ¹H NMR spectrum, showing downfield shifted H-3 and H-4 in the quinic acid moiety, δ 5.63 (dt, *J* = 4.0, 3.8 Hz) and 5.00 (dt, *J* = 8.8, 3.1 Hz) (Supplementary data). These three compounds are present as sodium salt as supported by their ESI-MS data ([M+Na]⁺ at *m/z* 561.3, [M+H]⁺ at *m/z* 539.1, [M–H][–] at *m/z* 536.9, and [M–Na][–] at *m/z* 514.9) (Supplementary data).

Compounds 4 and 5 were identified as the corresponding methyl ester of 1 and 3, respectively, as verified by almost identical ¹H NMR spectrum except for the presence of an additional methyl singlet at δ 3.68 and 3.75 (Supplementary data) [17,18]. The ¹³C NMR spectra of 4 and 5, showing a respective methyl signal at δ 53.0 (4) and 52.9 (5), and ESI-MS, both showing [M–H][–] at *m/z* 529.0, also supported this structure elucidation.

These major dicaffeoylquinic acids have been found in several food sources, such as kumquat, passion fruit, sweet granadilla [19], and sweet potato [20], and have been reported to possess several bioactivities *in vitro*, e.g. antioxidant, α-glucosidase inhibitory activity [21], and aldose reductase inhibitory activity [22]. Besides, these derivatives also display hypotensive [23] and anti-thrombotic [24] effects *in vivo*. The

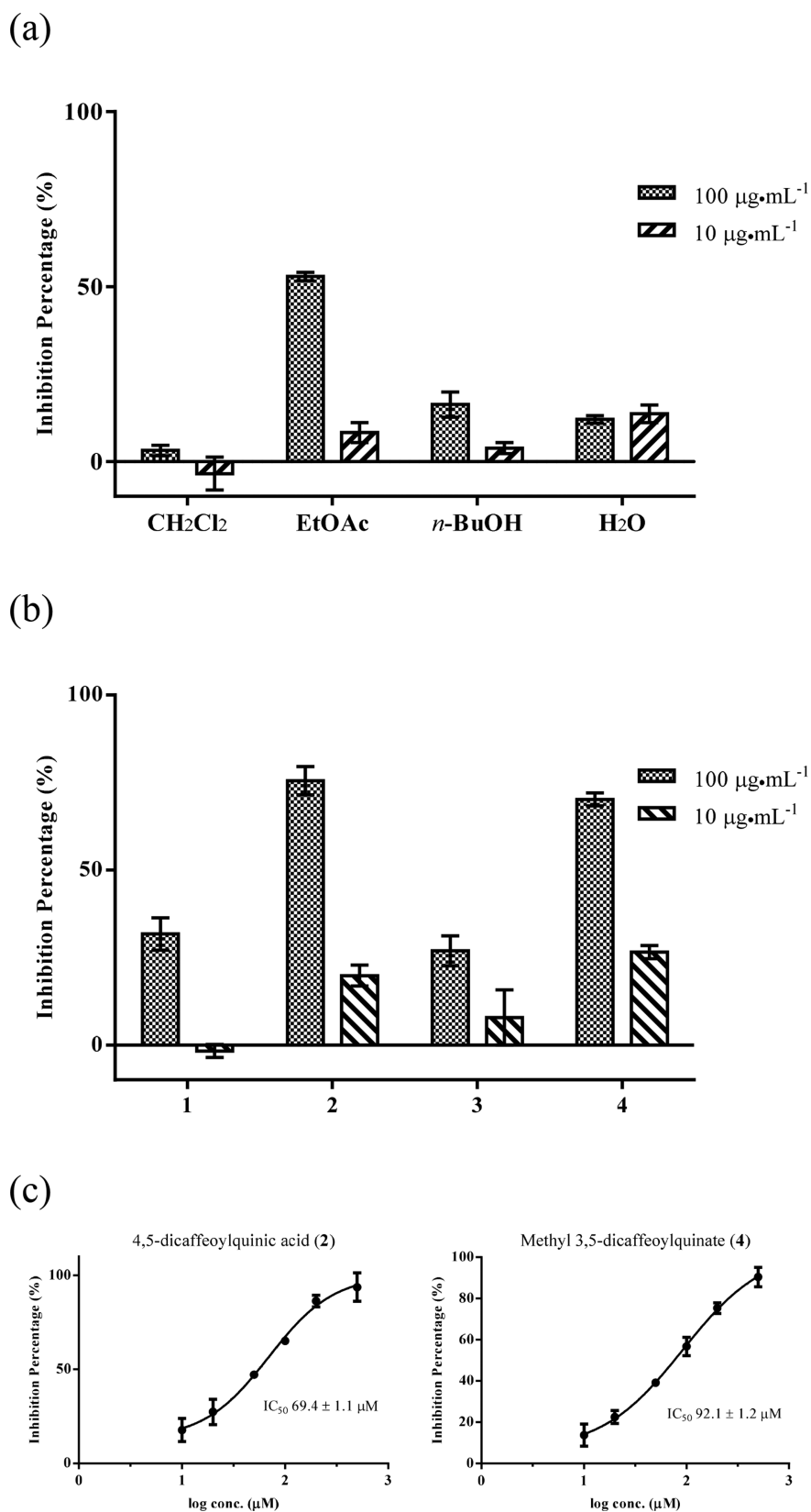
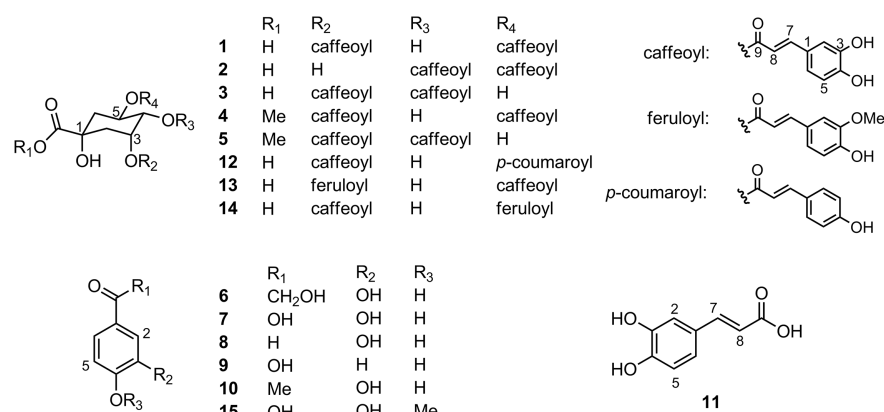


Fig. 1 – Inhibitory activity of the *H. suaveolens* seeds against xanthine oxidase (allopurinol IC₅₀ 28.4 ± 1.1 µM): (a) the EtOH extract, soluble in CH₂Cl₂, EtOAc, *n*-BuOH, and water (100 and 10 µg mL⁻¹); (b) compounds 1–4; (c) dose–response curve and IC₅₀ values of compounds 2 and 4.

Fig. 2 – Structures of compounds 1–15 from *H. suaveolens* seeds.Table 1 – ^1H NMR spectroscopic data (δ /ppm, m, J) of compounds 1 and 12–14.

Proton	1 ^a	12 ^b	13 ^b	14 ^b
2	2.31 dd (13.9, 3.8) 2.15 dd (13.9, 6.9)	2.31 dd (13.7, 3.7) 2.15 dd (13.4, 7.1)	2.31 dd (13.9, 3.5) 2.16 dd (13.8, 6.8)	2.32 dd (13.8, 3.8) 2.15 dd (13.6, 6.7)
3	5.42 dt (6.8, 3.7)	5.42 dt (6.8, 3.7)	5.43 dt (6.5, 3.5)	5.43 dt (7.0, 3.7)
4	3.96 dd (7.4, 3.4)	3.97 dd (7.3, 3.2)	3.97 dd (7.4, 3.2)	3.97 dd (7.2, 3.2)
5	5.38 td (7.2, 4.7)	5.38 td (6.7, 4.4)	5.39 m	5.38 td (7.1, 4.1)
6	2.24 dd (13.6, 7.6) 2.20 dd (14.0, 4.1)	2.24 dd (13.9, 7.3) 2.20 dd (13.9, 3.6)	2.24 m 2.20 m	2.25 dd (13.8, 7.2) 2.20 dd (13.5, 3.8)
2'	7.06 d (2.2)	7.06 d (1.9)	7.21 d (1.6)	7.06 d (2.0)
3'-OMe	—	—	3.90 s	—
5'	6.771 d (8.2)	6.771 d (8.2)	6.81 d (8.1)	6.771 d (8.2)
6'	6.96 dd (8.3, 2.0)	6.96 dd (8.2, 1.9)	7.08 dd (8.1, 1.6)	6.96 dd (8.2, 2.0)
7'	7.61 d (15.9)	7.61 d (16.1)	7.67 d (15.9)	7.61 d (15.9)
8'	6.34 d (15.9)	6.34 d (15.8)	6.44 d (15.9)	6.34 d (15.9)
2''	7.05 d (2.2)	7.48 d (8.6)	7.05 d (2.0)	7.21 d (1.7)
3''	—	6.80 d (8.6)	—	—
3''-OMe	—	—	—	3.90 s
5''	6.773 d (8.2)	6.80 d (8.6)	6.774 d (8.2)	6.81 d (8.2)
6''	6.97 dd (8.3, 2.0)	7.48 d (8.6)	6.97 dd (8.2, 1.9)	7.10 dd (8.2, 1.7)
7''	7.57 d (15.8)	7.64 d (16.1)	7.57 d (15.8)	7.63 d (15.8)
8''	6.26 d (16.0)	6.32 d (15.8)	6.26 d (15.8)	6.35 d (15.9)

^a Data obtained from analyzing the general ^1H NMR spectra (CD_3OD , 600 MHz).^b Data obtained from analyzing the ^1H NMR spectra using a multiple solvent suppression pulse program (CD_3OD , 600 MHz).

current chemical investigation demonstrates that *H. suaveolens* seed contains several bioactive ingredients beneficial to health.

3.2. Identification of minors by assistance of HPLC-SPE-NMR and HPLC-MS

As the amounts of two bioactive fractions (frs. E3 and E4) from Sephadex LH-20 column were limited, HPLC-DAD-SPE-TT-NMR hyphenation assisted by HPLC-MS was applied to characterize the chemical constituents in these fractions. Under the optimized HPLC conditions, base-line separation of frs. E3 and E4 was achieved, as that shown in Fig. S1 (Supplementary data) for fr. E3, Fig. 3 and Fig. S2 (Supplementary data) for fr. E4. This effort led to identification of five compounds (6–10) from 1.2 mg of fr. E3 and seven compounds (1, 3, 7, 11–14) from 3.3 mg of fr. E4, based on analysis of ^1H NMR spectra

(Fig. 4, fr. E4), UV, CD, and ESI-MS data (Tables 1 and 2 and Supplementary data).

The *n*-BuOH soluble fraction, displaying 15.18% inhibitory activity against xanthine oxidase at $100\ \mu\text{g}\ \text{ml}^{-1}$, was separated by a Sephadex LH-20 column to give compounds 1–3 containing fractions (frs. B6–8), confirmed on the basis of HPLC retention time and UV data in HPLC-DAD analysis (Fig. S3, Supplementary data). HPLC-SPE-TT-NMR analysis of fr. B5 (3.1 mg) identified 15 in addition to 1 (Fig. S3, Table S4, Supplementary data).

Compounds 6–10 and 15 were simple phenolics and were established as 1-(3',4'-dihydroxyphenyl)-2-hydroxyethan-1-one (6) [25], 3,4-dihydroxybenzoic acid (7) [26], 3,4-dihydroxybenzaldehyde (8) [27], 4-hydroxybenzoic acid (9) [26], 3',4'-dihydroxyacetophenone (10) [28], and isovanillic acid (15) [29], respectively. Compound 11 was identified as caffeic acid [30].

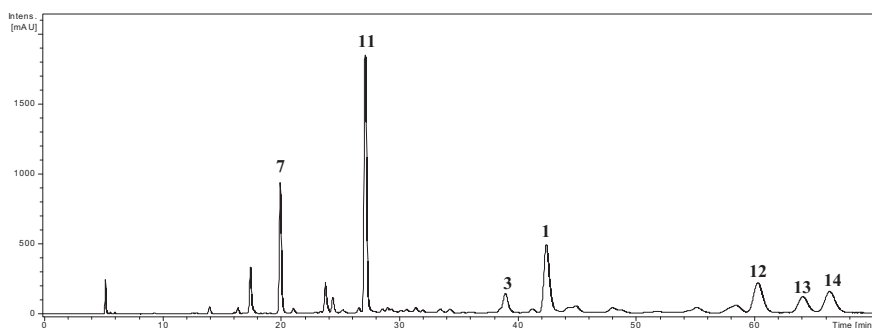
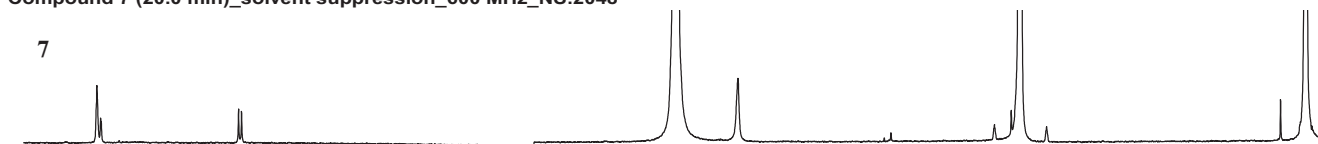


Fig. 3 – RP-18 HPLC chromatogram of fr. E4, detected at 280 nm, under an optimized condition as indicated in Section 2.4 for HPLC-SPE-NMR. Intens., intensity.

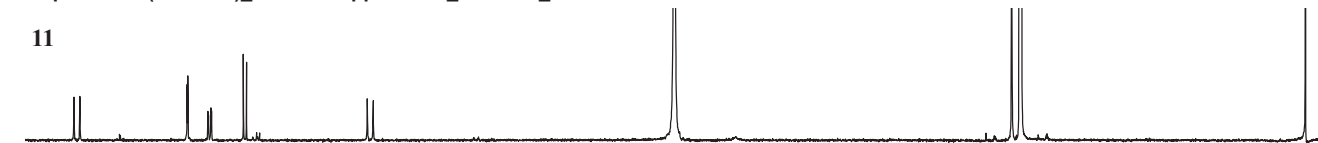
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7



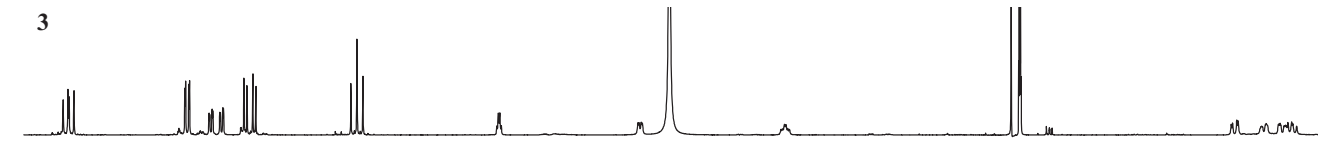
Compound 11 (27.2 min)_solvent suppression_600 MHz_NS:2048

11



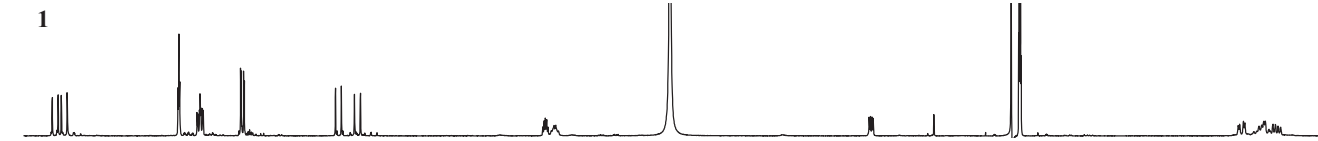
Compound 3 (38.9 min)_standard pulse program_600 MHz_NS:32

3



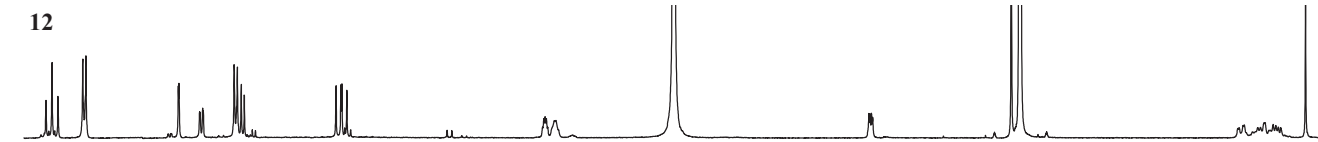
Compound 1 (42.4 min)_standard pulse program_600 MHz_NS:32

1



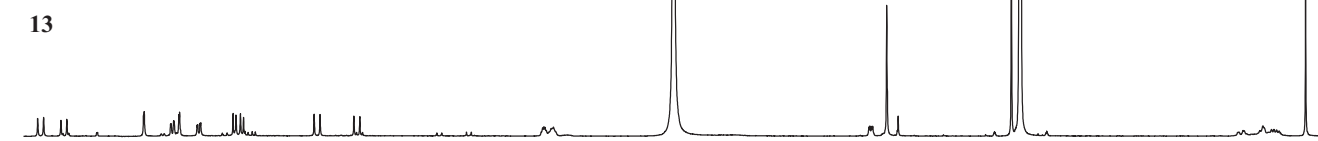
Compound 12 (60.2 min)_solvent suppression_600 MHz_NS:2048

12



Compound 13 (63.9 min)_solvent suppression_600 MHz_NS:2048

13



Compound 14 (66.2 min)_solvent suppression_600 MHz_NS:2048

14

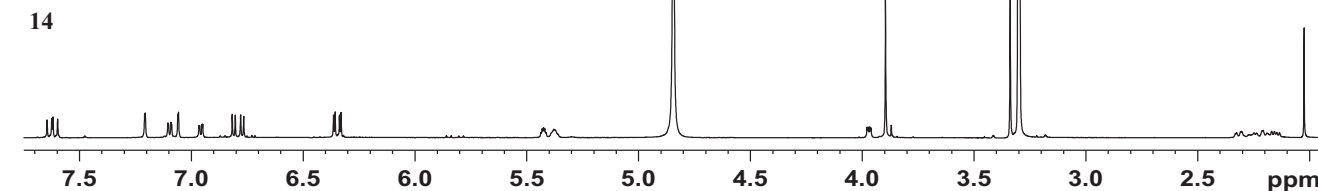


Fig. 4 – ¹H NMR spectra of compounds 1, 3, 7, and 11–14 (CD₃OD), adopted from HPLC-SPE-TT-NMR of fr. E4.

Table 2 – HPLC retention time, ESI-MS, and UV data of compounds in fr. E4.

Compound	t _R (min)	[M–H] [–] m/z	λ _{max} (nm) ^a
7	20.0	152.9	228, 261, 294
11	27.2	178.8	239, 324
3	38.9	514.9	243, 324
1	42.4	514.9	242, 326
12	60.2	498.9	232, 316
13	63.9	528.9	240, 326
14	66.2	528.9	240, 326

^a Recorded in CH₃CN–0.1% HCO₂H_(aq).

The ¹H NMR spectrum of **12** was similar to that of **1** except for the replacement of an AMX system in the aromatic region by an AA'XX' system (Fig. 4). The ¹H NMR spectrum of **13** is close resemblance to that of **14**, both of which were similar to that of **1** except for the presence of an additional methoxy singlet (Fig. 4). The NOESY spectrum of **14** (Fig. S7, Supplementary data) showed correlation of the methoxy

singlet (δ 3.90) to an aryl proton doublet (H-2, δ 7.21, *J* = 1.7 Hz), designating a 3-OMe group. The ESI-MS spectrum of **12** showed [M–H][–] at *m/z* 498.9, being 16 amu less than that of **1**, while that of **13** and **14** showing the same pseudo molecular ion [M–H][–] at *m/z* 528.9, 14 amu more than that of **1**. Based on these data, compound **12** was O-caffeoyl-O-*p*-coumaroyl quinic acid while the isomeric **13** and **14** were O-caffeoyl-O-feruloyl quinic acids.

Compounds **12–14** had been elucidated as 3-O-caffeoyl-5-O-*p*-coumaroylquinic acid, 3-O-feruloyl-5-O-caffeoylquinic acid, and 3-O-caffeoyl-5-O-feruloylquinic acid, respectively, mostly based on LC–MSⁿ data [31–33]. The location of *p*-coumaroyl, caffeoyl, and feruloyl residues at either C-3 or C-5 of the quinic acid moiety, however, was not well clarified. Thus, despite the location of the phenylpropenoyl groups in **13** had been determined on the basis of 2D-NMR spectroscopic analysis [34], the great similarity between the ¹H NMR spectra of **13** and **14** (Fig. 4) hampered their identification. In this study, such issue was solved by comparison of their ¹H NMR data, particularly those of olefinic protons (Fig. 5), with that of

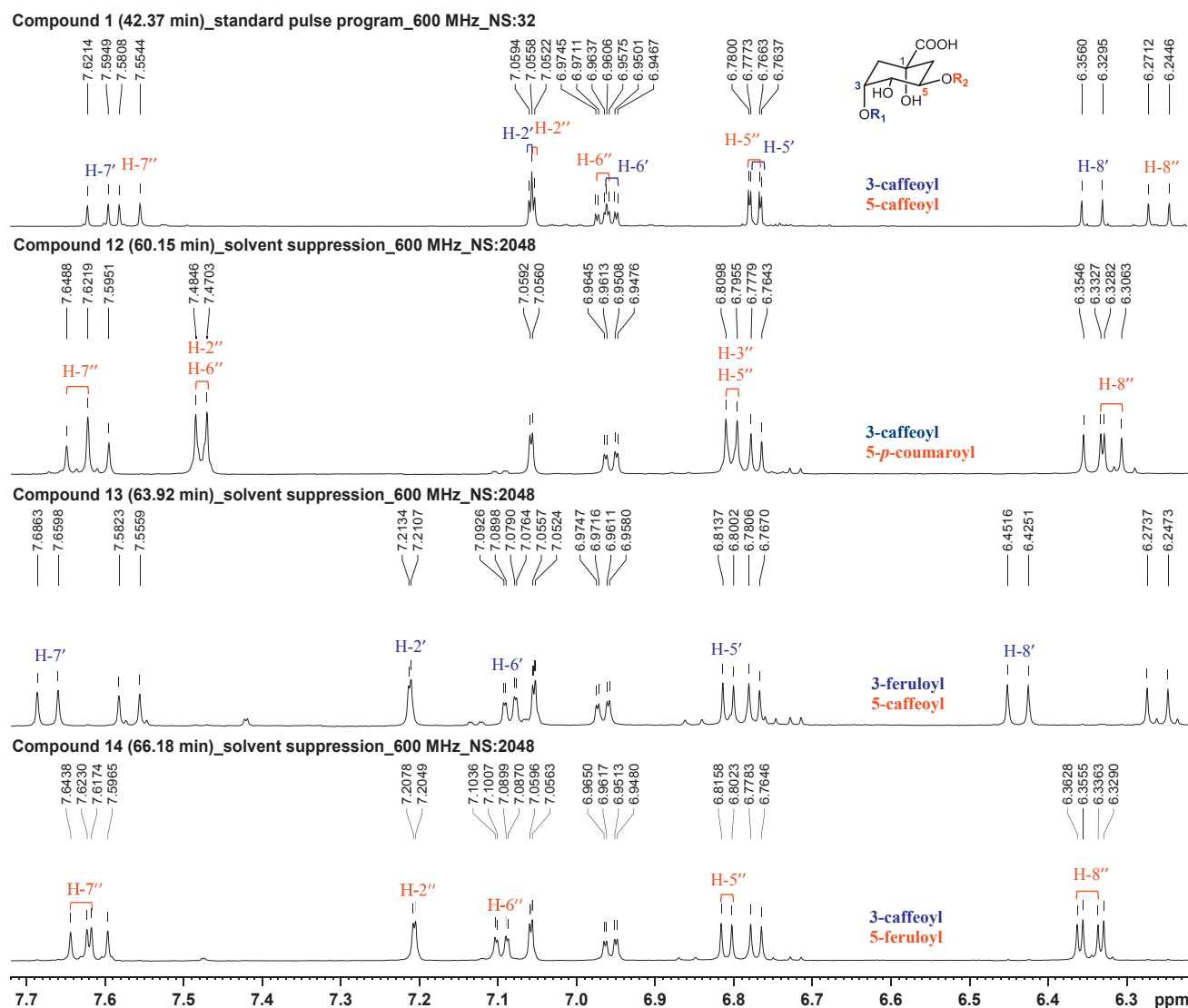
**Fig. 5 – ¹H NMR comparison of compounds 1 and 12–14 in the region of δ 6.22–7.72 ppm (CD₃OD).**

Table 3 – Regression equations, estimated amounts (in EtOH extract), and content (in dry seeds, w/w) of 1–3.

Compound	Regression equation ^a	R ²	In EtOH extract (50 µg) ^b		Content (%) ^c
			Mean peak area (mAu × s)	Estimated amounts (µg)	
1	$y = 8749x + 338.79$	0.9999	16,681.69	1.9	0.10%
2	$y = 7942.3x - 1004.8$	0.9995	10,827.09	1.5	0.08%
3	$y = 8405.9x + 187.82$	0.9999	2546.27	0.3	0.02%

^a The regression equation: $y = ax + b$; x , the amounts of compound (µg); y , peak area (mAu × s).

^b EtOH extract was prepared as 5.0 mg·mL⁻¹ solution; 10 µL/injection (50 µg).

^c Content (%) of whole plant material, e.g. 1: 1.9/50*30.9 g (EtOH extract)/1.2 kg (seed weight) ≈ 0.1%.

sodium 3,5-dicaffeoylquinic acid (1) (Table 1), which was assigned unambiguously by 2D-NMR spectroscopic analysis (HMBC, COSY) [15]. That is the ¹H NMR data of both caffeoyl residues at C-3 and C-5 positions were distinguishable, especially for the *trans*-coupled olefinic protons, i.e. δ_{H-7} 7.61 and $\delta_{H-8'}$ 6.34 (3-caffeoyl) vs. $\delta_{H-7'}$ 7.57 and $\delta_{H-8''}$ 6.26 (5-caffeoyl). Such comparison thus allowed designation of the caffeoyl residue substituted at C-3 for 12 and 14 but at C-5 for 13. Complete ¹H NMR assignment of these compounds was made as listed in Table 1.

3.3. Quantitative analysis of EtOH extract

The contents of three major constituents (1–3) in the EtOH extract of *H. suaveolens* seeds were determined quantitatively by reverse-phase HPLC analysis (Fig. S8, Supplementary data). The regression equations for linear standard curve of 1–3 (Fig. S9, Supplementary data) were established at concentration range, 0.1–10 µg. Based on these, the content of 1–3 in *H. suaveolens* seeds was determined to be 0.10%, 0.08%, and 0.02%, respectively (Table 3). This result could demonstrate that *H. suaveolens* seed is abundant in bioactive dicaffeoylquinic acids, which was described in Section 3.1.

3.4. Xanthine oxidase inhibitory activity

Among these isolates, four majors (1–4) were also assayed against xanthine oxidase *in vitro* (Fig. 1). Sodium 4,5-dicaffeoylquinic acid (2, 0.08% in dry seeds) and methyl 3,5-dicaffeoylquinic acid (4, 0.004% in dry seeds) which showed better inhibitory activity were selected to determine the IC₅₀ value, calculated from the dose–response curve. The IC₅₀ values of 2 and 4 were 69.4 ± 1.1 µM and 92.1 ± 1.2 µM, respectively (c.f. allopurinol IC₅₀ 28.4 ± 1.1 µM). The result also indicated that the location of the caffeoyl substitution affected the activity, i.e. 4,5-dicaffeoyl (2) > 3,5-dicaffeoyl (1) and 3,4-dicaffeoyl (3), and the corresponding methyl ester could enhance the inhibitory activity (4 > 1), which was consistent with that reported in literature [35]. Although poor bioavailability of caffeoylquinic acids has been reported previously, the hydrolysis of caffeoylquinic acids by human intestinal microbiota to form caffeic acid, which displays moderate xanthine oxidase inhibitory activity and better bioavailability, may enhance the absorption of bioactive compounds [35–37]. Moreover, xanthine oxidase highly distributes in liver and intestine [38], indicating that caffeoylquinic acids themselves would act directly in the intestine as hypouricemic agents. As oral administration of methyl 4,5-dicaffeoylquinic acid has been demonstrated to have good hypouricemic effect in potassium

oxonate-treated rat [39], the *H. suaveolens* seed, rich in dicaffeoylquinic acids, may provide a good source of healthy supplement beneficial for the hyperuricemic persons.

4. Conclusion

This first chemical investigation on *H. suaveolens* seed, commonly used as drink substance, led to the identification of 15 compounds via combination of Sephadex LH-20, reverse-phase chromatography, and HPLC-SPE-TT-NMR hyphenation. Of these, caffeoylquinic acid derivatives (1–5, and 12–14) and caffeic acid (11) are xanthine oxidase inhibitors, especially sodium 4,5-dicaffeoylquinic acid (2) and methyl 3,5-dicaffeoylquinic acid (4). This work also provides solid ¹H NMR data for identification of positional isomers of quinic acids substituted with heterogeneous phenylpropenoyl groups. This study demonstrates the *H. suaveolens* seed, rich in dicaffeoylquinic acids, to be a potential nutraceutical with benefit for the gout patients.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jfda.2019.05.006>.

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