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ORIGINAL PAPER

Identification of flavanones from peel of *Citrus changshan-huyou* Y. B. Chang, by HPLC–MS and NMR

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Abstract High-performance liquid chromatography coupled with a UV photodiode-array detector and a mass spectrometer (HPLC–MS) was used to analyze the constituents of an extract of *Citrus changshan-huyou* Y. B. Chang peel. Structures of two flavanones were identified based on their abundant $[M+H]^+$ ions, UV spectra, and ^{1}HNMR and ^{13}CNMR spectrometer as 5,4′-dihydroxy flavanone-7-*O*-α-glucoside (naringenin-7-*O*-α-glucoside) and 5,3′-dihydroxy-4′-methoxy flavanone-7-*O*-α-glucoside (hesperetin-7-*O*-α-glucoside). The two flavanones were first identified from peel of *Citrus changshan-huyou* Y. B. Chang.

Keywords Flavanones · Identification · *Citrus changshan-huyou* Y. B Chang

Introduction

A number of *Citrus* species have been recorded in the Chinese pharmacopoeia as appropriate for medical use. As a special species of genus from China, *Citrus changshan-huyou* Y. B. Chang is a cross bred species of *Citrus grandis* (L.) O Sbeck and *Citrus sinensis* (L.) O Sbeck [1]. Many compounds present in *Citrus* fruits have been reported to be biological active, including antiviral, anti-cancer, anti-inflammatory, anti-allergenic and analgesic activity [2, 3]. Also, some *Citrus* compounds have been shown to inhibit the growth of fungi and bacterial [4, 5]. Many potentially health promoting effects have been ascribed to the *Citrus* flavonoids [6]. For example, Hesperetin and naringenin from lemon (*Citrus limon*) and other *Citrus* species showed analgesic, anti-inflammatory and antioxidant properties [7–9]; Diosmin is an important flavonoid in *Citrus* used in the treatment of several illnesses of the circulatory system. It improves muscular tone and vascular resistance to inflammatory processes; It possesses anti-hemorroidal, anti-oxidant and anti-lipid peroxidation properties and protects against free radicals [10, 11]. *Citrus* flavonoids, which occur principally in the peel, have been studied for a century, and more than 60 flavonoids have been isolated and structurally determined from sour orange (*Citrus aurantium* L.), grapefruit (*Citrus paradisi* Mact. (Rutaceae)), pummelo (*Citrus grandis* L. Osbeck) and other *Citrus* species [12], but little attention has been paid on *Citrus changshan-huyou* Y. B. Chang. In our previous studies, flavonoids extract from peel of *Citrus changshan-huyou* Y. B. Chang showed good antioxidant and antibacterial activities, and flavonoids from the peel of *Citrus changshan-huyou* Y. B. Chang was proved to have an inhibitory role to T cell and B cell proliferation reaction [13]. Due to the importance of flavonoids as contributors of beneficial health effects of fruits and vegetables, the identification and structural determination of such compounds occurring in plant tissue or other biological systems play an important role in many areas of science. But up to now, only Xuemei Zhao [14] identified the following six flavonoids from peel of *Citrus changshan-huyou* Y. B. Chang: Naringenin, Hesperidin, 5-hydroxy-3′,4′,6,7,8-pentamethoxyflavone, 3′,4′,5,6,7,8-hexamethoxyflavone, 4′,5,6,7,8-pentamethoxyflavone, and 5-hydroxy-3′,4′,3,6,7,8-haxamethoxyflavone. In order to expound the pharmacological mechanism of peel of *Citrus changshan-huyou* Y. B. Chang, the present investigation was undertaken to isolate and identify other flavanoids from

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peel of *Citrus changshan-huyou* Y. B. Chang by HPLC–MS and NMR methods.

Materials and methods

Reagents

Ethanol and acetic acid were of analytical grade and purchased from WuLian Chemical Factory (Shanghai, China). Methanol, Tetramethylsilane (TMS) and deuterated acetone (CD_3COCD_3) were of HPLC grade (Sigma, USA), Reverse osmosis Milli-Q water (18M) (Millipore, USA) was used for all solutions and dilutions.

Plant material and sample preparation

Citrus changshan-huyou Y. B. Chang was purchased from local market and was identified by *Citrus* Research Lab of Huazhong Agriculture University, Wuhan, China. The peel was dried at 60 °C in the oven, finely ground and 100 g of the powder was extracted with 1000 ml 60% aqueous ethanol at 50 °C for 2 h, the ethanol solution was filtered and concentrated at vacuum to remove the solvent. The concentrated solution was applied onto a glass column (30 mm × 800 mm, i.d.) packed with AB-8 macroporous resin (chemical plant of Nankai University, Tianjin, China). Deioned water was adjusted to pH 4.0 by 1% HCl and used to remove sugar and other soluble impurity until the elution was nearly no color and 30% aqueous ethanol was used to elute the target flavonoids. After eluting, solvent was removed using a rotary evaporator under vacuum, and the residue was lyophilized. Ten milligrams of the lyophilized sample was redissolved in 10 ml of methanol and filtered over 0.45 μm cellulose-nitrate membrane filters for HPLC analysis.

Partial hydrolysis

Hundred milligrams of the above lyophilized sample was dissolved in 35 ml of methanol and added to the flask, 5 ml of 3 mol/L HCl was added. This solution was refluxed at 85 °C for 2 h on a water bath. After cooling, the sample was diluted to 50 ml with methanol and filtered over 0.45 μm cellulose-nitrate membrane filters for HPLC analysis.

HPLC analysis

The chromatographic separation of compounds was performed on a HP HPLC series 1100 (Hewlett Packard, Waldbronn, Germany) equipped with CHEMSTATION software, a degasser G1322A, a binary gradient pump G1312A, a thermoautosampler G1329/1330A, and a diode array detection system G1315A. The column used was a 5 μm Hypersil ODS C_{18} (4.60 mm × 200 mm, i.d.). The column was operated at a temperature of 25 °C. The mobile phase consisted of methanol/acetic acid/water (40:1:59, v/v/v) at a flow rate of 1.0 ml/min. The injection volume for all samples was 10 μl, monitoring was performed at 280 nm.

Isolation of pure compounds by semi-preparative HPLC

Pure compounds for NMR analysis were isolated from the extract by means of repeated semi-preparative HPLC. The column used was a Skim-pack prep—ODS (20 mm × 250 mm, i.d.) (Shimadzu, Kyoto, Japan). The mobile phase was the same above with isocratic flow rate of 8 ml/min, and injection volume of 100 μl. The purity of the compounds was examined by analytical HPLC–DAD.

HPLC–MS analysis

HPLC mass spectrometry was carried out on a HP HPLC series 1100 (Hewlett-Packard, Waldbronn, Germany) combined with a mass spectrometric detector LCQ ion trap instrument (Finnigan MAT. San Jose, CA, USA) with an electrospray source. Conditions: electrospray ionization (positive ion mode); the electrospray voltage is set to 5.0 kV; the capillary temperature is 210 °C; the full scan mass spectra of the flavanones from *m*/*z* 100–2000 u were measured using 500 ms for collection time and three micro scans were summed. The HPLC is connected to the mass spectrometer via the UV cell outlet.

NMR spectra

^{1}HNMR and ^{13}CNMR spectra were recorded at 600 MHz on Varian Unity Inova-600 (Varian, USA). Tetramethylsilane (TMS) was used as internal standard and deuterated acetone (CD_3COCD_3) as solvent. The sample for NMR analysis was obtained by semi-preparative HPLC.

Results and discussion

The UV-Vis spectra of flavones exhibit two major absorption peaks: band I (usually 330–380 nm) and band II (usually 240–280 nm) [15]. Figures 1 and 2 showed the HPLC and total ion chromatograms of flavonoids from *Citrus changshan-huyou* Y. B. Chang using the band II absorption at 280 nm, and UV-Vis spectra confirmed the identification of peaks 1–6 as flavanones due to the band I absorption at 330–340 nm. After partial hydrolysis, three new peaks (peaks 4–6, t_R 15.23, 26.93, and 33.66 min, respectively) were detected, which suggested that these new peaks may be the aglycone of peaks 1–3 (t_R 5.386, 8.496, and 10.296 min, respectively) correspondingly.

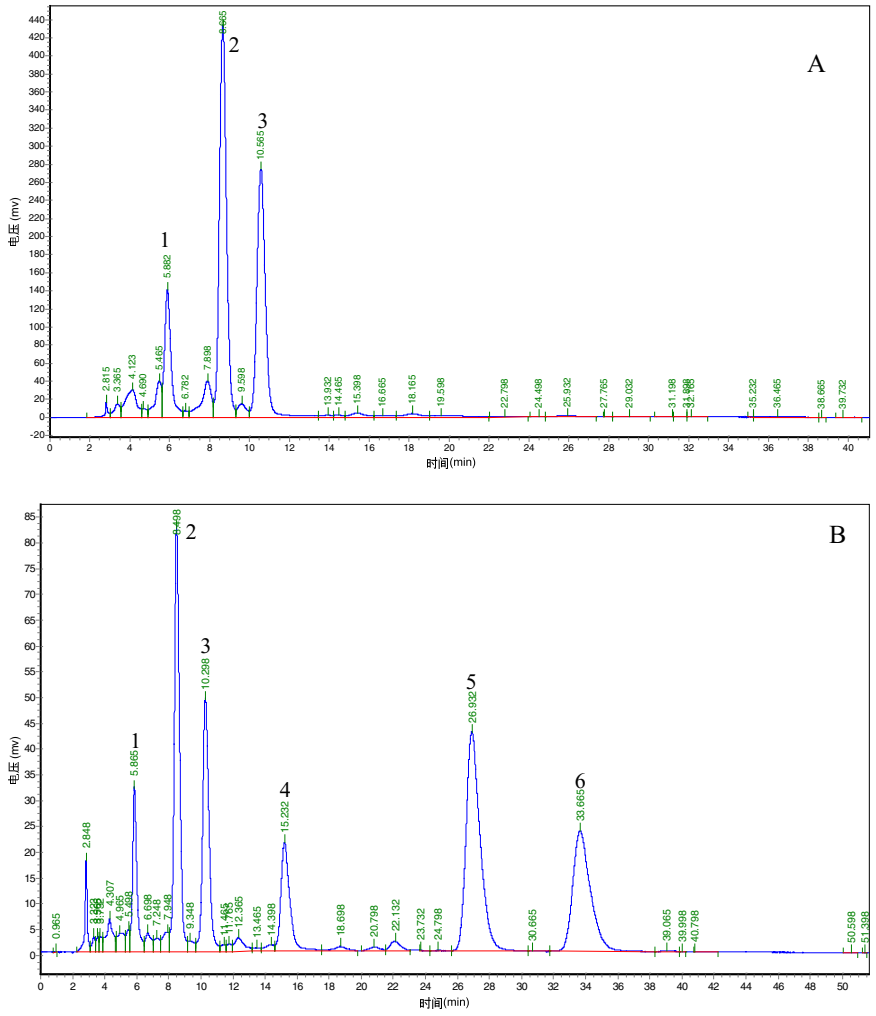


Fig. 1 HPLC chromatogram of flavonoids from peel of *Citrus changshan-huyou* Y. B. Chang before **A** and after partial hydrolysis **B**

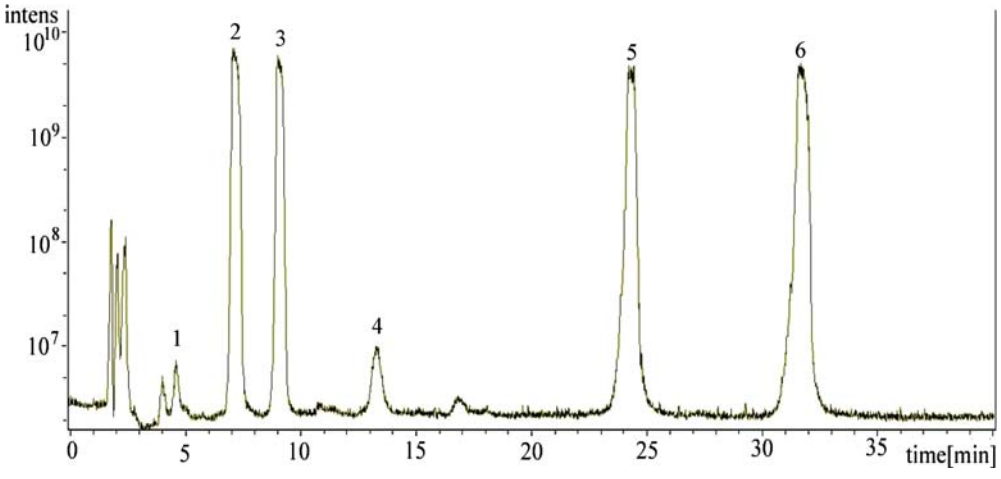


Fig. 2 Total ion chromatograns of flavonoids from peel of *Citrus changshan-huyou* Y. B. Chang after partial hydrolysis

Figure 3 confirmed the above suggestion, all characteristic aglycone ions of peaks 1–3 clearly showed a characteristic loss of 161 u indicative of a hexose sugar, peak 1 showed both an intense molecular ion $[M+H]^+$ at *m/z* 449 and an intense aglycone molecular ion $[A]^+$ at *m/z* 288, while peak 4 showed an only intense aglycone molecular ion $[A]^+$ at *m/z* 288. Peak 2 showed both an intense molecular ion $[M+H]^+$ at *m/z* 433 and an intense aglycone molecular ion $[A]^+$ at *m/z* 272, correspondingly, peak 5 showed an only intense aglycone molecular ion $[A]^+$ at *m/z* 272. Peak 3 showed

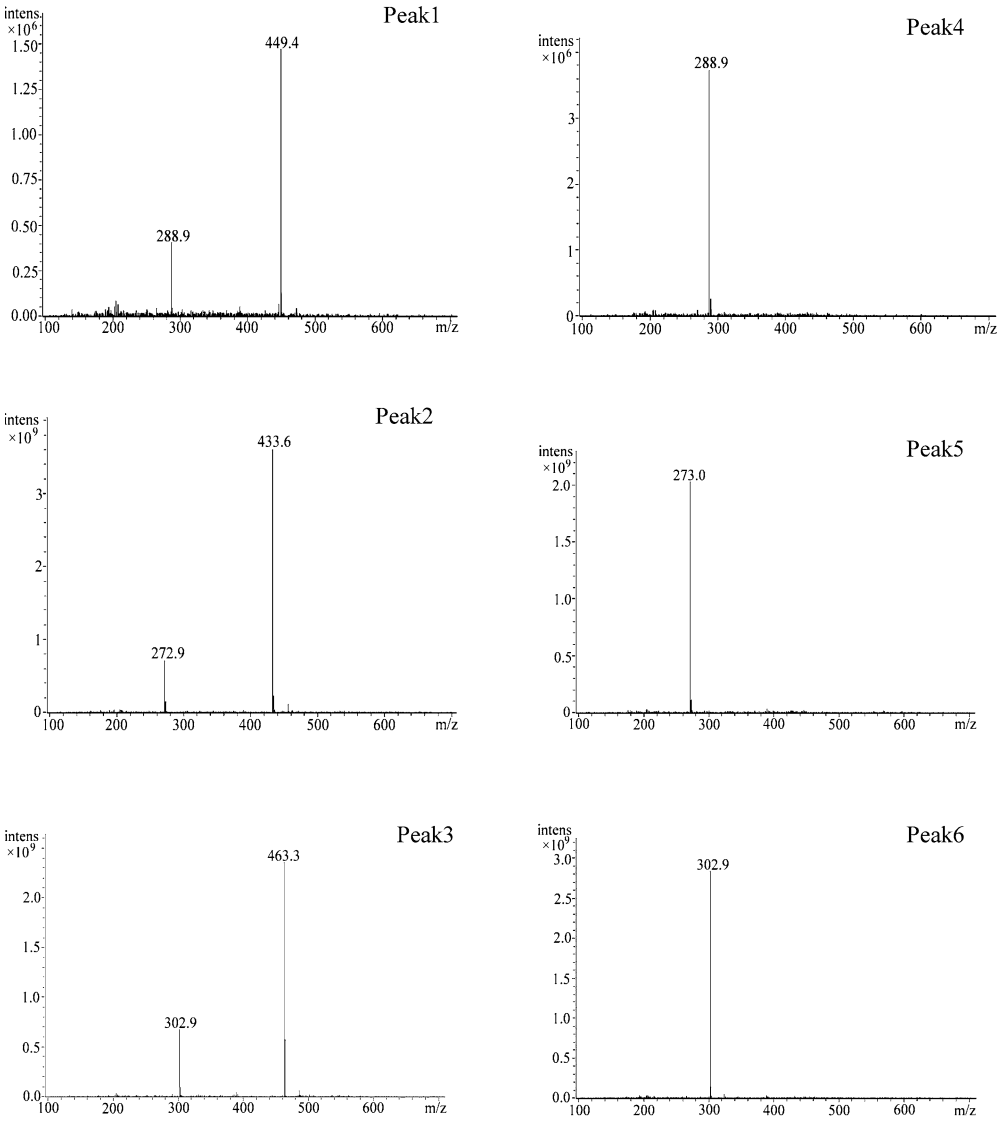


Fig. 3 ESI-MS spectrum of peaks 1–6

both an intense molecular ion $[M+H]^+$ at *m/z* 463 and an intense aglycone molecular ion $[A]^+$ at *m/z* 302, resulting from the elimination of 161 u, that is a indicative of a hexose sugar, meanwhile, peak 6 showed an only intense aglycone molecular ion $[A]^+$ at *m/z* 302.

In order to confirm the structure of the ions at *m/z* 433 (peak 2) and *m/z* 463 (peak 3), NMR spectra was performed. The NMR spectrum of peak 2 was typical of a flavanone (Table 1), the ^{1}HNMR spectrum showed a downfield signal (δ12.068) due to a hydrogen bonded hydroxy group at 5 position, the aromatic protons of meta-coupled doublets at δ6.125 and δ6.152 corresponded to 6, 8-protons of ring. Chemical shifts between δ3.204 and δ3.862 due to protons of glucose at 2–6 positions. The chemical shift of the proton of glucose at 1 position was between δ5.048 and δ5.080, which suggested the α substituent. The data of ^{1}HNMR showed the presence of para-substituted at 5, 7 and 4′ positions. ^{13}C NMR spectrum showed signals of one carbonyl carbon (δ197.280), three oxygenated aromatic carbons (δ164.112, δ166.013 and δ158.108), and six carbons of glucose (δ100.221, δ73.729, δ76.898, δ70.33, δ77.193, δ61.73). All the UV-spectra, $[M+H]^+$ ions and NMR data supported that the structure of compound peak 2 is 5,4′-dihydroxy flavanone-7-*O*-α-glucoside (naringenin-7-*O*-α-glucoside). Peak 3 was determined as flavanone from its similar spectral data (^{1}HNMR, ^{13}CNMR) (Table 2) to those of peak 2 except with an additional signals at δ4.037 in ^{1}HNMR and δ55.643 in ^{13}CNMR, which suggested the presence of $-OCH_3$. All these data supported that the structure of compound peak 3 is 5,3′-dihydroxy-4′-methoxy flavanone-7-*O*-α-glucoside (hesperetin-7-*O*-α-glucoside) (Fig. 4). Because of the lack of sample, structure of peak 1 could not be identified by NMR, the possible structure of peak 4 was 3′, 4′, 5, 7-tetrahydroxy flavanone from its UV-spectra and $[M+H]^+$

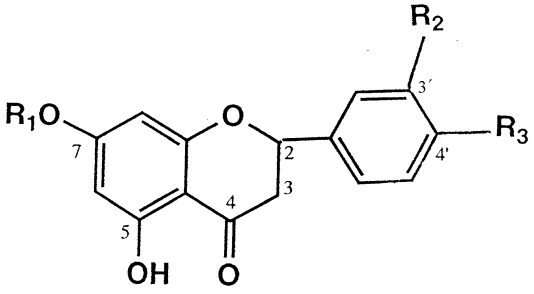
Table 1 ^{1}HNMR and ^{13}CNMR spectral data of peak 2 (CD_3COCD_3)

^{1}HNMR (δppm)		^{13}CNMR (δppm)			
2-H	5.495, 5.474	2-C	79.418	3′-C	128.451
3-H	2.878	3-C	42.851	4′-C	158.108
6-H	6.125	4-C	197.280	5′-C	130.120
8-H	6.152	5-C	164.112	Glc-1-C	100.221
5-OH	12.068	6-C	96.892	Glc-2-C	73.729
2′-H	7.380	7-C	166.013	Glc-3-C	76.989
3′,5′-H	6.897, 6.883	8-C	95.309	Glc-4-C	70.33
6′-H	7.394	9-C	163.427	Glc-5-C	77.193
Glc H-1	5.080, 5.067, 5.062, 5.048	10-C	103.776	Glc-6-C	61.73
Glc	2-6H	3.204–3.862	1′-C	129.875	
			2′,6′-C	115.498	

Table 2 ^{1}HNMR and ^{13}CNMR spectral data of peak 3 (CD_3COCD_3)

^{1}HNMR (δppm)		^{13}CNMR (δppm)			
2-H	5.488, 5.467	2-C	79.271	4′-C	146.902
3-H	2.858	3-C	42.906	5′-C	111.639
6-H	6.124	4-C	196.151	6′-C	118.190
8-H	6.170	5-C	164.476	Glc-1-C	100.350
5-OH	12.059	6-C	96.863	Glc-2-C	73.709
2′,6′-H	7.338	7-C	166.008	Glc-3-C	76.955
5′-H	7.039, 6.875	8-C	95.823	Glc-4-C	70.302
Glc H-1	5.080, 5.067, 5.062, 5.048	9-C	163.547	Glc-5-C	77.241
Glc 2-6	3.204-3.862	10-C	103.775	Glc-6-C	61.694
OCH_3	4.037(3H)	1′-C	131.915	OCH_3-C	55.643
Glc 2-6H	3.176-3.852	2′-C	113.725		
		3′-C	148.006		

Fig. 4 Structures of compounds peak 2 and peak 3



Peak number	compound	R_1	R_2	R_3
2	naringenin- 7-O- α -glucoside	glucosyl	H	OH
3	hesperetin-7-O- α -glucoside	glucosyl	OH	OCH_3

ions data, and peak 1 was its glycoside. The actual structure of peak 1 should be determined further.

Conclusion

To our knowledge, this is the first study that reports 5,4′-dihydroxy flavanone-7-*O*-*α*-glucoside (naringenin-7-*O*-*α*-glucoside) and 5,3′-dihydroxy-4′-methoxy flavanone-7-O-*α*-glucoside (hesperetin-7-*O*-*α*-glucoside) presence in peel of *Citrus changshan-huyou* Y. B. Chang. These compounds may play an important role in the antioxidant and antibacterial activities of extract from peel of *Citrus changshan-huyou* Y. B. Chang. The results show the potential of *Citrus changshan-huyou* Y. B. Chang for providing flavonoids of pharmaceutical interest from the by-products of industrial processing.

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