

Flavonoids from the Leaves of *Citrus aurantium* (Sour Orange) and *Citrus sinensis* (Sweet Orange)

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Polymethoxylated flavonoids and flavonoid O- and C-glycosides were isolated and identified from extracts of the leaves of *Citrus aurantium* var. *amara* L (sour orange) and *Citrus sinensis* L (sweet orange). Altogether twenty-three flavonoids were obtained from the two species (Table-1). This is the first report of four of the polymethoxylated flavonoids from leaves of *C. sinensis*, namely tetra-O-methyl scutallrein, queratagetin, hexamethyl ether, isosinensetin, and sinensetin; the latter two were also obtained for the first time from the leaves of *C. aurantium*. This is also the first report of several C-glycosides of apigenin and diosmetin from the two species (see Table-1). Furthermore, this is the first report of neodiosmin, poncirin, narirutin glucoside and naringin glucoside from the leaves of *C. aurantium*.

INTRODUCTION

The flavonoids of the orange subfamily Aurantioideae, family Rutaceae, have attracted the attention of generations of chemical researchers, beginning with the first description of hesperidin by Lebreon (1828)¹. Citrus fruit has been collected and used by man for centuries for medicinal, herbal and agricultural purposes. Many citrus flavonoids function as natural antioxidants, free radical scavengers and many also chelate pro-oxidant metals, reducing their capacity to produce free radicals¹. However, most reports describe flavonoids from peel oil and juice of oranges.²⁻⁹ Thus because of the paucity of reports of citrus leaf flavonoids¹⁰⁻¹³ we undertook a detailed investigation of the flavonoids in the leaves of the two citrus species *C. aurantium* and *C. sinensis*.

EXPERIMENTAL

Plant Material

Leaf samples of *Citrus aurantium* (sour orange) and *Citrus sinensis* (sweet orange) were obtained from fruiting trees cultivated at the Agricultural Crops Research Institute, Giza, Egypt in October 1996. The plants were identified by Dr. M. El-Gabaily, and herbarium specimens are deposited in the Herbarium of the Pharmacognosy Department of the Faculty of Pharmacy, Helwan University, Cairo, Egypt.

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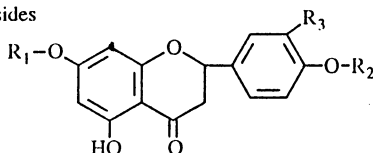
TABLE-1
 FLAVONOIDS FROM LEAVES OF *CITRUS AURANTIUM* (SOUR ORANGE)
 AND *C. SINENSIS* (SWEET ORANGE)

Retention time (min)	Compound	Concentration in mg	
		<i>C. aurantium</i>	<i>C. sinensis</i>
16.00	Narirutin glucoside	—	6.7
16.84	Naringin glucoside*	7.3	—
18.32	Vicenin*, †	57.7	25.8
19.49	Eroicitrin	—	10.1
20.11	Rhamnosyl vitexin*, †	24.0	5.1
20.80	Neeroicitrin	24.4	—
21.40	Vitexin 4'-rhamnoside*, †	12.7	9.2
22.01	2''-O-Xylovitexin*, †	12.5	5.8
22.72	Vitexin*	126.2	63.5
23.36	Isovitexin*, †	70.4	34.9
23.89	Narirutin	5.8	10.4
24.41	Naringin	89.3	—
24.78	Diosmetin 6,8-C-diglucoside*, †	62.7	97.3
25.46	Diosmetin 6-C-glucoside*	82.5	—
26.24	Hesperidin	90.0	344.9
26.56	Neohesperidin	93.6	—
27.27	Isorhiofolin	—	99.7
27.74	Rhiofolin	139.3	—
28.65	Diosmetin 8-C-glucoside*, †	133.4	89.2
28.98	Diosmin	—	16.4
29.22	Neodiosmin*	109.2	—
30.33	Isosakurantin-7-O-rutinoside	45.1	4.5
32.09	Isosakurantin-7-O-Neohesperidoside*	47.3	—
46.01	Queretagetin hexamethyl ether†	—	76.8
47.01	Isosinensetin*, †	102.1	87.3
48.03	Sinensetin*, †	80.4	96.5
48.93	Nobelitin	176.0	230.5
50.36	Tetra-O-methyl scutallarin†	—	205.6
51.98	Tangeritin	112.1	60.0
52.20	5-O-Demethyl nobelitin	110.0	152.3
	Total flavonoid material from 2 kg of dried leaves	1814.0 mg	1732.5 mg

*First report from leaves of *C. aurantium*

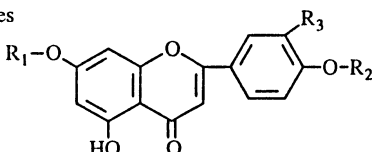
†First report from leaves of *C. sinensis*

Flavanone glycosides



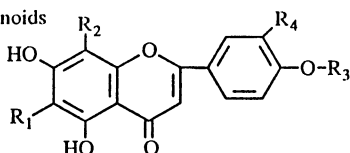
R ₁ =RUT	R ₂ =R ₃ =H		Narirutin
R ₁ =NEO	R ₂ =R ₃ =H		Naringin
R ₁ =RUT	R ₂ =glucosyl	R ₃ =H	Narirutin glucoside
R ₁ =NEO	R ₂ =glucosyl	R ₃ =H	Naringin glucoside
R ₁ =RUT	R ₂ =H	R ₃ =OH	Eriocitrin
R ₁ =NEO	R ₂ =H	R ₃ =OH	Neocierocitrin
R ₁ =RUT	R ₂ =CH ₃	R ₃ =OH	Hesperidin
R ₁ =NI:O	R ₂ =CH ₃	R ₃ =OH	Neohesperidin
R ₁ =RUT	R ₂ =CH ₃	R ₃ =H	Didymin
R ₁ =NEO	R ₂ =CH ₃	R ₃ =H	Poncirin

Flavone glycosides



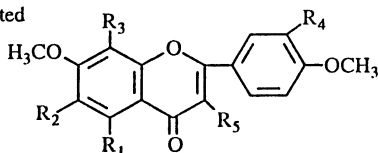
R ₁ =RUT	R ₂ =CH ₃	R ₃ =OH	Diosmin
R ₁ =NEO	R ₂ =CH ₃	R ₃ =OH	Neodiosmin
R ₁ =RUT	R ₂ =R ₃ =H		Isorhiofolin
R ₁ =NEO	R ₂ =R ₃ =H		Rhiofolin

C-glycoside flavonoids



R ₁ =glucosyl	R ₂ =R ₃ =R ₄ =H			Isovitexin
R ₁ =R ₃ =R ₄ =H	R ₂ =glucosyl			Vitexin
R ₁ =R ₂ =glucosyl	R ₃ =R ₄ =H			Vicenin
R ₁ =R ₄ =H	R ₂ =glucosyl	R ₃ =rhamnosyl		Vitexin-4'-rhamnoside
R ₁ =R ₃ =R ₄ =H	R ₂ =rhamnosyl			Rhamnosyl vitexin
R ₁ =R ₃ =R ₄ =H	R ₂ =xyloglucosyl			2''-O-Xylovitexin
R ₁ =glucosyl	R ₂ =H	R ₃ =CH ₃	R ₄ =OH	Diosmetin-8-C-glucoside
R ₁ =H	R ₂ =glucosyl	R ₃ =CH ₃	R ₄ =OH	Diosmetin-6-C-glucoside
R ₁ =R ₂ =glucosyl		R ₃ =CH ₃	R ₄ =OH	Diosmetin-6,8-C-glucoside

Polymethoxylated flavonoids



R ₁ =R ₂ =R ₃ =R ₄ =R ₅ =OCH ₃			Quercetagenin hexa-methyl ether
R ₁ =OH	R ₂ =R ₃ =R ₄ =OCH ₃	R ₅ =H	5-O-Demethyl nobiletin
R ₁ =R ₂ =R ₃ =R ₄ =OCH ₃	R ₅ =H		Nobiletin
R ₁ =R ₂ =R ₄ =OCH ₃	R ₃ =R ₅ =H		Sinensetin
R ₁ =R ₃ =R ₄ =OCH ₃	R ₂ =R ₅ =H		Isosinensetin
R ₁ =R ₂ =OCH ₃	R ₃ =R ₄ =R ₅ =H		Tetra-O-methyl scutellarein
R ₁ =R ₂ =R ₃ =OCH ₃	R ₄ =R ₅ =H		Tangeretin

Authentic reference compounds

Quercetagetin hexamethyl ether, isosinensetin, sinensetin, nobelitin, tetra-O-methyl scutellarein, tangerein, 5-O-dimethyl-nobelitin, diosmetin 6-C-glucoside, diosmetin 8-C-glucoside, diosmetin 6,8-C-diglucoside, diosmin, neodiosmin, hesperidin, neohesperidin, didymin, poncirin, vitexin, isovitexin, xylovitexin, vicenin, vitexin-4'-rhamnoside, rhamnosyl vitexin, rhoifolin, isorhoifolin, naringin, narirutin, naringin glucoside, narirutin glucoside, eriocitrin, and neoeriocitrin were obtained from either the Department of Botany, The University of Texas at Austin or from The Citrus and Subtropical Products Laboratory, USDA, Winter Haven, Florida.

Instruments

UV-visible spectrophotometer Shimadzu 1601; CI-MS MAT 112, 70 eV; Varian-50 NMR¹. The HPLC apparatus consisted of Beckman 110B pumps, autosampler 507e, a C-18 Allsphere ODS-2 (5 μ \times 250 mm) and a UV Hewlett Packard 1040 M photodiode array detector. The gradient elution schedule consisted of 90% water (having 1% H₃PO₄) and 10% acetonitrile at zero time followed by a linear gradient to 100% acetonitrile in 60 min and maintained for 10 min. Finally the gradient was brought back to 90% water in 10 min at a flow rate of 0.75 mL/min.

Extraction, isolation and identification

Dried and powdered leaves (2 kg) of both sweet and sour oranges were extracted with 95% aqueous ethanol followed by 70% aqueous ethanol. The combined two extracts for each species were evaporated to a small volume *in vacuo*. The two aqueous syrups from leaves of sweet and sour oranges were each extracted successively with *n*-hexane (to yield 9.3 and 12.7 g residues, respectively), methylene chloride (19.9 and 16.5 g), ethyl acetate (7.0 and 5.5 g) and *n*-butanol (20.0 and 27.0 g), respectively; the aqueous material remaining yielded 12.9 and 8.2 g of brown syrups, respectively.

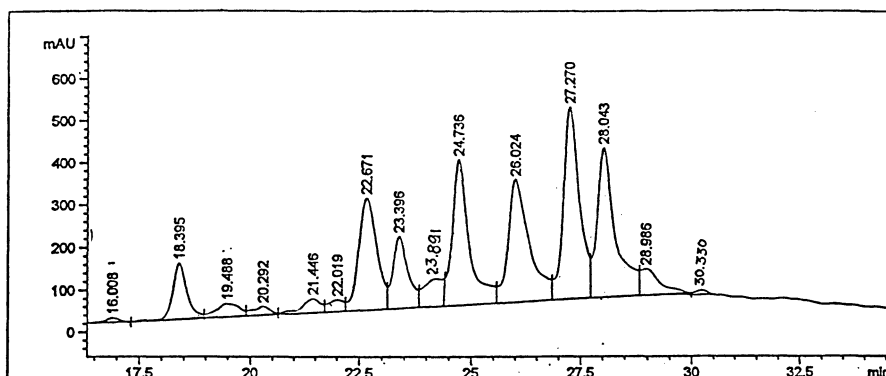


Fig. 1 Flavonoid glycoside chromatogram at 330 nm of *n*-butanol extract of the leaves of *C. sinensis*.

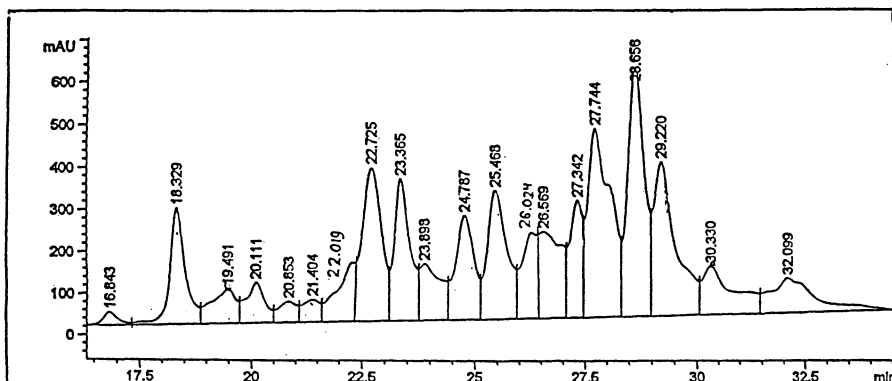


Fig. 2 Flavonoid glycoside chromatogram at 330 nm of *n*-butanol extract of the leaves of *C. aurantium*.

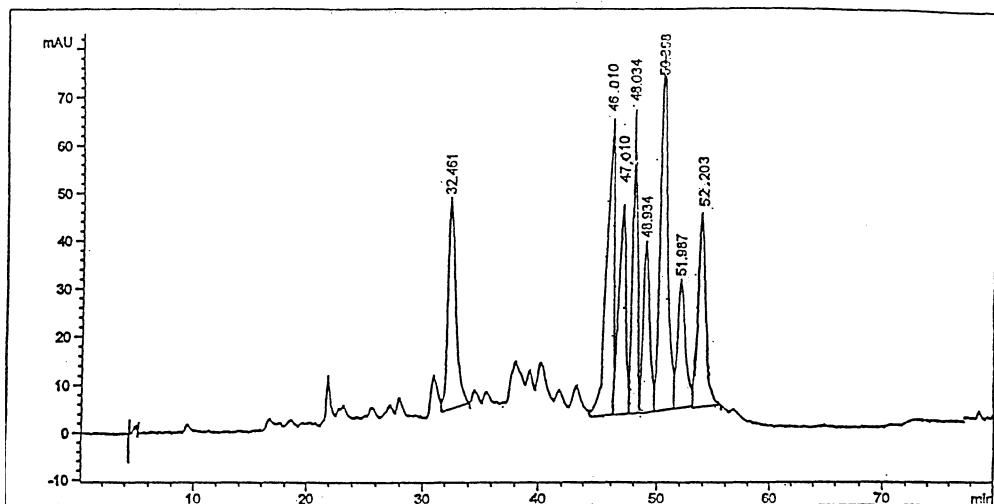


Fig. 3 Polymethoxylated flavonoid chromatogram at 330 nm of dichloromethane extract of the leaves of *C. sinensis*.

Two-dimensional paper chromatography technique¹⁴ using the solvent systems [BuOH:HOAc:H₂O (4:1:5)] and HOAc (15%) showed that the *n*-hexane fractions contained non-polar chlorophyll, terpenes, coumarins and a few polymethoxylated flavonoids, while the dichloromethane fractions contained coumarins and all the polymethoxylated flavonoids. The ethyl acetate fractions contained some of the di-O-glucosides and a few C-glycosides while all of the O-glucosides and the C-glycosides were detected in the *n*-butanol fractions from both species.

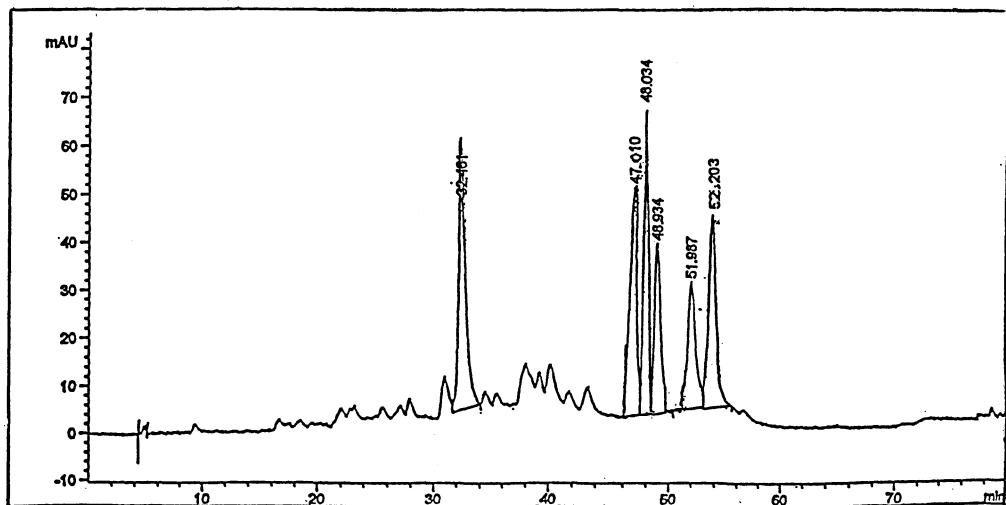


Fig. 4 Polymethoxylated flavonoid chromatogram at 330 nm of dichloromethane extract of the leaves of *C. aurantium*.

Silica gel (Merck 60A) columns were used for separation of the polymethoxylated flavonoids from the dichloromethane fractions. The columns were eluted with dichloromethane increasing the polarity gradually with 10% methanol, with a flow rate of 4 mL/min. Fractions with similar TLC patterns were combined using an *n*-butanol-acetic acid-water mixture 6:1:2 as a solvent system, and the plates were visualized under UV at 365 nm before and after exposure to NH_3 .

The *n*-butanol fractions of both citrus species were used for separation of the O- and C-glucosides on silica gel columns eluted with *n*-butanol and increasing the polarity with methanol in 10% steps up to 100% methanol. Further separation and purification of the compounds were done on Sephadex LH-20 columns eluted with water and increasing in methanol 10% steps. Identification of the compounds was carried out by HPLC technique comparisons with authentic standards for both the retention times and the UV absorption spectra (Table-1). Further confirmation of the structures of the compounds was carried out by MS, NMR, and UV.

RESULTS AND DISCUSSION

Table-1 presents the concentrations of the flavonoids in the extracts and the HPLC retention times of all the isolated flavonoids, all previously known from peel oil and juice of oranges. The table also denotes the compounds which are reported for the first time from the leaves of either or both species. The data show that flavonoids with rutinosyl groups are eluted before the flavonoids with neohesperiosyl groups in accordance with previous report¹³. As also previously reported¹⁵ the flavanone nucleus with a neohesperidosyl group such as in naringin and neohesperidin appears to be responsible for the bitter taste found in sour orange fruits. Furthermore, citrus fruits can be categorized as predominantly

containing either rutinosides or neohesperidosides^{13, 16}. Thus, we also found that the neohesperidin, poncinrin, naringin, neoeriocitrin and naringin glucoside (neohesperidoside flavonones), as well as neodiosimin and rhoifolin (which are neohesperidoside flavones without taste) are present in higher concentration in leaves of sour oranges relative to sweet oranges, while the flavonoid glycosides with the rutinosyl group, also without taste, hesperidin, diosmin, isorhoifolin, narirutin, narirutin glucoside and eriocitrin are more concentrated in leaves of the sweet orange. Hesperidin represents the major flavonoid in the leaves of *C. sinensis* (sweet orange) and its concentration is 1.7% in the *n*-butanol extract, more than 0.4% of its concentration in the peels⁴; since the peels are considered a rich source for hesperidin, the abundant leaves also represent an important source for this compound.

This is the first report of the isolation of so many C-glycosides in high concentrations from both citrus species; these include vitexin, isovitexin, vicenin, vitexin 4'-rhamnoside, 2''-O-xylovitexin, rhamnosyl vitexin, 6,8-C-diglucosyl diosmetin, diosmetin 8-C-glucoside and diosmetin 6-C-glucoside. Vitexin was previously obtained from the leaves of *C. sinensis* in low concentration¹¹. The percentage of the C-glycosides obtained from the leaves of *C. aurantium* is higher than obtained from leaves of *C. sinensis* (2.5 and 1.7%, respectively).

The polymethoxylated flavonoids, isosinensetin, sinensetin, tetra-O-methyl scutallarein, and quercetagenin hexamethyl ether were previously reported² from the oil peel of both citrus species. However, this is the first report of these polymethoxylated flavonoids from the leaf extract of *C. sinensis*; only sinensetin and isosinensetin were newly obtained from the leaf extract of *C. aurantium*. Nobelitin, 5-O-demethyl nobelitin, and tangeretin, which were obtained in this study in high yield from both citrus species, all were previously reported from leaf extracts¹⁰

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