Limonoids from *Citrus reticulata*

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The seeds of *Citrus reticulata* afforded the new limonoid derivative, isolimonexic acid methyl ether, in addition to the the previously isolated limonin, deacetylnomilin, obacunone and ichangin. The structure elucidation was achieved primarily through 1D and 2-D-NMR analyses. The marginal antimalarial activity of isolimonexic acid methyl ether is reported.

Key words: Citrus, Limonoids, Isolimonexic Acid Methyl Ether

Introduction

Limonoids comprise a group of highly oxygenated tetracyclic triterpene derivatives, which are widely distributed in plants of Rutaceae and Meliaceae (Champagne *et al.*, 1992; Nakatani, 2001). There are about fifty reported limonoid aglycones, of which thirty-six compounds were isolated from plants of the genus *Citrus* and related genera (Berhow *et al.*, 2000). These water-insoluble aglycones are usually responsible for the bitter taste of citrus juices. More than seventeen limonoid glycosides were also reported so far. Limonoid glycosides are not bitter in taste and freely soluble in water. Beside their economic importance in the processing of citrus fruits, limonoids have a wide array of interesting biological activities. Limonoids are gaining more current interest as potential anticancer agents. Limonoids are reported to inhibit chemically-induced tumorigenesis formation in the mouth, stomach, small intestine, colon, lung, and skin of experimental animals (Champagne *et al.*, 1992; Berhow *et al.*, 2000). They also reported to inhibit proliferation of breast cancer cells *in vitro* (Berhow *et al.*, 2000) and moult activity in mosquito *Culex quinquefasciatus* larvae (Jayaprakasha *et al.*, 1997). Obacunone and limonin, the known citrus limonoids, were recently reported to inhibit azomethane-induced colon carcinogenesis in rats (Tanaka *et al.*, 2000). Limonoids also induce GST, a detoxifying enzyme in mice and rats. Limonoids were reported active as insect repellents and antifeedants and they also disrupt insects' growth and inhibit their reproduction (Berhow *et al.*, 2000). The present communication describes the isolation of the new natural product isolimonexic acid methyl ether (**5**) from the seeds of *Citrus reticulata*, along with the known limonin (**1**), deacetylnomilin (**2**), obacunone (**3**) and ichangin (**4**). In addition, their antibacterial, antifungal activities are reported along with their effect on *Plasmodium falciparum.*

Experimental

General experimental procedure

Melting points (uncorrected) were obtained using an Electrothermal 9100 instrument; UV spectra were obtained in MeOH using a Shimadzu UV-1601PC spectrophotometer; IR spectra as KBr disks on a Perkin-Elmer 8508 spectrophotometer; NMR spectra were recorded in DMSO-d_6 on a Bruker AMX-NMR spectrometer, operating at 500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C NMR. The HRFTMS data were measured using a Bioapex FT-ICR mass spectrometer with electrospray ionization.

Plant material

Mandarin fruits (*Citrus reticulata*, Rutaceae) were purchased from Mansoura district in January 1999, identified by Prof. Nabil R. Samrah, Prof. of Pomology, Faculty of Agriculture, Mansoura University, Mansoura, Egypt. A voucher specimen is kept at the Dept. of Pharmacognosy, Faculty of Pharmacy, Mansoura, Egypt.

Extraction and isolation

Seeds were collected, dried and powdered. The powdered seeds (1.9 kg) were exhaustively extracted with ethanol. The alcoholic extract was evaporated *in vacuo* to afford 495 g. The latter was defatted with light petroleum (b.p. 60–80) to afford a non-polar fraction (108 g) and a polar fraction (387 g.). The latter was dissolved in 500 ml water and extracted with ethyl acetate, which was evaporated *in vacuo*. The resinous ethyl acetate extract (22 g) was column chromatographed over Silica gel (300 g) column (3.5 × 90 cm). The column was gradiently eluted with CH_2Cl_2-acetone mixture and fractions were monitored by TLC using pre-coated Silica gel GF_{254} (Merck) and the same previous mixture of solvents for development. Spots were visualized by spraying with vanillin/H_2SO_4 followed by heating at 105 °C for 30 sec., a procedure which gave invariably reddish-brown spots. Fractions containing a major compound with R_f 0.98 (10% acetone in CH_2Cl_2) were evaporated to dryness to give 550 mg which were further purified by C. C. (Silica gel) with isocratic elution with CH_2Cl_2 containing 0.01% acetic acid to afford compound **3** (30 mg). Fractions showing a spots at R_f 0.8 were evaporated *in vacuo* to give 3.2 g of impure crystalline residue. Repeated C. C. purification of 0.2 g of the latter residue afforded 100 mg of compound **1**. Fractions containing one spot with R_f 0.40 (10% acetone in CH_2Cl_2) were evaporated and crystallized to afford 20 mg of colorless needles (compound **2**). Fractions containing one spot with R_f 0.32 (10% acetone in CH_2Cl_2) were evaporated and crystallized to afford 10 mg of colorless needles (compound **5**). Fractions containing one spot with R_f 0.22 (10% acetone in CH_2Cl_2) were evaporated and crystallized to afford 30 mg of colorless needles (compound **4**).

Limonin (**1**): mp 118–120° C; $[\alpha]_D^{25}$ – 1.1° (*c* 1.21, acetone); FABMS, $[M + H]^+$ at *m/z* 471, $C_{26}H_{30}O_8$; IR υ_{max} (KBr) 3154, 2960, 1761 (lactones), 1719 (C = O), 1647, 1503, 1285, 1030, 875 cm^{-1}. ^{1}H-NMR (500 MHz, DMSO-d_6): δ 4.10 (*br s*, H-1), 2.26 (*dd*, *J* = 14.8, 3.2 Hz, H-2a), 2.65 (*dd*, *J* = 14.8, 3.2 Hz, H-2b), 2.47 (*dd*, *J* = 15.0, 2.8 Hz, H-5), 2.75 (*dd*, *J* = 15.0, 2.8 Hz, H-6a), 3.10 (*t*, *J* = 15.0 Hz, H-6b), 2.55 (*dd*, *J* = 10.0, 2.0 Hz, H-9), 1.78 (*m*, H-11a), 1.70 (*m*, H-11b), 1.26 (*m*, H-12a), 1.73 (*m*, H-12b), 4.09 (*s*, H-15), 5.45 (*s*, *H*-17), 1.09 (3H, s, H-18), 4.46 (*d*, *J* = 13 Hz, H-19a), 4.90 (*d* , *J* = 13 Hz, H-19b), 7.63 (*br s*, H-21), 6.48 (*br s*, H-22), 7.69 (*br s*, H-23), 0.98 (*s*, H-24), 1.17 (3H, *s*, H-25), 1.01 (3H, *s*, H-26), ^{13}C-NMR, data for compounds **1**–**5** are listed in Table I.

Deacetylnomilin (**2**): mp 253–255° C *dec*; $[\alpha]_D^{25}$ – 2.7° (*c* 1.20, acetone); IR υ_{max} (KBr) 3400, 3000–2900, 1770–1700 (lactone and C = O), 1460, 1390, 1290, 1120, 1080, 1030, 870 cm^{-1}. ^{1}H-NMR (500 MHz, DMSO-d_6): δ 3.63 (*t*, H-1), 2.98 (*t*, *J* = 2.0 Hz, H-2a), 3.30 (*dd*, *J* = 14.0, 2.0 Hz, H-2b), 2.42 (*dd*, *J* = 15.0, 2.8 Hz, H-5), 2.28 (*dd*, *J* = 15.0, 2.8 Hz, H-6a), 2.65 (*t*, *J* = 15.0 Hz, H-6b), 2.67 (*dd*, *J* = 10.0, 2.0 Hz, H-9), 1.40 (*m*, H-11a), 1.67 (*m*, H-11b), 1.70 (*m*, H-12a), 1.73 (*m*, H-12b), 3.75 (*s*, H-15), 5.35 (*s*, H-17), 1.98 (3H, s, H-18), 1.12 (*s*, H-19), 7.62 (*br s*, H-21), 6.65 (*br s*, H-22), 7.71 (*br s*, H-23), 1.09 (*s*, H-24), 1.26 (3H, *s*, H-25), 1.44 (3H, *s*, H-26).

Obacunone (**3**), mp 212–215° C; IR υ_{max} (KBr) 3400, 3000–2900, 1754 (lactone), 1711 (C = O), 1460, 1290, 1160, 880 cm^{-1}. ^{1}H-NMR (500 MHz, DMSO-d_6): δ 6.70 (*d*, *J* = 11.8 Hz, H-1), 5.81 (*d*, *J* = 11.8 Hz, H-2), 2.66 (*dd*, *J* = 13.5, 5.0 Hz, H-5), 2.20 (*dd*, *J* = 13.8, 4.8 Hz, H-H-6a), 3.02 (*t*, *J* = 14.0 Hz, H-6b), 2.10 (*dd*, *J* = 10.0, 2.0 Hz, H-9), 1.70 (*m*, H-11a), 1.77 (*m*, H-11b), 1.77 (*m*, H-12a), 1.80 (*m*, H-12b), 3.75 (*s*, H-15), 5.41 (*s*, H-17), 0.99 (3H, *s*, H-18), 1.34 (3H *s*, H-19), 7.63 (*br s*, H-21), 6.48 (*br s*, H-22), 7.70 (*br s*, H-23), 1.14 (3H, *s*, H-24), 1.30 (3H, *s*, H-25), 1.38 (3H, *s*, H-26).

Ichangin (**4**), mp 198–199° C; $[\alpha]_D^{25}$ – 6.3° (*c* 1.21 acetone); IR υ_{max} (KBr) 3100–3600 br, 2940, 1760–1700 br, 1280, 1030, 868 cm^{-1}. ^{1}H-NMR (500 MHz, DMSO-d_6): δ 3.80 (*m*, H-1), 2.60 (*m*, H-2a), 2.72 (*dd*, *J* = 13, 9.5 Hz, H-2b), 2.61 (*m*, H-5), 2.20 (*dd*, *J* = 13.5, 2.0 Hz, H-6a), 2.85 (*t*, *J* = 13.5 Hz, H-6b), 2.18 (*dd*, *J* = 9.8, 1.0 Hz, H-9), 3.69 (*s*, H-15), 5.38 (*s*, H-17), 0.98 (3H, *s*, H-18), 4.65 (*d*d, *J* = 13.0 Hz, H-19-a), 4.90 (*d*, *J* = 13.0 Hz, H-19b), 7.60 (*br s*, H-21), 6.40 (*br s*, H-22), 7.65 (*br s*, H-23), 1.15 (3H, *s*, H-24), 1.18 (3H, *s*, H-25), 1.10 (3H, *s*, H-26).

Isolimonexic acid methyl ether (**5**), $C_{27}H_{32}O_{10}$; HRFTMS *m/z* calcd for $C_{27}H_{33}O_{10}$ $(M + H)^+$ 517.2074, found 517.2012; mp, 166–167° C; $[\alpha]_D^{25}$ – 4.1° (*c* 0.22, methanol); UV λ_{max} (log ε) (MeOH) 205 (4.01), 269 (2.54), 275 (2.56) nm; IR υ_{max} (KBr), 3150, 2950, 1761–1719 br strong, 1290, 1120, 1030 cm^{-1}. ^{1}H-NMR (500 MHz, DMSO-d_6): δ 4.11 (*br s*, H-1), 2.26 (*dd*, *J* = 14.8, 3.2 Hz, H-2a), 2.65 (*dd*, *J* = 14.8, 3.2 Hz, H-2b), 2.49 (*m*, H-5), 2.99 (*m*,

Table I. ^{13}C-NMR Data of the isolated limonoids.[a]

C #	**1**	**2**	**3**	**4**	**5**
1	78.8	68.4	158.8	70.3	78.3
2	36.1	39.2	122.3	38.2	35.6
3	170.3	170.8	167.6	171.5	170.1
4	79.5	83.8	84.7	71.7	83.8
5	58.4	49.4	56.6	49.6	48.5
6	36.6	38.9	40.2	39.0	36.1
7	208.1	208.7	209.1	209.5	207.8
8	50.3	52.0	53.0	52.1	50.2
9	46.9	43.7	48.9	48.9	46.3
10	45.3	44.2	43.5	46.3	45.2
11	19.7	16.7	19.6	21.4	17.7
12	29.3	31.2	32.4	32.8	28.7
13	37.7	36.8	37.7	36.3	37.8
14	64.9	65.8	66.2	65.2	66.1
15	54.1	52.6	53.5	52.0	52.6
16	167.4	167.2	167.9	166.9	167.2
17	77.9	77.5	78.3	77.4	77.6
18	17.6	20.2	21.3	20.6	19.7
19	66.7	15.9	17.1	67.8	64.8
20	120.3	120.2	120.9	120.1	161.5
21	143.4	143.3	144.2	143.4	103.1
22	110.2	110.2	111.1	110.2	123.1
23	141.7	141.6	142.5	141.5	168.7
24	17.1	16.1	17.2	15.7	16.4
25	29.8	33.0	32.5	32.7	29.6
26	21.4	23.1	27.4	26.1	21.4
-OCH_3					56.6

[a] In DMSO-d_6, 125 MHz. Carbon multiplicities were determined by DEPT 135° experiments.

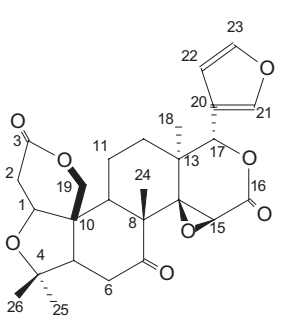
Hz, H-6a), 3.12 (*t*, *J* = 15.0 Hz, H-6b), 2.50 (*dd*, *J* = 10.0, 2.0 Hz, H-9), 3.81 (*s*, H-15), 5.10 (*s*, H-17), 1.09 (3H, s, H-18), 4.42 (*d*, *J* = 13.0 Hz, H-19-a), 4.92 (*d*, *J* = 13.0 Hz, H-19b), 5.96 (*br s*, H-21), 6.39 (*br s*, H-22), 1.12 (*s*, H-24), 1.44 (3H, *s*, H-25), 0.98 (3H, *s*, H-26), 3.45 (3H, *s*, -OCH_3).

Anti-malarial activity

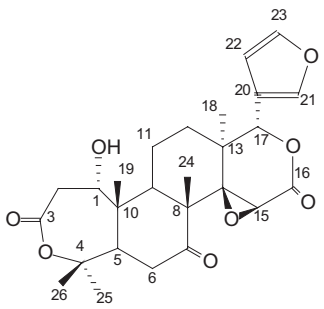
Stock cultures of the Sierra Leone D6 clone (chloroquine-sensitive, mefloquine-resistant) and Indochina W2 clone (chloroquine-resistant, mefloquine-sensitive) were obtained from the Department of Pharmacognosy, University of Mississippi culture collection and maintained using a modification of Trager and Jensen (1976). The detailed methodology is reported by El Sayed *et al.* (1996).

Results and Discussion

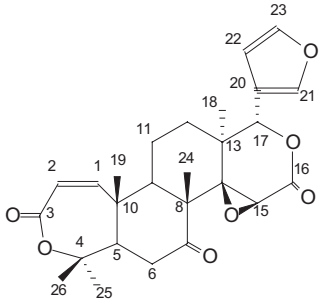
The alcoholic extract of the seeds of *Citrus reticulatea* afforded a bitter-tasting mixture whose components gave an Ehrlich-positive reaction on TLC, which is characteristic of limonoids (Bennett and Herman, 1989). Column chromatographic fractionation of the crude mixture resulted in the isolation of five pure compounds **1**–**5** (Fig. 1). Compound **1** revealed a molecular ion $[M + H]^+$ at *m/z* = 471 equivalent to the molecular formula $C_{26}H_{30}O_8$ and 12 degrees of unsaturation (DBE). Spectral data of **1** indicated the presence of 2 lactones, one ketone, 4 methyls and a furan ring. All physical and spectral data were consistent with those reported for limonin (Dreyer *et al.*, 1976). Compound **2** revealed a molecular formula $C_{26}H_{32}O_8$ and 11 DBE units, suggesting one ring less than limonin. NMR data of **2** possessed the same features of **1** except for the replacement of C-1 methine signal at δ 78.8 and C-19 methylene signal at δ 66.7 with the methine signal at δ 68.4 and the methyl carbon at δ 15.9, respectively. In the HMQC, the latter 2 signals were correlated to the proton triplet at δ 3.63 and the methyl singlet at δ 1.12, respectively. On the other hand, the HMBC spectrum of **2** showed cross peak correlations between H-1 and the quaternary C-3 carbonyl (δ 170.8) and C-10 (δ 44.2). The methyl singlet H_3-19 also show HMBC correlations with C-1,



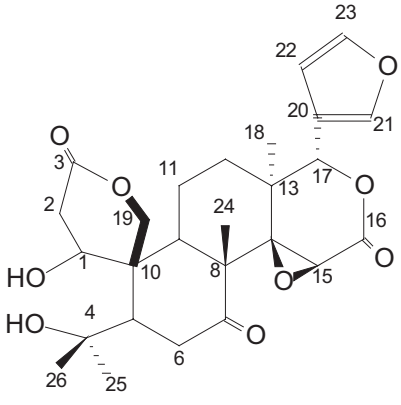
1 Limonin



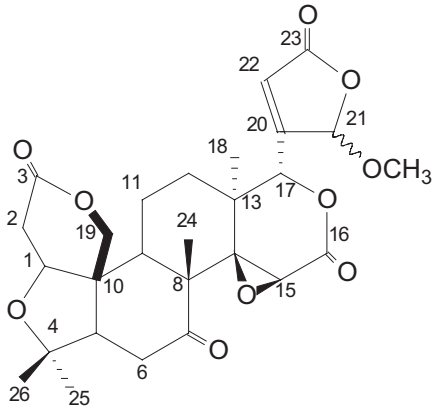
2 Deacetylnomilin



3 Obacunone



4 Ichangin



5 Isolimonexic acid methyl ether

Fig. 1. Limonoids from *Citrus reticulata.*

C-5, C-9, and C-10. Consequently, the structure of **2** was determined to be deacetylnomilin (Bennett and Hasegawa, 1981). Compound **3** possessed a molecular formula of $C_{26}H_{30}O_7$, *i. e.*, 18 Daltons less than **2** (probably due to loss of H_2O unit). The ^{13}C-NMR spectrum of compound **3** was similar to that of **2** except for the formation of $\Delta^{1,2}$ system. The olefinic methine signals at δ 158.8 and 122.3, which showed cross peaks at δ 6.70 (d, J = 11.8) and 5.81 (d, J = 11.8) in the HMQC spectrum were assigned to C-1 and C-2, respectively. This was confirmed by HMBC correlations between H-1 and both C-3 (δ 167.6) and C-19 (δ 17.1). Meanwhile, H-2 doublet showed HMBC correlation with C-10 (δ 43.5). These data were in full agreement to those reported for obacunone (Dreyer *et al.*, 1976).

Compound **4** revealed $[M + H]^+$ at *m/z* 489 equivalent to the molecular formula $C_{26}H_{32}O_9$ and 11 degrees of unsaturation, *i. e.*, with 18 mass units more than that of limonin. The ^{13}C-NMR data of **4** indicated an upfield shifts of C-1 (8.5 ppm) and C-4 (7.8 ppm), compared with those of limonin. These data suggested that the ether linkage between C-1 and C-4 was opened. Comparison of the data of **4** and those of Ichangin (Bennett *et al.*, 1991) revealed that the two compounds are identical.

The HR-FTMS of compound **5** revealed a molecular ion peak $[M + H]^+$ at *m/z* 517.2012, sug-

gesting the molecular formula $C_{27}H_{32}O_{10}$ and 12 DBE units. The NMR spectral data of **5** were somewhat similar to those of limonin except for the furan moiety which is replaced in case of **5** by a 5-methoxy-2(5H)-furanone moiety. The HMQC data of **5** exhibited a proton singlet at δ 3.45, which is correlated with the methyl carbon at δ 56.6, indicating the presence of a methoxyl group. This methoxy singlet showed ^3J-HMBC correlation with the methine carbon at δ 103.1, which in turn was correlated with the proton singlet at δ 5.96 and was assigned to C-21. The proton H-21 showed ^3J-HMBC correlation with the olefinic methine carbon at δ 123.1 (correlated with the broad proton singlet at δ 6.39), assigned to C-22. Both H-21 and H-22 showed ^3J- and ^2J-HMBC correlations, respectively, with the quaternary carbonyl signal at δ 168.7, which was assigned to C-23. The proton singlet of H-17 (δ 5.10) showed ^3J-HMBC correlation with H-22 (δ 6.39) and ^2J-HMBC correlation with the quaternary carbon at δ 161.5, assigned to C-20. The downfield shifting of the carbon C-20 indicated its β-location to the carbonyl C-23, and hence confirmed the assigned isolimonexic acid skeleton rather than limonexic acid at which the carbonyl is at C-21 and the methoxy is at C-23. The splitting pattern and the lack of any DQF-COSY correlation between H-21 and H-22 further supported this fact (Lee *et al.*, 1999 and Ming *et al.*, 1987). The stereochemistry of the chiral center C-21 was left ambiguous since there was no enough NOESY data to support its assignment. It is worth noting that X-ray crystallography of the naturally occurring limonexic acid, reported from *Citrus nippokoreana*, usually occurs as a 23*S* and 23*R* epimeric mixture (Lee *et al.*, 1999). The natural identity of **5** was confirmed by TLC detection of this compound in the fresh seed ethanolic extract. This is further supported by the fact that limonexic acid was isolated from a natural source in which limonin was not detected by TLC, suggesting that these compounds exist in nature and are not artifacts due to oxidation of limonin (Lee *et al.*, 1999). Based on the fore-mentioned data, compound **5** was found to be isolimonexic acid methyl ether, a newly reported natural product.

All limonoids **1**–**5** were subjected to antifungal activities against *Candida albicans* (ATCC 90028) and *Cryptococcus neoformans* (ATCC 90113), and antibacterial against *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853) and *Bacillus subtilis* (ATCC 6633) and found inactive at a concentration of 500 μg/ml. Of all tested limonins, isolimonexic acid methyl ether (**5**) showed marginal activity against *Plasmodium falciparum* (D6 clone) and *P. falciparum* (W2 clone) with $IC_{50} > 4.76$ μg/ml, with selectivity index > 1 and without any cytotoxicity to Vero cells (Vero African Green Monkey kidney cells ATCC CCL 81).

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