Acridone alkaloids and flavones from the leaves of *Citrus reticulata*

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**ABSTRACT**

A new acridone alkaloid, reticarcidone A (1), decorated with an oxygenated isopentenyl group between C-1 and C-2, was isolated from the leaves of *Citrus reticulata* Blanco, together with nine known acridone alkaloids (2–10) and fifteen flavones compounds (11–25). The structure of those compounds were confirmed by analysis of comprehensive 1D and 2D NMR, and MS data. Reticarcidone A (1) was the first pyrano[2,3-a]acridone isolated from the genus *Citrus*. Some of these compounds showed moderate cytotoxicity against the five human tumor cell lines MCF-7, SMMC-7721, HL-60, A549 and SW480.

**ARTICLE HISTORY**

Received 13 December 2020
Accepted 11 January 2021

**KEYWORDS**

*Citrus reticulata*; acridone alkaloids; flavones; cytotoxic activity

1. Introduction

The genus *Citrus* is an important economic crops and belongs to the Rutaceae family, of which are widespread throughout the tropical and subtropical regions (Rendeiro...
et al. 2016). Various species of the Citrus genus are used as food, beverages, folk medicine and perfumes, which have important medical and economic value (Marín et al. 2007; Han et al. 2010). Mounts of diverse secondary metabolites have been identified from this genus, including flavones, coumarins, limonoids, acridone alkaloids and essential oil (Wu et al. 1983a; Gonzalez et al. 1988; Akiyoshi et al. 1990; Panthong et al. 2013). The flavonoids were the largest number of components in Citrus genus, which exhibited extensive biological activities, such as anti-depression, anti-anxiety, anti-bacteria, and anti-inflammatory (Lichius et al. 1994; Benavente-Garcia and Castillo 2008; Cuong et al. 2015).

Citrus reticulata Blanco, is widely distributed in Sichuan, Zhejiang, Jiangxi, Hunan, Guangdong provinces of China. Arcidone alkaloids, a group of important bioactive compounds, are mainly founded in the roots of Citrus genus (Dzierzbicka et al. 2001; Samuel et al. 2019). In this study, a new arcidone alkaloid (1), nine known acridone alkaloids (2–10) and fifteen flavones (11–25) compounds (Figure 1) were isolated from the leaves of C. reticulata. Some compounds displayed moderate cytotoxic activities. Herein, the details of the separation, structure elucidation and cytotoxic activity assessment of these compounds are described.

2. Results and discussion

The molecular formula C_{19}H_{17}NO_{4} of reticarcidone A (1) was assigned by the 13C NMR and HRESIMS data (m/z 322.1091 [M – H]−, calcd for C_{19}H_{16}NO_{4}, 322.1085), indicating 12 indices of hydrogen deficiency (IOHD). The 1H NMR data indicated the presence of one hydroxyl proton at δ_{H} 10.52, three conterminal aromatic ring protons at δ_{H} 7.15 (dd, J = 7.9 Hz), 7.23 (dd, J = 7.9, 1.0 Hz), and 7.73 (dd, J = 7.9, 1.0 Hz), one singlet aromatic ring protons at δ_{H} 5.70, and three singlet methyls at δ_{H} 3.96, 1.42, and 1.42. The 13C and DEPT NMR data (Supplementary material Table S1) revealed the existence of 19 carbon resonances that were attributed as ten quaternary carbons, six methines, and three methyls. Analysis of these data manifested the characteristic resonances of one aromatic ring with three substituents, one aromatic ring with five substituents, one carbonyl, and a double bond with Z-configuration. Further study of the NMR data of 1 suggested that the structure of 1 was similar to those of the known compound 5-hydroxynoracronycin (2), an acridone alkaloid derivative decorated with an isopentenyl group. This conclusion was verified via the 1H–1H COSY correlations of H-6/H-7/ H-8 and H-1′/H-2′, as well as the HMBC correlations of OH-5 with C-5/C-5a/C-6; H-8 with C-5a/C-8a/C-9; H-4 with C-2/C-3/C-4a/C-9a, and N-methyl to C-4a/C-5a; Me-4′ and Me-5′ with C-2′/C-3′ (Supplementary material Figure S1). The distinct difference between 1 and 2 was the appearance of the hydroxyl proton at δ_{H} 14.45 in 1, the replacement of a methine (δ_{C} 97.0) and N-methyl (δ_{C} 48.6) in 2 by a methine (δ_{C} 92.1) and N-methyl (δ_{C} 40.9) in 1, respectively. The HMBC correlations between H-1′/C-1, C-2, and C-3, and H-2′/C-2 indicated the presence of the linear pyrano[2,3-a]arcidine in 1, instead of the linear pyrano[2,3-c]arcidine in 2. Therefore, the structure of reticarcidone A (1) was determined as shown in Figure 1, which was known as the first pyrano[2,3-a]acridone isolated from the genus Citrus.
The known compounds were identified as 5-hydroxynoracronycin (2) (Wu et al. 1983b), citracidone-II (3) (Basa and Tripathy 1984), citracidone-I (4) (Furukawa et al. 1983), citracidone-III (5) (Furukawa et al. 1983), citrusamine (6) (Motoharu et al. 1987), 5-hydroxyarboriarborine (7) (Wijeratne et al. 1992), citpressine-I (8) (Wu et al. 1982), glycofolinine (9) (Ono et al. 1995), 1,6-dihydroxy-2,3,4-trimethoxy-9(10H)-acridone (10) (Braga et al. 2007), 5,6,4′-trihydroxypyranoflavone (11) (Sadasivam et al. 2018), citrusinol (12) (Wu 1987), 4′-hydroxyisolonchocarpin (13) (Ngadjui et al. 1999),

Figure 1. The structures of compounds 1–25.
citflavanone (14) (Khaomek et al. 2008), chalcone (15) (Sastry and Row 1961a), noble-
atin (16) (Nagase et al. 2005), tangeretin (17) (Horie et al. 1998), natsudaidai (18) (Wang et al. 2014), 3-hydroxytangeretin (19) (Horie et al. 1998), 5-hydroxy-6,7,8,3’,4’-pentamethoxyflavanone (20) (Sastry and Row 1961b), 5-hydroxy-6,7,8,4’-tetramethoxyflavanone (21) (Cuong et al. 2015), 5-hydroxy-4’,6,7,8-tetramethoxyflavone (22) (Horie et al. 1998), 5-demethylnobiletin (23) (Nagase et al. 2005), sudachitin (24) (Lichius et al. 1994) and sinensetin (25) (Gonzalez et al. 1988), by comparing the NMR data with those in the literature.

These isolated compounds were tested for their in vitro cytotoxic activities against five human tumor cell lines SMMC-7721, MCF-7, HL-60, A549, and SW480 by using MTT method (Cory et al. 1991). The results (Supplementary material Table S2) revealed that compound 2 and 18 exhibited cytotoxic activity against all the five human tumor cell lines. Specially, compound 20 was revealed to displayed cytotoxic effect against the SMMC-7721 with IC50 value of 1.868 ± 0.058 µM.

3. Experimental
3.1. General experimental procedures
IR spectra and UV spectra were obtained from a Bruker FT-IR Tensor-27 infrared spec-
trophotometer with KBr disks and a Shimadzu UV-2401PC spectrometer, respectively. HREIMS data were recorded on Agilent G6230 TOF mass spectrometers. 1H, 13C, DEPT, HSQC, 1H–1H COSY, and HMBC NMR data were obtained from a Bruker DRX-600 spectrometer using TMS as an internal standard. Agilent 1100 HPLC with a Zorbarx SB-C18 (9.4 × 250 mm) column and Waters 1525 HPLC with a Cosmosil Cholester (20 × 250 mm) column, respectively, were used for semi-preparative and preparative HPLC. Column chromatography was performed by using MCI gel (75–150 µm, Mitsubishi Chemical Corporation, Tokyo, Japan), Sephadex LH-20 (GE Healthcare, USA), and Silica gel (100–200 and 200–300 mesh, Qingdao Marine Chemical Co., Ltd., Qingdao, People’s Republic of China).

3.2. Plant material
The leaves of C. reticulata were collected from Pengshan, Meishan, Sichuan, People’s Republic of China, in March 2018. The specific name was authenticated by Dr. En-De Liu, and the voucher specimen (201803-L02) was deposited at the Kunming Institute of Botany.

3.3. Extraction and isolation
The air-dried leaves of C. reticulata (8.0 kg) were extracted with methanol (50 L, three times, two days/each time). The crude extract (1.2 kg) was subjected to a silica gel column chromatography eluted with chloroform/methanol (1:0, 10:1, 5:1, 0:1, v/v, gradient system) to get seven fractions A-G.

Fraction E (128 g) was separated on an MCI-gel column, eluted with methanol/water (60:40–100:0, gradient system, v/v), to yield nineteen fractions (Fr. E1–E19). Fraction E2
(3.4 g) was separated by a silica gel column chromatography eluted with chloroform/methanol (100:1, 50:1, 20:1, 10:1, gradient system, v/v) to afford seven fractions (Fr. E2a-E2g). Fraction E2b (58 mg) was separated by semi-preparative HPLC (methanol/water, 3.0 mL/min, 73:27, v/v) to afford compound 3 (13.1 mg) and 5 (20 mg). Fraction E2c (28 mg) was purified by semi-preparative HPLC (methanol/water, 3.0 mL/min, 70:30, v/v) to yield compound 10 (2.4 mg). Compounds 1 (2.1 mg), 8 (4.9 mg), 19 (10.0 mg), 20 (15.2 mg) were obtained from fractions E2d (222 mg) and E2f (48 mg) by repeated semi-preparative HPLC.

Fraction E4 (4.5 g) was applied to a silica gel column chromatography eluted with CHCl3/methanol (100:1, 50:1, 20:1, 10:1, gradient system, v/v) to afford three fractions (Fr. E4a-E4c). Fraction E4a (900 mg) was separated by preparative HPLC (methanol/water, 10.0 mL/min, 72:28, v/v) to afford compound 2 (400 mg). Compound 21 (36.9 mg) was obtained from fraction E4b (59 mg) by semi-preparative HPLC (ACN/H2O, 3.0 mL/min, 66:34, v/v). Fraction E6 (6.1 g) was subjected to a silica gel column chromatography eluted with CHCl3/methanol (40:1, 20:1, 10:1, 5:1, 0:1, gradient system, v/v) to afford nine fractions (Fr. E6a-E6i). Fraction E6a (1.64 g) was separated by a Sephadex LH-20 column (eluted by acetone) to afford ten fractions (Fr. E6a1-E6a10). Fraction E6a2 (565 mg) was separated by preparative HPLC (methanol/water, 10.0 mL/min, 65:35, v/v), followed by repeated semi-preparative HPLC to get compounds 4 (3.3 mg), 18 (272 mg) and 22 (205 mg). Compounds 9 (29 mg) and 17 (280 mg) were purified from fraction E6a5 (498 mg) by preparative HPLC (methanol/water, 10.0 mL/min, 75:35, v/v).

Fraction E7 (4.7 g) was subjected to a silica gel column chromatography eluted with CHCl3/methanol (20:1, 10:1, 7:1, 5:1, 2:1, 0:1, gradient system, v/v) to afford seven fractions (Fr. E7a-E7g). Fraction E7a (83 mg) was separated by semi-preparative HPLC (methanol/water, 3.0 mL/min, 52:48, v/v) to obtained compounds 12 (9.9 mg) and 16 (7.5 mg). Fraction E7c (166 mg) was separated by semi-preparative HPLC (ACN/H2O, 3.0 mL/min, 50:50, v/v) to obtained compounds 24 (37 mg). Fraction E9 (3.9 g) was subjected to a silica gel column chromatography eluted with petroleum ether/acetone (10:1, 7:1, 5:1, 2:1, 0:1, gradient system, v/v) to afford thirteen fractions (Fr. E9a-E9m). Fraction E9c (154 mg) was separated by semi-preparative HPLC (methanol/water, 3.0 mL/min, 65:35, v/v) to obtained compounds 14 (5.4 mg) and 15 (23 mg). Fraction E14 (2.0 g) was applied to a Sephadex LH-20 column (eluted by acetone) to afford five fractions (Fr. E14a-E14e). Fraction E14a (231 mg) was chromatographed on a silica gel column, eluted with petroleum ether/acetone (20:1, 10:1, 7:1, 5:1, 2:1, 0:1, gradient system, v/v) to yield six fractions (Fr. E14b1-E14b6). Compounds 13 (5.7 mg) and 23 (2.6 mg) were separated from fraction E14b2 (16.7 mg). Compounds 7 (18.8 mg), 6 (12 mg), 11 (41 mg) and 26 (3.6 mg) were obtained from fraction E14d (67 mg) and fraction E14e (109 mg).

3.4. Characterization

Reticarcidone A (1): yellow oil, IR (KBr) $\nu_{\text{max}}$ 3236, 2970, 1615, 1578, 1560, 1460, 1127 cm$^{-1}$; UV $\lambda_{\text{max}}$ 305 nm; HRESIMS: m/z 322.1091 [M – H]$^-$, calcd for C19H16NO4, 322.1085.
3.5. Cytotoxicity assay

The cytotoxic assay was evaluated by using the MTT method according to the previous procedure (Ye et al. 2018). The detailed process was described in the Supplementary Information.

4. Conclusions

The chemical study of the leaves of C. reticulata has resulted in the isolation of a new acridone alkaloid, reticarcidone A (1), which was decorated with the linear pyrano[2,3-a]acridone, as well as nine known acridone alkaloids and fifteen known flavones compounds. Ten of the isolated twenty-five compounds showed cytotoxic activity against the SMMC-7721 cell lines.

Disclosure statement

There are no conflicts to declare.

Funding

This research was funded by the Technical Program of China Tobacco Sichuan Industrial Co., Ltd. [hx201907].

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