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Seven new triterpenoids from the aerial parts of *Ilex cornuta* and protective effects against H_2O_2-induced myocardial cell injury

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ABSTRACT

Seven new triterpenoids (**1**–**7**), together with two known ones (**8**–**9**), were isolated from the aerial parts of*Ilex cornuta*. The leaves of *I. cornuta* are the major source of "Kudingcha", a popular herbal tea consumed in China and other countries. The structures of compounds **1**–**7** were determined as 20-*epi*-urs-12,18-dien-28-oic acid 3β-*O*-α-L-arabinopyranoside (**1**), 20-*epi*-urs-12,18-dien-28-oic acid 2′-*O*-acetyl-3β-*O*-α-L-arabinopyranoside (**2**), 20-*epi*-urs-12,18-dien-28-oic acid 3β-*O*-β-D-glucuronopyranoside-6-*O*-methyl ester (**3**), 3β,23-dihydroxy-20-*epi*-urs-12,18-dien-28-oic acid (**4**), 23-hydroxy-20-*epi*-urs-12,18-dien-28-oic acid 3β-*O*-α-L-arabinopyranoside (**5**), 23-hydroxy-20-*epi*-urs-12,18-dien-28-oic acid 3β-*O*-β-D-glucuronic acid (**6**), 23-hydroxy-20-*epi*-urs-12,18-dien-28-oic acid 3β-*O*-β-D-glucuronopyranoside-6-*O*-methyl ester (**7**), on the basis of spectroscopic analyses (IR, ESI–MS, HR-ESI–MS, 1D and 2D NMR) and chemical reactions. Protective effects against H_2O_2-induced H9c2 cardiomyocyte injury were tested in vitro for compounds **1**–**9**, and the data showed that compound **4** had significant cell-protective effect. Compounds **1**-**9** did not show significant DPPH radical scavenging activity.

1. Introduction

Ilex cornuta Lindl. et Paxt. (Aquifoliaceae family), a slow-growing evergreen shrub or small tree, is cultivated widely in South China. The leaves of *I. cornuta* are used to make the popular herbal tea, called as "Kudingcha" or bitter tea (Chau and Wu, 2006) in China. Commercially, Kudingcha is sold in the form of tea-bags packed with ground leaves (1 to 2 g). The leaf extracts are also used as ingredients in food or dietary supplement industries. Traditionally, Kudingcha has been used for treatment of sore throat, as an agent for weight loss (Ahai, 1985), and for the relief of hypertension (Jiangsu Medical College, 1975). It is believed that Kudingcha has potential benefits for reducing cardiovascular and oxidative stress-related diseases (Thuong et al., 2009). Recently, *I. cornuta* has been gaining research attention because of its potential health benefits. Studies showed that Kudingcha possesses antioxidant, antidiabetic, hepatoprotective, neuroprotective, anti-inflammatory, and diuretic effects (Jiangsu New Medical College, 1986; Qin et al., 1988; Puangpraphant and De Mejia, 2009). Previous phytochemical investigations showed that the whole plant of *I. cornuta* is a rich source of triterpenoids and flavonoids as well as their corresponding glycosides (Wang et al., 2014; Liao et al., 2013; Li et al., 2006). Previous studies on the aerial parts of *I. cornuta* have resulted in the isolation of a series of new triterpenoidal saponins, and some triterpenoidal saponins showed potential benefit for inhibition of peroxidative damage associated with ischemia and reperfusion (Li et al., 2014). As part of a continuous search for potentially active substances for the prevention of coronary artery disease, seven new triterpenoids (**1**-**7**) (Fig. 1), along with two known ones were obtained from the aerial parts of *I. Cornuta*. The isolation and structural elucidation of the new compounds, as well as the protective effects of the isolates against cardiomyocytes injury induced by H_2O_2 are reported herein. In addition, their radical scavenging activity was evaluated by DPPH assay.

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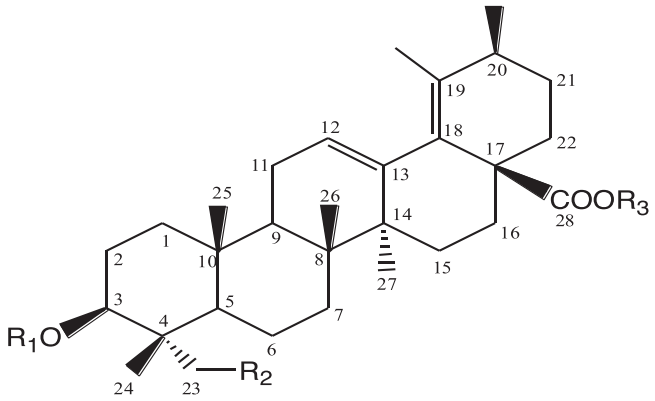

Fig. 1. Structures of the compounds **1**–**9**.

2. Results and discussion

2.1. Structure Identification of the isolates

A 50% EtOH extract of the aerial parts of *I. cornuta* was subjected to column chromatographic separations on macroporous resin D101, silica gel and octadecylsilane (ODS) silica gel, yielding seven new triterpenoids (**1**–**7**) and two known ones which were identified as 3β-hydroxy-20α(H)-urs-12,18-dien-28-oic acid (**8**) (Ali and Srivastava, 1990), 3β-*O*-α-L-arabinopyranosyl-20α(H)-urs-12,18-dien-28-oic acid 28-*O*-β-D-glucopyranoside (**9**) (Che et al., 2012) (Fig. 1).

Compound **1** was isolated as white powder, and its negative-ion HR-ESI–MS showed a quasimolecular $[M+Na]^+$ ion peak at *m*/*z* 609.3796, attributed to the molecular formula of $C_{35}H_{54}O_7Na$ (calc'd. 609.3767). The ^{1}H NMR spectrum showed the singlet resonances of six tertiary methyl groups at δ 0.97 (3H, s, Me-25), 1.06 (3H, s, Me-24), 1.12 (3H, s, Me-26), 1.23 (3H, s, Me-27), 1.38 (3H, s, Me-23), 1.97 (3H, s, Me-29), one methyl doublet at 1.21 (3H, d, *J* = 6.5 Hz Me-30), an olefinic proton signal at δ 5.73 (1H, m, H-12), and the signal of a oxygen-bearing methine at δ3.49 (1H, dd, *J* = 4.0, 12.0 Hz, H-3). The NMR spectra indicated that the aglycone of **1** was a 3β-hydroxy-20-*epi*-urs-12,18-dien-28-oic acid by comparing its spectroscopic data with those of compound **8** (Ali and Srivastava, 1990). In the ^{1}H NMR spectrum, an anomeric proton was observed at δ_H 4.90 (1H, d, *J* = 7.0 Hz), which showed HSQC correlation with the anomeric carbon at δ_C 107.7 (C-1 of arabinose (Ara)), indicating the presence of one sugar unit in the structure of compound **1**. The sugar residue yielded from acid hydrolysis of **1** was identified as L-arabinose by GC analysis. The location of arabinopyranosyl group was assigned at C-3 of the aglycone on the basis of the observations of the down field shift of H-3 at δ3.49, the correlation between δ_H 3.49 (H-3) and δ_C 107.7 (C-1 of Ara), as well as the correlation between δ_H 4.90 (H-1 of Ara) and δ_C 89.0 (C-3 of the aglycone) in the HMBC spectrum (Fig. 2). The α-configuration of arabinopyranosyl unit was inferred from the NOESY correlations between δ 4.90 (H-1 of Ara) and δ 4.26 (H-3 of Ara), 4.42 (H-4 of Ara) (Fig. 2). Thus, compound **1** was established as 20-*epi*-urs-12,18-dien-28-oic acid 3β-*O*-α-L-arabinopyranoside.

Compound **2** was obtained as white powder. Its molecular formula was determined to be $C_{37}H_{56}O_8$ according to the $[M-H]^-$ ion peak at *m*/*z*627.3891(calc'd. 627.3897). An anomeric proton observed at δ_H 4.83 (1H, d, *J* = 7.5 Hz) showed HSQC correlation with the anomeric carbon at δ_C 104.8 (C-1 of Ara), Which indicated the presence of one sugar unit in the structure of compound **2**. The structure of the sugar residue yielded from acid hydrolysis of **2** was identified as L-arabinose by GC analysis. Comparing the NMR spectrum of compound **2** with that of compound **1**, it was suggested that **2** was derived from the acetylation of **1**, The downfield shift of C-2 (+1.4 ppm) of Ara at δ_C 74.5 suggested that the acetyl (Ac) group was located at C-2 of arabinose, and the assignment was supported by the HMBC correlation between δ_H 5.98 (H-2 of Ara) and δ_C 170.1 (C-1 of Ac) (Fig. 2). Thus, compound **2** was elucidated as 20-*epi*-urs-12,18-dien-28-oic acid 3β-*O*-(2′-*O*-acetyl)-α-L-arabinopyranoside.

Compound **3** was obtained as white powder. Its molecular formula was determined to be $C_{37}H_{56}O_9$ based on the positive HR-ESI–MS. An anomeric proton observed at δ_H 5.13 (1H, d, *J* = 14.0 Hz, H-1 of GlcA) showed HSQC correlation to the anomeric carbon at δ_C 107.5 (C-1 of GlcA), indicating the presence of a sugar unit in the structure of compound **3**. The sugar residue obtained from the acid hydrolysis of **3** was identified as D-glucuronic acid by the GC analysis. The ^{13}C NMR spectrum of **3** was very similar to that of urs-12,18-dien-28-oic acid 3β-*O*-β-D-glucuronopyranoside-6-*O*-methyl ester, and the main differences arising from the significant downfield shifts of C-18 (+11.4) at δ 134.8 and C-22 (+1.1) at δ 35.9 due to the γ-effect of the 30-β (axial) methyl group that were observed when compared with urs-12,18-dien-28-oic acid 3β-*O*-β-D-glucuronopyranoside-6-*O*-methyl ester which possesses a 30-*α* (equatorial) methyl group instead (Hidaka et al., 1987). Moreover, the β orientation assignment of Me-30 was also confirmed by comparing its spectroscopic data with those of compound **1**. Therefore, the structure of **3** was elucidated as 20-*epi*-urs-12,18-dien-28-oic acid 3β-*O*-β-D-glucuronopyranoside-6-*O*-methyl ester.

Compound **4** was obtained as white powder. Its molecular formula was assigned as $C_{30}H_{46}O_4$ on the basis of its positive HR-ESI–MS $[M+Na]^+$ ion peak at *m*/*z*493.3318 (calc'd. 493.3294). The ^{1}H NMR spectrum showed the singlet resonances of five tertiary methyl groups at δ 1.08(3H, s, Me-27), 1.89 (3H, s, Me-29), 1.09 (3H, s, Me-26), 1.07 (3H, s, Me-24), 1.01 (3H, s, Me-25), one methyl doublet at 1.12 (3H, d, *J* = 10.0 Me-30), an olefinic proton signal at δ 5.69 (1H, m, H-12), and the signals of a hydroxymethylene group at δ 4.23 (1H, m, H-23), 3.76 (1H, d, *J* = 15.0 Hz, H-23), as well as the signal of a oxygen-bearing methine at δ4.28 (1H, dd, *J* = 5.0, 10.0 Hz, H-3). The ^{13}C NMR showed resonances for 30 carbon atoms, whose multiplicity patterns were revealed from the DEPT and HSQC experiments as six methyls, ten methylenes, five methines, and nine quaternary carbons. It showed four olefinic C-atoms at δ_C 126.4, 134.5, 135.6, 139.6, one oxymethylene group at δ_C 67.6, corresponding to C-23, and one oxymethine carbon at δ 73.1, corresponding to C-3, as well as one carboxyl group at δ_C 178.8, corresponding to C-28. The ^{13}C NMR spectrum of compound **4** was similar to that of compound **8** except for the significant chemical shift change of C-23 (+38.5 ppm) at δ_C 67.6, suggesting the methyl group in compound **8** was replaced by the hydroxymethylene group in compound **4.** The assignment was confirmed by the observation of HMBC correlations between C-23 at δ_C 67.6 and Me-24 at δ_H 1.07, and between C-24 at δ_C 13.2 and Me-23 at δ_H 3.76. Thus, compound **4** was identified as 3β,23-dihydroxy-20-*epi*-urs-12,18-dien-28-oic acid.

Compound **5** was isolated as white powder. Its HR-ESI–MS $[M+HCOOH-H]^-$ ion peak at *m*/*z* 647.3801 (calc'd. 647.3795)