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Ceanothane- and lupane-type triterpene esters from the roots of *Hovenia dulcis* and their antiproliferative activity on HSC-T6 cells

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ABSTRACT

Three ceanothane-type and three lupane-type triterpenoids, as well as three known compounds, were isolated from the roots of *Hovenia dulcis* (Rhamnaceae), based on LC–MS dereplication. The previously undescribed compounds were determined to be 27-*O*-protocatechuoyl-3-dehydroxyisoceanothanolic acid, 27-*O*-protocatechuoyl-3-dehydroxycolubrinic acid, 27-*O*-protocatechuoyl-3-dehydroxyepicolubrinic acid, 27-*O*-protocatechuoylbetulinic acid, 27-*O*-*p*-hydroxybenzoylbetulinic acid, and 27-*O*-syringoylbetulinic acid by 1D and 2D NMR spectroscopic and HR mass spectrometric data analysis. The isolates were examined for their antiproliferative activity in HSC-T6 hepatic stellate cells; compounds **1**, **2**, **3**, and **6** showed IC_{50} values in the range of 15–50 μM.

1. Introduction

Hovenia dulcis Thunb. (Rhamnaceae), also known as the oriental raisin tree, is used commonly as a pharmaceutical and nutraceutical supplement in East Asia. Recent studies have shown that the extracts of the seeds and fruits of *H. dulcis* exhibited various therapeutic effects, such as anti-inflammatory, antiadipogenic, and hepatoprotective activities (Hase et al., 1997; Kim et al., 2014; Park et al., 2016b). Triterpenoidal saponins, flavonoids, alkaloids and phenolic glycosides have been isolated from the bark, leaves, fruits, and seeds of *H. dulcis* (Ding et al., 1997; Park et al., 2016a; Xu et al., 2003; Yoshikawa et al., 1996, 1997). However, the chemical constituents of the roots of *H. dulcis* have not been well investigated.

In a previous phytochemical investigation on the roots of *Ziziphus jujuba*, another species in the Rhamnaceae family, it was revealed that lupane- and ceanothane-type triterpenoids were the major chemical constituents in the roots (Kang et al., 2016). Ceanothane-type triterpenoids, most of which have been isolated from Rhamnaceae plant species, are rearranged lupane-type triterpenoid derivatives possessing a five-membered A-ring (Grishko et al., 2015). The genus *Hovenia* is phylogenetically close to *Ziziphus*, belonging to the Paliureae tribe in the large family Rhamnaceae (Richardson et al., 2000). Thus, from a chemotaxonomic perspective, it was inferred that the roots of *H. dulcis* would show a phytochemical composition similar to the roots of *Z. jujuba*. UHPLC–Q/TOF–MS analysis of *H. dulcis* root extracts supported this suggestion by exhibiting many chromatographic peaks showing the molecular formula expected for triterpenic acids or their phenolic esters. Dereplication was performed based on an in-house triterpenoid library to target unidentified chromatographic peaks, which were expected to be previously undescribed compounds. Dereplication-based targeted isolation for the unidentified peaks was performed, and as a result, three ceanothane-type triterpenoids (**1**–**3**) and three lupane-type triterpenoids (**4**–**6**), along with three previously known lupane-type triterpenoids (**7**–**9**), were isolated from the roots of *H. dulcis*. Herein, the dereplication, isolation, structural elucidation, and biological evaluation of the compounds are described.

2. Results and discussion

The roots of *H. dulcis* were extracted in an ultrasonicator with MeOH to yield a crude extract. The methanolic extract was analyzed by UHPLC–Q/TOF–MS, and dereplication was performed based on an in-house triterpenoid library built with previously isolated compounds (Kang et al., 2016). Many chromatographic peaks with high intensities were tentatively identified by comparing their

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retention times and MS spectra, as shown in Fig. 1 and Table 1. Among triterpenoids previously isolated from the roots of *Z. jujuba,* 24-hydroxyceanothic acid (**a**), ceanothetric acid 2-methyl ester (**b**), ceanothic acid (**c**), epiceanothic acid (**g**), betulinic acid (**h**), epiceanothic acid 2-methyl ester (**i**), 3-*O*-methyl zizyberanalic acid (**k**), and zizyberenalic acid (**l**) were observed in the methanolic extract of the roots of *H. dulcis*. 3-Dehydroxy ceanothetric acid (**d**), 3-*O*-protocatechuoylceanothic acid (**e**), alphitolic acid (**f**), and 3-*O*-protocatechuoylceanothic acid 2-methyl ester (**j**) were not observed in *H. dulcis*, suggesting that the triterpenoid composition of *H. dulcis* roots is different from that of *Z. jujuba* roots. Conversely, the LC–MS chromatogram of the *H. dulcis* extract exhibited several chromatographic peaks that were not observed in *Z. jujuba.* The peak with the highest intensity, **A**, was not identified in the previous study; however, it was tentatively identified as ceanothenic acid by comparing its relative retention time and MS spectrum with that of the reference (Guo et al., 2011). After the dereplication, many chromatographic peaks that eluted between 6.50 and 13.00 min were not identified; therefore, further isolation was performed targeting these peaks.

The water-suspended crude extract of *H. dulcis* was sequentially fractionated with CH_2Cl_2, EtOAc, and *n*-BuOH. The triterpenoids-enriched CH_2Cl_2 fraction was subjected to a series of column chromatography and Sephadex-LH20 column chromatography and purified by preparative HPLC to yield nine triterpenoids (**1**–**9**). The chemical structures of the isolated compounds are shown in Fig. 2. Compounds **7**–**9** were identified, by comparing their spectroscopic data with those in the literature, to be 27-*O*-vanilloylbetulinic acid (**7**) (Schühly et al., 1999), 3-*O*-*trans*-*p*-coumaroylalphitolic acid (**8**), and 3-*O*-*cis*-*p*-coumaroylalphitolic acid (**9**) (Yagi et al., 1978). The full assignments of their ^1H and ^{13}C NMR spectroscopic data, which are listed in Table S1 (Supplementary Data), were also performed.

Compound **1** exhibited a deprotonated molecular ion at *m/z* 607.3622 ($[M-H]^-$, calcd for $C_{37}H_{51}O_7$, 607.3622) in the HRESIMS, suggesting a molecular formula of $C_{37}H_{52}O_7$. The ^1H NMR spectrum of **1** showed a characteristic isopropenyl group [δ_H 4.96 (1H, br s, H-29a), 4.75 (1H, br s, H-29b), and 1.76 (3H, s, H-30)], suggesting that **1** is a lupane- or ceanothane-type triterpenoid derivative (Table 2). Four additional tertiary methyl groups [δ_H 1.16 (H-26), 0.97 (H-24), 0.90 (H-25), and 0.89 (H-23)] and two oxygenated methylene groups [δ_H 4.09 (1H, dd, J = 10.2, 4.9 Hz, H-2a) and 3.79(1H, m, H-2b); δ_H 5.21 and 5.05 (1H each, d, J = 12.8 Hz, H-27a and 27b)] were also observed in the ^1H NMR spectrum, which indicated that one of the methyl moieties was substituted with an oxygen-containing functionality. An aromatic ABX system [δ_H 8.14 (1H, d, J = 2.1 Hz, H-2′), 7.94 (1H, dd, J = 8.2, 2.1 Hz, H-6′), and 7.99 (1H, d, J = 8.2 Hz, H-5′)] for the 3,4-disubstituted benzoyl moiety was also observed in the ^1H NMR spectrum, indicating the presence of a protocatechuoyl moiety, supported by the HRESIMS fragment ion at *m/z* 153.0185 (calcd for $C_7H_5O_4$, 153.0188). The HMBC spectrum of **1** showed that the oxygenated methylene H-27 correlated with C-8 (δ_C 43.0), C-13 (δ_C 40.4), C-14 (δ_C 47.4), C-15 (δ_C 25.9), and C-7′ (δ_C 167.5), which indicated the esterification of the protocatechuoyl moiety at C-27 (Fig. 3). The ^1H-^1H COSY spectrum revealed vicinal proton couplings between H-2 and a methine proton at δ_H 2.03 (1H, m, H-1) and between H-1 and a methylene group at δ_H 2.02 and 1.95 (1H, each, m, H-3), suggesting a pentacyclic A-ring structure of a ceanothane-type triterpenoid. This was confirmed by the HMBC correlations of H-25 with C-1 (δ_C 52.6) and of H-23 and H-24 with C-3 (δ_C 43.0). The HMBC spectrum also exhibited a correlation between H-18 (δ_H 2.06) and C-28 (δ_C 179.5), which indicated the position of the carboxylic acid group at C-28. The relative configuration at C-1 was determined by a ROESY experiment. H-1 showed a spatial correlation with the β-oriented H-25, while H-2a and H-2b showed a correlation with the α-oriented H-5 (δ_H 1.58) (Fig. 4). These NOE correlations suggested the α-orientation of C-2. The combined spectroscopic data analysis was used to elucidate the structure of **1** as 2α-hydroxymethyl-A(1)-norlup-20(29)-en-27-*O*-

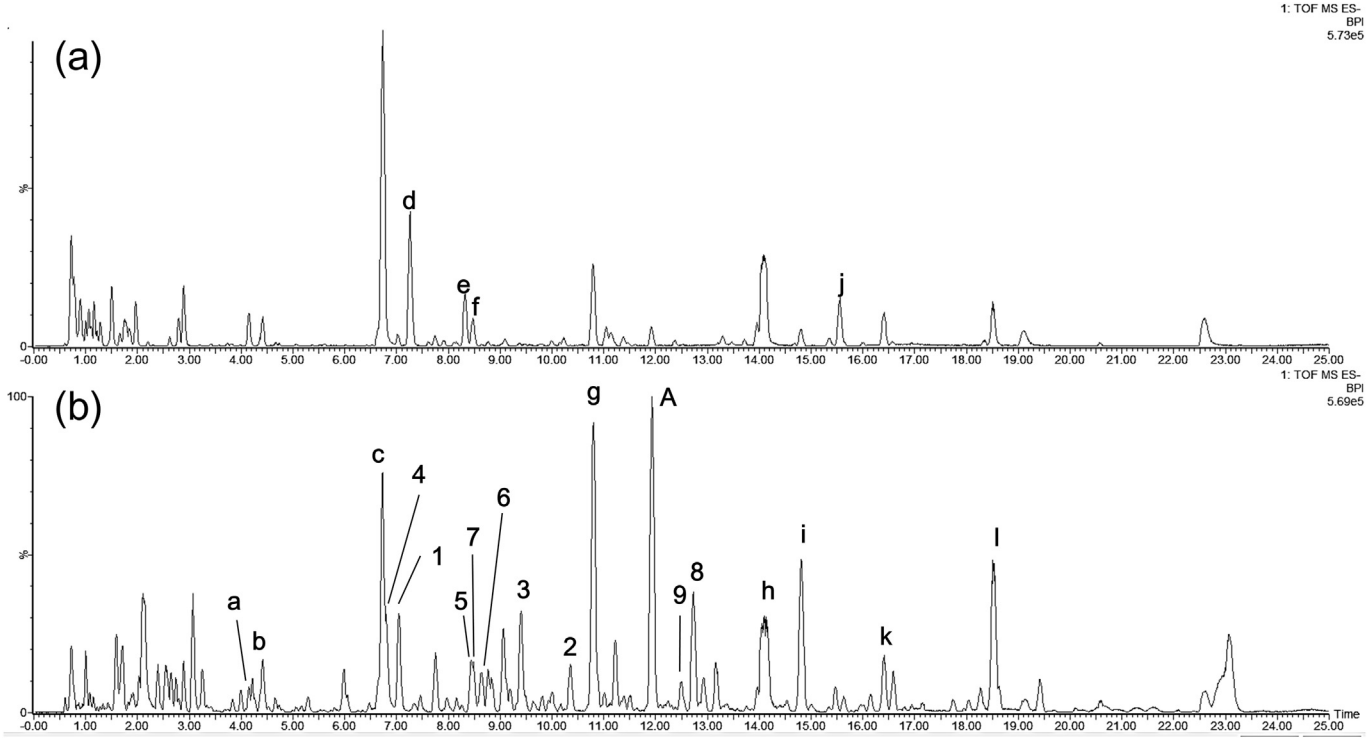

Fig. 1. UHPLC–Q/TOF–MS base peak ion (BPI) chromatograms of the methanolic extracts of (a) *Z. jujuba* and (b) *H. dulcis* roots. Peak numbers designate the isolated compounds **1**–**9** and dereplicated compounds **a**–**m**.