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Isolation and Structure Elucidation of Two Triterpene Acids from the Leaves of *Perilla frutescens*

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Purification of the dichloromethane (CH2Cl2) fraction of the aqueous alcoholic extract of the leaves of *Perilla frutescens* resulted in the isolation of two triterpene acids namely corosolic acid and tomentic acid. The structures of isolated compounds were characterized on the basis of extensive spectral data (1D and 2D NMR; and MS) and in comparison with their physical and spectral data reported earlier

Keyword: *Perilla frutescens*, Triterpene acids, Isolation and purification, NMR, MS, Structure elucidation.

1. Introduction

Perilla frutescens Britt. var. *acuta* Kudo is an annual herbaceous plant native to Asia. The leaves of *P. frutescens* are used in Asian gourmet food is a medicinal herb of Iabiatae[1]. *P. frutescens* (Japanese name; Shiso) is important in Japanese cooking as one of the popular garnishes of that country and also used as a food colorant. The leaves are treated with table-salt to remove the harshness prior to the application as a colorant, suggests the existence of some water-soluble principles in addition to flavonoids. The major constituent of *P.frutescens* is perillaldehyde and several other constituents were reported [2-4]. As a part of our research to discover natural sweeteners, we have recently reported several diterpene glycosides from *S. rebaudiana* and *R. suavissimus*; triterpene glycosides from *Siraitia grosvenorii*; phenolic glycosides and sterols from *R. suavissimus*[5-12].

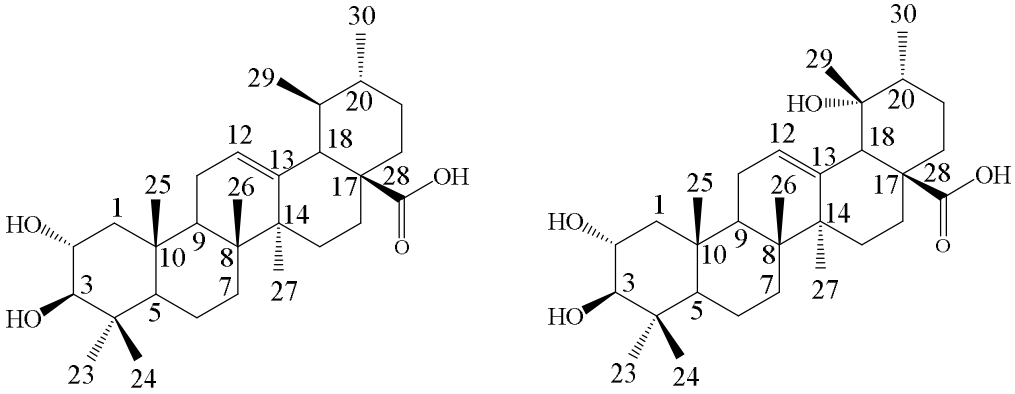
This paper describes the isolation and structure elucidation of two triterpene acids namely corosolic acid (**1**) and tomentic acid acid (**2**) (Figure 1) on the basis of extensive NMR and mass spectroscopic data and in comparison of their physical and spectral properties reported from the literature.

2. Materials and Methods

2.1 General Instrumentation

Melting points were measured using a SRS Optimelt MPA 100 instrument and are uncorrected. Optical rotations were recorded using a Rudolph Autopol V at 25°C and NMR spectra were acquired on a Varian Unity Plus 600 MHz instrument using standard pulse sequences at ambient temperature. Chemical shifts are given in δ (ppm), and coupling constants are reported in Hz. HRMS data was generated with a

Thermo LTQ Orbitrap Discovery mass spectrometer in the positive positive ion mode electrospray. Instrument was mass calibrated with a mixture of Ultramark 1621, MRFA [a peptide], and caffeine immediately prior to accurate mass measurements of the samples.



Corosolic acid (1) **Tomentic acid (2)**

Figure 1: Structures of Corosolic acid (1), and Tomentic acid (2)

Samples were diluted with water:acetonitrile:methanol (1:2:2) and prepared a stock solution of 50 ul concentration for each sample. Each sample (25 ul) was introduced via infusion using the onboard syringe pump at a flow injection rate of 120 ul/min. Low pressure chromatography was performed on a Biotage Flash system using a C-18 cartridge (40+ M, 35-70 µm). TLC was performed on Baker Si-C_{18}F plates and identification of the spots on the TLC plate was carried out by spraying 10% H_2SO_4 in EtOH and heating the plate at about 80 ^oC.

2.2 Plant Material

The commercial extract of the aqueous/alcoholic (4:1) extract of the leaves of *P. frutescens* was purchased from Naturomic LLC, Anaheim, CA, USA. A voucher specimen was deposited at The Coca-Cola Company, No: VSPC-3166-165.

2.3 Isolation and purification of Aromatic phenol acids (1-2)

The aqueous extract of the leaves of *P. frutescens* (15 g) was suspended in 100 ml water and extracted successively with *n*-hexane (3 x 100 ml), CH_2Cl_2 (3 x 100 ml) and *n*-BuOH (2 x 100 ml). The CH_2Cl_2 layer was concentrated under vacuum furnished a residue (2.6 g) which was purified on a Biotage flash chromatography system using C-18 (100 g) column (solvent system: gradient from 60-40 MeOH-water to 100% MeOH at 50 ml/min, detection at UV 210 nm) for 60 min by collecting 80 fractions. Fractions 54-60 were combined to get a residue 0.36 g respectively, which on repeated purification using the gradient 90% MeOH-water to 100% MeOH on a C-18 (10 g) column at 10 ml/min for 40 min resulted corosolic acid (**1**, 35 mg), and tomentic acid (**2**, 63 mg), respectively.

2.4 Identification of Corosolic acid (1), and Tomentic acid (2)

Corosolic acid (1) White powder; mp 250-253^oC; EIMS m/z: 473 $[M+H]^+$; ^1H NMR (pyridine-d_5, 600 MHz): δ 5.42 (1H, br s, H-12), 4.04 (1H, ddd, *J* = 10.8, 8.6, 4.2 Hz, H-2), 3.36 (1H, d, *J* = 9.1 Hz, H-3), 2.62 (1H, d, *J* = 11.1 Hz, H-18), 1.26 (3H, s, H-23), 1.24 (3H, s, H-27), 1.11 (3H, s, H-24), 1.04 (3H, s, H-25), 1.02 (3H, d, J = 6.6 Hz, H- 30), 0.98 (3H, s, H-26), 0.94 (3H, d, *J* = 6.4 Hz, H-29); ^{13}C NMR (pyridine-d_5, 150 MHz), see Table 1 [13].

Tomentic acid (2) White powder; mp 273-274°C; ESIMS m/z: 489 $[M+H]^+$; ^1H NMR (pyridine-d_5, 600 MHz): δ 5.56 (1H, br s, H-12), 4.82 (1H, br s, 19-OH), 4.06 (1H, ddd, *J* = 11.4, 9.4, 4.6 Hz, H-2), 3.36 (1H, d, *J* = 9.3 Hz, H-3), 3.04 (1H, s, H-18), 1.68 (3H, s, H-27), 1.42 (3H, s, H-29), 1.31 (3H, s, H- 23), 1.15 (3H, d, *J* = 6.4 Hz, H-30), 1.10 (3H, s, H-26), 1.07 (3H, s, H-24), 1.02 (3H, s, H-25); ^{13}C NMR (pyridine-d_5, 150 MHz), see Table 1 [14].

3. Results and Discussion

Compound **1** was isolated as a white powder. The mass spectral data of compound **1** gave a molecular ion peak at m/z 473 corresponding to its $(M+H)^+$ ion suggesting the molecular formula as $C_{30}H_{48}O_2$, which was supported by the ^{13}C NMR spectral data. Liebermann-Burchard reaction indicated compound **1** is having a terpenoid skeleton[15-16]. The ^1H NMR spectra of compound **1** showed the presence of five methyl signals at 0.98, 1.04, 1.11, 1.24, and 1.26; two methyl doublets that appeared at δ 0.94 and 1.02. The ^1H NMR spectra of compound **1** also showed two oxymethine protons resonating at δ 3.36 and 4.04; and a trisubstituted olefinic proton at δ 5.42. The presence of five methyl singlets and two methyl doublets suggested that compound **1** belongs to ursane type triterpenoid having two secondary hydroxyl groups and a trisubstituted double bond between C-12/C-13. The ^{13}C NMR values for all the protons and carbons were assigned on the basis of HMQC and HMBC correlations as reported and were given in Table 1.

The appearance of a carbonyl group resonating at δ 180.1 in the ^{13}C NMR spectral data of **1** suggested the presence of an acid functional group and its location was identified at C-28 by the key COSY and HMBC correlations as shown in Figure 3. A close study of COSY and HMBC spectrum of **1** indicated that the two oxymethine groups are adjacent to each other. A search in literature found that the physical and spectral characteristics of **1** were consistent to the reported literature values of corosolic acid that confirmed by the key COSY and HMBC correlations as shown in Figure 2.

Table 1. C NMR chemical shift values for Corosolic acid[13] (**1**) and Tometnic acid (**2**) recorded in C_5D_5N [a-c].

Position	1	2
1	47.8	47.7
2	69.3	69.3
3	84.4	84.6
4	39.8	40.1
5	56.0	56.3
6	18.9	19.1
7	33.8	33.7
8	40.3	40.8
9	48.6	48.1
10	38.4	38.8
11	24.9	24.7
12	125.6	125.8
13	139.4	140.1
14	42.9	42.3
15	29.3	29.8
16	24.0	26.3
17	48.2	48.8
18	53.1	55.1
19	39.1	73.2
20	39.5	42.5
21	31.6	27.6
22	37.5	39.1
23	29.4	29.6
24	17.1	18.2
25	18.1	17.3
26	18.3	18.0
27	24.8	24.9
28	180.1	180.2
29	17.3	17.2
30	22.1	27.3

a. assignments made on the basis of COSY, HMQC and HMBC correlations; **b.** Chemical shift values are in δ (ppm); **C.** Coupling constants are in Hz.

Compound **2** was also isolated as a white powder and its mass spectral data suggested the molecular formula as $C_{30}H_{48}O_3$, 16 amu more to **1** Compound **2** also showed positive Liebermann-Burchard reaction for terpenes as in **1**. The ^1H NMR spectra of compound **2** also showed the presence of five methyl signals, two methyl doublets, two oxygenated methine signals, and a trisubstituted olefinic proton as in **1**. The presence of an additional hydroxyl group together with the absence of any primary and secondary hydroxyl protons in the ^1H NMR spectral data of **2** suggested the presence of a tertiary hydroxyl group in its structure.

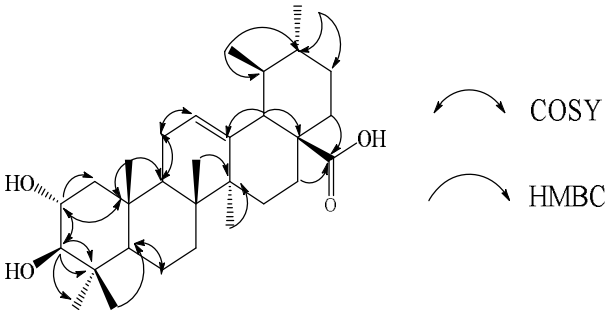


Figure 2: Key COSY and HMBC correlations of Corosolic acid (1)

This indicates the presence of tertiary hydroxyl group resonating at δ 73.2 in the ^{13}C NMR spectral data of **1** at either C-19 or C-20 positions. The ^{13}C NMR values for all the protons and carbons were assigned on the basis of HMQC and HMBC correlations as reported and were given in Table 1. The key COSY and HMBC correlations as shown in Figure 3 suggested the presence of tertiary hydroxyl group at C-19 position as in tomentic acid. Therefore, the structure of **2** was suggested to be tormentic acid (2α,3β,19α-trihydroxy-urs-12-en-28-oic acid) and its NMR spectral showed a good agreement with reported data.

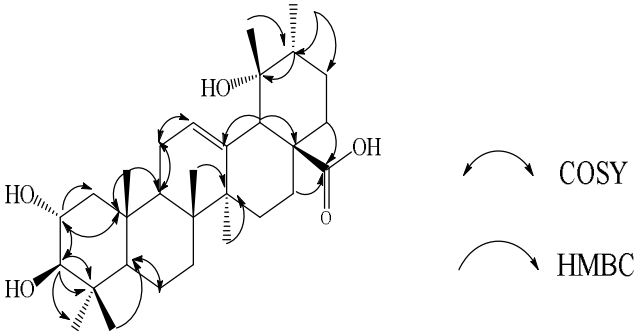


Figure 3: Key COSY and HMBC correlations of Tomentic acid (2)

4. Conclusion

Two triterpene acids were isolated from the commercial aqueous alcoholic extract of the leaves of *P. frutescens*. The structures of the two isolated compounds were identified as corosolic acid (**1**), and tomentic acid (**2**), on the basis of extensive spectroscopic studies and by comparing their physical properties and spectral data reported in the literature.

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