SHORT REPORT

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Two New Monoterpenes from *Tithonia diversifolia* and Their Anti-Hyperglycemic Activity

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Abstract: Two new monoterpenes: ((1*S*,2*R*,3*R*,5*S*)-2-hydroxymethyl-6,6-dimethylbicyclo[3.1.1]heptane-2,3-diol (**1**) and (3*R*)-6,6-dimethyl-4-methylenebicyclo[3.1.1]heptane-1,3-diol-3-*O*-*β*-D-glucopyranoside (**2**), along with three known compounds, namely, sobrerol (**3**), (1*R*,2*S*,5*S*)-2,8-p-menth-diol (**4**) and (l*R*,5*S*)-10-hydroxyverbenon (**5**), were isolated from aerial part of *Tithonia diversifolia*. Their structures were determined on the basis of spectroscopic analyses (IR, HR-ESI-MS/MS, 1D/2D NMR). Under the concentration of 10 µg/mL, compounds **1** and **3** significantly increased glucose uptake in 3T3-L1 adipocytes without significant toxic effects *in vitro*.

Keywords: *Tithonia diversifolia*; Monoterpene; 3T3-L1 adipocytes; Anti-hyperglycemic activity.

1. Plant Source

Tithonia diversifolia (Hemsl.) A. Gray (Asteraceae: Heliantheae), known as Mexican sunflower, is a native plant of Mexico and Central America [1]. As our continuing research on antidiabetic active metabolites from *T. diversifolia*, we report the isolation and identification of two new moneterpenes and three known moneterpenes. Furthermore, the anti-hyperglycemic activities of compounds **1–5** were evaluated for glucose uptake in 3T3-L1 adipocytes.

The aerial parts of *T. diversifolia* (Hemsl.) A. Gray were collected in Mengzi of Yunnan province, China in September 2007 and identified by Prof. Wansheng Chen (Department of Pharmacy, Changzheng Hospital, Second Military Medical University). A voucher specimen (NO.TD20070927) was deposited in the Department of Pharmacognosy of Second Military Medical University in Shanghai, P.R China.

2. Previous Studies

T. diversifolia is used traditionally for the treatment of malaria, fever or wound in Mexico. Modern pharmacological investigations revealed that it has extensive bioactivities including antimalarial [2], antidiabetic [3], anti-inflammatory [4], and anticancer [5]. Phytochemical studies on this species have resulted in the isolation of sesquiterpene lactones, chromenes, and flavones [6-8].

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3. Present Study

The dried aerial parts (21 kg) of *Tithonia diversifolia* were percolated with 80% EtOH at room temperature. The EtOH extract was concentrated to an aqueous residue (2.48 kg) and suspended with water. The water layer was extracted with petroleum ether, EtOAc and n-BuOH. The EtOAc fraction (128.0 g) was separated by column chromatography using silica gel as a packing agent and petroleum ether-EtOAc as a solvent mixture, which resulted in six major fractions (1-6). Fraction 2 (6.5 g) was further separated by chromatography using Sephadex LH-20 and silica gel to obtain compound **3** (49.1 mg) [9]. Fraction 3 (28.6 g) was subject to MCI gel column chromatography, eluting with a mixture of MeOH-H_2O, which gave six parts (3.1-3.6). Fraction 3.1(4.6 g) was separated by a silica gel column chromatography to obtain five parts (3.1.1-3.1.5). The subfractions 3.1.3(0.6 g) and 3.1.2(1.0 g) were further separated by using preparative TLC to yield compounds **1** (9.8 mg) and **4** (12.6 mg) [9]. Fraction 3.1.5 (2.1 g) was further fractionated using Sephadex LH-20, which gave five parts (3.1.5.1-3.1.5.5), and Fraction 3.1.5.5 (0.6 g) was purified by a silica gel column chromatography to obtain compound **5** (28.1 mg) [10]. Fraction 6 (23.7 g) was subject to MCI gel column chromatography to yield four parts (6.1-6.4). Then, the subfraction 6.1(3.5 g) was subjected to ODS silica gel column chromatography to give compound **2** (38.2 mg).

(+)-*2-hydroxymethyl-6,6-dimethylbicyclo[3.1.1]heptane-2α,3α-diol* (**1**): Colorless raphide; $[\alpha]_D^{20}$ =+18°(c=0.14, acetone); IR (KBr): 3420, 3348, 3001, 2978, 2953, 2923, 2868, 1644, 1577,1419, 1384, 1174, 1029, 1002, 971, 886, 863, 758 cm^{-1}; ^{1}H-NMR (600 MHz, $CDCl_3$): δ1.82(dd, J=5.4 and 6.0 Hz, H-1), 4.31(dd, J=2.4, 9.0 Hz, H-3), 2.60(ddt, J=2.4, 9.0, 13.8 Hz, H-4a), 1.68(dt, J=3.0, 13.8 Hz, H-4b), 1.95(ddt, J=2.4, 3.0, 5.4 Hz, H-5), 2.24(ddt, J=2.4, 6.0, 13.2 Hz, H-7a),1.24(d, J=13.2 Hz,H-7b), 1.25(s, H-8), 1.04(s, H-9), 4.16(d, F=10.8 Hz, H-10a), 3.26(d, J=10.8 Hz, H-10b). ^{13}C-NMR (150 MHz, $CDCl_3$): δ49.7(C-1), 80.2(C-2), 74.4(C-3), 37.2(C-4), 40.6(C-5), 38.7(C-6), 26.9(C-7), 27.1(C-8), 23.7(C-9), 68.3(C-10). HR-ESI-MS: *m/z* 209.1119 $[M+Na]^+$ (calcd for $C_{10}H_{18}NaO_3$, 209.1148).

(-)-*6,6-dimethyl-4-methylenebicyclo[3.1.1]heptane-1α,3α-diol-3-O-β-D-glucopyranoside* (**2**); $[\alpha]_D^{20}$ =-44.1°(c=0.24, MeOH); ^{1}H-NMR (600 MHz, pyridine-d_5):δ2.81(d, J=12.0 Hz, H-2a), 2.43(dd, J=7.8, 12.0 Hz, H-2b), 4.88(dd, J=7.2 Hz, H-3), 2.36(d, J=6.6 Hz, H-5), 2.48(d, J=15.2 Hz, H-7a), 2.48(dd, J=6.6, 15.2 Hz, H-7b),0.74(s, H-8), 1.35(s, H-9), 5.25(s, H-10a), 5.04(s, H-10b), 5.12(d, J=7.8 Hz, H-1′), 4.05(dd, J=7.8, 8.4 Hz, H-2′), 4.21(dd, J=8.4, 9.6 Hz, H-3′), 4.18(d, J=9.6 Hz, H-4′), 3.89(d, J=6.6 Hz, H-5′), 4.54(d, J=11.4 Hz, H-6′a), 4.34(dd, J=6.0, 11.4 Hz, H-6′b).^{13}C-NMR (150 MHz, pyridine-d_5):δ73.9(C-1), 35.3(C-2), 72.9(C-3), 146.7(C-4), 45.1(C-5), 47.6(C-6), 40.5(C-7), 20.0(C-8), 21.9(C-9), 114.8(C-10), 99.1(C-1′), 74.9(C-2′), 78.6(C-3′), 71.7(C-4′), 78.1(C-5′), 62.7(C-6′). HR-ESI-MS: *m/z* 375.1669 $[M+COOH]^-$, 353.1575 $[M+Na]^+$.

Bioactivity Test- Agar diffusion test: The differentiated 3T3-L1 adipocytes, plated into 96-well plates were pre-incubated with DMEM, containing 0.2% BSA, and then incubated with various concentrations of the compounds **1–5** (10μg/mL) for 12 hours. The amounts of glucose uptake were calculated by the glucose concentrations of blank wells, subtracting the remaining glucose in the cell-plated wells. Meanwhile, MTT assay was performed to monitor the cell proliferation and adjust the glucose uptake values.

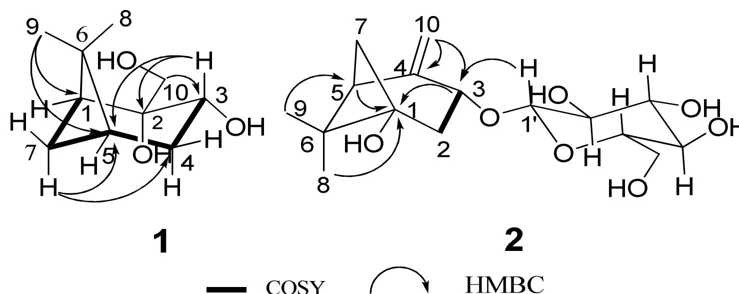

Figure 1. ^{1}H-^{1}H COSY correlations and the key HMBC correlations of compound **1-2**

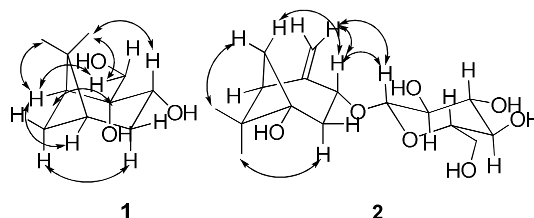

Figure 2. Key ROESY correlations of compound **1** and **2**

Compound **1** was obtained as a needle crystal. The HR-ESI-MS (*m/z* 219.1119 $[M+Na]^+$) and NMR data (Table 1) explained the molecular formula of compound **1** as $C_{10}H_{18}O_3$, indicating two degrees of unsaturation. The structure of the compound was established through detailed analyses of its ^1H and ^{13}C NMR spectra, including 2D NMR. The ^{13}C NMR spectrum of **1** exhibited 10 signals, together with the information from a DEPT spectrum, corresponding to two methyl, three methylenes, three methines, and two quaternary carbons. One oxygenated methane (δ_C 74.4), one oxygenated methylene (δ_C 68.3) and one oxygenated quaternary carbon (δ_C 80.2) were among the signals. The ^1H-NMR spectrum of compound **1** displayed two methyls at δ_H 1.04 (s, CH_3-9) and δ_H 1.25(s, CH_3-8), assigned to δ_C 23.7 (C-9) and δ_C 27.1 (C-8), respectively, according to the HSQC correlations; an oxygenated methylene group at δ_H 4.16 (d, *J*=10.8 Hz, H-10a) and δ_H 3.26(d, *J*=10.8 Hz, H-10b), showing correlation with δ_C 68.3 (C-10) in the HSQC and one oxygenated methane at δ_H 4.31 (dd, *J*=2.4, 9.0 Hz, H-3), assigned to δ_C 74.4 (C-3), which were further confirmed by the HSQC correlations. The ^1H- and ^{13}C-NMR spectroscopic data (Table 1) as well as the observed HSQC and HMBC correlations (*Fig 1*) suggested that compound **1** is a pinane monoterpene. The methine signal at δ_C 49.7(C-1) was attached at δ_C 26.9 (C-7) due to the ^1H-^1H COSY correlation of δ_H 1.82 (H-1) to δ_H 2.24 (H-7). Similarly, δ_C 26.9 (C-7) attached to δ_C 40.6 (C-5); δ_C 40.6 (C-5) attached to δ_C 37.2 (C-4) and δ_C 37.2(C-4) attached to δ_C 74.4 (C-3) were further confirmed by the ^1H-^1H COSY correlations (H-7/H-5, H-5/H-4 and H-4/H-3). The oxygenated quaternary carbon at δ_C 80.2 was assigned to C-2, which showed correlations with H-1 (δ_H 1.82), H-3 (δ_H 4.31) and H-7 (δ_H 2.24) by the HMBC measurements. The remaining quaternary carbon at δ_C 38.7 was assigned to C-6, which showed correlations with H-1 (δ_H 1.82) and H-4 (δ_H 1.68) in the HMBC. The oxygenated methylene (δ_C 68.3) was attached to C-2 (δ_C 80.2), confirming by the HMBC correlations from H-10b (δ_H 3.26) to C-1 (δ_C 49.7), C-2 (δ_C 80.2) and C-3 (δ_C 68.3). The two methyl C-8 (δ_C 27.1) and C-9 (δ_C 23.7) were located at the C-6, which was identified by the HMBC correlations from H-8 (δ_H 1.25) and H-9 (δ_H 1.04) to C-1 (δ_C 49.7), C-6 (δ_C 38.7) and C-5 (δ_C 40.6). The α-orientation of the hydroxyl groups at C-2 and C-3 were established by the ROESY correlations of the H-8/H-10a and H-3/H-8. Furthermore, the ROESY correlations of H-1/H-5, H-1/H-10 indicated that the H-1 and H-5 had α-orientations, then the configuration of compound **1** could be established as shown in Fig 1. Thus, based on the above evidences, compound **1** was assigned as (+)-2-hydroxymethyl-6,6-dimethylbicyclo[3.1.1]heptane-2α,3α-diol. Compound **2** was obtained as a colorless oil. The positive-ion HR-ESI-MS showed a quasimolecular ion at m/z 353.1572 $[M+Na]^+$, and negative-ion HR-ESI-MS at *m/z* 375.1669 $[M+COOH]^-$ corresponding to a molecular formula of $C_{16}H_{26}O_7$, requiring four degrees of unsaturation. The ^1H-NMR spectrum of compound **2** displayed two methyl groups at δ_H 1.35(s) and δ_H 0.74(s), respectively, two olefinic protons as broad singlets at δ_H 5.04(br, s) and 5.25(br, s) and an oxygenated methine at δ_H 4.88(*J*=7.2 Hz). The ^{13}C-NMR spectrum exhibited five oxygenated methine carbons at δ_C 99.1, 74.9, 78.6, 71.7 and 78.1, and one methylene carbon at δ_C 62.7, indicating the presence of a glucose moiety, which was identified by GC analysis as D-glucose. Other carbon signals were identified by a DEPT experiment as two methyl, three methylene, three methane and two quaternary carbons, including one oxygenated methine group at δ_C 72.9 and one oxygenated carbonyl carbon at δ_C 73.9. The above assignments were characteristic for a glucopyranosyl moiety attached to β-pinene aglycone. The oxygenated carbonyl methine signal at δ_C 72.9 was assigned to C-3 due to the HMBC correlations with H-10 (δ_H 5.25 and 5.04), H-5 (δ_H 2.36) and H-2 (δ_H 2.43). The glucose moiety was located at the C-3, which was confirmed by the HMBC correlations of H-1' (δ_H 5.12) to C-3. The *β*-anomeric configuration for the glucose was determined by a large coupling constant of H-1' (δ_H 5.12, d, *J*=7.8 Hz). The oxygenated carbonyl carbon signal at δ_C 73.9 was assigned to C-1 due to

the HMBC correlations of H-2 (δ_H 2.81 and 2.43), H-3 (δ_H 4.88), H-5 (δ_H 2.36), H-7 (δ_H 2.48), H-9 (δ_H 1.35) and H-8 (δ_H 0.74) to the carbonyl carbon. The remaining carbons and protons were confirmed at their respective positions based on analyses of the ^{1}H-NMR, ^{13}C-NMR, HMBC and HSQC dates. Structure of compound **2** was confirmed by a ROESY experiment (*Fig 2*). The hydroxyl groups at C-1 and C-3 were assigned as α-orientations by the ROESY correlations of H7a/H3 and H10/H3. The α-orientation of the H-5 was confirmed by the ROESY correlations of H7a/H3, H7b/H9 and H8/H2. Then, compound **2** was assigned as (-)-6,6-dimethyl-4-Methylenebicyclo[3.1.1]heptane-1α,3α-diol-3-O-β-D-glucopyranoside. Compounds **1–5** were evaluated for their anti-hyperglycemic activity based on glucose uptake in differentiated 3T3-L1 adipocytes. 10 μg/mL of compounds **1** and **3** significantly increased glucose uptakes of 3T3-L1 adipocytes by 1.2- and 1.6- fold compared with the basal level, respectively. Cell viability was assayed by the MTT method, which indicated that the five compounds were not cytotoxic to fully differentiated 3T3-L1 adipocytes at this concentration.

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