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# Four new prenylflavonol glycosides from the leaves of *Cyclocarya paliurus*

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#### ABSTRACT

Four new prenylflavonol glycosides (1–4) along with two known analogues (**5–6**) were isolated from the leaves of *Cyclocarya paliurus* for the first time. The structures of these compounds were characterized by comprehensive analysis of 1 D, 2 D NMR, HRESIMS, UV data and enzymatic hydrolysis. In bioassays, compounds **1–4** were evaluated for inhibitory effects on xanthine oxidase (XOD) and effects on the inhibition of nitric oxide (NO) production in lipopolysaccharide (LPS) induced RAW264.7 cells. Moreover, compounds **1** and **2** showed outstanding XOD inhibitions with IC<sub>50</sub> values of  $18.16 \pm 3.91$  and  $37.65 \pm 5.67 \,\mu$ M, and exhibited inhibitions against LPS-induced NO production with IC<sub>50</sub> values of  $80.50 \pm 3.09$  and  $82.28 \pm 2.87 \,\mu$ M.

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*Cyclocarya paliurus;* prenylflavonol glycosides; xanthine oxidase; nitric oxide; RAW264.7 cells



#### 1. Introduction

Cyclocarya paliurus (Batal.) Iljinsk is a single species belonging to family Juglandaceae, widely distributed in south and southeast areas of China (Ramallo et al. 2006). Its leaves have been consumed as traditional tea in China for a long time due to its palatable taste and many curative effects, such as relieving arthrophlogosis, analgesic, antiinflammation, hypoglycemic, antioxidant and so on (Xie et al. 2015, Xuan et al. 2019, Sun et al. 2020). The flavonoids were considered as one of the main active composition in C. paliurus, such as quercetin and its glycoside derivatives (Fang et al. 2011, Liu et al. 2016, Li et al. 2020, Lin et al. 2020), while only a few phytochemical investigations about C. paliurus were focused on flavonoids. To explore structurally intriguing and bioactive flavonoids from C. paliurus, the systematic study on the n-BuOH fraction of C. paliurus was carried out. Finally, four new prenylflavonol glycosides, Icarisids L-O (1-4), together with two known analogues (5-6) were isolated and identified (Figure 1). Herein, the isolation, structure elucidation, and biological evaluations were reported.

#### 2. Results and discussion

#### 2.1. Chemistry

Compound **1** was obtained as yellow, amorphous powder. Its chemical formula:  $C_{33}H_{42}O_{17}$  was determined by HRESIMS and <sup>13</sup>C NMR spectrum. The <sup>1</sup>H NMR data (Supplementry material Table S1) displayed four aromatic protons at  $\delta_{H}$  8.01 (d, J = 9.2 Hz, 2H), 7.10 (d, J = 8.8 Hz, 2H) suggested the presence of a *p*-substituted benzene ring B; an aromatic methine at  $\delta_{H}$  6.66 (s, 1H) indicated the existence of a *penta*-substituted ring A; a methoxy group signal at  $\delta_{H}$  3.86 (s, 3H). Besides, The <sup>1</sup>H NMR and <sup>13</sup>C NMR data (Supplementry material Table S1 and S2) displayed a series of signals at [ $\delta_{H}$  3.00, 2.88 (overlapped, 2H), 3.44 (overlapped, 1H), 1.17 (d, J = 3.6 Hz, 6H);  $\delta_{C}$  24.3 (CH<sub>2</sub>-11), 77.4 (CH-12), 71.9 (C-13), 26.0 (CH<sub>3</sub>-14), 25.0 (CH<sub>3</sub>-15)] ascribed to a 2,3-



Figure 1. Chemical structures of compounds 1-6.

dihydroxy-3-methylbutyl moiety; a set of signals at  $[\delta_{\rm H}$  5.23 (br s)/ $\delta_{\rm C}$  102.3 (CH-1'')] and [ $\delta_{\rm H}$  4.92 (d, 1H, J=6.8 Hz)/101.3 (CH-1<sup>'''</sup>)] indicated the existence of two sugar moieties. The monosaccharides obtained by enzymatic hydrolysis were identified by GC-MS analysis with authentic samples as L-rhamnose and D-glucose. And combining with the coupling constants of terminal protons, the absolute configurations of sugars were determined as  $\alpha$ -L-rhamnopyranosyl and  $\beta$ -D-glucopyranoside. The HMBC correlations (Supplementry material Figure S1) between H-1'' ( $\delta_{\rm H}$  5.23)/C-3 ( $\delta_{\rm C}$  134.7), H-1''' $(\delta_{\rm H}$  4.92)/C-7 ( $\delta_{\rm C}$  161.5) indicated the  $\alpha$ -L-rhamnopyranosyl was linked at C-3 and the  $\beta$ -D-glucopyranoside was linked at C-7; the correlations between 4'-OCH<sub>3</sub> ( $\delta_{\rm H}$  3.86) and  $\delta_{\rm C}$  C-4' (161.6) suggested C-4' was substituted by a methoxy group. The location of 2,3-dihydroxy-3-methylbutyl moiety to C-8 was proved by the cross-peaks of H-11 ( $\delta_{\rm H}$ 3.00, 2.88) with C-7 ( $\delta_{C}$  161.5), C-8 ( $\delta_{C}$  107.8), C-9 ( $\delta_{C}$  153.5), and C-12 ( $\delta_{C}$  77.4). Moreover, the correlations between H-6 ( $\delta_{H}$  6.66) and C-5 ( $\delta_{C}$  159.2), C-7 ( $\delta_{C}$  161.5), C-8 ( $\delta_{\rm C}$  107.8), C-10 ( $\delta_{\rm C}$  105.8) confirmed the proton singlet at  $\delta_{\rm H}$  6.66 was assignable as H-6. Besides, enzymatic hydrolysis of **1** by snailase afforded an aglycone (**X1**),  $[\alpha]_{D}^{25}$ -27.8 (MeOH). The configuration at C-12 was deduced as "S" on the basis of optical rotation and <sup>13</sup>C-NMR chemical shift data comparison with reported analogues (Yukinori et al. 1994). Therefore, the structure of 1 was established as 8-[(S)-2,3-dihydroxy-3-methylbutyl]-5-hydroxy-4'-methoxy-3-O- $\alpha$ -L-rhamnopyranosyl-flavonol-7-O- $\beta$ -Dglucopyranoside, and named as Icarisid L.

Compound **2** was obtained as yellow, amorphous powder. Its chemical formula:  $C_{33}H_{42}O_{17}$  was determined by HRESIMS. The 1 D/2D NMR spectra of **2** was almost same as **1** except for the signals of side-chain. The <sup>13</sup>C NMR spectrum of **2** displayed signals at  $\delta_{C}$  27.1 (CH<sub>3</sub>-14) and  $\delta_{C}$  23.3 (CH<sub>3</sub>-15) which were significantly different from that of compound **1** [ $\delta_{C}$  26.0 (CH<sub>3</sub>-14) and  $\delta_{C}$  25.0 (CH<sub>3</sub>-15)], indicated that the configuration at C-12 was different. Besides, enzymatic hydrolysis of **2** afforded an aglycone (**X2**), [ $\alpha$ ]  $_{D}^{25}$  24.6 (MeOH). The configuration at C-12 was deduced as "*R*" on the basis of optical rotation and <sup>13</sup>C-NMR chemical shift data comparison with reported analogues (Yukinori et al. 1994). Therefore, the structure of **2** was established as 8-[(*R*)-2,3-dihydroxy-3-methylbutyl]-5-hydroxy-4'-methoxy-3-O- $\alpha$ -*L*-rhamnopyranosyl-flavonol-7-O- $\beta$ -*D*-glucopyranoside, and named as lcarisid M.

Compound **3** was obtained as yellow, amorphous powder. Its chemical formula:  $C_{39}H_{52}O_{21}$  was determined by HRESIMS. The 1D/2D NMR spectra of **3** were almost same as **2**, except for the existence of another  $\alpha$ -rhamnopyranosyl moiety. Further enzymatic hydrolysis and GC-MS analysis determined the glycosyl moieties were  $\alpha$ -*L*-rhamnopyranose and  $\beta$ -*D*-glucupyranose. The HMBC (Supplementry material Figure S1) correlations between H-1<sup>''''</sup> ( $\delta_{H}$  4.91) and C-2<sup>''</sup> ( $\delta_{C}$  75.6) suggested that rhamnopyranosyl' was linked at C-2<sup>''</sup>. Besides, enzymatic hydrolysis of **3** also afforded an aglycone (**X3**), [ $\alpha$ ]<sup>25</sup><sub>D</sub> 26.3 (MeOH), which indicated the configuration at C-12 was deduced as "*R*". Therefore, the structure of **3** was established as 8-[(*R*)-2,3-dihydroxy-3-methylbutyl]-5-hydroxy-4'-methoxy-3-O- $\alpha$ -*L*-rhamnopyranosyl-(1 $\rightarrow$ 2)- $\alpha$ -*L*-rhamnopyranosyl-flavonol-7-O- $\beta$ -*D*-glucopyranoside, and named as Icarisid N.

Compound **4** was obtained as yellow, amorphous powder. Its chemical formula:  $C_{38}H_{48}O_{20}$  was determined by HRESIMS data. The 1 D/2D NMR spectra of **4** was basically same as **3**, except for the rhamnopyranosyl' was replaced by a  $\beta$ -xylopyranose and the change of side-chain. The <sup>1</sup>H and <sup>13</sup>C NMR data (Supplementry material Table S1 and S2) displayed a set of signals at [ $\delta_{\rm H}$  2.99, 2.87 (overlapped, 2H), 4.17 (m, 1H), 4.86, 4.72 (s, each 1H), 1.75 (s, 3H);  $\delta_{\rm C}$  29.2 (CH<sub>2</sub>-11), 73.7 (CH-12), 148.4 (C-13), 109.6 (CH<sub>2</sub>-14), 17.8 (CH<sub>3</sub>-15)] indicated the side chain at C-8 was a 2-hydroxy-3-methylbut unit. The HMBC (Supplementry material Figure S1) correlations between H-1<sup>''''</sup> ( $\delta_{\rm H}$  4.12) and C-2<sup>''</sup> ( $\delta_{\rm C}$  80.8) suggested that  $\beta$ -xylopyranose was linked at C-2<sup>''</sup>. Further enzymatic hydrolysis and GC-MS analysis determined the glycosyl moieties were  $\alpha$ -*L*-rhamnopyranose,  $\beta$ -*D*-glucupyranose and  $\beta$ -*D*-xylopyranose. Enzymatic hydrolysis of **4** also afforded an aglycone (**X4**), [ $\alpha$ ]<sup>25</sup><sub>D</sub> -19.6 (MeOH). Moreover, because of the 2-hydroxy-3-methylbut group did not contribute much to Electronic circular dichroism (ECD), the rotation of **X4** was finally deduced as "-" by its specific rotation (-19.6, MeOH). Therefore, the structure of compound **4** was established as 8-[(-)-2-hydroxy-3-methylbut]-5-hydroxyl-4'-methoxy-3-O- $\beta$ -*D*-xylopyranose-(1 $\rightarrow$ 2)- $\alpha$ -*L*-rhamnopyranosyl-flavonol-7-O- $\beta$ -*D*-glucopyranoside, and named as Icarisid O.

Together with two known analogues (5-6) were identified by comparison of their reported spectroscopic data as diphylloside B (5) (Liang et al. 1993) and epimedoside E (6) (Su et al. 2018).

## 2.2. Bioassay

The xanthine oxidase (XOD) inhibitory effects of **1-4** and allopurinol (positive control) have been evaluated by measuring the production of superoxide ion and uric acid, and the results (see Supplementry material Table S3) indicated that compounds **1** and **2** showed outstanding inhibitions on XOD with the IC<sub>50</sub> values of  $18.16 \pm 3.91$  and  $37.65 \pm 5.67 \,\mu$ M. Besides, the anti-inflammation activities of **1-4** and indometacin (positive control) were evaluated in the RAW 264.7 cells, and the results were listed in Supplementry material Table S4. Compounds **1** and **2** exhibited inhibitory activities on lipopolysaccharide (LPS) induced NO production with IC<sub>50</sub> values of  $80.50 \pm 3.09$  and  $82.28 \pm 2.87 \,\mu$ M.

# 3. Experimental

#### 3.1. General experimental procedures

Optical rotations were measured on a JASCO model 1020 polarimeter (Horiba, Tokyo, Japan) at room temperature. NMR spectra were measured on Bruker AVANCE 400 spectrometer (Bruker, Karlsruhe, Germany) using dimethyl sulfoxide (DMSO- $d_6$ ) as solvent and tetra methylsilane (TMS) as an internal standard. High resolution electrospray ionization mass spectroscopy (HRESIMS) was recorded with an Agilent Technologies liquid chromatograph connected to Q-TOF mass spectra. Gas Chromatography-Mass Spectrometer (GC-MS) was measured on GCMS-QP2010 Ultra (Shimadzu Corporation). Analytical HPLC experiments was carried out on an Agilent 1200 with YMC-Pack ODS-C18 column (250 × 4.6 mm, 5  $\mu$ m, YMC Co. Ltd., Kyoto, Japan). Column chromatographic (CC) separations were carried out on Macroporous resin HPD-100 (Zhengzhou Qinshi Technology Co., Ltd), polyamide (80-100 or 30-60 mesh, Taizhou Luqiao Sijia Biochemical Plastics Factory, Taizhou, China), HW-40C (TOYOPEARL, TOSOH, Japan) and

ODS-AA (50  $\mu$ m, YMC). Semi-preparative HPLC separations were conducted by using Agilent 1260 system with an ODS-C18 column (250  $\times$  10 mm, 5  $\mu$ m, YMC). All the analytic reagents were analytical grade.

#### 3.2. Plant material

The leaves of *Cyclocarya paliurus* (Batal.) Iljinsk were collected from Xinning, Shaoyang, Hunan Province. A voucher specimen (No. 20160820) was identified by Prof. Kangping Xu from Xiangya School of Pharmaceutical Sciences, Central South University, and deposited in Xiangya School of Pharmaceutical Sciences, Central South University.

#### 3.3. Extraction and isolation

Air-dried leaves of C. paliurus (10 Kg) were powdered and extracted with 70% EtOH- $H_2O$  (100 L  $\times$  2h  $\times$  2) under reflux conditions, and were dried under reduced pressure to get crude extract then suspended in water and successively partitioned with CH<sub>2</sub>Cl<sub>2</sub>, EtOAc and n-BuOH. The n-BuOH extraction (445 g) was subjected to column chromatography on Macroporous resin HPD-100 with EtOH-H<sub>2</sub>O to obtain five fractions Fr.A-E (0-95%, stepwise). Fr.C (107 g) was separated by a polyamide column using EtOH-H<sub>2</sub>O to afforded six sub-fractions Fr.C1-C6 (0-95%, stepwise). Fr.C1 (22.9 g) was separated to twelve fractions Fr.C1-C12 by HW-40C eluted with MeOH-H<sub>2</sub>O (from 0:100 to 70:30). Fr.C1.2 was purified by ODS column with MeOH-H<sub>2</sub>O (10-75%, stepwise) and obtained 45 sub-fractions (Fr.C1.2.1-Fr.C1.2.45). Fr.C1.2.29 was separated by Semipreparative HPLC (ACN-H<sub>2</sub>O 24%, v/v, 220 nm, 3 mL/min) to give compound 1 (9.7 mg). Fr.C1.3 was purified by the ODS column with MeOH-H<sub>2</sub>O (10-75%, stepwise) and obtained 28 sub-fractions (Fr.C1.3.1-Fr.C1.3.28). Fr.C1.3.12 was separated by Semi-preparative HPLC (ACN-H<sub>2</sub>O 18%, v/v, 220 nm, 3 mL/min) to give compounds 2 (7.8 mg) and 3 (4.9 mg). Fr.C1.3.15 was separated by Semi-preparative HPLC (ACN-H<sub>2</sub>O 21%, v/ v, 220 nm, 3 mL/min) to give compounds 5 (13.1 mg) and 6 (21.4 mg). Fr.C1.3.17 was separated by Semi-preparative HPLC (ACN-H<sub>2</sub>O 24%, v/v, 0.1% CH<sub>3</sub>COOH, 220 nm, 3 mL/min) to give compound **4** (6.4 mg).

#### 3.3.1. Icarisid L (1)

Yellow amorphous powder;  $[\alpha]_D^{25}$  –127.0 (c 0.07, MeOH); UV (MeOH)  $\lambda_{max}$  (log  $\varepsilon$ ) nm: 204 (4.10), 271 (2.30), 316 (1.29), and 350 (1.05) nm; <sup>1</sup>H and <sup>13</sup>C NMR data (400 and 100 MHz in DMSO- $d_6$ ) see Supplementry material Tables S1 and S2; HRESIMS, m/z 711.2500 [M + H]<sup>+</sup> (calcd for C<sub>33</sub>H<sub>43</sub>O<sub>17</sub>, 711.2500).

#### 3.3.2. Icarisid M (2)

Yellow amorphous powder;  $[\alpha]_D^{25}$  –111.3 (c 0.09, MeOH); UV (MeOH)  $\lambda_{max}$  (log  $\varepsilon$ ) nm: 200 (3.70), 271 (1.05), 316 (0.59), and 350 (0.48) nm; <sup>1</sup>H and <sup>13</sup>C NMR data (400 and 100 MHz in DMSO-*d*<sub>6</sub>) see Supplementry material Tables S1 and S2; HRESIMS, *m*/*z* 711.2505 [M + H]<sup>+</sup> (calcd for C<sub>33</sub>H<sub>43</sub>O<sub>17</sub> 711.2500).

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## 3.3.3. Icarisid N (3)

Yellow amorphous powder;  $[\alpha]_D^{25} - 141.7$  (*c* 0.07, MeOH); UV (MeOH)  $\lambda_{max}$  (log  $\varepsilon$ ) nm: 199 (3.75), 271 (1.13), 316 (0.63), and 350 (0.52) nm; <sup>1</sup>H and <sup>13</sup>C NMR data (400 and 100 MHz in DMSO-*d*<sub>6</sub>) see Supplementry material Tables S1 and S2; HRESIMS, *m*/*z* 857.3083 [M + H]<sup>+</sup> (calcd for C<sub>39</sub>H<sub>53</sub>O<sub>21</sub>, 857.3079).

#### 3.3.4. Icarisid O (4)

Yellow amorphous powder;  $[\alpha]_D^{25}$  –165.3 (c 0.1, MeOH); UV (MeOH)  $\lambda_{max}$  (log  $\varepsilon$ ) nm: 203 (3.88), 270 (1.42), 320 (0.77), and 349 (0.71) nm; <sup>1</sup>H and <sup>13</sup>C NMR data (400 and 100 MHz in DMSO- $d_6$ ) see Supplementry material Tables S1 and S2; HRESIMS, m/z 825.2822 [M + H]<sup>+</sup> (calcd for C<sub>38</sub>H<sub>49</sub>O<sub>20</sub>, 825.2817).

#### 3.4. Enzymatic hydrolysis

The sugar units of compounds were identified by enzymatic hydrolysis and followed by GC-MS analysis. This part was conducted according to published method with some modifications (Jia et al. 2010).

#### 3.5. XOD activity bioassay

The production of superoxide ion was detected by the nitrite method in 96-well plates (Shang et al. 2006). The mixture was consisted of  $10 \,\mu$ L compound solution (100, 33.33, 11.11, 3.70, 1.23 and 0.41  $\mu$ M),  $10 \,\mu$ L Nitrotetrazolium Blue chloride (NBT) solution (50  $\mu$ M) and 40  $\mu$ L XOD (20 U/L) as the substrate, incubated at 37 °C for 10 min. The reaction was initiated by adding 40  $\mu$ L xanthine (120  $\mu$ M), and incubated at 37 °C for another 30 min. Finally, the reaction was stopped with adding 50  $\mu$ L HCl (2 M), the absorbance value was detected at 560 nm. The uric acid content was measured according to published method with some modifications (Liu et al. 2017). The method was similar to the method above, excepted for the NBT solution was replaced by phosphate buffer and final absorbance value was detected at 295 nm. Allopurinol was used as the positive control.

#### **3.6.** NO production bioassay

According to the published method with some modifications (Yang et al. 2011), RAW 264.7 cells were cultured in RPMI-1640 medium, and were plated at 8000 cells per well in a 96-well plate and incubated for 24 h under 5% CO<sub>2</sub> atmosphere at 37°C. After incubated for 24 h, the cells were pre-treated with different compounds (100, 33.33, 11.11, 3.70, 1.23 and 0.41  $\mu$ M) for 1 h, and then stimulated with LPS (1  $\mu$ g/mL) for 23 h. The final concentration of DMSO should not exceed 0.1% in the culture medium. The supernatant (50  $\mu$ L) was harvested and measured by Griess reagent. Subsequently, nitrite content was measured at 540 nm. Indometacin was used as a positive control.

#### 4. Conclusion

The research reported four new and two known prenylflavonol glycosides from *Cyclocarya paliurus* for the first time. The bioassays suggested that compounds **1** and **2** exhibited outstanding inhibitory activities on XOD and moderate inhibitions on LPS-induced NO production. The discovery of these compounds enriched the chemical constituents of flavonoids in *C. paliurus*. And the bioassay results proposed new insights for the usage of *C. paliurus*.

#### **Disclosure statement**

No potential conflict of interest was reported by the authors.

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