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5,6-dihydroxypyranoflavone, a New Flavonoid with an Oxidized Prenyl Group from Dietary Plant *Citrus hystrix*

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Abstract: *Citrus hystrix* has been widely used as beverages, traditional condiments and folk medicines. In this study, 5,6-dihydroxypyranoflavone (**1**), a new flavonoid with an oxidized prenyl group, and three known phenols (**2-4**), were separated from the leaves of this plant. Their structures were elucidated using comprehensive spectroscopic data including 1D NMR, 2D NMR and mass spectral analysis. Additionally, 5,6-dihydroxypyranoflavone (**1**) exhibited moderate antioxidant effect against DPPH and ABTS free radicals with IC_{50} values of 18.28 ± 0.36 and 12.46 ± 0.82 μM, respectively.The results suggested that the dietary plant *Citrus hystrix* could be considered as a potential source of natural antioxidants.

Keywords: *Citrus hystrix*; dietary plant; flavonoid; antioxidant.

1. Plant Source

The leaves of *Citrus hystrix* were collected from Xishuangbanna, Yunnan Province, P. R. China, in September 2009 and identified by Mr. Longteng Cui. The voucher specimen (TY091101) was deposited in School of Physical Education, Yunnan Minzu University.

2. Previous Studies

The genus *Citrus* (Rutaceae), a very significant medicinal or economic crops, are widespread throughout the tropical and subtropical regions [1-3]. Various species are used as traditional condiments, folk medicines, beverages, perfumes and so on [3]. Among them, *Citrus hystrix* (commonly called as wild or kaffir lime), widely distributed in south-east Asia, has been consumed as both healthy condiments and folk medicines [2,3]. *C. hystrix* are often used to make juice and side dish or used as dietary acidulant (such as curries). Furthermore, the whole fruits and leaves are used as various inflammatory ailments in the folk medicinal system [4]. Previously phytochemical investigations on this plant resulted in the isolation of diversified furanocoumarins, flavonoids and

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arcidone alkaloids [4-7]. These chemical components were found to exhibit various bioactivities, such as antioxidant, anti-microbial, anti-fungal, anti-tumor, hepato and cardio protective effects [2-7].

3. Present Study

The air-dried and powdered leaves of *C. hystrix* (2.8 kg) were extracted three times with 95% ethanol aq. (each 15 L) at r.t. and filtered. Then, the filtrate was evaporated by rotary evaporator. The resulting residue (320.0 g) was suspended in water (1 L), and further partitioned three times using ethyl acetate (each 1 L). The ethyl acetate-soluble fraction (58.0 g) was purified on column chromatography (CC) on silica gel (petroleum ether/ethyl acetate: gradient system from ratio 1:0 to 0:1) to give six main fractions I-VI. Fraction III (petroleum ether/ethyl acetate: ratio 8:2, 12.6 g) was subjected into subfractions III1-III6 by RP-C_{18} column (methanol/water: gradient elution from ratio 50:50 to 1:0). Finally, subfraction III3 (1.4 g) was separated through preparative HPLC (flow: 15 mL/min, methanol/water: ratio 80:20, detector UV: λ_{max} 202 and 254 nm) and followed by semi-preparative HPLC (flow: 3 mL/min, acetonitrile/water: ratio 65:35, DAD-detector UV: λ_{max} 202, 210, 220, 254, 280, and 360 nm) to yield **1** (21 mg, R_t = 11.4 min). Fraction VI (ethyl acetate, 4.8 g) was subjected by RP-C_{18} column (methanol/water: gradient elution from ratio 20:80 to 50:50) and followed by semi-preparative HPLC (flow: 3 mL/min, methanol/water, DAD-detector UV: λ_{max} 202, 210, 220, 254, 280, and 360 nm) to yield **2** (13 mg), **3** (9 mg), and **4** (5 mg), respectively.

*5,6-Dihydroxypyranoflavone (**1**)*: $C_{20}H_{16}O_5$, isolated as a yellow amorphous powder, UV(MeOH) λ_{max} (log ε): 228 (3.62), 371 (4.02) nm; ^{1}H NMR and ^{13}C NMR data (DMSO-*d*6 and $CDCl_3$, 400 and 100 MHz, see Table 1); Positive ESI-MS: *m/z* 337 $[M + H]^+$; Positive HR-ESI-MS: *m/z* 337.1071 $[M + H]^+$ (calcd for $C_{20}H_{17}O_5$, 337.1071).

*Synthesis of 5,6-dihydroxypyranoflavone (**1**)*: baicalein (1 equiv, 135 mg, 0.5 mmol) and 3-methyl-2-butenal (2 equiv, 84 mg, 1.0 mmol) were dissolved in anhydrous pyridine (2 mL), and the reaction was performed by stirring the mixture under nitrogen at 110 ^{o}C for 10 hours. Then, the solution was reduced under a vacuum. The resulting mixture was directly subjected to silica gel column eluted with petroleum ether/ethyl acetate (ratio 8:2) to afford compound **1** as a yellow solid (59 mg, 0.175 mmol, 35%).

DPPH and ABTS radical scavenging assays: the antioxidant potential of 5,6-dihydroxypyranoflavone (**1**) was evaluated using DPPH and ABTS free radical scavenging assays [8-11] with ascorbic acid as a positive control (10.64 ± 0.14 and 7.16 ± 0.28 μM, respectively) (see SI). Compound **1** exhibited moderate inhibitory effects against DPPH free radical and ABTS free radical with IC_{50} values of 18.28 ± 0.36 and 12.46 ± 0.82 μM, respectively. The results suggested that 5,6-dihydroxypyranoflavone could be considered as a potential natural antioxidant from dietary plant *C. hystrix*.

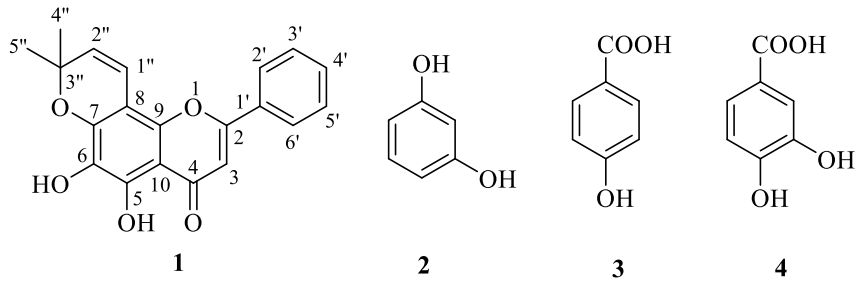


Figure 1. Chemical structures of compounds **1**-**4** from *C. hystrix*

Compound **1**-**4** (Figure 1) were isolated from the leaves of *C. hystrix*. Compound **1** (5,6-dihydroxypyranoflavone) was identified by its spectroscopic data as a new flavonoid, while the known compounds, resorcinol (**2**) [12], *p*-dihydroxybenzoic acid (**3**) [13] and protocatechuate (**4**) [14], were confirmed by the comparison of their data with literature.

Compound **1**, a yellow amorphous powder, its molecular formula $C_{20}H_{16}O_5$ was revealed by its HR-ESI-MS (Figure S1) at *m/z 337*.1071 $[M + H]^+$ (calcd. for $C_{20}H_{17}O_5$, 337.1076), requiring thirteen degrees of unsaturation. The ^1H NMR spectrum (Table 1) indicated the presence of a hydroxyl proton at δ_H 12.77 (s), two conterminal aromatic protons signals at δ_H 6.86 (J = 8.9 Hz) and 5.76 (J = 8.9 Hz), a mono-substituted benzene moiety (δ_H 7.54-8.03, 5H), one singlet aromatic ring protons at δ_H 6.93 (s), and a sharp singlet at δ_H 1.41 (6H, s) for a geminal dimethyl groups.

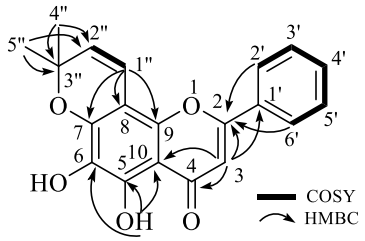


Figure 2. Selected HMBC correlations and key ^1H-^1H COSY correlations of **1**

The ^{13}C NMR data and DEPT spectra (see Table 1) exhibited twenty carbon signals categorized into two sp^3 methyl's [δ_C 27.6 (2C)], eight olefinic methines [δ_C 104.6, 114.7, 126.4 (2C), 128.4, 129.2 (2C), and 132.0], and ten quaternary carbons (δ_C 78.0, 101.2, 104.7, 129.8, 130.8, 144.2, 147.3, 147.5, 162.9, and 182.4) including one carbonyl carbon, one oxygen-containing sp^3 quaternary carbon, and eight sp^2 quaternary carbons (including five oxygenated). Further analysis of its 1D NMR data suggested that its structure was very similar to those of 5,6,4'-trihydroxypyranoflavone, a previously isolated pyranoflavanone from the title plant [2]. The main difference was that the hydroxyl group at C-4' position in 5,6,4'-trihydroxypyranoflavone was replaced by an aromatic proton in 5,6-dihydroxypyranoflavone (**1**) [2]. Subsequently, the 2D NMR data provided reliably evidence for the structural identification (Figure 2). Firstly, the flavone nucleus was established by the observed HMBC correlations from H-3 (δ_H 6.93) to C-2 (δ_C 162.9), C-4 (δ_C 182.4), C-10 (δ_C 104.7), and C-1' (δ_C 130.8), and from H-2'/H-6' (δ_H 8.03) to C-2 (δ_C 162.9). Furthermore, the fusion of pyran ring between C-7 and C-8 was confirmed by the HMBC interactions from H-1" (δ_H 6.86) of the pyran ring to C-7 (δ_C 147.5), C-8 (δ_C 101.2), and C-9 (δ_C 144.2) (Figure 2). Finally, the location of the hydroxyl group at C-5 were inferred from the HMBC interactions of 5-OH (δ_H 12.77) with C-5 (δ_C 147.3), C-6 (δ_C 129.8), and C-10 (δ_C 104.7). based on those evidences, the compound **1** was assigned as 5,6-dihydroxypyranoflavone (Figure 1), a new flavonoid with an oxidized prenyl group isolated from nature for the first time.

Biosynthetically, 5,6-dihydroxypyranoflavone might be formed via a condensation reaction between a flavonoid (baicalein) and a dimethylallyl pyrophosphate (DMAPP) (Figure 3) in nature.

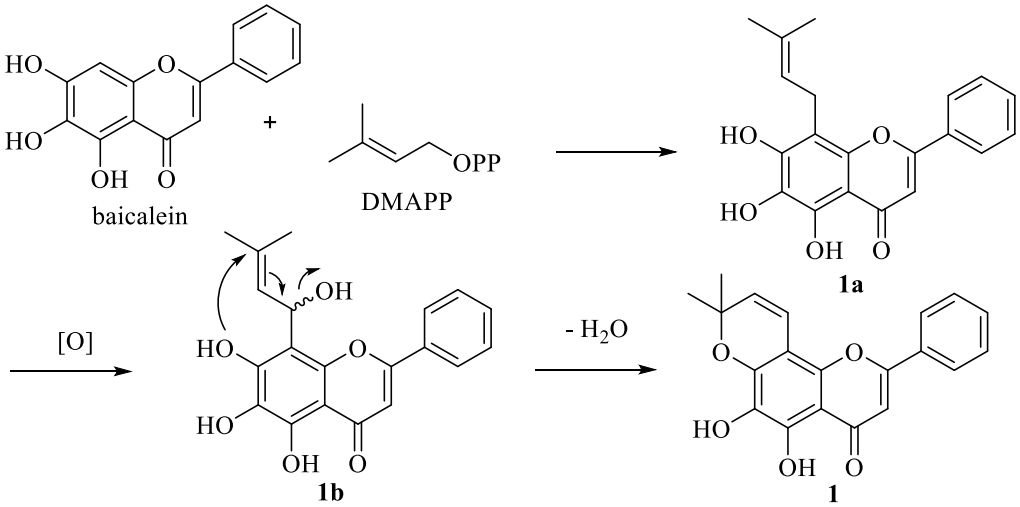
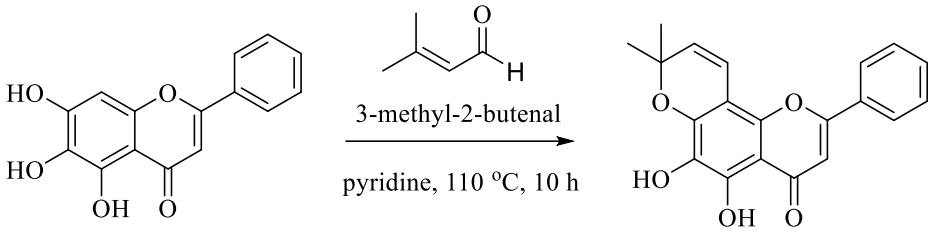


Figure 3. Hypothetical biogenetic pathway of compound **1**

Finally, the structure of 5,6-dihydroxypyranoflavone was further confirmed by a single-step biomimetic synthesis from baicalein and 3-methyl-2-butenal with a yield of 35% (Figure 4). The NMR spectral data of synthetic 5,6-dihydroxypyranoflavone was in full agreement with those of the natural product (see supporting information).



baicalein **1**

Figure 4. Synthesis of 5,6-dihydroxypyranoflavone (**1**). Reagents and conditions: baicalein (1 equiv), 3-methyl-2-butenal (2 equiv), pyridine as solvent, 110 °C, 10 h, 35% for **1**

Table 1. ^{1}H and ^{13}C NMR Data of Compound **1** (δ in ppm, 400 MHz and 100 MHz)

No.	**1**			
	$\delta_C{}^a$	$\delta_H{}^a$	$\delta_C{}^b$	$\delta_H{}^b$
2	162.9 s	-	163.9 s	-
3	104.6 d	6.93 s	105.5 d	6.67 s
4	182.4 s	-	183.0 s	-
5	147.3 s	-	146.2 s	-
6	129.8 s	-	129.2 s	-
7	147.5 s	-	146.8 s	-
8	101.2 s	-	101.6 s	-
9	144.2 s	-	145.6 s	-
10	104.7 s	-	105.5 s	-
1'	130.8 s	-	131.8 s	-
2'/6'	127.3 d	8.03 overlapped	126.4 d	7.88 dd (7.8, 1.8)
3'/5'	129.2 d	7.53 overlapped	129.3 d	7.54 overlapped
4'	132.0 d	7.53 overlapped	132.0 d	7.54 overlapped
1"	114.7 d	6.86 d (8.9)	115.1 d	6.85 d (10.0)
2"	128.4 d	5.76 d (8.9)	128.1 d	5.67 d (10.0)
3"	78.0 s	-	79.0 s	-
4"/5"	27.6 q	1.41 s	28.3 q	1.56 s
5-OH	-	12.77 s	-	12.75 s

[a] Data were recorded in DMSO-d_6. [b] Data were recorded in $CDCl_3$

In conclusion, we isolated a new pyranoflavone from the Citrus hystrix for the first time from nature and its chemical structure was determined as 5,6-dihydroxypyranoflavone (**1**) by using 1D, 2D NMR techniques and mass spectral data. The structure of the compound (**1**) was also confirmed by semisynthesis of it from baicalein.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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