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A rare prenylated isoflavone-quinone from the roots of *Flemingia philippinensis*

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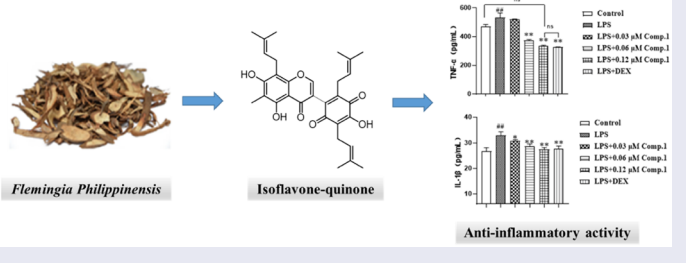
ABSTRACT

In order to make more rational use of *Flemingia Philippinensis*, a systematic separation from the roots of *F. philippinensis* was performed in the current study. The investigation of chemical constituents resulted in the isolation of a rare prenylated isoflavone-quinone, fleminquinone A (**1**), together with four known analogues (**2–5**). Their structures were established by extensive physical and spectroscopic data analysis. Anti-inflammatory activities of the isolated compounds were evaluated in lipopolysaccharide induced mouse mononuclear macrophage leukemia cells RAW 264.7 model. Compound **1** exhibited significant inhibitory effects on LPS-induced NO production and COX-2. Compound **1** also significantly affected the levels of inflammatory cytokines.



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Flemingia Philippinensis; isoflavone-quinone; anti-inflammatory activity; inflammatory cytokines; structure elucidation

1. Introduction

The genus *Flemingia* contains more than 40 species in world, which are distributed in tropical regions of Asia, Africa and Oceania, including 16 species and 1 variety in China. Most plants of the genus *Flemingia* can reduce inflammation and relieve pain, so they are widely used for the treatment of rheumatism, chronic nephritis, fall and beat injury, swelling poison, toothache, and gynecological diseases in the

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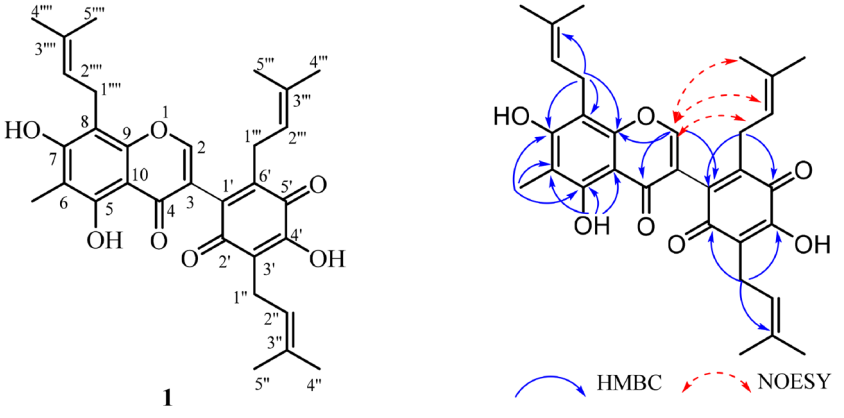
folk [1]. *Flemingia Philippinensis* is one of the most studied plants of the genus *Flemingia*. It is widely distributed in southwest, south central and southeast provinces in China. In addition to medicinal use for the treatment of rheumatoid arthritis and chronic nephritis, the folk also mixed *F. Philippinensis* with various ingredients stew for diet to relieve muscle fatigue and maintain the normal function of muscles and bones. The isoflavones, as the main and characteristic components, along with flavonoid glycosides, chromenones, and benzofurans are obtained from *F. philippinensis* based on previous studies [2–5]. These components displayed a wide variety of biological activities including neutrophil elastase inhibitory activity, tyrosinase inhibitory activity, anti-cancer activity, anti-inflammatory activity and so on [6–10].

Previously, we reported four new prenylated isoflavanones from *F. philippinensis* with significant β-amyloid aggregation inhibition activities [2]. In order to make more rational use of *F. philippinensis* resources and develop novel anti-inflammatory agents from natural origin, a systematic separation to seek the chemical compositions of the CH_2Cl_2 extract from the roots of *F. philippinensis* was performed in the current study. Herein, we report the isolation, structural elucidation, and anti-inflammatory activity of a rare prenylated isoflavone-quinone.

2. Results and discussion

The HPLC-DAD analysis on the CH_2Cl_2-soluble fraction of the 95% EtOH extract of *F. philippinensis* roots exhibited the characteristic UV absorption of isoflavanones at approximately 260 nm [11]. The CH_2Cl_2 extract was purified by successive column chromatography using silica gel, Sephadex LH-20, ODS and semi-preparative HPLC which permitted the isolation of one new (**1**) (Figure 1) and four known isoflavanones (**2**–**5**).

Compound **1** was isolated as yellow powder. The molecular formula, $C_{31}H_{34}O_7$, was deduced from its HRESIMS data (*m/z* 519.2389 $[M+H]^+$), requiring 15 degrees of unsaturation. The UV absorption maxima of this compound at 212 and 268 nm were suggestive of an isoflavonoid derivative [11]. Its IR spectrum showed absorption bands of a conjugated ketone carbonyl (1640 cm^{-1}) and a hydrogen-bonded hydroxy



1

Figure 1. Structure, key HMBC and NOESY correlations of compound **1**

group (3439 cm^{-1}). The ^1H NMR spectrum of **1** displayed signals due to a chelate hydroxyl group at δ_H 12.61 (1H, brs, OH-5), as well as a characteristic olefinic proton at δ_H 7.66 (1H, s, H-2), suggestive of an isoflavonoids nucleus. In addition, the characteristic signals of one methyl at δ_H 2.13 (3H, s, H-11), and three typical prenyls at δ_H 3.16 (2H, d, J = 6.9 Hz, H-1"), 5.14 (1H, t, J = 6.9 Hz, H-2"), 1.66 (3H, s, H-4") and 1.73 (3H, s, H-5"), 3.27 (1H, dd, J = 14.1, 6.9 Hz, H-1'"a), 3.02 (1H, dd, J = 14.1, 6.9 Hz, H-1'"b), 4.93 (1H, t, J = 6.9 Hz, H-2'"), 1.59 (3H, s, H-4'") and 1.51 (3H, s, H-5'"), and 3.50 (2H, d, J = 6.8 Hz, H-1""), 5.21 (1H, t, J = 6.8 Hz, H-2""), 1.78 (3H, s, H-4"") and 1.85 (3H, s, H-5"") were also observed. The ^{13}C NMR spectrum showed 31 carbon resonances which were classified as 21 olefinic carbons including three carbonyl carbons at δc 185.8, 183.5 and 180.0, three methylene carbons, and seven methyls. Comparison of the NMR spectral data of **1** with those of licoriquinone B [11] indicated that they are structural analogues, but the major difference is the appearence of a more prenyl group. By analyzing the above NMR data, the structure was suggested to be an isoflavone-quinone, and one of the aromatic rings (B ring) was oxidized to be a *p*-benzoquinone ring. The location of the prenyl was determined by the HMBC experiment. HMBC correlations (Figure 1) of H-2 with C-9 (δ_C 152.8), C-4 (δ_C 180.0), and C-1' (δ_C 136.5), and of H-1"" with C-9, C-8 (δ_C 104.5) and C-7 (δ_C 159.6) indicated that a prenyl was linked to C-8 of A ring. NOESY correlations (Figure 1) of H-2 with H-1'"a, H-1'"b, H-2'" and H-5'" suggested that the second prenyl was condensed to C-6' of B ring. This assignment was also supported by the HMBC correlations of H-2, H-1'"a and H-1'"b with C-1' (δ_C 136.5). The third prenyl was condensed to C-3' of B ring because of H-1" correlations with C-2' (δ_C 185.8) and C-4' (δ_C 151.0) by HMBC experiments. Meanwhile, a methyl at δ_H 2.13 (s, H-11) was assigned to connection with C-6 and two OH groups were placed at C-7 and C-5, respectively, by δ_H 2.13 correlations with C-7, C-6 (δ_C 108.2) and C-5 (δ_C 158.0) in the HMBC spectrum.

Consequently, complete analysis of the ^1H and ^{13}C NMR, HSQC, HMBC data established the structure of compound **1** as 6-methyl-5,7,4'-trihydroxy-8,3',6'- triprenylisoflavonquinone, and named fleminquinone A.

The isoflavone-quinone represent a rare group of secondary metabolites from plants, and only a few structures have been reported so far [11–14]. The previous study revealed that certain isoflavones are prone to oxidative degradation into isoflavone-quinones, and the proposed biogenetic mechanism of oxidation of the isoflavans into the quinones was outlined. Additionally, our findings further enriched the structural resources of isoflavone-quinone from natural origin. By comparing the spectroscopic properties with reported data, four known isoflavanones were elucidated as philippinones A-C (**2**-**4**) [2], 5,7,3'-trihydroxy-2'-(3-methylbut-2-enyl)-4',5'-(3,3-dimethylpyrano) isoflavone (**5**) [15], respectively

Considering the traditional uses of *F. philippinensis* and a goal to obtain a lead compound of anti-inflammatory activity, all isolated compounds (**1**–**5**) were tested individually for NO production inhibition in LPS-stimulated RAW 264.7 macrophages. To explore the content of NO in the process of inflammatory regulation, the concentration of NO was measured by use of the method of Griess reagent. The isolated compounds **1**-–**5** showed significant NO inhibitory effects with IC_{50} values of 0.15 ± 0.06, 10.34 ± 2.3, 13.42 ± 2.4, 7.28 ± 1.1, 17.19 ± 1.9 μM, respectively.

Using dexamethasone as the positive control (DEX, IC_{50} = 0.64 nM), compound **1** exhibited more potent inhibitory activity against NO production among these compounds. Therefore, we speculate that the *p*-benzoquinone moiety in B ring may be the active group. As known from the experimental results in Figure 2(A), the content of NO was increased very significantly ($p < 0.01$) in LPS group comparing with the control group; and compared with the LPS group, the concentration of NO in 0.03, 0.06, and 0.12 μM of compound **1** groups were all very significantly ($p < 0.01$) decreased in a dose-dependent manner.

Using DEX as the positive control, we also tested the effects of compound **1** on the concentration of inflammatory cytokines in LPS-stimulated RAW 264.7 macrophage cells. The levels of the inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-10 after LPS administration are shown in Figure 2(B–E). Compared to the LPS-treated group, the present results showed that compound **1** induced significant decrease ($p < 0.01$) in TNF-α, IL-1β, and IL-6 production and increases ($p < 0.01$) in IL-10 production. Compound **1** significantly down-regulated the levels of inflammatory cytokines IL-1β, IL-6 and TNF-α (Figure 2(B–E)) and up-regulated the levels of

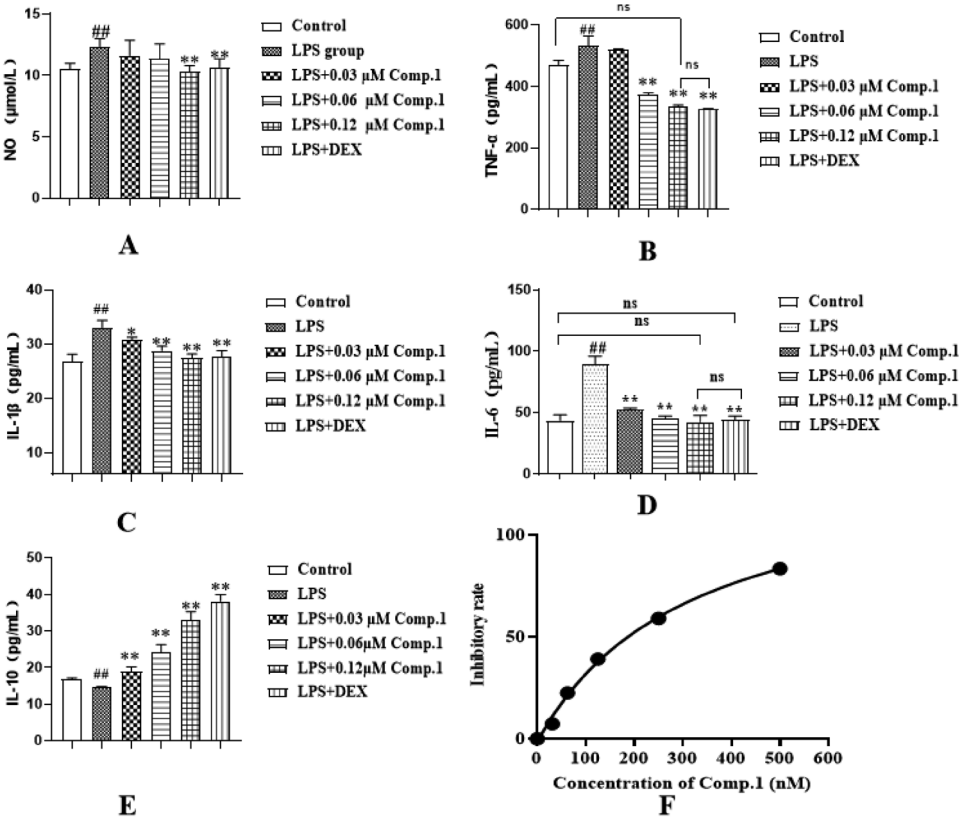


Figure 2. Results of the content of NO (**A**), TNF-α (**B**), IL-1β (**C**), IL-6 (**D**), and IL-10 (**E**) in the supernatant of LPS-induced RAW 264.7 cells (mean ± *SD*, $n = 5$) and the inhibitory rate curve of compound **1** against COX-2 enzyme (**F**). *Note*. ##Represents a significant difference with the blank control group ($p < 0.01$). **Represents a significant difference with the model control group ($p < 0.01$), DEX: dexamethasone.

inflammatory cytokines IL-10 in a dose-dependent manner in the concentration range of 0.03–0.12 μM. The research showed that compound **1** could suppress the inflammatory response directly by decreasing the production of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 and by increasing the content of anti-inflammatory cytokine IL-10.

COX-2 enzyme plays a key role in the production and development of inflammation and is an important action target for anti-inflammatory drugs. Thus we also tested the COX-2 enzyme inhibitory effects of compound **1** in RAW 264.7 cells. The results revealed compound **1** also inhibited the activity of COX-2 enzyme in a dose-dependent manner in the concentration range of 31-500 nM. The inhibition rate curve of the compound **1** against COX-2 is shown in Figure 2(F). The IC_{50} value of compound **1** against COX-2 was 182 nM, using celecoxib as the positive control (IC_{50} = 42 nM).

In conclusion, a rare isoflavone-quinone derivative was isolated, which was rarely reported from *F. philippinensis*. The biological evaluation of the isoflavone-quinone shown great application potential as a lead compound from natural sources in the research and development of anti-inflammatory drugs.

3. Experimental

3.1. General experimental procedures

The UV spectra were conducted on a Shimadzu UV-2201 spectrometer (Shimadzu, Tokyo, Japan). The FT-IR spectra were obtained on a Bruker IFS-55 spectrometer by a KBr disk method (Bruker Corporation, Karlsruhe, Germany). ESIMS were conducted on an Agilent 1100 ion trap spectrometer (Agilent Technologies Inc., Palo Alto, CA). HRESIMS were recorded on a Bruker microTOF-Q mass spectrometer. NMR spectra were recorded on Bruker AV-400 NMR spectrometers (Bruker Corporation, Karlsruhe, Germany). Sephadex LH-20 was purchased from GE Healthcare (Boston, MA). The chromatographic silica gel (200–300 mesh) was purchased from Qingdao Ocean Chemical Factory (Qingdao, China) and ODS (50 μm) was purchased from YMC Co. LTD. (Kyoto, Japan). Semi-preparative HPLC was conducted on an Agilent 1260 (Agilent Technologies Inc., Palo Alto, California, USA) with a DAD detector and equipped with a C-18 column (20 mm × 250 mm, 5 μm, YMC Co. LTD., Japan).

3.2. Plant material

The roots of *Flemingia philippinensis* were purchased in October 2020 from Quanzhou County, Guilin City, Guangxi Province, China. The material was identified by Prof. Wei Ning (College of Horticulture, Shenyang Agricultural University, Shenyang, China). The voucher sample (FPA-2020101501) was deposited in the Department of Animal Pharmacy, Shenyang Agricultural University, Shenyang, China. Molecular Device microplate reader was performed on a SpectraMax plus 384 (MD, USA). Dexamethasone was purchased from Dalian Meilun Biotechnology Co., LTD (Dalian, China). The ELISA kits of inflammatory cytokines (IL-1β, IL-6, IL-10 and TNF-α)

were manufactured by Thermo Fisher Scientific Co., LTD (Waltham, MA). COX-2 kit was manufactured by Shanghai Biyuntian Biotechnology Co., LTD (Shanghai, China).

3.3. Extraction and isolation

The air-dried roots of *F. philippinensis* (24.5 kg) were extracted with 95% (v/v) alcohol to give an alcohol extract. The alcohol solvents were evaporated under reduced pressure to produce a crude extract. The residue was then suspended in water (10 L) and partitioned successively with petroleum ether (PE, 3 × 10 L), CH_2Cl_2 (3 × 10 L), and *n*-butanol (3 × 10 L). The CH_2Cl_2 extract (430 g) was subjected to silica gel column chromatography (CC), eluted with a gradient system of PE-acetone (from 100:0 to 0:100, v/v) to give ten fractions A-J. Fraction D (33 g) was further separated by silica gel CC using *n*-hexane-acetone mixtures of increasing polarity (from 100:10 to 100:30, v/v) to give ten sub-fractions, designated as D1–D10.

Purification of subfraction D8 through ODS CC using MeOH-H_2O (from 40:60 to 95:5, v/v) as eluent with a gradient system afforded eight fractions D8a-D8h. Fraction D8e (3 g) was further purified by semi-preparative RP-HPLC eluted with acetonitrile-water (from 70:30 to 90:10, v/v, 0.1% trifluoroacetic acid) to yield **2** (7.2 mg, t_R 18.0 min), **3** (6.2 mg, t_R 21.0 min), and **4** (9.5 mg, t_R 27.0 min).

Separation of fraction D9 through Sephadex LH-20 CC (CH_2Cl_2: MeOH = 1:1) provided seven subfractions D9a-D9g. Subfraction D9c was further chromatographed by semi-preparative RP-HPLC eluted with methanol-water (85:15, v/v, 0.1% trifluoroacetic acid) to obtain **1** (5.8 mg, t_R 22.0 min) and **5** (8.2 mg, t_R 26.0 min).

3.3.1. Fleminquinone A (1)

Pale yellow powder (in MeOH); UV (MeOH) λ_{max} 268, 212 nm; IR (KBr) ν_{max} 3439, 2924, 1640, 1442, 1384, 1260, 1168, 580 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ_H 12.61 (1H, brs, OH-5), 7.66 (1H, s, H-2), 5.21 (1H, t, J = 6.8 Hz, H-2''''), 5.14 (1H, t, J = 6.9 Hz, H-2''), 4.93 (1H, t, J = 6.9 Hz, H-2'''), 3.50 (2H, d, J = 6.8 Hz, H-1''''), 3.27 (1H, dd, J = 14.1, 6.9 Hz, H-1'''a), 3.16 (2H, d, J = 6.9 Hz, H-1''), 3.02 (1H, dd, J = 14.1, 6.9 Hz, H-1'''b), 2.13 (3H, s, H-11), 1.85 (3H, s, H-5''''), 1.78 (3H, s, H-4''''), 1.73 (3H, s, H-5''), 1.66 (3H, s, H-4''), 1.59 (3H, s, H-4''') and 1.51 (3H, s, H-5'''); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 185.8 (C-2'), 183.5 (C-5'), 180.0 (C-4), 159.6 (C-7), 158.0 (C-5), 154.6 (C-2), 152.8 (C-9), 151.0 (C-4'), 143.8 (C-6'), 136.5 (C-1'), 136.1 (C-3''''), 134.6 (C-3'''), 133.9 (C-3''), 121.1 (C-2''''), 120.5 (C-3'), 119.7 (C-2''), 119.5 (C-2'''), 116.5 (C-3), 108.2 (C-6), 105.4 (C-10), 104.5 (C-8), 27.2 (C-1'''), 26.0 (C-4''''), 25.9 (C-4''), 25.8 (C-4'''), 22.6 (C-1''), 22.0 (C-1''''), 18.1 (C-5''''), 18.0 (C-5''), 18.0 (C-5'''), 7.5 (C-11); HRESIMS *m/z* 519.2389 $[M+H]^+$ (calcd for $C_{31}H_{35}O_7$, 519.2383).

3.4. Anti-inflammatory assays

3.4.1. The culture of LPS-activated RAW 264.7 cells

The cells were thawed in 37 °C for about 3 min to melt them quickly and completely, and then centrifuged for 5 min at 15,000 g, to discard the supernatant. Then, we mixed the cells in DMEM high glucose medium with 10% FBS, transferred them to

25 cm^2 cell culture bottle, and cultured them in a carbon dioxide incubator at 37 °C with 5% CO_2. We used an inverted microscope to observe the growth of RAW 264.7 cells. When the cells adhesion growth exceeded 80%, the cells were seeded in 96-well plates (150 L/well) under 5% CO_2 at 37 °C and incubated with LPS (1 μg/ml) for 1 h.

3.4.2. NO production inhibition assays

The nitrite concentration in the medium was measured as an indicator of NO production in accordance with the Griess reaction [10]. The NO inhibitory effects of **1–5** were tested in LPS-activated RAW 264.7 cells. Cells (1×10^5 cells/ml) were seeded in 96-well plates (150 μl/well) and incubated for 24 h. After stimulation with LPS, the compounds were added at different concentrations; then, cells were incubated for 24 h. The level of nitrite formation in supernatants was measured using a Griess reagent by measuring the absorbance at 540 nm. The percent inhibition for nitrite production was determined by comparing it with the vehicle control. IC_{50} values were calculated from dose curves, and the dexamethasone (DEX) was used as the positive control. Nitrite concentrations and inhibitory rates were calculated by the calibration curve prepared with sodium nitrite standards. Experiments were conducted in triplicate, and data were presented as mean ± SD of three independent experiments.

3.4.3. COX-2 inhibition assays

COX-2 assay buffer, Milli-Q grade water, DMSO, and other appropriate solvents were used to prepare appropriate concentrations of compound **1**. The effect of compound **1** on COX-2 enzyme activity was determined by fluorescence assay. Groups included the blank control group, 100% enzyme active control group, compound **1** at different concentrations (31.25, 62.5, 125, 250, and 500 nM) groups, and positive control group (50 nM Celecoxib). Corresponding reagents were added to the 96-well plate successively. You can refer to the instruction manual of the COX-2 inhibitor screening kit for the other steps. The relative fluorescence unit was measured by using a microplate reader. The inhibition of COX-2 enzyme activity by compound **1** was analyzed by GraphPad Prism 8, and the IC_{50} value was obtained.

3.4.4. Inflammatory cytokine assays

RAW 264.7 cells (5×10^5 cells/ml) were cultured in a 96-well plate (150 μl/well) for 48 h and were incubated with LPS for 1 h followed by the addition of compound **1** (0.03, 0.06, and 0.12 μM) and DEX (1 nM) for 1 h. The cell supernatant was collected, and the concentration of the cytokines IL-1β, IL-6, IL-10, and TNF-α were determined by ELISA. The other steps were carried out by referring to the instruction manual of ELISA kit. Experimental data were presented as mean ± SD of five independent experiments.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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