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The chemical constituents of *Gentiana macrophylla* pall. under acidic condition using gentiopicroside-rich secoiridoids extract

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ABSTRACT

The chemical constituents of *Gentiana macrophylla* Pall. under acidic condition were explored using gentiopicroside-rich secoiridoids extract treated with hydrochloric acid. 2 new secoiridoid-like structures gentiagenin A (**1**) and B (**2**), together with 4 known compounds, swerimilegenin G (**3**), 4-hydroxyindan-1-one (**4**), erythrocentaurin (**5**) and swerimilegenin E (**6**) were identified using 1D, 2D NMR and HR-ESI-MS spectral technology. In the activity evaluation, the 2 new compounds, such as gentiagenin A (**1**) and B (**2**), exhibited stronger inhibitory effect on NO production in RAW264.7 macrophage cells than that of gentiopicroside.

1. Introduction

Secoiridoids are a group of characteristic compounds that widely distribute in *Gentiana* herbs (Venditti et al., 2013) used in the treatment of arthralgia, stroke, and osteoarthritis (Jia et al., 2012). The gentiopicroside type secoiridoids, especially gentiopicroside, have been proved to display wide range of bioactivities including inhibition of phospholipase A_2, COX-2, lipopolysaccharide-induced NO production in cell models and the protective effect against inflammatory reaction in whole animal models (Zhang et al., 2018; Zhao et al., 2015). *Gentiana macrophylla* Pall. is the good resource of secoiridoids, which was reported to be rich in gentiopicroside, swertiamarin and other similar secoiridoid components (Jiang et al., 2010; Pan et al., 2016; Yuan et al., 2017; Zhang et al., 2018). This phytochemical characteristic suggests the significance of the intensive chemical research of *Gentiana macrophylla* in anti-inflammatory agents' discovery. However, the gentiopicroside-type secoiridoids possess the unstable structures of vinyl ether bond and glycosidic bond, which will lead to the decomposition and conversion reactions under certain conditions (i.e. acidic or alkali environments, glycosidase or microbial degradation), (Ikeda et al., 1984; Wang et al., 2009; Wu et al., 2017; Zeng et al., 2013). It has been reported that the structure of gentiopicroside will be transformed during the separation process under alkali condition (Popov et al., 1988) and lead to the artificial products of gentiannine and gentianaldehyde (Yang and Song, 1999). Meanwhile, gentiopicroside was reported to give gentioline and gentianaldehyde following the glycosidase hydrolysis (El-Sedawy et al., 1989; Wang et al., 2007; Yang and Song, 2000). These reports indicated that the phytochemistry research on *Gentiana macrophylla* or other secoiridoid-rich herbs might be disturbed by possible artifacts generated during extraction and separation in the presence of acid, alkali or even the plant origin enzyme.

On the other hand, although the artificial product disturbs the original analysis of herb constituents, it can also provide the new chemical skeletons for lead compounds searching. Besides the common chromatographic separation, a new methodology called "diversity-enhanced extracts" has been developed recently (Ignatenko et al., 2013; Kikuchi et al., 2014). In which, the bioactive natural product extract with known or similar structures as whole are conducted to the chemical reaction aims to natural product-like skeletons discovery. Considering the unstable property of gentiopicroside and similar secoiridioids in *Gentiana macrophylla*, the secoiridoid-rich extract could be conducted to structure transform according to aforementioned methodology for new bioactive skeletons finding and avoid the difficulty in the minor secoiridoids isolation. To explore the possible artificial products of secoiridoids in *Gentiana macrophylla* during separation in acidic condition and obtain the new bioactive anti-inflammatory compounds, we reported herein the chemical constituent research of gentiopicroside-rich secoiridoids extract treated with hydrochloric acid. As a result, 2 secoiridoid-like compounds with new skeleton and 4 known constituents were identified. In the subsequent anti-inflammatory effect evaluation, 2 new compounds were detected to exhibit stronger inhibitory effect on NO production in RAW264.7 macrophage cells than that of gentiopicroside.

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Table 1
^{1}H (400 Hz) and ^{13}C (100 Hz) NMR data for compounds **1** and **2** (in CD_3OD).

Position	1		2	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1	4.68, m	101.4	4.57, m	98.1
2				
3	4.66, m	73.0	4.99, d (4.0)	67.2
4		176.3		175.4
5		135.6		133.9
6	2.38, m	28.1	2.33, m	27.4
	2.09, m		2.19, m	
7		210.3		210.9
8	2.47, m	36.2	2.43, m	35.6
9	2.57, m	26.9	2.62, m	27.2
10	1.43, d (7.0)	19.2	1.39, d (8.0)	18.3
11	3.49, s	56.7	3.42, s	55.6

2. Results and discussion

2.1. Identification

Compound **1** was isolated as yellow amorphous powder with the fluorescence quenching under UV light. Its molecular formula was assigned as $C_{10}H_{14}O_3$ with four degrees of unsaturation on the basis of HR-ESI-MS analysis (*m/z* 183.0973 $[M+H]^+$, calcd for $C_{10}H_{15}O_3$: 183.1021). The IR data indicated the presence of carbonyl group at 1720.07 cm^{-1}. The ^{1}H NMR spectrum (Table 1) showed one methyl group with CH_3-CH-segment at δ_H 1.43 (3H, d, J = 7.0 Hz, H-10), one methoxy group at δ_H 3.49 (3H, s, H-11), one ketal methine at δ_H 4.68 (1H, m, H-1), one oxygenated methine at δ_H 4.66 (1H, m, H-3), and three methylene signals at δ_H 2.57 (2H, m, H-9), δ_H 2.47 (2H, m, H-8), δ_H 2.38 (1H, m, H-6) and δ_H 2.09 (1H, m, H-6), respectively. In the ^{13}C NMR (DEPT) spectrum (Table 1), the elucidation of CH_3-CH-segment and methoxy group was confirmed by the methyl signals at δ_C 19.2, δ_C 56.7, and δ_C 73.0, respectively. From the methine signal at δ_C 101.4, a ketal moiety can be elucidated, which accounted for one unsaturation degree. Furthermore, the quaternary carbon signals at δ_C 210.3, δ_C 176.3 and δ_C 135.6 indicated an α, β-unsaturated carbonyl moiety with two degree of unsaturation (Fig. 1). Moreover, the rest three methylene signals observed at δ_C 36.2, δ_C 28.1 and δ_C 26.9 proposed other ring structure, which possessed one degree of unsaturation and suggested two-ring system of compound **1**.

The methyl group at δ_H 1.43 correlated with δ_C 19.2 in the HSQC spectrum and also showed cross peaks with δ_C 73.0 and δ_C 176.3 in the HMBC experiment (Fig. 2). On the other hand, the methoxy group at δ_H 3.49 showed HMBC correlation with methine signal at δ_C 101.4 indicating that the methoxy group was located at ketal moiety (Fig. 2). In the HSQC spectrum, the signal at δ_H 4.68 showed cross peak with δ_C 101.4 and this proton also correlated with δ_C 73.0 in HMBC experiment suggested that the methyl and methoxy groups were substituted at the same ketal structure. Moreover, HMBC correlation peaks were also observed between δ_H 4.68 with δ_C 28.1 and δ_C 135.6 (Fig. 2). In the HSQC spectrum, the carbon signal δ_C 28.1 correlated with protons at δ_H 2.38 and δ_H 2.09, these two signals also showed cross peaks with δ_H 4.68 in the ^{1}H-^{1}H COSY spectrum indicating the direct coupling system of these two proton groups in the ketal skeleton (Fig. 2). In addition, since the proton signal at δ_H 4.66 correlated with δ_C 73.0 in HSQC spectrum and also showed cross peaks with δ_C 19.2 and δ_C 176.3 in the HMBC spectrum (Fig. 2), a hexatomic ketal structure with α, β-unsaturated carbonyl moiety could be deduced. This elucidation can be further confirmed from the correlations between δ_H 2.38 and δ_H 2.09 with δ_C 101.4, δ_C 135.6 and δ_C 176.3 in HMBC experiment (Fig. 2). Furthermore, the other two methylene groups can be assigned as δ 2.57, δ 26.9 and δ 2.47, δ 36.2 according to their cross peaks in the HSQC spectrum. Their direct coupling can also be observed in ^{1}H-^{1}H COSY experiment (Fig. 2). In the HMBC spectrum, δ_H 2.47 correlated with δ_C 26.9, δ_C 135.6, δ_C 176.3 and δ_C 210.3, δ_H 2.57 correlated with δ_C 36.2, δ_C 135.6, δ_C 176.3 and δ_C 210.3, respectively (Fig. 2). On the basis of aforementioned spectroscopic data, the planar structure of compound **1** was ascertained (Fig. 1).

In order to determine the orientation of C-1 and C-3, the NOE experiment was further carried out. The NOE correlations between methyl group at C-10 position with H-3 and methoxy group at C-11 position with H-1 (Fig. 2) indicated the different sides of methyl and methoxy groups in the pyran structure, which was same with the literature reported (Suyama et al., 2013). Meanwhile, since no notable cotton effects were observed in CD spectrum (Fig. S21), the racemic mixture of **1** was deduced. Thus, compound **1** was identified as gentiagenin A (Fig. 1), whose spectra data was summarized in Table 1.

Compound **2** was also obtained as yellow amorphous powder with

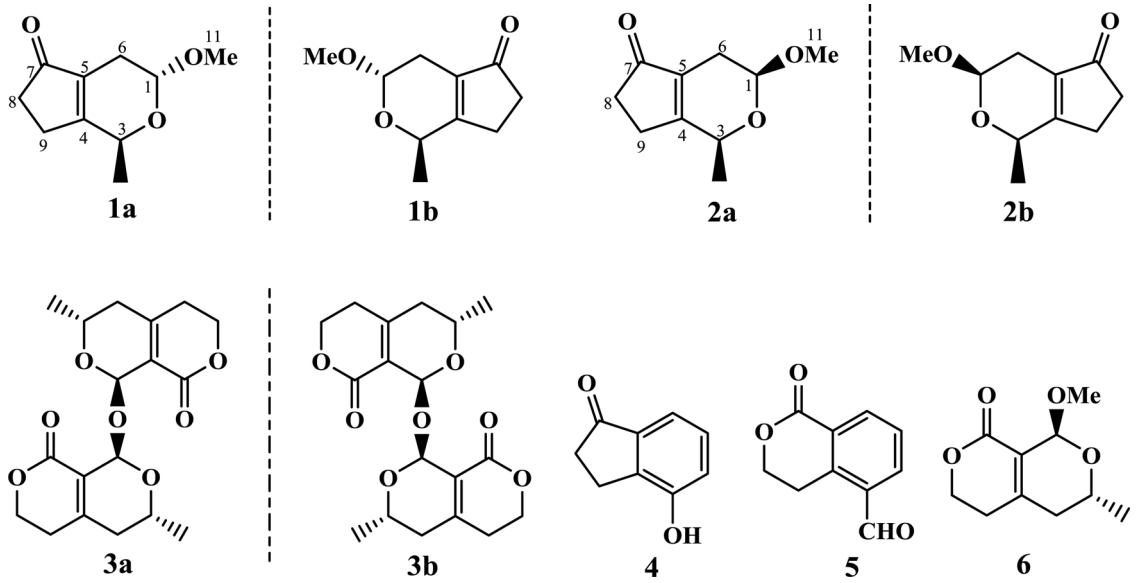

Fig. 1. Structures of isolated compounds **1-6.**

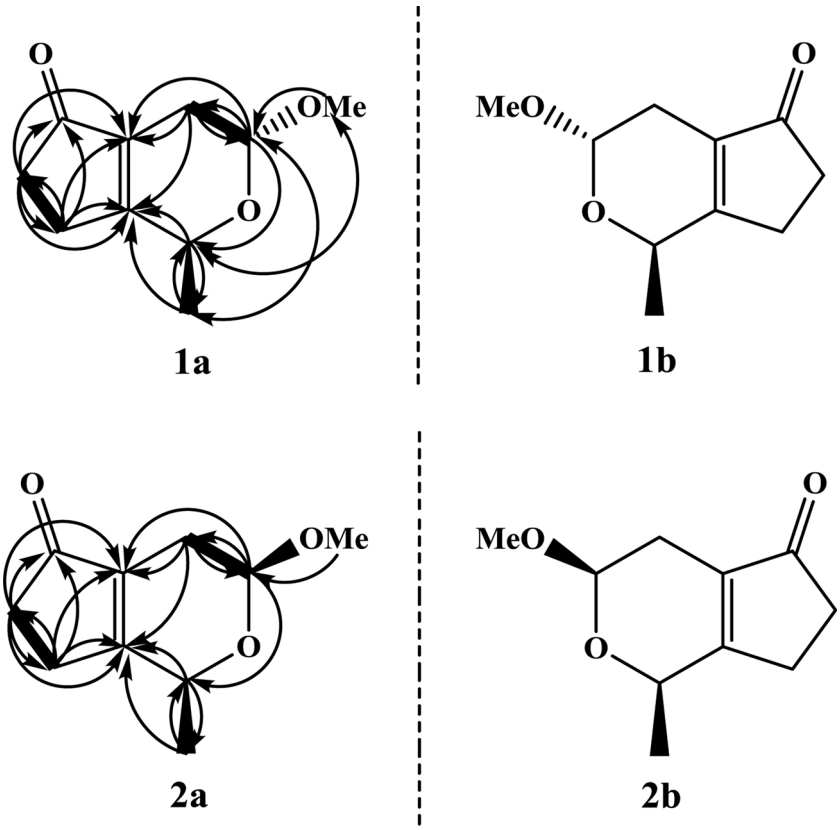

Fig. 2. Key HMBC (→), ^{1}H-^{1}H COSY (-) and NOE (↔) correlations of compounds 1 and 2.

the fluorescence quenching under UV light. Its quasi-molecular ion was shown in HR-ESI-MS analysis (*m/z* 183.1009 $[M+H]^+$, calcd for $C_{10}H_{15}O_3$: 183.1021) to give the same molecular formula of $C_{10}H_{14}O_3$ with that of compound **1**. The IR spectrum showed C–H stretching vibration and carbonyl group at 2977.42 and 1719.72 cm^{-1}. The ^{1}H NMR, ^{13}C NMR (DEPT) spectra (Table 1) and 2D NMR correlations (Fig. 2) of compound **2** resembled to those of **1**, which revealed the same skeleton of **2** with gentiagenin A. Since there is no correlation peaks can be observed in NOE experiment (Fig. 2), an epimer structure of **1**, i.e. the same sides of methyl and methoxy groups substituted in the pyran structure of **2** could be characterized (Fig. 1). Similar with that of compound **1**, no cotton peaks can be detected in its CD spectrum (Fig. S21), the compound **2** was also deduced to be racemic mixture. Consequently, gentiagenin B was assigned as shown (Fig. 1), its spectra data was summarized in Table 1.

The known compounds were identified as swerimilegenin G (**3**) (Geng et al., 2013), 4-hydroxyindan-1-one (**4**) (Louden and Razdan, 1954), erythrocentaurin (**5**) (Nie and He, 1984) and swerimilegenin E (**6**) (Geng et al., 2013) (Fig. 1), respectively, in which, compound **3** was also determined as raceme by means of the X-ray analysis (Fig. S22).

From the structure point of view, it seems that compounds **1** and **2** could not be generated from gentiopicroside or similar secoiridoids through glycosidic linkage hydrolysis and the following oxidation or rearrangement reactions. On the other hand, the methoxy substituent is usually found in previous reported secoiridoid constituents (Geng et al., 2013; Hou et al., 2020), which may be biosynthesized in plant tissues or formed as artifact of original constituents in eluent (Madmanang et al., 2016). Considering the abundant other composition in extract (80 g) and sufficient reaction time (20 h) in methanol solvent, the newly identified structures were proposed to be generated from unknown constituents through certain reaction mechanism or even obtained as artifact form of the primary compounds in secoiridoid extract during isolation process.

2.2. Bioactivity evaluation

In the MTT test, no significant difference was detected among sample groups with that of control group in the concentration range of 1～100 μg/mL, which indicated no cytotoxic effects of isolated compounds on RAW264.7 cells.

In the anti-inflammatory evaluation, 6 isolated compounds and gentiopicroside exhibited inhibitory effects on NO production in a concentration-dependent manner in LPS-stimulated RAW264.7 cells (Table 2). The NO production inhibitory effect of gentiagenin A (**1**), gentiagenin B (**2**) and swerimilegenin E (**6**) were stronger than that of gentiopicroside (IC_{50}: 44.8 ± 7.8 μM) with the IC_{50} values of 24.3 ± 3.2 μM, 28.2 ± 2.6 μM and 29.3 ± 4.3 μM, respectively. Swerimilegenin G (**3**) gave the similar bioactivity compare to gentiopicroside with the IC_{50} value of 49.1 ± 7.9 μM. On the other hand, the inhibitory effect of 4-hydroxyindan-1-one (**4**) and erythrocentaurin (**5**)

Table 2
Inhibitory effects of compounds **1–6** and gentiopicroside against LPS-stimulated NO production in RAW264.7 macrophages.

Compounds	NO production (IC_{50}, μM)[a]
1	24.3 ± 3.2
2	28.2 ± 2.6
3	49.1 ± 7.9
4	50.1 ± 6.4
5	55.5 ± 4.7
6	29.3 ± 4.3
gentiopicroside	44.8 ± 7.8
Aspirin[b]	23.5 ± 2.4

[a] Each value represents as mean ± SD of five independent experiments.
[b] Aspirin was used as a positive control.

were slightly weaker than gentiopicroside, which gave the IC_{50} values of 50.1 ± 6.4 μM and 55.5 ± 4.7 μM.

3. Experimental

3.1. General experimental procedures

The IR spectra were measured on a Nicolet 6700 FT-IR spectrometer using KBr discs. CD spectra were obtained using a JASCO *J*-85 spectra polarimeter (Jasco, Tokyo, Japan). HR-ESI-MS were taken on a Waters Q-TOF MS spectrometer (Waters, Karlsruhe, America). X-ray crystal graphic analysis using Cu Mo radiation was performed on an Agilent X-ray single crystal diffractometer (Agilent, Oxford, England). The NMR spectra were recorded on a Bruker 400/600 MHz (AVANCE III /AV600, Berne, Switzerland) instrument for compounds in $CDCl_3$, CD_3OD, DMSO as solvents. Chemical shifts are expressed in δ (ppm), and are referenced to the residual solvent signals. Column chromatography was performed using silica gel (200–300 mesh, Branch of Qingdao Haiyang Chemical Co., Ltd., Qindao, China). Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and ODS silica gel (Merck, Darmstad, Germany) were used for column chromatography. TLC was performed with pre-coated silica gel GF_{254} glass plates (Branch of Qingdao Haiyang Chemical Co., Ltd., Qingdao, China). The HPLC analysis and preparation HPLC experiment were performed by Shimadzu HPLC instrument (Shimadzu, Kyoto, Japan) equipped with YMC-Pack ODS analysis (200 mm × 4.6 mm, 5 μm) or preparation (250 mm × 21.2 mm, 10 μm) column and the UV detector.

3.2. Preparation of gentiopicroside-rich iridoids extract from Gentiana macrophylla

Gentiana macrophylla was obtained from An Guo (Hebei, China), and identified by Dr. Guang Chen from Beijing University of Chemical Technology. A voucher specimen (Reference number 2016−04) was deposited at College of Life Science and Technology, Beijing University of Chemical Technology. The whole *G. macrophylla* (10 kg) was extracted twice with 70 % EtOH under reflux. for 2 h. After removal of the solvent by evaporation, the combined extracts (2300 g) were suspended in H_2O and partitioned with $CHCl_3$ (280 g) and n-BuOH (1200 g) successively. The n-BuOH extract was chromatographed on silica gel eluting with $CHCl_3$-MeOH gradient (50:1, 25:1, 10:1, 5:1, 2:1, and 1:1) to yield seven fractions (1–7) under the TLC detection. Fraction 4 was evaporated to remove the solvent to get gentiopicroside-rich iridoids extract (230 g). The extract was then detected and analyzed by TLC and HPLC using gentiopicroside as standard (a standard was provided by the National Institute on Drug Abuse of China with the Batch number of 0578–201605), whose gentiopicroside content was finally determined as 83 % (mobile phase: MeOH/H_2O 30/70; flow rate: 0.7 mL/min; 254 nm).

3.3. Acidic conversion of gentiopicroside-rich iridoids extract

Few gentiopicroside-rich iridoids extract was divided into several groups for preliminary conversion experiment. In which, the extract was dissolved in pure water and adjusted pH value into 3, 4, 5, 6 by hydrochloric acid. The solutions were then heated into 100 ˚C under boiling water bath condition. Although the conversion process can be detected in each solution, more obvious conversion products can be clearly detected in pH = 3 solution, which was then determined as the acidic treratment condition.

Then added 80 g gentiopicroside-rich iridoids extract into 450 mL pure water and adjusted pH value to 3 by hydrochloric acid. The conversion solution was refluxed under 100 ˚C with boiling water bath and the solution was sampled every 30 min to detect the conversion process. After 20 h reaction, the extract was detected to fully converse under the HPLC analysis. After cooling by airing, the conversion product was then adjusted pH to 7 using NaOH for further separation.

3.4. Isolation of acidic conversion products

After removal of solvent by evaporation, the acidic conversion extract (80 g) was separated using silica gel column chromatography (600 g, 9 × 100 cm column; CH_2Cl_2/MeOH gradient) to yield 8 fractions (1–8) based on TLC profiles. The fraction 2 was chromatographed on ODS column eluted by MeOH/H_2O to give 7 fractions (1–7). The fraction 2.2 was subjected on Sephadex LH-20 using MeOH/H_2O as eluent to afford a mixture. The mixture was further subjected to semi-preparative HPLC using MeOH/H_2O (30/70) as the mobile phase at a flow rate of 5.0 mL/min to give **1** (27.4 mg) and **2** (26.5 mg). The fraction 2.4 was then purified by Sephadex LH-20 to obtain compound **3** (38.5 mg). The fraction 2.5 was separated by Sephadxe LH-20 and further purified with semi-preparative HPLC using MeOH/H_2O (30/70) as the mobile phase at a flow rate of 5.0 mL/min to give compound **5** (26.9 mg). The fraction 3 was submitted to passage over ODS column eluted by MeOH/H_2O to afford 4 fractions. The fraction 3.2 was applied to semi-preparative HPLC using MeOH/H_2O (25/75) as the mobile phase at a flow rate of 5.0 mL/min to afford compounds **4** (28.1 mg) and **6** (27.4 mg).

3.4.1. Compound 1

Yellow amorphous powder; UV (MeOH) λ_{max} (log ε): 234 (3.59) nm; IR ν_{max} (KBr): 2977.93 and 1720.07 cm^{-1}; ^1H and ^{13}C NMR data: see Table 1. HR-ESI-MS: 183.0973 $[M+H]^+$ (calcd. for $C_{10}H_{15}O_3$: 183.1021).

3.4.2. Compound 2

Yellow amorphous powder; UV (MeOH) λ_{max} (log ε): 234 (3.59) nm; IR ν_{max} (KBr): 2977.42 and 1719.72 cm^{-1}; ^1H and ^{13}C NMR data: see Table 1. HR-ESI-MS: 183.1009 $[M+H]^+$ (calcd. for $C_{10}H_{15}O_3$: 183.1021).

3.5. Cell cultures

RAW264.7 macrophage cells were obtained from Institute of Biophysics, Chinese Academy of Sciences (Beijing, China). The cells were cultured in DMEM containing L-Glutamine, Sodium Pyruvate (110 mg/L), 10 % FBS, 1% penicillin (100 IU/mL) and streptomycin (10 mg/mL) at 37 ˚C in a humidified atmosphere of 95 % air-5% CO_2.

3.6. Cell viability

The impact of each compound on cell viability was detected using MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide) assay. RAW264.7 cells were seeded in 96 well plates (1 × 10^5 cells/mL) at 37 ˚C. After overnight growth, cells were treated with various concentrations of each compound (100, 50, 10, 5, 1 μg/mL) for 1 h, followed in the presence or absence of LPS (100 ng/mL) for the next 24 h. Then, sucked out 50 μL of supernatant fluid, 50 μL of MTT (5 mg/mL) was added to cultures, followed by incubation at 37 ˚C for 4 h. The supernatant fluid was poured out and added 150 μL of dimethyl sulfoxide (DMSO) for the solubilization. The OD at 570 nm was measured by a microplate reader. According to the OD_{570} values, the cell viability was calculated as follows:

$$Cell\ Viability = \frac{OD(sample\ group)}{OD(control\ group)} \times 100\%$$

3.7. Determination of NO content

The anti-inflammatory effects of 6 conversion products were evaluated by means of the NO inhibition rate detection in LPS-stimulated RAW264.7 cells. The analysis was divided into 4 groups: control group, LPS modle group, sample group (each compound was formulated into

five concentrations: 100 μg/mL, 50 μg/mL, 10 μg/mL, 5 μg/mL, 1 μg/mL) and positive control group. RAW264.7 cells were seeded in 96 well plates (1×10^5 cells/mL) at 37 °C. After overnight growth, cells were treated with various concentrations of each compound for 1 h, followed in the presence or absence of LPS (100 ng/mL) for the next 24 h. Then, sucked out 50 μL of supernatant fluid and added the Griess reagent and measured the optical density (OD) at 540 nm on a microplate reader. By comparison with the standard curve, the NO concentration can be calculated. According to the OD value, the NO inhibition rate of each compound was also calculated as follows:

$$NO\ Inhibition\ Rate = \frac{OD(\mathrm{mod}el\ group) - OD(sample\ group)}{OD(\mathrm{mod}el\ group) - OD(control\ group)} \times 100\%$$

Statistical analysis Data were expressed as means ± SD (n = 5). Differences between means of each group were assessed by one-way analysis of variance (ANOVA) using SPSS 13.0 statistical software. A p-value < 0.05 was considered statistically significant.

Declaration of Competing Interest

The authors have no conflicts of interesting to declare.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phytol.2020.07.004.

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