Phenolic Compounds from the Rhizomes of *Smilax china* L. and Their Anti-Inflammatory Activity

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**Abstract**: A new triflavanoid, kandelin B-5 (1), was isolated from the rhizomes of *Smilax china* L., together with six known phenylpropanoid substituted flavan-3-ols (2−7), nine flavonoids (8−16), two stilbenoids (17, 18), and two other compounds (19, 20). The structure of compound 1 was determined on the basis of 1D, 2D NMR and HR-ESI-MS data, as well as chemical method. Compounds 2−5, 8−12, 15, 17, and 19 were evaluated for anti-inflammatory activity. Only compounds 10, 15 and 17 showed slightly IL-1β expression inhibitory activities on LPS induced THP-1 cells, with inhibition rate of 15.8%, 37.3%, and 35.8%, respectively, at concentration of 50 µg/mL.

**Keywords**: *Smilax china*; phenolic compounds; anti-inflammatory activity

1. **Introduction**

*Smilax china* L. (Liliaceae), a perennial climbing deciduous shrub, is widely distributed in Southern China, and Southeast Asian countries. The leaves of *S. china* are used as detoxication agent in folk China [1]; while the rhizomes of *S. china*, called “Jin Gang Teng”, are collected by Chinese Pharmacopoeia with the efficacy of carminative, diaphoretic, and circulative [2]. Previous studies on the title species have disclosed the presence of steroidal saponins [3], flavonoids [4,5], phenylpropanoids [6], and stilbenoids [7,8]. These isolated compounds showed a wide spectrum of activities, such as immunosuppressive activity [9], anti-oxidative activities [10], anti-inflammatory activities [3,11].

The pharmaceutical preparations of *S. china* are widely used in clinic for the treatment of chronic pelvic inflammatory disease, a kind of chronic inflammation in the female genital organs, connective tissues and pelvic peritoneum. Recently, we reported five known flavonoids from the anti-chronic pelvic inflammation fraction of *S. china* using high speed counter current chromatography [12,13]. Further detail chemical investigation on the title species led to the isolation of a new triflavanoid (1) and 19 known phenolic compounds (2−20) (Figure 1). Compounds 2−5, 8−12, 15, 17, and 19 were evaluated for their anti-inflammatory activity. Herein we describe the isolation and structure elucidation of these compounds as well as their anti-inflammatory activities.
Figure 1. Chemical structures of 1–20.
2. Results and Discussion

The ethanol extract of the rhizomes of *S. china* was partitioned with Ethyl Acetate (EtOAc), and then subjected to column chromatography (CC) over MCI gel CHF 20, Toyopearl HW 40F, silica gel, and preparative HPLC to yield a new triflavanoid (1), together with 19 known compounds. The known compounds were identified as cinchonain Ila (2) [14], cinchonain Ib (3) [14], cinchonain Ia (4) [15], cinchonain lb (5) [15], catechin-[8,7-e]-4β-(3,4-dihydroxyphenyl)-dihyro-2(3H)-pyranone (6) [15], catechin-[8,7-e]-4α-(3,4-dihydroxyphenyl)-dihyro-2(3H)-pyranone (7) [15], engeletin (8) [16], astilbin (9) [17], neoaestilbin (10) [17], isoastilbin (11) [17], isoeaestilbin (12) [17], quercetin-3-O-α-L-rhamnopyranoside (13) [12], luteolin-3-O-α-L-rhamnopyranoside (14) [18], (-)-epicatechin (15), 5,7,4′-trihydroxyflavonone (16), scirpusin A (17) [4], resveratrol (18) [4], chlorogenic acid (19) [19], and protocatechuic acid (20) [19], by comparison of their physicochemical data with those reported in the literature. Of them, compounds 2–3, 14, 16, and 20 were isolated from the genus *Smilax* for the first time.

Compound 1 was isolated as white amorphous powder. The molecular formula, C_{53}H_{44}O_{21}, was determined on the basis of the HRESIMS data (m/z 1027.2285 [M − H]^−; calcd. 1027.2297). The 1H-NMR data of 1 showed three singlets at δ_H 6.10, 5.90, 5.90, arising from the A-rings, three pairs of signals due to the flavan C_2-H and C_3-H at δ_H 5.17 (br.s, H-2), 4.48 (d, J = 7.4 Hz, H-2′), 3.83 (m, H-3′), 3.73 (d, J = 9.2 Hz, H-2′′), 3.71 (br.s, H-3), and 3.41 (m, H-3′′), two singlet methine signals at δ 4.51 (s, H-4′), 4.34 (s, H-4), and a methylene signal at δ_H 2.40 (br.d, J = 15.6 Hz, H-4′′α), 2.05 (dd, J = 7.0, 15.6 Hz, H-4′′β). The connectivities of these protons on C-rings were identified by 1H-1H COSY experiment (Figure 2). The above evidence indicated the presence of a triflavanoid moiety, which was consistent with 13C-NMR data. The 13C-NMR showed three aromatic methine carbons at δ_C 95.7, 95.5, 94.3, nine aliphatic carbons δ_C 82.1, 81.4, 75.6, 70.8, 68.2, 66.7, 36.3, 35.9, 30.7, and overlapped carbon signals arising from four 3,4-dihydroxybenzyl rings. The chemical shifts of the C-2 carbons, as well as the coupling patterns of the C_2 proton signals suggested that the triflavanoid contains an epicatechin and two catechin moieties [20]. The 13C-NMR coupled with HSQC data showed the presence of an ester (δ_C 169.0), a methine (δ_C 33.6; δ_H 3.81, m), and a methylene (δ_C 37.0; δ_H 2.84 (dd, J = 6.0, 16.0 Hz), 2.25(dd, J = 10.0, 16.0 Hz)), suggesting the existence of a phenylpropanoid moiety. The phenylpropanoid moiety was connected with A-ring of the upper flavan-3-ol unit since all the protons on A rings showed singlets in the 1H-NMR spectrum [20]. Additionally, the phenylpropanoid moiety was presumed to be connected with C-8 position of upper flavan-3-ol unit as indicated by the carbon chemical shift of C-6 and C-8 [δ_C: 96.0 (C-6), 105.2 (C-8) for C-8 substituted isomer; δ_C: 106.2 (C-6), 99.4 (C-8) for C-6 substituted isomer] [21]. The two singlet methine signals at δ 4.51 (H-4′), 4.34 (H-4) indicated two β linkages of three flavan-3-ol units [14]. The 1D NMR of compound 1 was very similar to that of cinchonain Ila (2) or Iib (3), suggesting the C(4), C(8′)-linkages of flavan-3-ol moieties. The obviously up-field shifted of H-2″ in the terminal catechin unit as comparing with catechin, attribute to the deshielding effect of phenyl group on the middle flavan-3-ol unit, suggested the C(4′), C(8″)-linkages of middle and terminal flavan-3-ol units.

The constitution and points of the interflavanoid linkages in 1 were confirmed by hydrochloric acid-catalyzed degradation with cysteamine (Figure 3). Complete degradation of 1 afforded 4β-(2-aminoethylthio)-cinchonain lb (1a), 4β-(2-aminoethylthio)-catechin (1b) [22], and catechin (1c), establishing that it consists of cinchonain lb and catechin units. 1a showed strong positive cotton effects at 230 nm and negative cotton effect at 250 nm, indicating that the phenyl group of phenylpropanoid unit was α-oriented. Thus, the structure of 1 was elucidated as showed in Figure 1.
Interleukin-1β (IL-1β), an important mediator of the inflammatory response, is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. Compounds 2–5, 8–12, 15, 17, and 19 were evaluated for their IL-1β expression inhibitory activities on lipopolysaccharide (LPS) induced THP-1 cells. Compounds 10, 15, and 17 showed slightly inhibitory activities, with inhibition rate of 15.8%, 37.3%, and 35.8%, respectively, at concentration of 50 µg/mL. The other compounds showed no obvious activity at the same concentration. CCK-8 results revealed that these tested compounds showed no obvious cytotoxicities towards THP-1 cells at same concentration, indicating that the anti-inflammatory activities of compounds 10, 15, and 17 were not resulted from cytotoxic effects. Previous studies revealed the unique immunosuppressive activity of astilbin (9) [23]; While our results suggested that astilbin (9) and its isomers (10–12) didn’t directly inhibit the production of proinflammatory cytokines IL-1β. (-)-Epicatechin and stilbene are reported to show anti-inflammatory activities [24]. Consistently, our results showed that (-)-Epicatechin (15) and stilbene dimer, scirpusin A (17), inhibited IL-1β expression on the LPS induced THP-1 cells.

3. Materials and Methods

3.1. General Experimental Procedures

Optical rotation was measured with an MCP-500 polarimeter (Anton Paar). The UV spectrum was recorded with a TU-1810DSC UV-Vis spectrometer (Puxi Tongyong). IR spectrum was measured on a IR Affinity-1 spectrophotometer (Shimadzu); CD was measured on a Chirascan™ (Applied Photophysics Ltd., Leatherhead, UK). NMR was acquired on an AV-400 spectrometer (Bruker) with
TMS as internal standard, J in Hz; HRESIMS spectra were measured on the Orbitrap Fusion high resolution mass spectrometer (Thermo) equipped with ESI source. Preparative or semi-preparative HPLC were performed on a HPLC system equipped with a Waters 1525 pump and a Waters 2487 Dual Wavelength Detector using a Thermo hypersil C18 column (5 µm, 150 × 25.0 mm, i.d.) or Thermo hypersil C18 column (5 µm, 250 × 10 mm, i.d.). Open column chromatography was performed using silica gel (200–300 mesh, Qingdao Marine Chemical, Qingdao, China), Sephadex LH-20 (25–100 µm, Fiji), MCI gel CHP 20P (75–150 µm, Fiji), Toyopearl HW 40F (TOSOH, Tokyo, Japan) or ODS (40–63 µm, Merck, Kenilworth, NJ, USA).

3.2. Plant Materials

The rhizomes of *S. china* were purchased from Shenzhen Hongen Pharmaceutical Company, and were identified by one of the authors (L.B. Hou). The voucher specimen of this material (NO. SMU-NPC-201301) was deposited in Natural Product Chemistry Lab, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou, China.

3.3. Extraction and Isolation

The rhizomes of *S. china* (6.0 kg) were refluxed with 95% ethanol at 75 °C for three hours, three times and filtered. The combined filtrate was concentrated to yield crude extracts (1.2 kg). The crude extracts were suspended in H2O, and then partitioned with EtOAc and n-BuOH consecutively. A portion (8.5 g) of the EtOAc-solution fraction (150 g) was subjected to MCI gel CHP 20P (MeOH/H2O, 1:9–10:0) CC to give seven fractions A-G. Fraction A (0.21 g) was chromatographed over MCI gel CHP 20P (MeOH/H2O, 1:9–4:6), and ODS (MeOH/H2O, 2:8–5:5) to afford 20 (4.0 mg). Fraction B (0.25 g) was chromatographed over Toyopearl HW 40F (MeOH/H2O, 3:7–8:2) to yield four sub-fractions, B1–B4. Sub-fraction B1 was subjected to MCI gel CHP 20P (MeOH/H2O, 2:8–6:4) to yield 19 (4.0 mg). Sub-fraction B2 was chromatographed over ODS (MeOH/H2O, 2:8–6:4), Toyopearl HW 40F (MeOH/H2O, 3:7–7:3), and Sephadex LH-20 (100% MeOH) to yield 15 (9.0 mg). Sub-fraction B4 was purified by preparative HPLC (gradient model, MeOH/H2O, 20–80% over 30 min, MeOH and H2O contained 0.1% TFA) to yield 1 (16 mg). Fraction C (4.08 g) was chromatographed over MCI gel CHP 20P (MeOH/H2O, 3:7–9:1) to yield three sub-fractions, C1-C3. Sub-fraction C2 was purified by Toyopearl HW 40F (MeOH/H2O, 4:6–1:0) CC, and preparative HPLC (gradient model, MeOH/H2O, 20–80% over 30 min, MeOH and H2O contained 0.1% TFA) to afford 2 (21 mg), and 3 (26 mg). Sub-fraction C3 was subjected to CC over Toyopearl HW 40F (MeOH/H2O, 4:6–1:0) to yield three sub-fractions, C3-1, C3-2, C3-3. Sub-fraction C3-2 was purified by semi-preparative HPLC (35% MeOH, isocratic model) to afford 9 (8 mg), 10 (40 mg), 11 (6 mg), and 12 (5 mg). Sub-fractions C3-3 was purified by semi-preparative HPLC (gradient model, MeOH/H2O, 20–80% over 30 min, MeOH and H2O contained 0.1% TFA) to yield 4 (21 mg), 5 (15 mg), 6 (4 mg), and 7 (6 mg). Fraction D (1.0 g) was chromatographed over MCI gel CHP 20P (MeOH/H2O, 4:6–1:0), Toyopearl HW 40F (MeOH/H2O, 4:6–1:0), and Sephadex LH-20 (100% MeOH) to afford 13 (42 mg). Fraction E (1.0 g) was chromatographed over MCI gel CHP 20P (MeOH/H2O, 4:6–8:2), Toyopearl HW 40F (MeOH/H2O, 4:6–8:2) to yield 8 (30 mg), and 16 (9.0 mg). Fraction F (0.4 g) was subjected to CC over MCI gel CHP20P (MeOH/H2O, 6:4–1:0), Sephadex LH-20 (100% MeOH), and silica gel (DCM/MeOH, 9:1–8:2) to afford 14 (12 mg), 17 (5 mg), and 18 (9 mg).

Kandelin B-5 (1): Amorphous powder, [α]D25 = 86.5 (c = 0.18, MeOH); UV (MeOH), λmax (logε): 227 (4.55), 282 (4.03) nm; IR(KBr)νmax 3350, 1743, 1616, 1521, 1448, 1284, 1109 cm−1; 1H-NMR (400 MHz, DMSO-d6) and 13C-NMR (100 MHz, DMSO-d6): see Table 1; (-)-HRESIMS: 1027.2285 [M − H]− (Calcd for C54H43O21, 1027.2297).
Table 1. $^1$H- (400 MHz) and $^{13}$C-NMR (100 MHz) data of 1 (in DMSO-$d_6$, $J$ in Hz, $\delta$ in ppm).

| Position | $\delta$H | $\delta$C |
|----------|-----------|-----------|
| 2        | 5.17 br.s | 75.6      |
| 3        | 3.71 br.s | 70.8      |
| 4        | 4.34 br.s | 36.3      |
| 6        | 5.90 s    | 94.0      |
| 8        |           | 108.0     |
| 10       |           | 99.5      |
| 2'       | 4.48 d (7.4) | 81.4 |
| 3'       | 3.38 m    | 66.7      |
| 4'       | 4.51 br.s | 35.9      |
| 6'       | 5.90 s    | 95.1      |
| 8'       |           | 108.0     |
| 10'      |           | 103.6     |
| 2''      | 3.73 d (9.2) | 82.1 |
| 3''      | 3.41 m    | 68.2      |
| 4''      | 2.84 dd (6.0, 16.0) | 30.7 |
| 6''      | 2.25 dd (10.0, 16.0) | 95.7 |
| 8''      |           | 107.7     |
| 10''     |           | 100.4     |
| $\alpha$ | 2.40 br.d (15.6) | 38.1 |
| $\beta$  | 2.05 dd (7.0, 15.6) | 33.9 |
| -COO-    |           | 169.0     |

3.4. Thiolysis of Compound 1

A mixture of compound 1 (8 mg), cysteamine (20 mg), and 1 M aqueous hydrochloric acid (3 mL) was refluxed for 2 h with stirring. After removal of the solvent by evaporation under reduced pressure, the oily residue was applied to a Sephadex LH-20 column (DCM/MeOH, 1:1), and was further purified by semi-preparative HPLC (gradient model, MeOH/H$_2$O, 10–90% over 30 min) to afford: 1a (0.9 mg), 1b (0.6 mg), and 1c (0.4 mg).

4β-(2-aminoethylthio)-cinchonain Ib (1a): Amorphous powder, $[\alpha]_{D}^{25} = 41.8$ (c = 0.09, MeOH); $^1$H-NMR (400 MHz, Acetone-$d_6$): $\delta$ 6.91 (1H, d, $J$ = 1.9 Hz, H-2'), 6.74 (2H, s, H-5', 6'), 6.71 (2H, m, H-2'', 5''), 6.53 (1H, dd, $J$ = 2.0, 8.1 Hz, H-6''), 6.23 (1H, s, H-6), 5.27 (1H, br.s, H-2), 4.45 (1H, m, H-7''), 4.17 (1H, br.s, H-3), 3.07 (1H, dd, $J$ = 6.9, 15.9 Hz, H-8'a), 4.45 (1H, m, H-7''), 4.17 (1H, br.s, H-3), 3.07 (1H, dd, $J$ = 6.9, 15.9 Hz, H-8'a), 2.90 (1H, br.d, $J$ = 15.9 Hz, H-8'b), 3.80-2.80 (4H, m, S-CH$_2$-CH$_2$-N); (-)-ESIMS: $m/z$ 526 [M − H$^-$].

4β-(2-aminoethylthio)-catechin (1b): Amorphous powder, $[\alpha]_{D}^{25} = 33.2$ (c = 0.06, MeOH); $^1$H-NMR (400 MHz, Acetone-$d_6$): $\delta$ 6.91 (1H, d, $J$ = 1.9 Hz, H-2'), 6.75 (1H, d, $J$ = 8.1 Hz, H-5'), 6.70 (1H, dd, $J$ = 1.9, 8.1 Hz, H-6'), 5.91 (2H, s, H-6, 8), 5.31 (1H, d, $J$ = 9.3 Hz, H-2), 4.00 (1H, m, H-3), 3.88 (1H, d, $J$ = 6.6 Hz, H-4), 3.60-2.40 (4H, m, S-CH$_2$-CH$_2$-N).

catechin (1c): Amorphous powder; $^1$H-NMR (400 MHz, Acetone-$d_6$): $\delta$ 6.91 (1H, d, $J$ = 1.9 Hz, H-2'), 6.81 (1H, d, $J$ = 8.1 Hz, H-5'), 6.77 (1H, dd, $J$ = 1.9, 8.1 Hz, H-6'), 6.04 (1H, d, $J$ = 1.9 Hz, H-8), 5.89 (1H, d, $J$ = 1.9 Hz, H-6), 4.57 (1H, d, $J$ = 7.8 Hz, H-2), 4.00 (1H, m, H-3), 2.93 (1H, dd, $J$ = 5.5, 16.0 Hz, H-4a), 2.90 (1H, dd, $J$ = 8.4, 16.0 Hz, H-4b).

3.5. Quantitation of Cytokine IL-1β

The human acute monocytic leukemia (THP-1) cells (Shanghai Cell Bank, C.A.S., Shanghai, China) were cultured in Dulbecco’s Modified Eagles Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) at 37 °C in a humidified atmosphere with 5% CO$_2$. The cells were seeded into 96-well plates with density of $1 \times 10^5$ cells per milliliter. Thereafter, cells were treated with sample solutions diluted with cell sustainable medium. After incubation for 1 h, the cells were treated with 1 μg/mL of LPS for another 24 h. The cells were treated with LPS alone or LPS plus dexamethasone as blank control.
and positive control, respectively. The supernatant was collected by centrifugation (14,000 rpm, 4 °C). The quantity of IL-1β in culture supernatant was determined by using Enzyme Linked Immunosorbent Assay (ELISA) kit specific for IL-1β [25]. The inhibition rates were calculated according to the formula:

\[
\text{Inhibition rate} = \left(\frac{C - T}{C}\right) \times 100\% \tag{1}
\]

where C is the average quantity of IL-1β of the blank control and T is the average quantity of IL-1β of the group with the test compound.

3.6. Cytotoxicity Assay

The effect of compounds 2–5, 8–12, 15, 17, and 19, on the viability of THP-1 cells were evaluated using the Cell Counting Kit-8 (CCK-8) according to the manufacturer’s instructions. Briefly, THP-1 cells were seeded into 96-well plates at a density of 2.5 × 10⁴ cells/well in the presence of sample solutions (50, 100, 200 µM, respectively) or absence of compounds for 48 h. Thereafter, 10 µL CCK-8 solutions were added to each well for further incubation at 37 °C in 4 h. The optical density was measured at a wavelength of 450 nm using a microplate reader [26]. Cell viability was expressed as a percentage of the control.

4. Conclusions

In this study, a new triflavanoid, kandelin B-5 (1), was isolated from the rhizomes of Smilax china L., together with six known phenylpropanoid substituted flavan-3-ols (2–7), nine flavonoids (8–16), two stilbenoids (17,18), and two other compounds (19,20). The structure of compound 1 was determined on the basis of 1D, 2D NMR and HR-ESI-MS data, as well as chemical method. Compounds 2–5, 8–12, 15, 17, and 19 were evaluated for anti-inflammatory activities. Only neoastilbin (10), (-)-epicatechin (15), and scirpusin A (17) showed slight IL-1β expression inhibitory activities on LPS induced THP-1 cells.

Supplementary Materials: Supplementary materials are available online. The NMR spectra of 1; the 1H-NMR, ESIMS, and CD spectra of hydrolysis products of 1.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Sample Availability: Samples of the compounds are available from the authors.